DEVELOPMENT OF AN EFFICIENT METHOD FOR PRODUCTION OF α-MANGOSTIN REFERENCE STANDARD FROM *GARCINIA MANGOSTANA* L. RINDS USING LIQUID FLOW PROCESSING

A thesis submitted for the degree of

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By

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DECLARATION OF AUTHENCITY

I hereby declare that I am the sole author of this thesis

Sri Murhandini

ABSTRACT

Over centuries, world populations exclusively have used medicinal plants as therapeutic agents. Currently, some studies have proven that α-mangostin, a natural xanthone isolated from part of *Garcinia mangostana* L. tree especially from stem bark or fruit rinds has many potential activities including antioxidant, anti-bacterial, antivirus, anti-inflammatory, and anticancer that has resulted in many mangosteen products appearing on the market. To standardise and quality control mangosteen and herbal products in general, an acceptable reference standard which is isolated from the plant itself is required. Separation and purification techniques using liquid flow processing known as counter-current chromatography (CCC) are widely applied for this purpose. However, generally only a single injection of the sample into a CCC apparatus is used due to sample complexity. Multiple injections to increase the overall yield and scale of the purification are seldom used. But this has the advantage of reducing the cost of the purification process.

The purpose of this has been to develop an efficient method for production of α-mangostin reference standard from fruit rind of *Garcinia mangostana* L. using liquid flow processing. The experiment were conducted initially by sample loading studies at analytical scale using a Mini HPCCC (17.4 mL coil, 0.8 mm bore) with a hexane/ethyl acetate/methanol/water (5:5:10:4 v/v) solvent system. Extract was prepared by overnight maceration of mangosteen rinds powder in 80% aqueous ethanol at 30°C. The extract in lower phase was injected up to 10 times without any replacement or topping up the stationary phase. The studies establsiehd 22.8 mg extract in 0.86 mL lower phase as the optimum amount of sample with multiple injections; and produced α-mangostin with 98.82% purity and 93.68% yield. Scaled up 8 times with 10 injections on Spectrum-CCC and 50 times with 7 injections on Midi-CCC gave α-mangostin with 99.24% purity and 96.35% yield; and 98.24% purity and 94.42% yield respectively. The concentration was then optimised nearly 3 times on Spectrum giving α-mangostin with 98.11% purity and 93.81% yield. These α-mangostin products with purity >98% can be accepted as reference standard for quality control of mangosteen based products, allowing the precise calibration of analytical instruments with this target compound. The purified α-mangostin was identified using commercial reference standard on HPLC and NMR.

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ABBREVIATIONS

CCC	Counter-current Chromatography
$-CH_3$	Methyl
GC	Gas Chromatography
DCCC	Droplet Counter-current Chromatography
CPC	Centrifugal Partition Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
-H	Hydrogen
HEMWat	Hexane/ethyl/acetate/methanol/water
HPCCC	High Performance Counter-current Chromatography
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LP	Lower phase of a two-phase system
mg	Milligram
min	Minute(s)
mL	Millilitre
NMR	Nuclear Magnetic Resonance
ppm	Part per million
S_F	The fraction of stationary phase in a CCC column, usually expressed as a percentage.
TFA	Tri-fluoroacetic Acid
TMS	Tetra-methyl-silane
α	Alpha
β	Beta
γ	Gamma
µg	Micro gram
µL	Micro litre
UP	Upper phase of a two phase system
µVolt*sec	Micro voltage second

Chapter 1 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Traditional Herbal Medicines and the need for standard material

The World Health Organization (WHO) defines traditional medicine as, "…the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness". Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products that contain as active ingredients parts of plants, or other plant materials, or combinations (WHO, 2000). To ensure consistent quality which the active ingredients have been identified in herbal medicines, WHO requires standardisation to contain a defined amount of the active ingredients. Therefore, standard material of the active ingredients from medicinal plants must be provided to meet the needs.

For centuries, medicinal plants have been used as traditional medicines to treat many diseases. Traditional use refers to the long historical use of these medicines. Their use is well established and widely acknowledged to be safe and effective, and may be accepted by national authorities (WHO, 2000). Currently, in spite of the exponential development of synthetic pharmaceutical chemistry, including combinatorial chemistry and microbial fermentation, 25% of prescribed medicines in developed countries are of plant origin. This percentage can reach 50% for the over-the-counter (OTC) market (drugs for self-medication). In fact, it is also estimated that natural products are used in the development of 44% of all new drugs, especially for the preparation of semi-synthetic derivatives. Nowadays, the pharmaceutical industry fully considers plants as a viable option for the discovery of new leads (Hostettmann, 2001).

Indonesia has its own traditional medicine which is a generations heritage called "Jamu". It is predominantly herbal medicine made from natural materials, such as parts of plants i.e. roots, leaves, bark, and fruit. Some times, there is also material from the bodies of animals, such as bile of goat or alligator (Anon[2]). The Policy of the Jamu is governed by the Ministry of Health; however the monitoring of the products were undertaken by the the National Agency of Drug and Food Control of the Republic of Indonesia (NADFC-RI).

To anticipate the increasing number of the natural products in the market of Indonesia, NADFC-RI needs to consider tools for controlling the products. Analytical methods for controlling the products should be developed especially to ensure the quality, safety and efficacy as a domain of the institute. However, to apply the methods either qualitatively or quantitatively, an acceptable reference standard needs to be provided. The standard can be an active compound (a biologically active molecule which is often the main ingredients in pharmaceuticals) or a marker compound (a compound used as an internal standard or to label particular batches) depending on the analytical purposes. Usually, a marker compound is used to ensure the content of the product, to avoid product counterfeiting, while an active compound is needed as a standard when the quality and efficacy of the product needs to be ensured. According to the WHO, if the identification of an active principle is not possible, then it should be sufficient to identify a characteristic substance or mixture of substances (e.g. "Chromatographic fingerprint") (WHO, 2000)

1.1.2 Preparation of standards; a role for Counter-current Chromatography (CCC)

Preparation of a marker or active compound from a medicinal plant is not a simple task because there will be a long procedure to extract and isolate, as well as purify and identify the compounds which can be very complex molecules. To determine and isolate such compounds from the medicinal plant, it is important to understand its physical and chemical properties. The target may be a major compound that is easily found and isolated; otherwise an advanced purification method should be developed when the compound is difficult to separate. Using a classical method like column chromatography is not always satisfactory due to the possibility of column blockage with particulates from the crude sample, column properties changing with time and the expense of replacing the column material. There will be occasions when an alternative and efficient method therefore needs to be developed, and liquid flow processing known as counter-current chromatography (CCC) seems to be suitable for this purpose. CCC is an excellent alternative to avoid the problems associated with solid-phase adsorbents and to preserve the chemical integrity of mixtures subjected to fractionation. Furthermore, the advancing technology of CCC is easy and predictable to be scaled up (Doshi, 2010; Sutherland, 2009).

1.1.3 The preparation of α-mangostin as a standard using CCC

To develop an efficient method for the production of α-mangostin, the study described in this thesis was undertaken to develop a one step isolation and purification process of to obtain α-mangostin as a standard material from fruit rinds of *Garcinia mangostana* L., using liquid flow processing (CCC). Importantly, the method developed is different from existing methods that have previously been performed. This research was undertaken initially on an analytical scale CCC instrument (17.4 mL coil; 0.8 mm bore) using Mini High Performance Counter Current Chromatography (HPCCC) and an aqueous ethanol extract of mangosteen fruit rinds as a sample. The sample was prepared by overnight maceration of mangosteen rinds powder in 80% aqueous ethanol at 30°C. To produce a high throughput of α-mangostin, the method was developed and optimised by increasing volume and concentration of sample with multiple injections up to 10 times without any replacement or topping up the stationary phase. The developed method was then scaled up on semi preparative Spectrum HPCCC instrument (143.5mL coil; 1.6 mm bore) and on preparative Midi HPCCC instrument (912.5 mL coil; 4 mm bore). Using this method has some advantages such as simpler; reduce cycle time and less solvent use. Moreover, using this method provided α-mangostin with high purity, yield and recovery as well as throughput.

1.2 LITERATURE REVIEW

1.2.1 Mangosteen and mangostin

Among tropical fruits, *Garcinia mangostana* L (mangosteen) is the most popular and valued in the family Clusiaceae (Morton, 1987). The origin of the mangosteen is not exactly known, but it is believed to come from the Sunda Islands and the Moluccas of Indonesia. The mangosteen tree can be described as a slow-growing tree, erect with a pyramidal crown; and attains 20 to 82 ft (6-25 m) in height. The fruit, capped by the top calyx at the stem end and with 4 to 8 triangular, flat remnants of the stigma in a rosette at the apex, is round, dark-purple to red-purple and smooth externally; 1 1/3 to 3 in (3.4-7.5 cm) in diameter. The rind is 1/4 to 3/8 in (6-10 mm) thick, red in cross-section, purplish-

white on the inside. The flesh is juicy, slightly acid in flavour and delicious (Morton, 1987). Mangosteen fruit can be obtained as fresh fruit, packed in cans, or made into syrup/juice.

Figure 1.1 Mangosteen fruits

Mangosteen thrives in most of Southeast Asia countries such as Indonesia, Thailand, Malaysia and Myanmar which have tropical climates in the whole year. People in those countries, especially in Thailand, have traditional used the hull of mangosteen as a medicine for skin infection, wounds, and diarrhoea for many years. In Indonesia, it is traditionally used for treating haemorrhoid, thrush and wounds as well. The rinds of the fruit are used as a natural dye for textiles and the trunk is used as building materials, and for firewood/crafts (Obolskiy, 2009).

Reviews of mangosteen have been carried out especially in the area of phytochemical and pharmacological effect, other traditionally applications, including the therapy of various diseases such as dysentery, urinary disorders, cystitis and gonorrhoea (Obolskiy, 2009). Currently, some new interesting properties are being reported and research on the mangosteen area has been growing rapidly, not only in Asian countries where mangosteen originates but also in USA and European countries. It has been reported that phytochemical and pharmacological studies on xanthone and their derivatives isolated from the mangosteen fruit rind are the most widely performed to explore the benefit properties and efficacy of mangosteen as the queen of tropical fruit. Nevertheless, the research on toxicity is still very limited and preliminary toxicity studies need to be improved to ensure safety in the use of Mangosteen products (Ajayi, 2007).

It has been reported that a 40% ethanol extract of mangosteen hull could inhibit both histamine release and prostaglandin E2 synthesis (Nakatani, 2002). This suggests that

the crude extract is promising for both anti allergy and anti inflammatory agent. Other researchers have claimed that the crude methanol extract from the pericarp of *Garcinia mangostana* had anti-proliferative, apoptotic and antioxidative properties, so it has the potential as a natural cancer chemoprevention agent (Moongkarndi, 2004). Furthermore the crude ethanol extract showed α-glucosidase inhibitory activity and elicited a reduction of postprandial blood glucose levels that is a potential for anti-diabetes (Ryu, 2011). In vitro cytotoxicity studies of lyophilized hot water and juice extract of the fruit rind of mangosteen against K562, P3HR1, Raji, and U937 leukemic cells have exhibited a potent anti-leukemic activity for the hot water extract (Chianga, 2004).

The latest in scientific research confirms that mangosteen contains a class of naturally occurring polyphenolic compounds known as xanthones. Isolation and identification of xanthones and their derivatives: α-mangostin, 8-desoxygartanin, gartanin, β-mangostin, 3-mangostin, and 9-hydroxycalabaxanthone has been reported (Walker, 2007). The results of several research projects show that xanthone has properties which include antioxidant activities (Suvarnacuta, 2011; Zarena and Sankar, 2009 [a, b]; Zarena and Sankar, 2012), analgesic and anti-inflammatory (Cui, 2010), anti cancer therapeutics (Tangpong, 2011), and Alzheimer's disease (Moongkarndi, 2010). Many scientists have reviewed the medicinal properties of *Garcinia mangostana* L. extract for antioxidant, antitumor, anti allergic, anti-inflammatory, antibacterial and antiviral activities (Caverri, 2008; Dembitsky, 2011; Kapoor, 2009). It has even been mentioned by other reviewers that mangosteen xanthones are effective for treating gastrointestinal disturbances and for wound-healing including antifungal, anti malarial, and anti-HIV (Kinghorn, 2011). This seems to suggest a potential for development as an anti-virus medicine in the future, especially for anti HIV-AIDS virus which no effective treatment is currently available.

The most medicinally important xanthones isolated from mangosteen are α-mangostin and γ-mangostin. Mangostin with molecular formula: $C_{24}H_{26}O_6$; Mol. Wt. = 410.46, is a natural organic compound isolated from the mangosteen plant. It is a yellow colour, crystalline solid with a xanthone core structure (The Merck Index, 13th edition 2001). This mangostin has density of 1.265 g/cm^3; melting point of 180-182 ºC, boiling point of 640.1 ºC at 760 mmHg and flash point of 220.3 ºC. It is a specific chemical

compound in mangosteen so it can be used as a marker for the analysis of mangosteen. The chemical structures of α, β, and γ-mangostin are as follows:

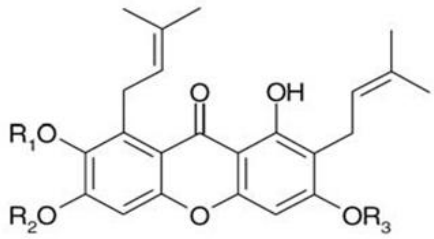

Figure 1.2 Chemical Structures of Mangostins (Pothitirat and Gritsanapan, 2008)

Alpha-mangostin: R1 = CH3, R2 = R3 = H

Beta-mangostin: R1 = R3 = CH3, R2 = H

Gamma-mangostin: R1 = R2 = R3 = H

Mangostin has been shown to have an analgesic and anti-inflammatory effect (Cui, 2010; Chena, 2008), antioxidant and neuroprotective effect (Chaverri, 2009; Chin, 2008; Abundis, 2010), cytoprotective effect (Sampath and Vijayaragavan, 2008), anti-acne producing bacteria (Pothitirat, 2009), anti-melanoma agents (Wang, 2011), abscess and skin infection (Tewtrakul, 2009), inflammation, pain and neuropsychiatric symptoms effects (Sukma, 2011), moderate inhibitory effects on cAMP phosphodiesterase (Chairungsrilerd, 1996), Alzheimer's disease (Moongkarndi, 2010), potent inhibitory activity of prostaglandin E2 (Nakatani, 2002), renoprotective effect against cisplatin-induced renal damage in rats (Pérez, 2010), preventive and therapeutic application for cancer treatment (Matsumoto, 2004), reduced [Ca2+] elevation by suppressed Ca2+ influx/ Inhibitory effect on rat basophilic leukaemia RBL-2H3 cell de-granulation (Itoh, 2008), in vitro cytotoxicity against human colon cancer DLD-1 cells and effective chemo-sensitizer (Nakagawa, 2007), and significant anti-mycobacterial activity against Mycobacterium tuberculosis (Arunrattiyakorn, 2011). Recent investigations of the anti tuberculosis potential of mangosteen revealed that tovophyllin B possesses a significant inhibitory activity against Mycobacterium tuberculosis (MIC = 25 lg/mL). Direct synthesis of α-mangostin has been established by researchers from Japan (Iikubo, 2009). After assessment they concluded that the α-mangostin was a potent inhibitor against the acidic sphingomyelinase cause of death

in early childhood. Mangosteen can also enhance the clinical effect of periodontal treatment (Rassameemasmaunga, 2008) and shows excellent apoptotic effects on head and neck squamous cell carcinoma (Kaomongkolgit, 2011). It can potentially be developed as a new medicine for anti-inflammatory and anti cancer.

On the other hand research on mangosteen, especially on pericarp or shells, has grown not only for pharmacological investigation but also for cosmetic application as ageing control, anti-acne and natural dye (Pothitirat, 2009; Zhou, 2011). Mangostin shell was effective in removing low concentrations of toxic metal such as lead, zinc and cobalt, so it might be useful for water purification or other related purposes (Zein, 2010). Since mangosteen has been shown to have strong antioxidant activity, research on mangosteen has developed in the area of nutraceutical and food additive, so that xanthones from mangosteen are claimed as phytonutrients, natural antioxidant, and food preservative (Kapoor, 2009; Dembitsky, 2011; Zhou, 2011). In addition, pelargonidin 3-glucoside that is contained in mangosteen was also investigated to be a natural colorant in food and use of this new natural colorant has increased (Zarena, 2012). Moreover, a group of Indian researchers have reported that the leaf extract of mangosteen can be used as a reducing agent when doing biologically synthesised nanoparticles that are highly effective against different multi-drug resistant human pathogens (Veerasamy, 2011).

Because of the widely varied potential benefits of Mangosteen and its derivatives for drug, traditional medicine, food supplement, cosmetic and food additives, it can be estimated that in future there will be more mangosteen products produced and offered by pharmaceutical and food supplement industries to meet market needs.

1.2.2 Isolation of mangostin

Separation and isolation of α-mangostin and other xanthones from mangosteen have been performed by different researchers in several ways in order to obtain the maximum amount of Component. However, they generally used maceration at room temperature with a wide variety of organic solvents such as methanol, ethanol, butanol, ethyl acetate, chloroform, hexane, and ethyl chloride for extraction and isolation; and used column chromatography for fractionation as well as purification. A α-mangostin isolation processing was developed and patented in the USA by Boehringer Ingelheim Pharma

Company in January 2006 using several steps with various aqueous alcohol and aromatic solvent of 1.2-ethanediol and toluene in preferable temperature range 60-80°C. The crude product was purified in a Kühni column and using this method, the quantity of α-mangostin extracted was 2.48% of the crude in the toluene extract (Sobotta, et. al., 2006).

An isolation method by Marquez-Valadez et al. (2009), repeated by Caverri (2009), used silica gel column chromatography eluted with hexane/CH_2Cl_2 (from 4:6 to 0.1) after macerating by CH_2Cl_2-MeOH (1:1). The amount of α-mangostin was 3.15% (Caverri, 2009). An extraction method has also been performed by Rahmania, in Research Center for Drug and Food of NADFC-RI (personal communication) which gave 1.66% of α-mangostin. The method employed macerating the crude mangosteen rinds powder in 80% ethanol in water at room temperature for 24 h with occasionally stirring. The maceration was repeated until colourless solvent was obtained and the macerates were pooled. The solvent was removed by rotary evaporator to yield a concentrated extract. The ethanol extract was then dissolved in 50% methanol in water and partitioned 3-4 times in a separating funnel using n-hexane for 15 min each to remove non polar compounds. The n-hexane phase was removed from the funnel and ethyl acetate added to the methanol-water phase and shaken. The addition of ethyl acetate was repeated 3-4 times until the layer of ethyl acetate was colourless. The ethyl acetate phase was collected and dried down using a rotary evaporator and then fractionated by column chromatography with silica gel 60 as the stationary phase and eluting with a number of n-hexane-ethyl acetate mixtures which had different concentration of n-hexane from 10% to 90%. There were 31 fractions divided into 6 combined fractions which had the same TLC profile from the eluent of n-hexane/ethyl acetate (8:2 v/v). Fractions with the same TLC profile were combined and the solvent was removed using a rotary evaporator at 40-50°C. The extract was crystallized and re-crystallised using methanol and n-hexane. The crystals were analysed by TLC. From the TLC analysis, 2 spots were observed therefore the crystal was purified further using Sepacore apparatus (an isocratic flash chromatography technique) with n-hexane/ethyl acetate (8:2 v/v) as a mobile phase and analysed again by TLC. The fraction which had only one spot on TLC was assumed as pure mangostin. The melting point was measured and the sample identified by UV-Vis Spectrophotometer, Fourier Transform Infrared

Spectroscopy (FTIR) and Nuclear Magnetic Resonance Spectroscopy (NMR) (Rahmania, 2009).

Ee et al. (2006), a group of researchers from Malaysia, have isolated and identified α-mangostin, β-mangostin and other xanthones from the finely ground stem bark of *Garcinia mangostana* L. They obtained 0.007% of α-mangostin and 0.005% of β-mangostin in the n-hexane extract of 1.5 kg mangosteen crude after purifying by column chromatography using hexane, hexane/dichloromethane, dichloromethane/ ethyl acetate and dichloromethane/methanol as the eluting solvents. The compounds were determined by spectroscopic methods such as ^{1}H NMR, ^{13}C NMR, and mass spectrometry (MS) (Ee, 2006).

1.2.3 Counter-current Chromatography

1.2.3.1 Distribution in two- Phase Systems

Counter-current chromatography (CCC) is a modern technology that is suitable to separate individual chemical compounds, or groups of compounds from complex mixtures. Therefore it is becoming widely used as a versatile method of purifying a variety of materials (Doshi, 2010; Sutherland, 2000). CCC is defined as a liquid chromatography (LC) technique that uses two immiscible liquid phases without any solid support (Berthod, 2009).

Conventional liquid chromatography employes a single phase to elute the analytes released from the adsorptive or liquid phase coated solid support. On the other hand, the CCC technique uses a two-phase solvent system made of a pair of mutually immiscible solvents, one used as the stationary phase and the other as the mobile phase. The use of two-phase solvent systems allows one to choose solvents from an enormous number of possible combinations (Ito, 2005). One major advantage of working with a liquid as opposed to a solid stationary phase is that the solutes have access to the whole volume of the stationary phase (Berthod, 2007).

A unique advantage of CCC is the ability to change the elution mode by simply selecting which of the two liquid phases is the mobile phase, effectively selecting either normal or reverse phase mode of elution. Scaling up CCC is also simple because CCC is mathematically linear and very predictable. The major challenge of CCC is to obtain a

stable support-free liquid stationary phase (Berthod, 2009). Migration through the column is controlled by the partition coefficient also known as the distribution ratio (K_D) (Conway, 1995).

The distribution ratio is a concentration ratio between compounds dissolved in the stationary phase divided by compounds dissolved in the mobile phase. A compound with K_D= 0 will not dissolve in the stationary phase and will come out with the solvent front in a time dictated by the volume of mobile phase. A compound with K_D= 1 will be equally soluble in both phases and therefore elute with the column volume in a time dictated by the column volume divided by the flow rate. This effect is measured by the following formula:

$$K_D = \frac{C_S}{C_M}$$

C_S is the concentration of a sample component dissolved in the stationary phase (SP) and C_M is the concentration of the same component dissolved in the mobile phase (MP). Good K_D values are between 0.5 and 2, if K_D<0.5 there will be loss of peak resolution. Otherwise, if K_D>2, long retention time and peak broadening will occur (Doshi, 2010).

Component elution of sample on CCC can be illustrated in the diagram below:

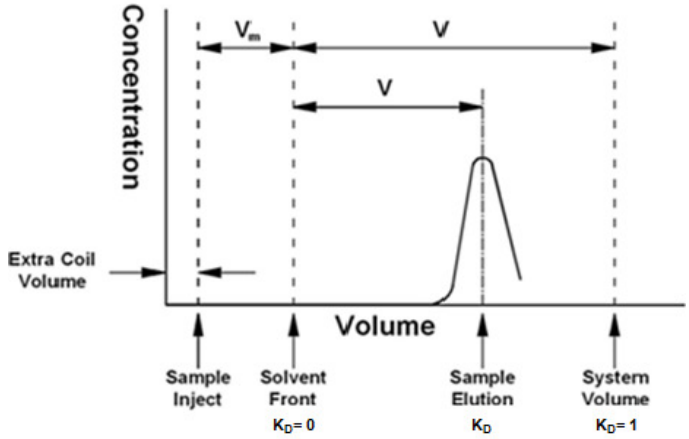

Figure 1.3 Diagram of Component Elution on CCC (Anon[6])

The diagram shows the theoretical elution of a compound with a partition coefficient/distribution ratio (K_D). The liquid–liquid partition ratio of the solute in the biphasic liquid system used to perform the CCC separation is the only parameter in the retention equation

$$V_R = V_M + K_D V_S$$

Where V_M is the mobile phase volume and V_S is the stationary phase volume inside the CCC apparatus. V_M corresponds to the hold-up volume in HPLC (Berthod, 2009). Since there is no solid support, the column volume (V_C) is calculated according to equation below:

$$V_C = V_M + V_S$$

To calculate the peak elution time (retention time) for any of Components is used the following equation:

$$t_R = \frac{V_C}{F}\left[1 + S_f\left(K_D - 1\right)\right]$$

Where V_C is the column volume, F is the MP flow rate, S_f is the SP retention and K_D is the distribution ratio. Running CCC with the same conditions will provide the same retention time of the target peak. Once the system is developed, prediction of elution times is easy (Conway, 1995).

On CCC running where two liquid-liquid phase systems are used, the column is simply filled with the stationary phase (SP) by pumping at a high flow rate without rotation until at least one complete coil volume. The mobile phase is pumped at the chosen flow rate with column rotation at the desired speed. The displaced volume of SP is measured using a graduated cylinder. When the displacement of SP ceases, the column has reached a state called hydrodynamic equilibrium and the column is ready for injection. Since the displacement volume and the column volume are known, the amount of stationary phase left in the column can be calculated and predicted exactly. The compounds will elute based

on their distribution ratio (partition coefficient). The stationary phase retention factor (S_f) is calculated using the following formula:

$$\%S_f = \frac{V_C - V_{DISP}}{V_C} \times 100$$

Where V_C is the known column/coil volume and V_{DISP} is the displaced volume of SP in a graduated cylinder. Larger retained stationary phase (S_f) values mean that the column capacity available for the separation is greater and resolution is enhanced. There are mainly two different ways to obtain a liquid stationary phase, using centrifugal forces, the hydrostatic way and the hydrodynamic way (Berthod, 2009).

In separations by chromatography, resolution is directly influenced by the ratio of SP volume (V_{SP}) and MP volume (V_{MP}) i.e. the higher the ratio, the better the resolution will be.

$$R_S \propto \frac{V_{SP}}{V_{MP}}$$

The efficiency of the separation can be expressed in terms of peak resolution (Rs) using a conventional equation,

$$R_S = \frac{2(V_{RA} - V_{RB})}{(W_A + W_B)} or \frac{2[(t_r)_A - (t_r)_B]}{W_A + W_B}$$

Where V, t and W indicate the retention volume, retention time and the peak width of the specified peaks, respectively (Ito and Yu, 2009). V_{RA} is retention volume of the earlier and V_{RB} is retention volume of the later eluted peaks. W_A and W_B are their respective base widths. The resolution of adjacent peaks is also given by:

$$R_S = \frac{2\Delta Z}{W_A + W_B}$$

Where ΔZ is the separation between peaks A and B; and W_A and W_B are the widths at the base of peaks A and B, respectively.

Acceptable resolution is on the order of R_S = 1.0, and baseline resolution between two peaks (as shown in the figure below) requires an R_S > 1.5

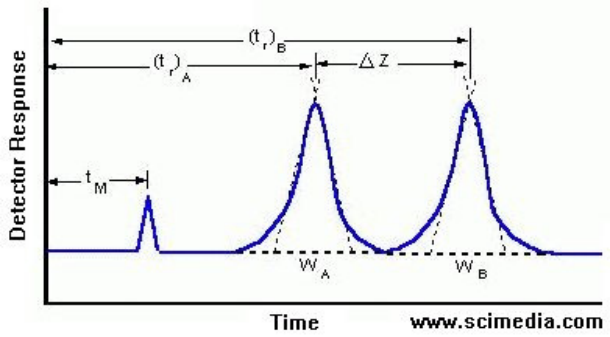

Figure 1.4 Chromatographic resolutions

Berthod et al demonstrated very well in the chromatograms of the figure below that chromatographic resolution declines dramatically as S_F decreases:

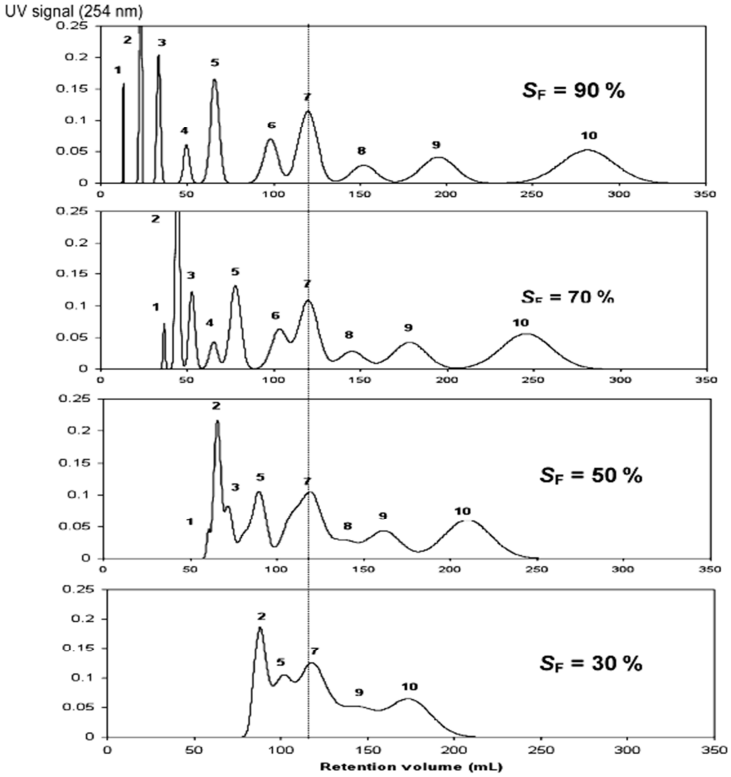

Figure 1.5 Correlation between retention volume of stationary phase (S_f) and chromatographic resolution on CCC (Berthod, 2009)

Comparison of chromatograms on Figure 1.5 obtained with the same hydrodynamic CCC column, the same biphasic liquid system, and the same sample containing 10 compounds. The volume of stationary phase retained decreased from 108 mL (S_F = 90 %) to 36 mL (S_F = 30 %). Column volume V_C = 120 mL (vertical dotted line), average efficiency 500 plates (Berthod, 2009).

1.2.3.2 Hydrostatic (CPC)

Centrifugal Partition Chromatographs (CPC) was first introduced in 1982. Its early use focused on determining octanol–water partition coefficients, the separation of natural products and the extraction of heavy metals. In 1990, Foucault and Nakanishi published a comparison of several aqueous two-phase systems for the fractionation of biopolymers using CPC (Sutherland, 2008).

There are two column types that can be used to retain a liquid stationary phase, the hydrostatic and the hydrodynamic designs. Classical hydrostatic is referred to droplet CCC columns which have a single rotational axis and use only gravity to maintain the liquid stationary phase, it takes very long elution times (days). Because of the efficiency reason, the droplet CCC column is no longer in use today. Modern hydrostatic is referred to CPC (Berthod, 2009). The performance of this CPC equipment far exceeds the original gravity stabilised Droplet Counter-current Chromatography (DCCC) due to using centrifugal force (circa 200g) but the construction mimics DCCC and this design concept does have flaws. CPC machines rotate around only one axis.

CPC is composed of a number of partition channels which are linked in cascade by ducts. The single-axis centrifuge generates centrifugal force to retain stationary phase while the mobile phases are passing. Mixing and settling take place in individual partition cells that compose a group of partition disks (Yoon, 2010). CPC is actually a continuous form of counter-current distribution where a series of chambers are fitted circumferentially on a disc which is rotated to create a centrifugal force field. Each chamber has a connection from the top of one to the bottom of the next. The chambers are initially filled with the stationary phase and then the mobile phase is pumped through displacing some of the stationary phase and creating a series of chambers with a retained volume of stationary

phase and cascade mixing much like a waterfall between the mobile phase and the retained stationary phase in each chamber (Sutherland, 2008).

As mentioned above, CPC is basically hydrostatic like Droplet CCC however; there are improvments in mixing and more theoretical plates with rotor spinning and one axis of gyration, using centrifugal field to hold a better stationary phase retained than in gravitational field, has constant g-field and also provides much faster separations than in DCCC (Murayama, 1982).

The hydrostatic CCC column can be described as a schematic of CPC below,

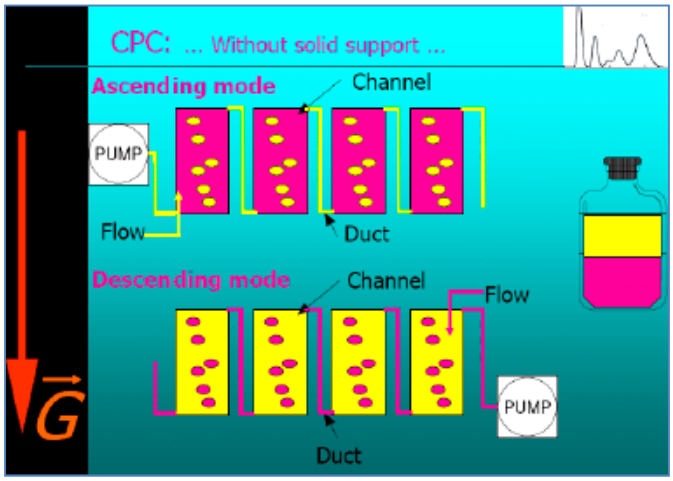

Figure 1.6 Schematic of CPC as a basic of Centrifugal Partition Chromatography (Doshi, 2009)

The schematic reflects the liquid motion in hydrostatic CCC columns in centrifugal partition chromatographs. CPC use centrifugal force to speed separation and achieves higher flow rates than DCCC (which relies on gravity). CPC can be operated in either descending or ascending mode, where the direction is relative to the force generated by the rotor rather than gravity. According to the fast and permanent evolution of the cells design, the efficiency and flow rate with low back pressure are improved (Doshi, 2009). There is a single axis of rotation producing constant centrifugal field and no phase exchanges in the connecting ducts. This design reduces the contact time for solute exchange with the stationary phase. It also builds a hydrostatic pressure that explains the significant pressure drop needed to operate hydrostatic centrifuges. All hydrostatic centrifuges contain two rotary seals; one at the top and the other one at the bottom. They are quiet to operate. CPC

instruments are generally operated at higher flow rates and higher back pressures (Berthod, 2009).

Helix CCC or toroidal coil CCC system is usually run under a centrifugal force. The dimensions of the coil are condensed to a design which is convenient for analytical separations. The coil is fitted around the periphery of the centrifugal bowl so that the radially acting centrifugal force field retains the stationary phase in one side of the coil, as in the basic hydrostatic system described above.

Ito and Yu have introduced a new configuration of the toroidal coil using an equilateral triangular core, which improves both retention of the stationary phase and peak resolution. The performance of this triangular helical tube has been demonstrated on the CCC separation of dipeptide samples with a two-phase solvent system composed of 1-butanol-acetic acid-water at a volume ratio of 4:1:5, using a rotary-seal-free continuous flow centrifuge system (Ito, 2009).

1.2.3.3 Hydrodynamic

A hydrodynamic design column has a variable and cyclic centrifugal field produced by the planetary rotation of the bobbin around its own axis and the central rotor axis. There is contact between the two liquid phases throughout the tubing. In the schematic below, the mobile phase is pictured in black and the stationary phase is white (Berthod, 2009).

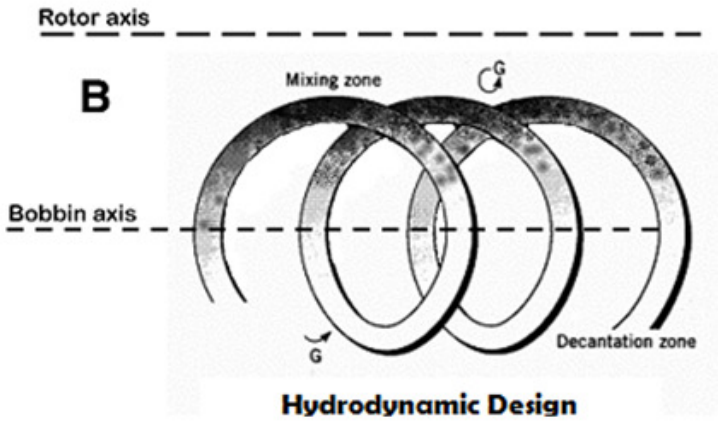

Figure 1.7 Schematic view of hydrodynamic design on the liquid motion in CCC (Berthod, 2009)

In a hydrodynamic column, there is a variable and cyclic centrifugal field produced by the planetary rotation of the bobbin around its own axis and the central rotor axis. (Berthod, 2009).

The hydrodynamic centrifuges used in the CCC columns have two rotational axes, a main axis and a planetary one which generates a variable centrifugal force field. There can be any number of planetary axes but the most common are single, double, and triple axes. Each planetary axis has a bobbin or spool mounted on it that contains the coils of continuously wound metal or plastics tubing. In hydrodynamic columns, it is important to know the ratio of the spool radius, *r*, over the rotor radius, *R*. This ratio was traditionally termed β. Since β is defined in LC as the phase ratio V_S/V_M so the CCC *beta ratio* should be noted $\beta = r/R$.

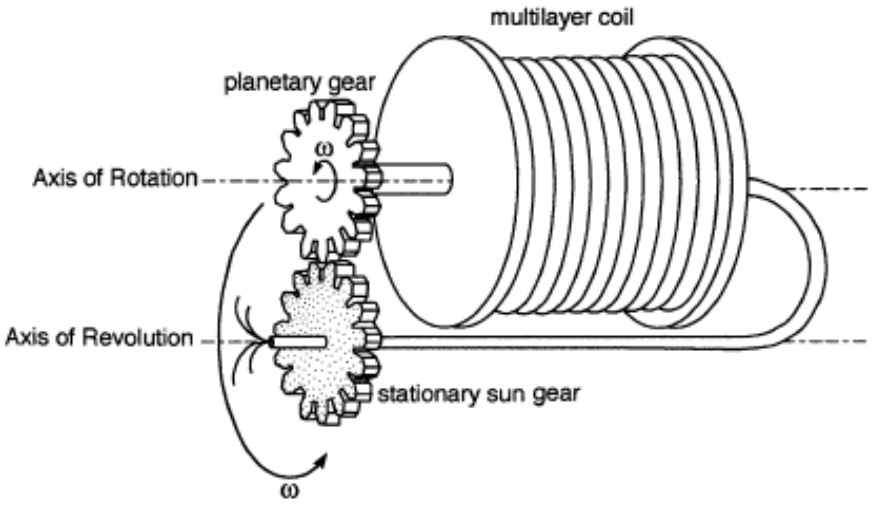

Figure 1.8 Schematic illustration of the type-J synchronous planetary motion of a multilayer coil separation column (Ito, 2005)

These machines have a continuous length of tubing, the column helically wound on a bobbin that rotates on its own axis and which itself rotates around a central axis to achieve a planetary motion. This motion sets up an oscillating hydrodynamic force field, which causes a mixing and settling step to occur with each revolution of the bobbin. This hydrodynamic force field also leads phases of differing density to travel to opposite ends of the coil; this phenomenon alone retains the SP. The benefit of this design is it operates at low pressure, which allows higher mobile phase flow rates and hence shorter separation

times. This apparatus has been developed to High Performance Counter Current Chromatography (HPCCC) which produces relatively high stationary phase retention across the complete range of biphasic systems used so there is reduced cycle time and hence increased throughput (Ito, 2005).

A simply installation of HPCCC can be figured as a schematic diagram as follows:

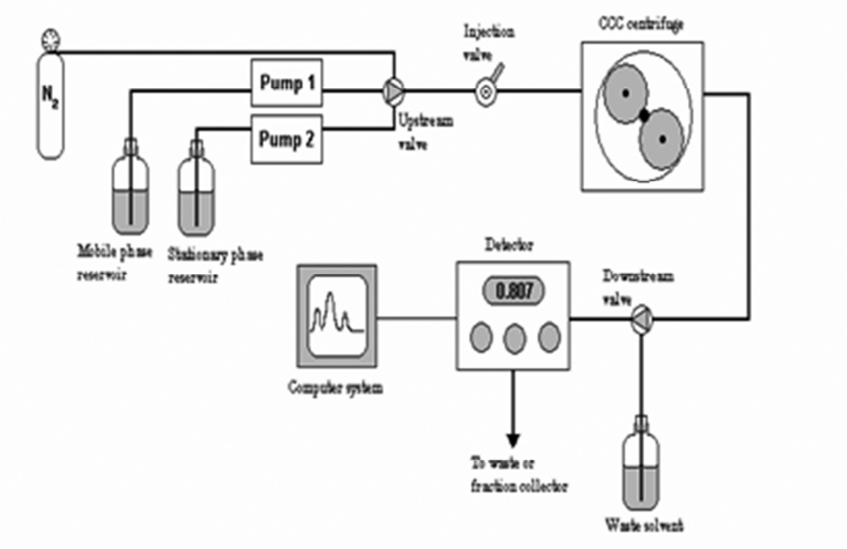

Figure 1.9 Schematic layout of a typical CCC set-up (Garrard, 2005)

The schematic diagram represents a simple installation of HPCCC unit. The HPCCC is just a different type of column, replacing the solid stationary phase (SP) column of HPLC or Flash. The primary alteration to the system is the fitting of a restrictor downstream of the pumps, if the pumps currently operate at high pressures, HPCCC works at significantly lower pressures than HPLC. Performing scale-up of purification between differing capacity HPCCC instruments is quick and simple. HPCCC instruments create the same operating conditions so simply using the volumetric ratio between the two column volumes can determine the new sample volume and mobile phase flow rate. A further significant advantage concerns sample solubility. Often a limiting factor with HPLC purifications, with HPCCC instruments particulates sample can be injected onto the column in either mobile, stationary or a mixture of both phases, without affecting the performance of the chromatography.

Counter-current chromatography benefits from a number of advantages when compared to the more traditional liquid-solid separation methods i.e. (i) no irreversible

adsorption; (ii) total recovery of injected sample; (iii) low risk of sample denaturation, (iv) simpler with fewer steps of purification process (Marston and Hostettmann, 2006).

In addition, there are several other strengths which researchers consider for CCC, such as CCC can cope with a wide range of radically different polarity compounds; allows particulates and extract solid samples; achieves > 99 % purity of Components from complex samples; takes extremely complex matrices, such as natural product extracts and heart cut target polarities, bio-actives etc without risk of on-column degradation or adsorption; uses exactly the same type of liquid pumps, injectors, switching valves fraction collectors etc as HPLC or flash chromatography; scale up is linear and predictable etc. (Doshi, 2010).

1.2.3.4 Use for natural products

Nowadays, research on natural products is rapidly growing, especially on isolation and purification of the active compounds from medicinal plants which will be developed as new natural medicines. Regarding these purposes, CCC has become a choice method and has made possible the separation of a number of biologically interesting natural products that are difficult or impossible to separate by other techniques. Crude extracts of plants or other organisms are often too complex for the direct analysis by HPLC. Certain materials may irreversibly bind to the packing material or may plug the column inlet filters, and hence reduce the column life. Those restrictions do not apply to analytical CCC, which represents an interesting method for enrichment and separation of various analytes (Berthod, 2009).

Applications of CCC in analytical chemistry and comparison with other separation and enrichment methods have shown that the techniques can be successfully used in the purification of plants and other natural products including fermentation. It is almost universally applicable on mg scale to multi-gram scale as a preparative purification technique for both polar and non polar organic materials as well as inorganic mixtures such as rare earths. It has been applied to many classes of compounds, including agricultural chemicals, alkaloids, amino acids, peptides, proteins, antibiotics, drug metabolites, dyes, food products, flavonoids, glycosides, herbicides, pesticides, pharmaceuticals, optical

isomers, saponins, tannins, metals and other inorganic materials. (Berthod, 2009; Conway, 1995).

Different types of hydrodynamic (High Speed CCC, cross-axis coil) and hydrostatic (toroidal coil) centrifuges can be used for separation and concentration of various compounds from plant and different natural products. The quantity of separated compounds may range from trace to gram amounts (Berthod, 2009).

Currently the reasons for increasing application of CCC in the separation of natural products include:

1. Bioactive natural products are frequently found in very small amounts and may be lost due to irreversible adsorption in conventional column chromatography.
2. CCC can enable savings in solvent consumption costs and once optimal separation conditions are selected for Components by a reduction in the total number of separation steps required (Yoon, 2010).

The new legislation on traditional herbal medicinal products in China requires them to be designated as medicines rather than functional foods. A recent review of the role of counter-current chromatography in the modernisation of Chinese herbal medicines clearly shows that the use of the CCC technology is growing in China, particularly for isolation and purification of natural products including group of flavonoids, alkaloids, polyphenol, terpenoid, coumarins etc. (Figure 1.10) (Sutherland, 2009).

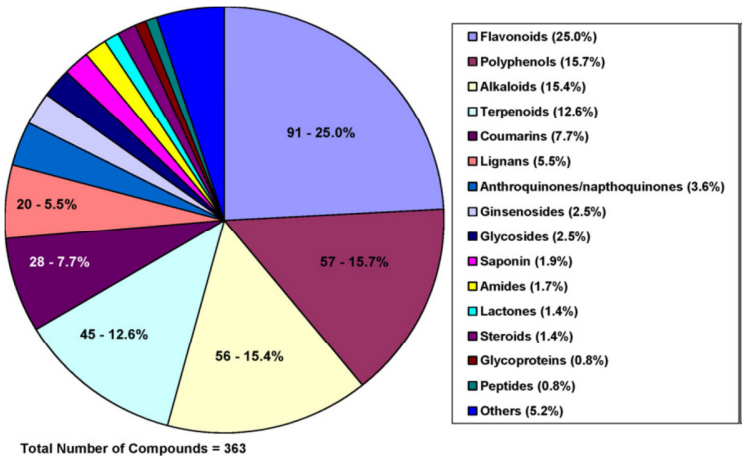

Figure 1.10 Pie chart showing the classification of the 363 different compounds isolated in the modernisation of Chinese herbal medicines (Sutherland, 2009).

In purification of Chinese Herbal Medicines, alkane/alcohol systems are now by far the most popular representing nearly 40% of the phase systems used. The usage has changed over time. Prior to the year 2000, chlorinated systems were used twice as frequently as alkane systems. The decrease in use recently reflects the need to avoid such solvents on health and safety grounds. By 2007 alkane systems were used in 70% of the papers. More recently, papers have been appearing which use acetonitrile systems and acetic acid systems (Sutherland, 2009). Probably the two most commonly used solvent systems in the area of natural products for aqueous-organic phase system have been ternary chloroform/methanol/water and the quaternary hexane/ethyl acetate/methanol/water systems (Conway, 1995).

1.2.3.5. Use for Proteins and Large Molecules

CCC has been employed for performing purification of biological samples including protein and larger structures, based on partitioning technique using aqueous–aqueous polymer phase systems. The most common solvents used in this technique are polyethylene glycol (PEG)–dextran and PEG–potassium phosphate systems. This partitioning of biological macromolecules was first established by Albertsson in the 1950s (Shibusawa, 2006). Currently in modern biotechnology, the technique is growing interest not only for downstream processing of enzymes but also in efficient methods for the large scale recovery and purification of fermentation products, such as intracellular enzymes and biologically active proteins which require low to medium purity but the absence of interfering activities for industrial catalysts; and the other is required high purity for analytical and medical applications (Hustedt, 1985).

When aqueous two-phase systems (ATPSs) and liquid–liquid partition-based counter-current chromatography (CCC) are employed for the separation of large bioactive molecules including proteins, DNAs and RNAs, it can avoid risks of sample loss, denaturation, and greatly reduce processing time. Biologically active large molecules, such as commercially important or potentially important therapeutic proteins, DNAs and RNAs, need to be prepared under benign physiochemical milieu to preserve their biological activities. There are at least two important factors affecting the maintenance of their

bioactivities: the media used for separating and purifying the required large molecules and the processing time for exposing these molecules in such media (Guan, 2010).

Some researchers have developed a new method for the separation of proteins using liquid-liquid partition using CCC. It has been reported that the cross-axis coil planet centrifuge (*X*-axis CPC) is useful for partitioning macromolecules with aqueous–aqueous polymer phase systems. Performance of the apparatus was evaluated on protein separation using an aqueous–aqueous polymer phase system composed of PEG 1000 and dibasic potassium phosphate with four multilayer coiled columns. This apparatus would be useful for the separation of various bioactive compounds with polymer phase systems (Shinomiya, 2006)

Study on the separation of a protein mixture containing of myoglobin, cytochrome *c*, and lysozyme has been done by HSCCC using a two-phase aqueous/reverse micelle-consisting organic solvent system. Both pH and potassium chloride (KCl) concentration gradients were applied in the separations. In addition to efficiency of separation, enrichment of protein was also examined at the same time. The study confirmed the feasibility of performing protein separation and enrichment in one chromatographic run in HSCCC. (Shen and Yu, 2007).

A review on the recent progress of protein and larger molecules separation in the industrial scale-up using liquid-liquid chromatography technique was done by Sutherland. This review considers recent developments in centrifugal liquid-liquid partition chromatography using aqueous two-phase solvent systems, a gentle host medium for biologicals, and the prospect for scale-up and eventual manufacture of high-value pharmaceutical products. With the results demonstrated in various machines from different manufacturer, the author has highlighted particularly the efficiency of each instrument when used in separation i.e. lysozyme and myoglobin. This case illustrates the important role of CCC in the use of drug and health supplement development (Sutherland, 2007).

1.2.4 Counter-current Chromatography and Mangostin

The major xanthones (α- and γ-mangostins) in mangosteen fruit pericarp (MFP) have recently been reported to be isolated at high purity in one step using high-performance centrifugal partition chromatography with a solvent system containing petroleum ether/

ethyl acetate/methanol/water (10:5:5:1).From a load of 200 mg crude extract of MFP 55.4 mg α-mangostin with 93.6% purity and 12.4 mg γ-mangostin with 98.4% purity were obtained, with yield of 86.3% and 76.3%, respectively. The xanthones were characterized by comparing the retention time and UV spectrum with commercially available standards (Shan, 2010).

Screening and fractionation method for major xanthones in pericarp of *Garcinia mangostana* has been developed using CPC directly interfaced with mass spectrometry. Extraction was done by pressurised liquid extraction with ethanol and separated at the semi-preparative scale by centrifugal partition chromatography (CPC) with a biphasic solvent system composed of heptane/ethyl acetate/methanol/water (2:1:2:1, v/v/v/v). Separation and identification of the compounds were applied by CPC-electro-spray ionisation MS coupling. An additional stream of ethanol/1 mol L^{-1} ammonium acetate (95:5, v/v) and a variable flow splitter were also included, and all the compounds in the solvents used in mobile phase for the CPC separation were analysed. The dual mode or elution–extrusion, which is less solvent-consuming and faster than the elution mode, was used without loss of ionisation and detection (Destandau, 2009; Yoon, 2010).

A method for extraction of α-mangostin and γ-mangostin from *Garcinia mangostana* was developed as a microwave-assisted extraction (MAE) by Fang, et al. The experiment conditions were optimised using orthogonal test and 5 g sample was extracted with the optimised conditions. Isolation and purification was performed by high-speed counter-current chromatography (HSCCC) with a two-phase solvent system consisting of petroleum ether/ethyl acetate/methanol/water (0.8:0.8:1:0.6, v/v) and resulting 75 mg of α-mangostin at 98.5% purity, and 16 mg of γ-mangostin at 98.1% purity from 360 mg crude extract of G. mangostana in less than 7 h. The purity of the two xanthones was identified by HPLC. Their structures were further characterised by ESI-MS, ^{1}H NMR and ^{13}C NMR (Fang, 2011).

Isolation and purification of major xanthones in another medicinal plant (*Swertia mussotii*) have also been performed on HSCCC using solvent system containing n-hexane/ethyl acetate/methanol/water (5:5:10:4, v/v/v/v). The experiment conditions were reverse phase running at flow rate of 1.5 mL/min, a rotation speed of 800 rpm and a temperature of 25°C. Using the described method, a 150 mg crude sample yielded 8mg of

methylswertianin, 21mg of swerchirin and 11mg of decussatin with purities of over 98%. The compounds isolated were determined by 1H-NMR and 13 C-NMR analyses (Jia, 2011).

1.2.5 High Performance Liquid Chromatography

1.2.5.1 Principle and Theory

Many scientists are very familiar with High Performance Liquid Chromatography (HPLC). This instrument is applicable for a wide range purposes; not only for chemical, pharmaceutical, cosmetics and food industry but also in agriculture, environment, mining and many other purposes including biomedical as well as veterinary, where the sample is blood and other biological fluids (Levin, 2001). In drug clinical trials, HPLC plays an important role for the determination of bioavailability and metabolite level of a drug in the human body. It is also important in forensic science to analyse an unknown sample obtained from a crime scene or victim. In phytochemical studies, chromatography is commonly used, particularly for fingerprinting analysis, extraction, isolation, purification and analysis of Components in medicinal plants or natural resources.

Chromatography was invented by a Russian botanist, Mikhail Tswett around 1906 when he separated pigments of a plant by organic solvent as a mobile phase and chalk as a stationary phase. In 1941, Martin and Synge developed liquid-liquid partition chromatography which led them to winning a Chemistry Nobel Prize in 1952 (Harris, 2005). The chromatography word comes from “chromos” meaning colour and “graphos” meaning writing, therefore literally “colour writing”. Now, it is considerably more sophisticated, however there are still some basic principles that must be applied for successful operation of a chromatography system.

Chromatography is 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 (Eti're, 1993). There are many different types of chromatography depending on the mobile and the stationary phases. Chromatography which uses liquid as the mobile phase is called liquid chromatography (LC) and gas chromatography (GC) is chromatography where gas is used as the mobile phase. According to the stationary phase, liquid chromatography is divided

into several types such as HPLC, Flash Chromatography (FC) and Supercritical Fluid Chromatography (SFC) when the stationary phase is a solid. On the other hand, Counter Current Chromatography (CCC) and Centrifugal Partition Chromatography (SPC) are the types of liquid-liquid chromatography. In addition, there are two types of GC i.e. Packed Column Gas Chromatography with a solid as a stationary phase and Gas Chromatography with liquid as a stationary phase.

In general, liquid chromatography can be defined as an analytical chromatographic technique that is useful for separating ions or molecules dissolved in a solvent. The sample is poured onto the top of the column followed by the solvent system. LC separation mechanism is due to differences in adsorption, ion exchange, partition, or size. If the sample solution is in contact with the solid or liquid stationary phase, the different solutes will interact with the other phase with different degrees and allow the mixture components to be separated from each other and determine the transit time of the solute through the column. Sometimes the column contains active solids (adsorption), ionic groups on a resin (ion-exchange), liquids on an inert solid support (partitioning), or porous inert particles (size-exclusion). The compounds are separated by accommodating the solution from the column effluent with the time (Tissue, 2000). The schematic can be seen in Figure 1.11.

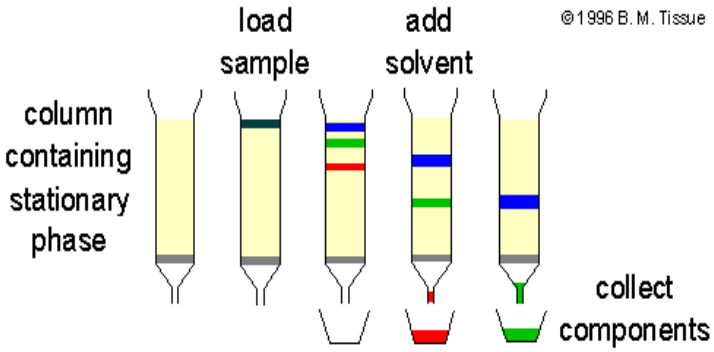

Figure 1.11 Schematic of a simple liquid chromatographic separation (Tissue, 2000)

Chromatography can be preparative or analytical. The aim of preparative chromatography is to isolate as much as possible of the desired component from a complex sample mixture. It can also serve to separate the components of a mixture for further use and to purify large scale of products. Furthermore, it is also used in ultra trace separations

where disposable columns are used once. Conventional LC is most commonly used for these purposes. Otherwise, analytical chromatography is used to obtain quantitative and qualitative information about the compound of interest (analytes) in a sample. Analytical chromatography is done normally with smaller amounts of material and is used for measuring the relative portions of analytes in a mixture. Analytical separations of solutions for detection or quantification normally use more sophisticated HPLC instruments (Levin, 2001; Lindholm, 2004).

As an analytical technique, HPLC is usually projected for the separation and determination of organic and inorganic solutes in any samples. However, HPLC can also be preparative because it is basically a highly improved form of column chromatography. The solvent is forced through under high pressures of up to 400 atmospheres on HPLC while column chromatography uses gravity. That makes HPLC much faster and allows a very small particle size for the column packing material, which provides a much greater surface area for interactions between the stationary phase and the molecules flowing past it. So a better separation of the mixture will be obtained. In addition, another major improvement concern the detection methods which can be used. These HPLC methods are highly automated and extremely sensitive (Clark, 2007). In principle, all LC and HPLC work in the same way, however HPLC is more efficient, sensitive and easier to operate. HPLC is now becoming one of the most dominant instruments in analytical chemistry, not just because of ability to separate, identify and quantify the compounds, but *parts per trillion* [ppt] sample may easily be identified by HPLC (Anon, 2011).

The instrumentation components in HPLC system are the pump, injector, detector and data station, whereas the chemical components are the mobile phases and the stationary phases (Levin, 2001). Sometimes, HPLC is connected to an auto sampler that makes it easier to inject and allows the analyst to analyse samples in large numbers (usually up to 100 samples) for unattended automatic operation. A schematic of the HPLC system can be seen in figure 1.12.

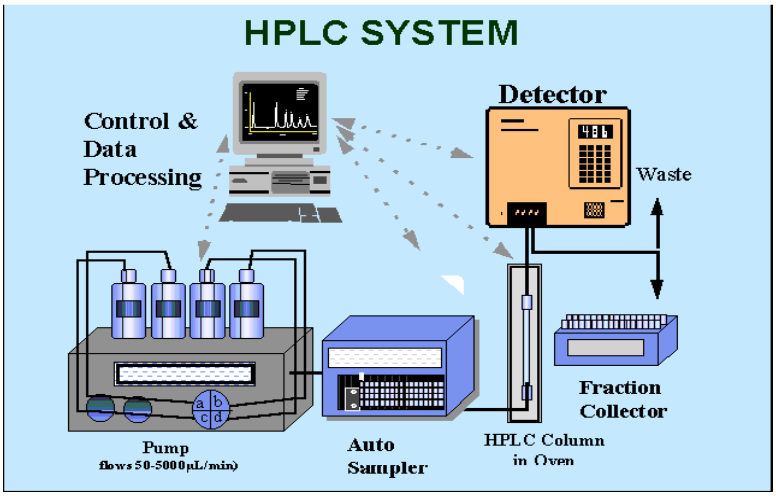

Figure 1.12 Schematic of an HPLC System (Levin, 2001).

Running an HPLC can be conducted by injection of liquid sample in a small volume into a column (stationary phase) where the individual components of the sample are moved down with mobile solvent forced at high pressure using a pump. These components are separated by the column packing contains various chemical and/or physical interactions between their molecules and the packing particles. These separated components are detected by a detector that measures their amount. This detector produces a chromatogram. An auto sampler is an important alternative device for the ease of the analyst when a lot of samples need to be analysed at the same time, such as in drug clinical trials analysis or further analysis for preparative chromatography fractions.

In many respects HPLC and Gas Chromatography (GC) are complementary techniques. HPLC is the recommended technique for wide range of sample types whose analysis by GC can be quite difficult. High boiling or non volatile samples, proteins, polymers, ionic compounds, and thermally unstable compounds are all candidates for analysis by HPLC. Volatile samples, especially in complex mixtures, on the other hand, are more appropriately analyzed by GC. HPLC typically offers more flexibility than does GC. These results in part come from the participation of the mobile phase (the solvent system) in the separation of chemistry of HPLC (Harris, 2005).

Based on interactions between the sample, stationary phase and mobile phase, most of the separations by HPLC can be placed in one of five categories called modes. (1)

Reverse-phase LC is based on distribution of the sample between a polar mobile phase (usually water-containing) and a non polar stationary phase. (2) Normal-phase LC is generally intended that the mobile phase is non polar compared to the stationary phase. In practice, normal-phase LC is often used synonymously with "adsorption' chromatography. It is driven by interaction between the sample and the solvent with polar active sites on the surface of the packing material. (3) Ion-exchange LC, as the name implies, depends on exchange of sample or buffer ions between the mobile phase buffer and charged groups on the stationary phase. (4) Ion-pair chromatography uses a reversed-phase column and a "soapy" ion-pair reagent to create an ion exchange system. (5) Size-exclusion chromatography separates on the basis of the extent to which the sample molecule can penetrate the pores of the packing material. As the name implies, the separation is based on molecular size (Dolan, 2007). There are seven basic considerations in choosing HPLC operating parameters to work with i.e. solubility, molecular weight, functional groups, sample matrix, levels in matrix, detection ability, and how the species differ (Levin, 2001).

Detection in HPLC can take advantage of a wide range of sample and solvent characteristics. Compared to GC that has Flame Ionization Detector (FID), LC lacks the sensitive/universal detector. Commonly used HPLC detection techniques include ultra violet (UV) or visible light absorbance, refractive index or conductivity monitoring, fluorescence measurement, amperometric or coulometric redox, chemical dramatisation, and even more elaborate techniques such as chemiluminescence or mass spectroscopy. The most common is the UV detector, known as photodiode array (PDA) when it can measure a wide range of UV wavelengths at once. UV detection requires the compounds to absorb UV light. Conjugation of the molecule does this i.e. an alternating double bond, single bond, double bond. Therefore aromatic compounds show the absorption but fats and sugars typically do not. For them, another detection method must be employed, such as evaporative light scattering detection (ELSD). This is another common detector which is destructive, so it is not used on preparative systems, but is universal as long as all components have a boiling point higher than that of the solvent used for the mobile phase. PDA detection, the most common HPLC detector, generates 3D data which can be viewed in 3 ways as 2 dimensional data with 2 axes.

1.2.5.2 Column Characteristic

The column is considered the "heart of the chromatograph". In conducting an analysis using the HPLC, selection of the column or stationary phase is very important for the purpose of analyte separation because an inappropriate column might cause inefficient separation. Characteristics of a good column are influenced by several factors such as pore size, particle shape and size distribution as well as the length and diameter of the column itself. Substrate materials inside the column are also critical in affecting of separation. Usually the column contains silica or polymeric substrates of different pore size. Pore size can be defined by the ability of the analyte molecules to penetrate inside the particle and interact with its inner surface (Levin, 2001). In this case, the column packing and the mobile phase are the most important factors in successful HPLC analysis.

Several types of column can be selected in the application of HPLC depending on the purpose. A column for analytical purposes usually has an internal diameter (i.d.) 1.0-4.6-mm; lengths 15–250 mm while for preparative purposes the i.d. > 4.6 mm ; lengths 50–250 mm. Capillary columns are also used for analytical HPLC and usually have i.d. 0.1-1.0 mm in various lengths. This column offers performance plus flexibility, versatility and ease of use. Nano column is a capillary column which has i.d. < 0.1 mm or sometimes stated as < 100 μm.

Materials of construction for the tubing usually are metal which is chemically inert to virtually all common solvents and buffers and easy to cut, however has disadvantages including limited pressure capability, permeability to air and oxygen and tendency to cold flow; stainless steels (the most popular gives high pressure capabilities); glass (mostly for bio-molecules) and polyether ether ketone (PEEK) that can be operated at elevated pressure, biocompatible and chemically inert to most solvents except tetra hydro furan (Dolan, 2007).

Another thing that is also important is the effect of the separation mode. The chemical and physical properties of the analyte should be considered too. Analytes with low to intermediate polarity and high solubility in low-polarity solvents will get a better separation in normal phase chromatography while water-soluble analytes are usually not good candidates for normal-phase chromatography. Reverse phase chromatography using a

RP-column is useful for the separation of compounds having high to intermediate polarity and ion-exchange chromatography separates analytes by their ionic functionality

1.2.5.3 Isocratic and Gradient

Solvent composition of the mobile phase in a chromatography creates the chemical environment for the interaction between the solutes and the stationary phase. Separation can be achieved by controlling and manipulating these interactions, which affect the relative retention times of the various sample components.

Mobile phase parameters which influence retention and separation in reverse-phase includes type of modifier, solvent strength, pH, type of buffer, ionic strength and ion-pairing reagents. Most separations on HPLC are done with reverse-phase, probably over 90%. In reverse-phase separations of organic molecules, separations are based on their degree of hydrophobicity. There is a correlation between the degree of lipophylicity and retention in the column. In normal-phase HPLC, the polar solutes elute later than non-polar lipophylic ones (Dolan, 2007).

There are two modes of operation for mobile phase composition:

1. *Isocratic Elution* which has constant solvent composition where mobile phase polarity stays constant throughout elution process. Isocratic elution creates increasing dispersion as a function of efficiency (N), void volume (V_O) and retention (k), causing lower sensitivity for more retained solutes. Isocratic elution is best for simple separations and often used in quality control applications that support a manufacturing process. This is equivalent to isothermal separations in GC.
2. *Gradient Elution* where mobile phase composition (and thus polarity) varies throughout elution process. Gradient elution focuses sample components at the column inlet and creates uniform dispersion of all solutes by reducing and eliminating the retention factor aspect. This is best for the analysis of complex samples and often used in method development for unknown mixtures. This is equivalent to temperature programming in GC. Linear gradients are most popular.

1.2.5.4 Acidic Additives to Mobile Phase

The acidity or alkalinity of the mobile phase can have a significant effect on separation. Acetic acid and triethylamine are two traditional mobile phase additives that are commonly used in reverse phase HPLC (Li, 2010). An acid mobile phase additive impacts the retention behaviour of pH-sensitive compounds.

The use of mobile phase additives might enhance the separation and resolution of the bioactive compounds in the HPLC analysis of a medicinal plant. However this must be considered with the nature of particle substrates contained in the column itself. The C_{18} column for example, which has a hydrocarbon bonded phase, will be damaged if the pH is higher than 8.0. That is why using sodium hydroxide solution as a mobile phase additive in C_{18} and C_8 columns must be done with care. On the other hand, the silica contained in the C_{18} column will dissolve when the pH is less than 2.0. Therefore, for longer life the column which has chemically bonded carbon and silica inside, the pH should be ranged between 2.0 to 8.0. It is also important to wash column from acid because leaving the column overnight with acid from the mobile solvent will make the silica porous and destroy the column in a short time (Dolan, 2007).

1.3 OBJECTIVES

1.3.1 General Objective

The increasing number of various herbal medicines and complementary products on the market requires reliable quality control and standardisation of the products to protect the consumers. This requires acceptable reference standards for qualitative and quantitative analysis of the products. This study aimed to develop an efficient method for isolation and purification of natural compounds from medicinal plants as reference materials for quality control and standardisation of the products using liquid flow processing The production of purified α-mangostin from *Garcinia mangostana* L. rinds in one step is used as an example.

1.3.2 Specific Objectives

- To provide a reference standard with high purity and yield for quality control and standardisation of natural based products

- To isolate and purify natural compounds from a medicinal plant using liquid flow processing
- To develop a method for isolation and purification of α-mangostin using liquid flow processing
- To optimise a method in analytical scale for the production of α-mangostin with high purity and yield with a simpler process, with reduced processing time and less solvent use, by Mini high performance counter-current chromatography (HPCCC)
- To scale up the production of purified α-mangostin using the optimised method from analytical scale to Spectrum HPCCC and Midi HPCCC
- To ensure the identity and purity of Components through identification and characterisation by High Performance Liquid Chromatography (HPLC) with comparation to a commercial reference standard, and Nuclear Magnetic Resonance (NMR) Spectroscopy

Chapter 2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Crude Sample

Figure 2.1 Mangosteen fruit, rinds powder and ethanol extract

The dry powder of mangosteen (*Garcinia mangostana* L.) fruit rinds, as a sample of this research, was purchased from Bina Agro Mandiri Yogyakarta Indonesia. The mangosteen sample was collected from Kulon Progo area in February 2011. The drying was performed in an oven at 60 °C for 3 hours to ensure the materials were completely dry, then were ground into powder and passed through a sieve with 90 mesh size. The dry mangosteen rind powder was stored in an airtight plastic container protected from light until used. The mangosteen was authenticated by Sujadmiko, Faculty of Biology Gadjah Mada University Yogyakarta Indonesia with certificate number 0300/T.Tb./I/2012.

2.1.2 Reference Standard

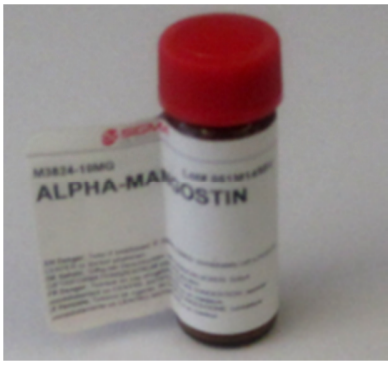

Figure 2.2 Reference standard of α-mangostin

Reference standard of α-mangostin with purity ≥98% was a product of China; with lot number #051M1495V; P code 10011345133; CAS number 6147-11-1; and was purchased from Sigma Aldrich (Gillingham, UK).

2.1.3 Chemicals and Consumables

Figure 2.3 Chemicals

Organic solvents such as ethanol, hexane, ethyl acetate, and methanol were analytical reagent grade. Methanol and trifluoroacetic acid (TFA) for mobile phase on HPLC were HPLC grade. Molecular sieve for drying methanol was general purpose grade type 4 A with nominal pore size 4 A. The chemicals were purchased from Fisher Chemicals (Loughborough, UK).

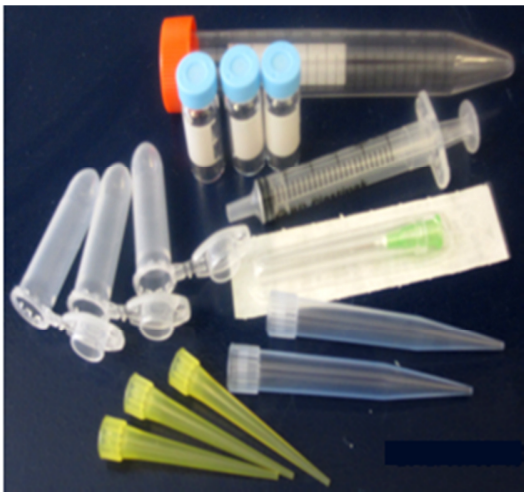

Figure 2.4 Consumables

Consumables i.e. HPLC vials, pipette tips, glass tubes and plastics tubes for fraction collection, were supplied from Fisher Scientific (Loughborough, UK). Other consumables were disposable syringes and needles (BD Plastipak^{TM}), filter paper (Whatman no 113), centrifuge tubes (Eppendorf) and deionised water and HPLC water was purified from a Purite Select Fusion pure water system (Thames, UK).

2.2 APPARATUS

2.2.1 HPCCC Instruments

HPCCC was made by Dynamic Extractions (Slough, UK), and employed for the separation and isolation of Components including sample loading studies and development of an efficient method for production of α-mangostin. There were 3 scales used in this project, with specifications presented on table 2.1. as follows:

Table 2.1 Specifications of HPCCC centrifuges

PARAMETERS	MINI HPCCC	SPECTRUM HPCCC	MIDI HPCCC
Image			
Instrument Scale	Analytical	Semi Preparative	Preparative
Coil volume (mL)	20	22 and 132	1000
Flow rate (mL/min)	0.5 - 2	0.5 - 12	10 - 80
Loading (g/run)	Up to 0.2	Up to 2	5 to 40
Rotational Speed (@240g (RPM)	2100	1600	1400
Approximate elution time for K_D=1 component (min)	20	20	20

2.2.2 High Performance Liquid Chromatography (HPLC)

Figure 2.5 Waters Alliance 2695 Separations Module HPLC

The HPLC was Waters Alliance 2695 Separations Module (Milford, MA, USA), equipped with isocratic and gradient pumps at a maximum flow rate of 10 mL/min. The instrument was also fitted with an auto sampler with 120 vials configured in five carousels of 24 vials each. Detection system was Waters Photodiode Array Detector 2996 and Empower^{2TM} Chromatography Data software was employed as a data processor. The Empower software is capable of automating method development, archiving methods and customizing data reports. This HPLC was used to analyse α-mangostin from crude extract and CCC fractions using a commercial reference standard.

2.2.3 Nuclear Magnetic Resonance Spectrometer

Figure 2.6 Brüker Avance III HD 600 MHz FT-NMR Spectrometer

The NMR was Brüker Avance III 600 MHz FT-NMR Spectrometer with Spin Works 3 Data Processor (Germany) housed and operated in the Department of Pharmacy and Chemistry, Kingston University. The Spin is a software package for acquiring, processing and analyzing NMR data, for streamlined convenience. This instrument was applied for the characterisation of α-mangostin isolated from the fruit rinds of *Garcinia mangostana* L.

2.2.4 Supporting Equipment

Figure 2.7 Supporting equipment e.g. (clockwise from top left) rotary concentrator, touch mixer, water bath and fraction collector

There were several items of supporting equipment that were used in this study, such as analytical balance (Sartorius), rotary evaporator (Büchi Rotavapor R205 with Büchi Heating Bath B-490), rotary concentrator (Eppendorf Concentrator 5301), fume cupboard (Zurich L 12-3), centrifuge (Sanyo MSE Micro Centaur), touch mixer (Top Mix FB 15024; Fisher Scientific), vacuum pump (Laboport), water bath (Grant), micropipette (Eppendorf), ultra sonic bath (Sonomatic Jencons), fraction collector for Mini and Spectrum HPCCC (Teledyne ISCO Foxy Jr.), fraction collector for Midi CCC (Gilson Model 202), Rotary wheel (Stuart Rotator SB3), HPLC Column: Agilent Zorbax Bonus RP C_{18} with dimension (3.5um; 4.6x150mm), buchner funnel, separating funnel and glassware (Fisher brand).

2.3 METHODOLOGIES

2.3.1 Optimisation of Analytical Method for α-Mangostin by HPLC

2.3.1.1 Preparation of HPLC Mobile Phase

Into 1000 ml methanol HPLC grade and 1000 ml fresh deionised water in separate containers was added 500 µL TFA each. The mixtures were well shaken and filtered using a Millipore filter with pore diameter of 0.45 µm under vacuum condition if needed. The mixtures were prepared freshly before use.

Figure 2.8 Methanol and water, to which 0.05% TFA was added for the HPLC mobile phase

2.3.1.2 Optimisation of analytical condition for α-Mangostin using HPLC

Optimisation of analytical condition was conducted on Waters Alliance 2695 Separations Module HPLC which was equipped with photo diode array (PDA) UV detector. An Agilent Zorbax Bonus RP-C_{18} (3.5µm; 4.6x150mm) column was selected among several columns as a stationary phase; run time 12 min; temperature 40°C; detection range at 210-400nm with a binary mobile phase consisting of 0.05% TFA in water (solvent D) and in methanol (solvent C) at a flow rate of 0.5 ml/min. The gradient elution program was as follows: 0-3.00 min, 95-100% C; 3.00-8.00 min, 100% C; next injection at 14 min after column equilibration. Data was collected over 12 min run time.

The optimised method was then used to assay the standard solution made from commercial α-mangostin reference standard and to create a calibration curve; then to analyse the crude extract as well as CCC fractions. The low limit of detection (LLOD) was determined based on the results of sample measurements that provided a peak height with 3 times base line noise i.e. signal to noise ratio S/N=3.

2.3.1.3 Preparation of Standard Solution I

Standard solution I for the calibration curve of α-mangostin was prepared in the following manner: Approximately 1.2 mg α-mangostin reference standard was weighed accurately, 5.0 mL dry methanol added by pipette. The mixture was weighed again in order to calculate the true volume of methanol added and mixed evenly, labelled as standard solution I; and 1 mL was transferred into an HPLC vial, analysed for the calibration curve I.

The true volume of methanol in the α-mangostin standard solution was calculated by the following formula:

$$V = \frac{M}{\rho}$$

V = volume; M = Mass; and ρ = density.

Methanol density is 0.7918 g cm−3 (value from Wikipedia), approximately 20°C which is likely to be the room temperature at the time

Dry methanol was prepared in a flask by adding some molecular sieves into methanol HPLC grade, shaken vigorously and left overnight.

2.3.1.4 Preparation for Standard Solution II

Standard solution II was a 25 times dilution of standard solution I. Preparation was carried out as follows: Into a volumetric flask, 2.0 mL α-mangostin standard solution I was pipetted accurately and dry methanol added up to 50.0 mL at temperature of 20°C. The solution was mixed evenly, labelled as standard solution II. 1 mL was transferred into an HPLC vial, analysed for the calibration curve II.

2.3.1.5 Preparation for Calibration Curve

To prepare calibration curves, the standard solution II was injected with successive volumes: 2, 5, 10, 15, 20, 25, 30, 40 µL and the standard solution I was injected with successive volumes: 2, 5, 10, 15, 20, 25 µL respectively into HPLC with optimised conditions as decribed on 2.3.1.2. The calibration curves were created by plotting the amount vs. peak area of each standard solution injected. The data from standard solution I was plotted for calibration curve I and the data from standard solution II was plotted for calibration curve II.

2.3.1.6 Preparation for Aqueous Ethanol Extract as Stock Solution

Figure 2.9 Preparation of aqueous ethanol extract with overnight maceration (left) and extract filtration under vaccum (right)

100 gram crude powder was macerated overnight in 700 ml of 80% ethanol at 30°C with occasionally stirring. The mixture was then filtered under vacuum using Whatman filter paper number 113. The pulp was added to 500 mL 80% ethanol, stirred, left for about 1 hour and filtered in the same manner. The filtrates were combined and the extract yield measured before drying down using rotary evaporator at 35 rpm, 45°C. To remove water from the syrup extract, about 200 mL methanol was added into the extract and drying continued. When completely dry, the extract was then re-dissolved in methanol or lower phase (LP) accordingly and used as a stock solution for further experiments.

2.3.1.7 Measurement of the Extract Yield

An empty vial was weighed accurately, 5.0 mL macerate added and weighed again. The macerate was dried down using a rotary concentrator and the dry extract was weighed again until stable. The yield was calculated as a percentage of dry extract in mangosteen rinds powder. The measurement was conducted in triplicate.

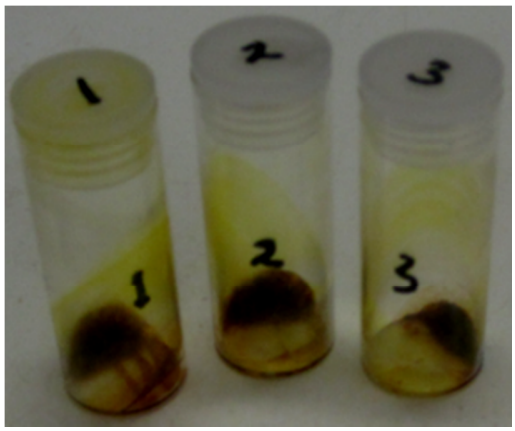

Figure 2.10 Dry extract from macerates

2.3.1.8 Crude Extract analysis on HPLC

Analysis of the crude extract was performed on HPLC in the following manner: 10-20 µL of stock solution in LP was transferred into an HPLC vial and dried down using a rotary concentrator. 1000 µL methanol was added and mixed well. 5 µL solution was then injected into HPLC and analysed under the same analytical conditions as used for the preparation of the calibration curve. The peak area of α-mangostin was calculated using the calibration curve especially to determine the recovery of α-mangostin injected on HPCCC. Sample from stock solution in methanol could be directly injected into HPLC without drying down the solvent.

2.3.2 Optimisation of an Efficient Method for Production of α-Mangostin using HPCCC

2.3.2.1 Solvent System Selection and Partition Coefficient (K_D) Measurement

Into a vial containing an accurate weight of approximately 5 mg of mangosteen crude material was added 5.0 mL of several HEMWat solvent systems from the literature study i.e. HEMWat (1:1:1:1 v/v), (5:5:10:4 v/v), (8:8:10:6 v/v) and (10:10:5:1 v/v).

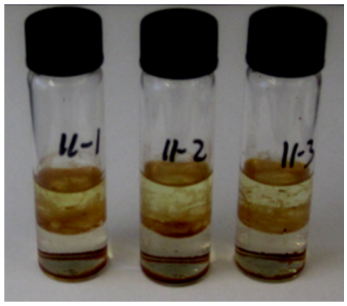

Figure 2.11 Partition coefficient measurement

The mixtures were shaken and then left to settle for about 30 min until the phases were completely separated. Into HPLC separate vials, 0.5 mL upper phase and 0.5 mL lower phase of each mixture was delivered respectively and the solvent dried down in a rotary concentrator. 1.0 mL methanol was added into the vial and mixed evenly. Before analysing in HPLC, if required the mixture was centrifuged at 13,000 rpm for 5 min to avoid column blockage by crude particles. The solvent system which had a suitable K_D=1 was selected as solvent system for this study. The experiment was done in triplicate

2.3.2.2 Settling Time Measurement

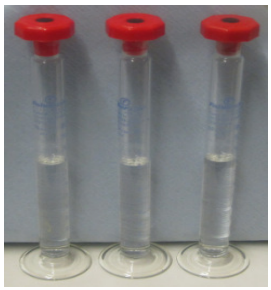

Figure 2.12 Settling time measurement using graduated cylinder

The procedure was as follows: the two phases of the chosen HEMWat solvent system were first equilibrated in a separating funnel; 2.0 ml of each phase (lower and upper phase), a total volume of 4.0 ml, was delivered into a graduated cylinder, which was then capped. The container was gently inverted for several times and then immediately placed it in an up-right position to measure the time required for the two phases to form clear layers with a distinct interface (Ito, 2005).

2.3.2.3 Extraction Kinetics Studies Using Rotary Wheel

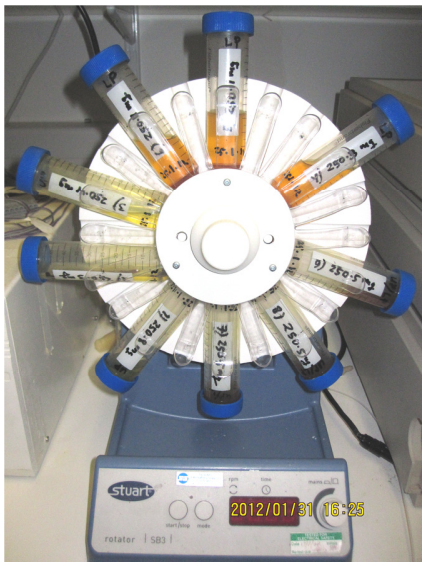

Figure 2.13 Extraction kinetic studies on a rotary wheel

Into several tubes containing an accurate weight of approximately 250 mg crude mangosteen powder each were added 25 mL UP, LP and UP+LP (50:50) respectively. The tubes were placed on a rotary wheel and were rotated for 30 min, 60 min, 90 min, 360 min, overnight (16 hours), and 2 days. In every completion time, 100 µL extract was taken from the tubes into an HPLC vial and dried down using rotary extractor. After completely drying, 1.0 mL methanol was added into vial and mixed evenly. The solution then was analyzed by HPLC using the same condition as in the calibration curve analysis with an injection volume of 10 µL each. The experiment was performed in triplicate.

2.3.2.4 CCC Procedure

2.3.2.4.1 Solvent System Preparation for CCC

Classical preparation of the HEMWat solvent system for CCC was conducted shortly before use by thoroughly mixing hexane/ethyl acetate/methanol/water in ratio of 5:5:10:4 (v/v) respectively. The mixture was equilibrated in a separating funnel at room temperature for about 30 min and the upper phase (UP) and lower phase (LP) separated. The upper phase was used for stationary phase and the lower phase used as mobile phase.

Figure 2.14 Solvent system preparations using a separating funnel

2.3.2.4.2 Sample Preparation for CCC

To prepare sample for CCC, the extract was dissolved in mobile phase (LP). The stock solution dissolved in methanol was dried down and re-dissolved in LP. The preparation was done by drying 5.0 mL of the stock solution in a rotary concentrator to remove the solvent. The dry extract was then re-dissolved in LP to the original volume of 5 mL and used for sample loading studies with dilution using the following equation,

$$C_1 \times V_1 = C_2 \times V_2$$

Where C_1 is initial concentration or molarity, V_1 is initial volume, C_2 is final concentration or molarity, and V_2 is final volume. Sample from stock solution in LP could be directly diluted at the same manner without drying.

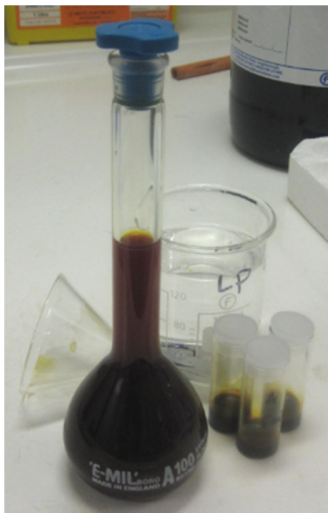

Figure 2.15 Dry extract from stock solution in methanol and the extract after re-dissolving in LP

2.3.2.4.3 Operating CCC

2.3.2.4.3.1 Analytical Scale Mini HPCCC Centrifuge

The Mini-CCC centrifuge used for analytical separations and the sample loading studies was previously described in detail by Janaway et al (2003). It has a rotor radius of 50mm, tubing bore of 0.8mm, mean β of 0.74 and a single bobbin of 5.4mL capacity with a counter weight. The Mini can be rotated up to a speed of 2100 rpm (246×*g*) and has a typical flow range for most organic/ aqueous phase systems of 0.5–2 mL/min for a separation, but the Mini column can cope with flow rates up to 10 mL/min (w.r.t. pressure) for refilling (Sutherland, 2009). The system was equipped with one Hewlett Packard series 1100 pump with 4 different lines that are suitable for 4 different solvents. Detection system was a Knauer K-2501 UV detector (Berlin, Germany) which was set at 240 nm to monitor the elution process. The fractions were collected using Teledyne ISCO Foxy Jr. fraction collector (Lincoln, USA) and the data was processed using Euchrome 2000 Data Processor.

The operating procedure for Mini was done with equilibration of the column before injection. A 50 mL graduated cylinder was placed at the end of the tubing (tail), then the stationary phase was initially pumped into the column at a flow rate of 5 mL/min with no

rotation until at least one complete coil volume of stationary phase came out into the cylinder. The coil was then rotated at 2100 rpm at temperature 25 °C. The mobile phase was pumped from head to tail at a flow rate of 1 mL/min. In order to observe the volume of stationary phase eluted from the column, the resulting effluent was collected in a 25 mL graduated cylinder. The hydrodynamic equilibrium was considered to be established when the stationary phase was no longer dripping into the cylinder (usually when the two phases volumes of the eluent were approximately equal) and the machine was ready to be injected. The sample solution was injected with the desired concentration up to 107 mg/mL or volume up to 1.8 mL into the column, the fraction collector started, and the recorder turned on. All fractions were collected at 1 min/tube within elution time. At the end of the run, the column was emptied of stationary phase by pumping the mobile phase at a higher flow rate without rotation. The stationary phase was collected and analysed on HPLC in the same manner as on fractions, to ensure all the mangostin had eluted. For multiple injections, the injection time was every 25 min. Concentration of α-mangostin was calculated by viewing peak area from the calibration curve after analysis by HPLC.

2.3.2.4.3.2 Semi Preparative Scale Spectrum HPCCC Centrifuge

The Spectrum HPCCC instrument was initially described by Guzlek et al (2009). The equipment that was employed in this project was manufactured by Dynamic Extractions (Slough, UK). The machine comes with two sets of two multilayer columns on two bobbins, i.e. four columns on two bobbins. These two sets were designated as analytical and semi-preparative columns, respectively. Their tubing bore was 0.8 mm for analytical coils and 1.6 mm for semi-preparative coils, respectively with 22 and 136mL total column volumes. In this study the semi-preparative columns were employed. The β-value of these coils varied from 0.52 to 0.86. The rotational speed can be varied up to 1600 rpm and the instrument, which has an integrated temperature controller, was set to 25 °C for all runs. The system was equipped with an Agilent Technologies 1200 series isocratic pump (California, USA) and detector using photodiode array detection system (Milford, MA, USA). The fractions were collected using Teledyne ISCO Foxy Jr. fraction collector (Lincoln, USA).

Column equilibrium must also be performed before operating Spectrum HPCCC. A 200 mL graduated cylinder was placed at the end of the tubing (tail), then the stationary phase was initially pumped into the column at a flow rate of 40 mL/min with no rotation until at least one complete coil volume of stationary phase came out into the cylinder. The mobile phase was pumped from head to tail at a flow rate of 4-8 mL/min while the centrifuge was rotated at 1600 rpm, 25 °C. In order to observe the volume of stationary phase eluted from the column, the resulting effluent was collected in a 100 mL graduated cylinder. The hydrodynamic equilibrium was reached when the stationary phase was no longer dripping into the cylinder and the machine was ready to be injected. The sample solution was injected with the desired concentration or volume into the column, the fraction collector started, and the recorder turned on. All fractions were collected at 1 min/tube within elution time. At the end of the run, the column was emptied from stationary phase by pushing out the stationary phase in each bobbin separately using compressed air at a low rotation (215 rpm). The stationary phase was collected in separate cylinder and analysed on HPLC in the same manner as on fractions. For multiple injections, the injection time was every 25 min. Concentration of α-mangostin was calculated by viewing peak area from the calibration curve after analysis by HPLC.

2.3.2.4.3.3 Preparative Scale Midi HPCCC Centrifuge

The Midi-CCC centrifuge was manufactured by Dynamic Extractions (Slough, UK) and the setup used for the study was described in detail by Hewitson et al. (2009). It has a rotor radius of 110mm, tubing bore of 4mm and two bobbins (columns) with a total capacity of 912.5 mL. The Midi can be rotated up to a speed of 1400rpm (241×*g*), has a typical flow range of 10–100 mL/min and a mean β value of 0.75 where β is the ratio of planet to rotor radius (Sutherland, 2009). This instrument was fitted with a sample injector, a Knauer pump K 1000 (Berlin, Germany) which has maximum flow rate 1000 mL/min and a Knauer detection system K 2501 UV detector (Berlin, Germany) as on Mini. Data processor was Euchrome 2000. The fraction collector was Gilson Model 202 (France). The wave length detection was set at 240 nm as optimum wave length of mangostin.

The operating procedure for Midi was done with equilibration of the column before injection. A 1000 mL graduated cylinder was placed at the end of the tubing (tail), then the

stationary phase was initially pumped into the column at a flow rate of 200 mL/min with no rotation until at least one complete coil volume of stationary phase came out into the cylinder. The coil was then rotated at 1400 rpm, 25 °C. The mobile phase was pumped from head to tail at a flow rate of 20-50 mL/min. In order to observe the volume of stationary phase eluted from the column, the resulting effluent was collected in a 500 mL graduated cylinder. The hydrodynamic equilibrium was considered to be established when the stationary phase was no longer dripping into the cylinder and the machine was ready to be injected. The 50 mL sample solution was injected with the desired concentration into the column, the fraction collector started, and the recorder turned on. All fractions were collected at 1 min/tube within elution time. The fractions were analysed on HPLC after drying down the solvent. At the end of the run, the column was emptied from stationary phase by pushing out the stationary phase in each bobbin separately using compressed air at low rotation (215 rpm). The stationary phase was collected and analysed on HPLC in the same manner. For multiple injections, the injection time was every 25 min. Concentration of α-mangostin was calculated by viewing peak area from the calibration curve after analysis by HPLC.

2.3.2.4.3.4 Procedure of analysis for CCC Fractions on HPLC

Figure 2.16 Fraction preparation for analysis on HPLC, fraction drying in a rotary concentrator

Analysis of the HPCCC fractions was carried out on HPLC in the following manner: 100 µL of the fraction was transferred into an HPLC vial and dried down using a rotary concentrator. 1000 µL methanol was added and mixed well. 5 µL solution was then injected on HPLC under the same analytical conditions for the preparation of the

calibration curve. All peak areas that appeared on the HPLC chromatogram were plotted on a fractogram (a reconstructed chromatogram). These data were used to calculate the purity and yield of α-mangostin. From the fractogram, the resolution (R_S) between α-mangstin and the nearest component peaks (component 3) was calculated using equation on page 13. The stationary phase extracted from the column was analysed on HPLC in the same manner.

2.3.2.5 Nuclear Magnetic Resonance (NMR) Spectroscopy Procedure

This NMR experiments were conducted in Kingston University London. The procedure which was done and reported by E Keaveney from School of Pharmacy and Chemistry was as follows:

1. Preparation of initial NMR standard solution, 1 ml of deuterated chloroform was added to the vial that contained the alpha-mangostin standard supplied by Sigma-Aldrich. The resulting solution was then filtered into an NMR tube through cotton wool in a disposable pipette. A drop of TMS (tetra methyl silane) was added to the NMR tube which was then shaken to disperse the TMS.
2. Preparation of second NMR standard solution: the initial NMR standard preparation (1) was transferred from the NMR tube to a sample vial. The solution was taken to dryness using a stream of nitrogen. 0.75 ml of acetonitrile was added to the sample vial using disposable pipettes. The sample was transferred back to the NMR tube by filtration through a cotton wool filter using disposable pipettes. A drop of TMS was added to the NMR tube and it was covered and shaken to fully disperse.
3. Preparation of sample: in a vial containing 8 mg sample (indicated pure α-mangostin) from a combined HPCCC fractions (EKSM23F2021) prepared by Brunel University London, was added 0.75 ml of acetonitrile using disposable pipette. The sample was transferred to a glass NMR tube by filtration through a cotton wool filter using disposable pipettes and labelled with the sample codes. A drop of TMS was added to the NMR tube, which was then shaken to fully disperse and analysed.

To analyse on NMR, the sample tube was then placed inside a cylindrically wound magnet and exposed to a pulsed magnetic fie$_{ld\ wh}$ich cause the nuclei within the sample to become first excited then relaxed. The resonance produces a signal characteristic to the type

of isotope in the sample which is then recorded. This signal is integrated to produce a series of spectral lines.

Chapter 3 RESULTS AND DISCUSSION

3.1 Optimisation of Analytical Method for α-Mangostin by HPLC

Table 3.1 Optimised conditions of HPLC

No	HPLC Parameters	Optimised Conditions of HPLC
1.	Column	Agilent Zorbax Bonus RP C_{18} with dimension (3.5um; 4.6x150mm)
2.	Temperature	40 ℃
3.	Flow rate	0.5 mL/min
4.	Mobile phase	Methanol with 0.05% TFA (C) Water with 0.05% TFA (D)
5.	Elution program	Gradient system: 0-3 min: 95-100% C; 3-8 min: 100% C;
6.	Run time	12 min
7.	Delay time	2 min
8.	Collecting data	12 min
9.	Wave length detection	PDA Max Plot 210 nm to 400 nm
10	Low limit of detection (LLOD)	0.000008 μg/μL (8 ppm)
11	Retention time of α-mangostin	6.00 – 6.50 min

As described previously, the optimisation of an analytical method for α-mangostin on HPLC began with a literature study. Based on previous research (Jia, 2007; Jujun, 2009; Syamsudin, 2010), a C_{18} column and methanol/water were selected as stationary phase and mobile phase respectively for analysing α-mangostin on HPLC.

In this research, the column selected was Agilent Zorbax Bonus RP-C_{18} with dimension 3.5μm; 4.6x150mm. The column had small particles size that provided a greater surface area for interactions between the stationary phase and the molecules flowing past it. So a better separation of the mixture was obtained (Clark, J., 2007). In addition, with an appropriate dimension of the column, it also produces a good pressure approximately 700 psi.

Analytical conditions were maintained at a temperature of 40 °C where it was a high enough temperature to reduce solvent viscosity and the chance of column over-pressure but non destructive to α-mangostin and other Components in the extract. The flow rate and elution program were set in order to create a chromatogram with good separation. The run time of 12 min was optimised to a minimum time that was able to elute all compounds in the extract. The delay time was needed to remove all compounds that might remain at the end of elution which interfere with the peaks from the next injection. When preparing the solvent system for HPLC, 0.05% trifluoroacetic acid (TFA) was added to methanol HPLC grade and to fresh ionised water separately as an ion pair reagent to remove tailing of the peaks.

3.2 Preparation of Standard Solution

Table 3.2 Calculation of **α-**mangostin standard solution for calibration curve

Parameters	**Results**
α-Mangostin	1.2 mg
Vial and 1.2 mg α-Mangostin	8.2759 g
Vial and 1.2 mg α-Mangostin + 5.0 mL dry Methanol	12.2217 g
Dry methanol	3.9458 g
True volume of dry methanol	4.9833 mL
Concentration of α-Mangostin standard solution I	$\frac{1200}{4.9833}$ µg/mL = 0.2408 µg/µL
Concentration of α-Mangostin standard solution II (25xdilution)	9.632 µg/mL = 0.0096 µg/µL

Standard solution I was prepared by adding 5.0 mL dry methanol into a vial containing an accurate weight of approximately 1.2 mg α-mangostin commercial reference standard. After adding the dry methanol, the vial was weighed again to ensure the true volume of dry methanol due to minimise errors that might occur in weighing of the reference standard. The dry methanol was prepared in a flask by adding some molecular sieve into methanol HPLC grade, shaken vigorously and left overnight. This molecular sieve was intended to absorb water that probably present in methanol since methanol is

hygroscopic. The true volume of methanol was calculated by V=M/ρ; where V is true volume of methanol; M is Mass; and ρ is density of methanol (0.7918 g cm−3). Standard solution II was 25 times dilution of standard solution I and prepared using the formula of $C_1 \times V_1 = C_2 \times V_2$ (2.3.2.4.2).

3.3 Calibration Curves

3.3.1 Calibration curve I

Table 3.3 Amount of standard solution I injected on HPLC vs. HPLC peak area

Volume (µL)	Amount (µg)	Peak Area (µVolt*sec)
2	0.4816	4921765
5	1.2040	15194643
10	2.4080	31753001
20	4.8160	64215307
25	6.0200	80014555

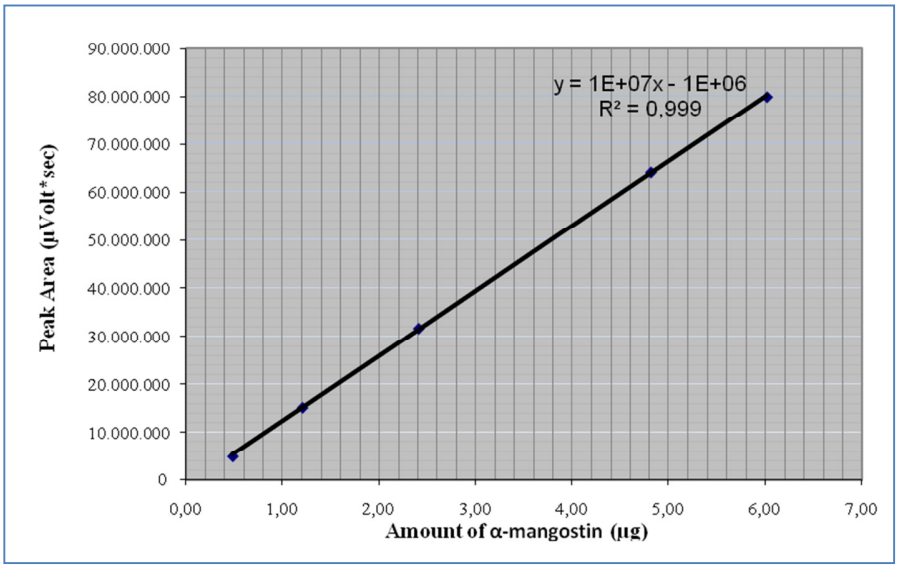

Figure 3.1 Calibration curve I, peak area vs. amount of **α-**mangostin

The calibration curve of α-mangostin was intentionally made from 2 different concentration of standard solutions to cope with a variety of concentrations of sample/fraction analysed,

which have peak areas up to 80,000,000 µVolt*sec. The sample with peak area ≥ 5,200,000 µVolt*sec was calculated using calibration curve I while peak area < 5,200,000 µVolt*sec was calculated using calibration curve II. Both calibration curves either from standard solution I or standard solution II had the regression equations with the same R^2=0.999 which mean both curves are linear and using the HPLC optimised method all *x* values were uniformly distributed either side of $\bar{x}$; similarly, all the *y* values was uniformly distributed about $\bar{y}$. These calibration curves were used to calculate the amount/concentration of α-mangostin in all samples including crude extract and HPCCC fractions during this study.

3.3.2 Calibration curve II

Table 3.4 Amount of standard solution II injected on HPLC vs. HPLC peak area

Volume (µL)	**Amount (µg)**	**Peak Area (µVolt*sec)**
2	0.0192	234364
5	0.0480	643434
10	0.0960	1295177
15	0.1440	1965049
20	0.1920	2629968
25	0.2400	3313694
30	0.2880	4017900
40	0.3840	5392568

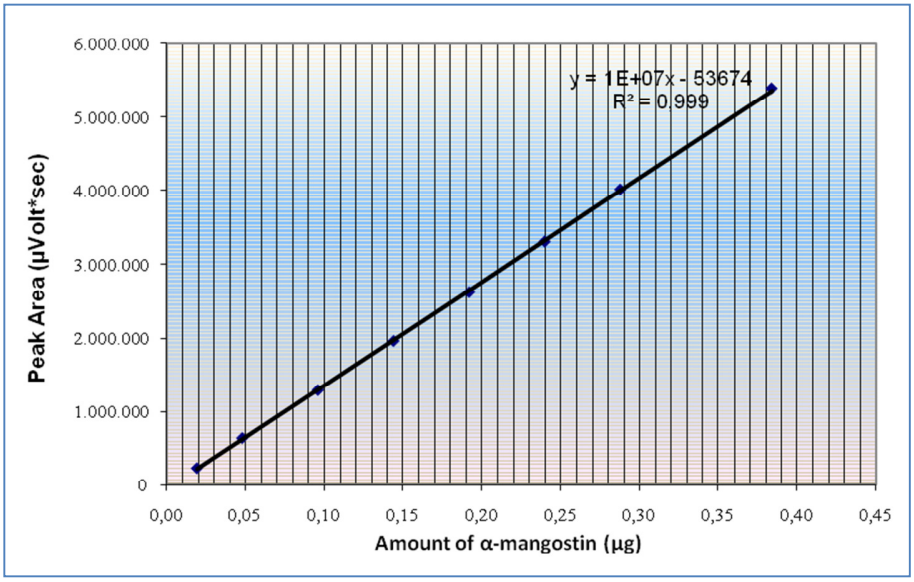

Figure 3.2 Calibration curve II, peak area vs. amount of **α-**mangostin

3.4 Preparation for 80% Aqueous Ethanol Extract as Stock Solution

Table 3.5 Sample preparation

No	Crude Material (g)	Dry Extract (g)	Stock Solution (mL)	Yield (%)
1	100	10.6721	62 (in methanol)	10.61
2	100	15.5898	55 (in methanol)	15.59
3	200	20.4970	100 (in LP)	10.25
	200	20.3816	100 (in LP)	10.19

In an initial study of this project, extraction of α-mangostin using 80% ethanol obtained a higher concentration of α-mangostin compare to methanol and ethyl acetate. So the solvent was chosen for extraction of α-mangostin from the fruit rinds powder.

Preparation of the ethanol extract was done with overnight maceration in 80% aqueous ethanol, the extract was then filtered under vacuum conditions and collected before measuring the yield and drying in a rotary evaporator. Stock solution was prepared by re-dissolving the dry extract in methanol or in LP. When using stock solution in methanol as a sample for separation on HPCCC, the methanol was removed by drying on rotary concentrator and then re-dissolved in LP. Otherwise, the stock solution in LP was directly

used as a sample on HPCCC without removing the solvent. It was noted; however that the stock solution in LP was not as stable as in methanol so it was prepared fresher. Moreover, the LP which contains ethyl acetate and hexane will absorbs UV; therefore it needed be eliminated from the extract or fraction before analysing on HPLC.

3.5 Measurement of the Ethanol Extract Yield of Mangostin from Crude Material

Table 3.6 Crude extracts yield measurement

No	Weight of empty vial (g)	Weight of vial with extract (g)	Weight of extract (g)	Average weight of extract in 5ml (g)	Total volume of solution (mL)	Total weight of dry extract (g)	Weight of crude material used (g)	Yield of man-gostin (%)
1	7.3126	7.3710	0.0584	0.0560	947	10.6060	100	10.61
	7.3158	7.3630	0.0472					
	7.1790	7.2415	0.0625					
2	7.2713	7.3271	0.0558	0.0670	1164	15.5976	100	15.59
	7.1546	7.2262	0.0716					
	7.2948	7.3683	0.0735					
3	7.3136	7.3670	0.0534	0.0533	1970	20.9870	200	10.25
	7.3211	7.3627	0.0416					
	7.1777	7.2425	0.0648					
4	7.2096	7.2624	0.0528	0.0530	1924	20.3816	200	10.19
	7.1232	7.1763	0.0531					
	7.3378	7.3908	0.0530					

Measurement of the yield of ethanol extract was conducted before drying the whole filtrates collected using a rotary evaporator. When weighing the dry extract for calculating the yield, it was done several times until the weight of the extract was stable to ensure the extract was completely dry; so the yield could be calculated accurately. The measurement of the yield was actually intended particularly to predict the amount of the extract that would be injected to HPCCC for the sample loading study. The range of mass/extract that

is possible to be injected depends on the scale of the machine. Table 3.6 shows that 4 different preparation of ethanol extracts gave the yields 10.19% to 15.59%.

3.6 Crude Extract and Fraction analysis on HPLC

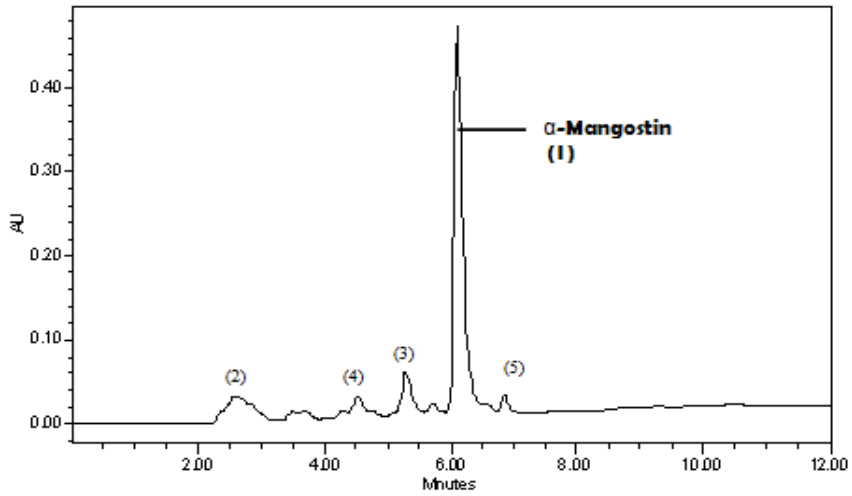

Figure 3.3 Chromatogram of mangosteen crude extract, analysed by HPLC with analytical conditions as in table 3.1, injection amount 5µL and concentration loaded approximately 0.5µg/µL

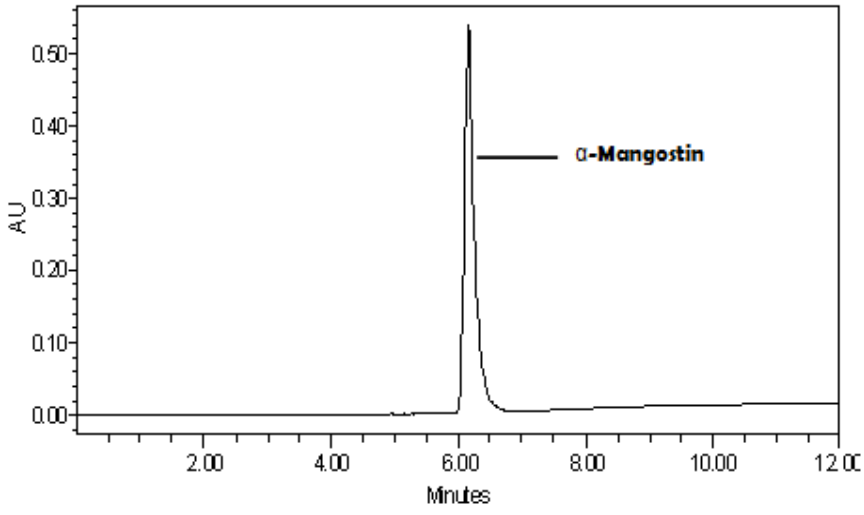

Figure 3.4 Chromatogram of purified α-mangostin in a sample fraction, analysed by HPLC with analytical conditions as on table 3.1, injection amount 5µL and concentration loaded approximately 0.1µg/µL.

The Chromatograms above show the peak of α-mangostin from analysis on HPLC. The α-mangostin peak was the major peak in the chromatogram of the crude extract (Figure 3.3.) indicating that α-mangostin is a major compound in the fruit rind of *Garcinia mangostana* L. The sample from a fraction presents a chromatogram with single peak (Figure 3.4). So it can be assumed that α-mangostin was isolated nearly pure using HPCCC according to HPLC peak area at this UV wavelength.

3.7 Solvent System Selection and Partition Coefficient (K_D) Measurement

Table 3.7 Partition coefficient (K_D) as the results of **2.3.2.1.**

No	Solvent System (v/v)	Concentration of α-mangostin in UP (Mean ± SD)	Concentration of α-mangostin in LP (Mean ± SD)	Partition Coefficient (K_D)
1	HEMWat (1:1:1:1)	0.0494 ± 0.0002	0.0024 ± 0.0001	19.65 - 21.37
2	HEMWat (5:5:10: 4)	0.0328 ± 0.0004	0.0333 ± 0.0009	0.97 – 1.00
3	HEMWat (8:8:10:6)	0.0570 ± 0.0001	0.0174 ± 0.0003	3.23 – 3.32
4	HEMWat (10:5:5:1)	0.0186 ± 0.0003	0.0458 ± 0.0008	0.40 – 0.40

Note that HEMWat refers to the solvent system consisting of hexane-ethyl acetate-methanol-water. Selection of the solvent system for the separation of compounds by HPCCC requires consideration of the partition coefficient (also called the distribution ratio) and the settling time of the phase system. It can be seen in the Table 3.7 that the hexane-ethyl acetate-methanol-water (HEMWat) (5:5:10:4 v/v) provided a partition coefficient (K_D) of 0.97 – 1.00. This meets the requirement of the suitable K_D value which is $0.5 \leq K_D \leq 2.0$ for separation using HPCCC. This phase system was selected as solvent system for isolation and purification of α-mangostin from the fruit rinds of *Garcinia mangostana* L. using HPCCC. If K_D=1, the analyte will elute at the retention volume equal to the column capacity regardless of the retention volume of the stationary phase (Ito, 2005). Having K_D=1 is a good point since the concentration of Component dissolved in UP is equal to the LP. The separation can also be conducted either in normal phase or reverse phase by simply

changing LP to stationary phase or mobile phase. In the present study, the separation was selected with reverse phase mode since this solvent system was prepared classically using a separating funnel it produced the LP volume almost 3 times more than the UP volume. So it was better to choose reverse phase mode to balance solvent usage where more mobile phase was needed than stationary phase.

3.8 Settling Time Measurement

Table 3.8 Settling time of HEMWat (5:5:10:4 v/v) solvent system (2.3.2.2)

No	Settling Time (sec)
1	9.45
2	8.37
3	9.12
Mean ± SD	8.98 ±0.55

The table above shows the average of settling time of solvent system selected i.e. HEMWat (5:5:10:4 v/v) was 8.98 sec. To provided satisfactory retention of the stationary phase over 50% of the total column capacity in a proper range of flow rates, the time required for the two phases to form clear layers with a distinct interface is less than 20 sec (Ito, 2005), so the solvent system selected met the requirement.

3.9 Study on Extraction Kinetics Using Rotary Wheel

Table 3.9 Percentage of α-mangostin extracted from the fruit rind of *Garcinia mangostana* L. in HEMWat (5:5:10:4 v/v)

No	Time	UP (%)	LP (%)	UP+LP (%)
1	30 min	2.77	2.79	2.82
2	60 min	2.7	2.91	2.94
3	90 min	2.79	2.91	2.96
4	180 min	2.98	3.02	3.08
5	360 min	2.94	3.17	3.05
6	Overnight	2.93	2.93	3.10
7	2 Days	3.22	3.09	3.22

Figure 3.5 below shows the extraction of approximately 0.025 g of rinds with 25 ml of either upper phase, lower phase or total phase that the α-mangostin extracted was approximately 2.8% of the rind in just 30 min after rotation on a rotary wheel. There was no significantly different percentage of α-mangostin extracted in UP, LP as well as UP+LP of the HEMWat (5:5:10:4 v/v) phase system. The percentage of α-mangostin then increased a little bit and reached at nearly 3% after 60 min rotation. This condition has remained relatively stable for 2 days rotation, with the variation observed being assumed to be simple experimental error.

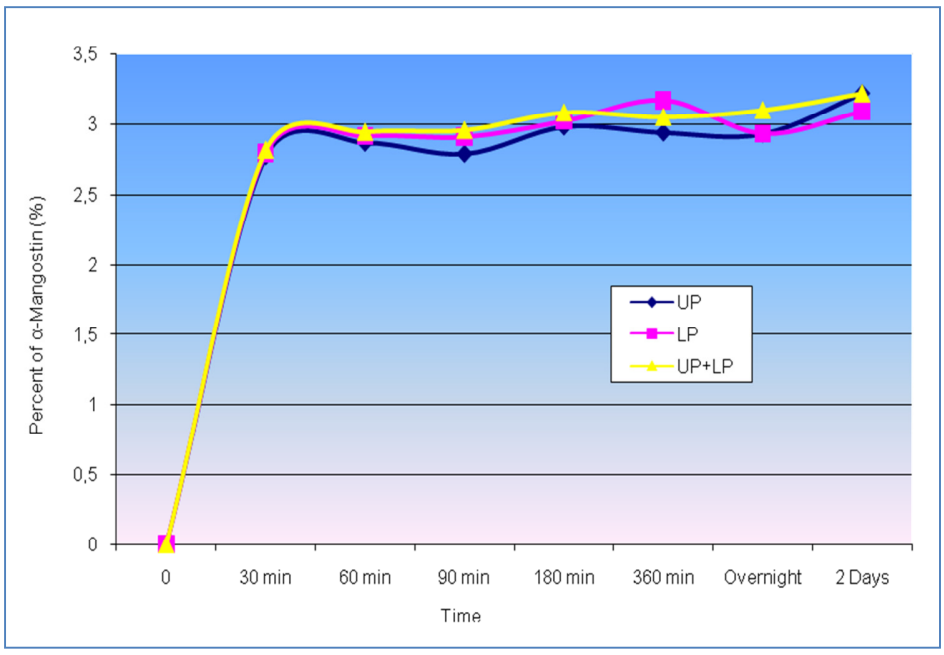

Figure 3.5. Extraction kinetic of α-mangostin from the fruit rinds of *Garcinia mangostana* L. (Percent vs. Time) with either upper, lower or totals phase system (HEMWat 5:5:10:4 v/v)

3.10 Sample Loading Study

Sample loading study was conducted on an analytical scale Mini HPCCC instrument. This method was a modification of the method described by Jia et al (2011) for the isolation of major xanthones in *Swertia mussotii* using Centrifugal Partition Chromatodraphy. The solvent system was selected based on the solvent that is commonly used for the isolation and separation of natural materials by liquid flow processing, which is

generally composed of hexane or heptane /ethyl acetate/methanol/water and is called HEMWat. Applying the solvent system of hexane/ethyl acetate/methanol/water (5:5:10:4 v/v) for isolation of α-mangostin from fruit rinds of *Garcinia mangostana* L. provided a suitable distribution ratio (K_D=1) as mentioned above. The running conditions which several parameters can be seen on Table 3.10 as follows:

Table 3.10 Optimised conditions of sample loading study

No	Parameters	Optimised Conditions
1	Instrument	Mini HPCCC
2	Column volume	17.4 mL
3	Bore	0.8 mm
4	Solvent system	HEMWat (5:5:10:4 v/v)*
5	Stationary phase	Upper phase
6	Mobile phase	Lower phase
7	Running mode	Reverse phase
8	Rotational speed	2100 rpm
9	Flow rate	1 mL/min
10	Temperature	25 °C
11	Run time per injection	25 min
12	Number of injections	Up to 10 x

**H stands for hexane*

Samples injected were a variety of extract amounts from 4.6 mg to 91.2 mg and volumes from 0.86 mL to 1.8 mL. The experiments were done with single and multiple injections up to 10 times with 25 min interval time between injections without any replacement or topping up of the stationary phase. The interval time was considered based on the elution time of α- mangostin that generally eluted after 17 min to 23 min using the method conditions above.

Table 3.11 below shows the results of sample loading study. Ethanol extract that was injected on Mini HPCCC up to 22.8 mg, showed a good separation and provided high

purities and yields with high recoveries. The purity reached at least 98% and yield over 97 % with recovery not less than 99% in average. The resolutions were also good for most injections from 4.6 mg – 22.8 mg crude extract. It can be seen on fractograms (an elution chromatogram recontructed from the off-line analysis data) at Figure 3.6 to Figure 3.10. below, the peaks of α- mangostin were separated well in most injections; however the resolution decreased a little with the increasing number of injections, as well as concentration of sample injected. This phenomenon probably occurs because more viscous/concentrated samples injected into the solvent system will create a resistance/shock of the solvent system that will cause a loss of stationary phase and dilation of the peak (Berthod, 2009). The multiple injections with a long running time affected the loss of stationary phase on Mini HPCCC. On the fractograms can be seen clearly that the distance between α- mangostin peaks and other components were getting closer and closer with the increasing number of injections of 9.1 mg sample injected in 10 injections (Figure 3.7). This also happened with sample injected up to 22.8 mg with 5 injections (Figure 3.8. to Figure 3.11). However, all of them still had good resolutions with a value above 1.5.

Sample injected with concentration 45 mg - 47 mg as shown on Figure.3.11 and Figure 3.12 provided acceptable resolution; however the purities and yields decreased and the recoveries were low. When the amount of injection was increased two times (91.2 mg); the peaks of α-mangostin and all other compounds lost resolution and became overlapping (Figure 3.13). It also affected the purity, yield, as well as recovery which was significantly down. For this reason the concentration of 22.8 mg ethanol extract (SM-22 on Figure 3.11) was selected as a sample injected for further study on development of an efficient method for productions of purified α- mangostin using liquid flow processing.

The percentage of stationary phase left was calculated and presented as initial % S_F as %S_F before injection and final %S_F as %S_F after the last injection. The %S_F can be seen on each fractogram on Figure 3.6 – Figure 3.22 below.

Table 3.11 Sample loading study on Mini HPCCC (17.4 mL coil; 0.8 mm bore)

Running Code	Sample amount per injection (mg)	Number of Injections	Resolution* (Mean ± SD)	Total α-Mangostin Isolated (mg)	Throughput Sample Process (g/h)	Throughput α-Mangostin Isolated (g/h)	Purity (Mean ± SD) (%)	Yield (Mean ± SD) (%)	Recovery (%)
SM-20	4.6	5	2.04 ± 0.29	6.6	0.01	0.003	99.59 ± 0.28	99.42 ± 0.53	99%
SM-16	9.1	10	1.98 ± 0.24	36.8	0.02	0.01	97.81 ± 0.57	97.17 ± 2.30	98.75
SM-17	13.7	5	2.24 ± 0.26	22.4	0.03	0.01	98.74 ± 0.88	98.54 ± 0.43	97.05
SM-18A	18.2	5	1.83 ± 0.39	29.5	0.04	0.01	98.50 ± 0.99	97.44 ± 1.27	99%
SM-19	22.8	5	1.82 ± 0.18	30,6	0.06	0.02	98.82 ± 0.18	93.68 ± 1.67	99%
SM-22	45.6	1	1.33	12.7	0.11	0.03	96.87	98.98	60.2
SM-23	47.8	1	1.44	14.2	0.11	0.03	97.85	95.61	57.5
SM-21	91.2	1	0.67	21.7	0.22	0.05	94.69	92.38	67.1

**Resolution of α-mangostin from the component 3*

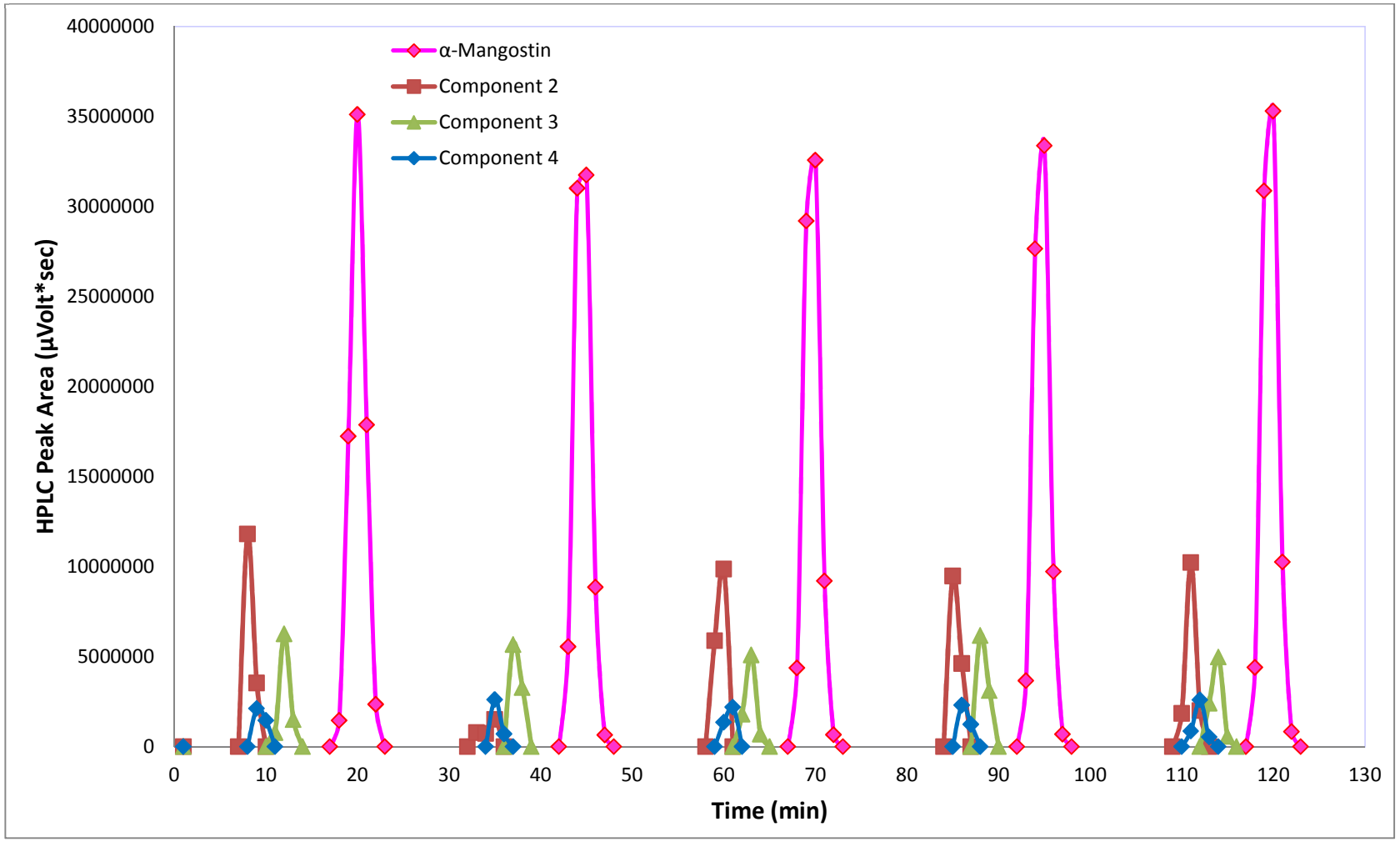

Figure 3.6 Fractogram of SM-20; Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100 rpm; 25°C; 1 mL/min; 5 consecutive injections of the same amount of 4.6 mg ethanol extract in 0.86 mL without any charge of the SP with initial to final $\%S_F$=69%

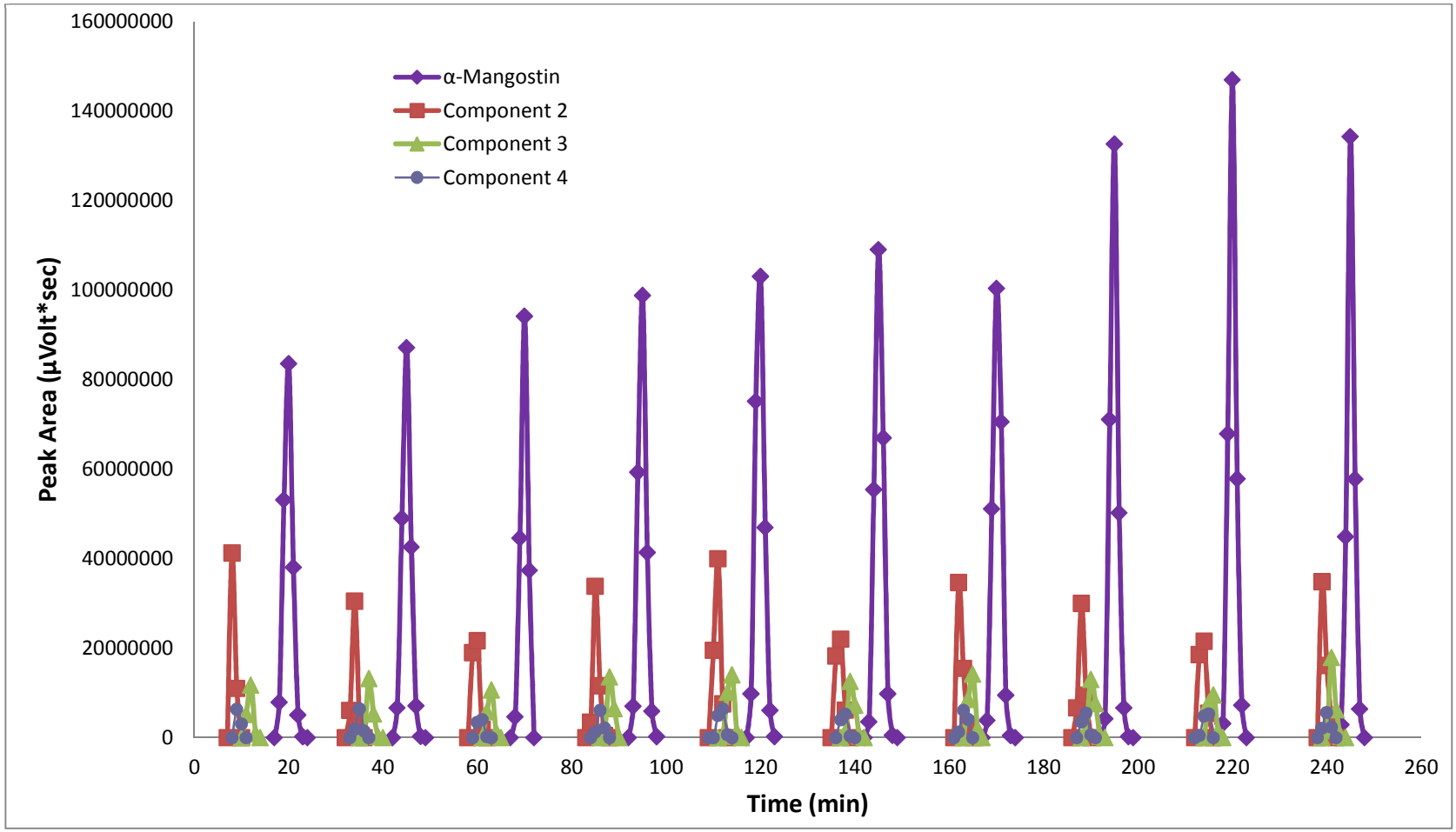

Figure 3.7 Fractogram of SM-16; Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100 rpm; 25°C; 1 mL/min; 10 consecutive injections of the same amount of 9.1 mg ethanol extract in 0.86 mL without any charge of the SP with initial $\%S_F$=69% and final $\%S_F$=68%

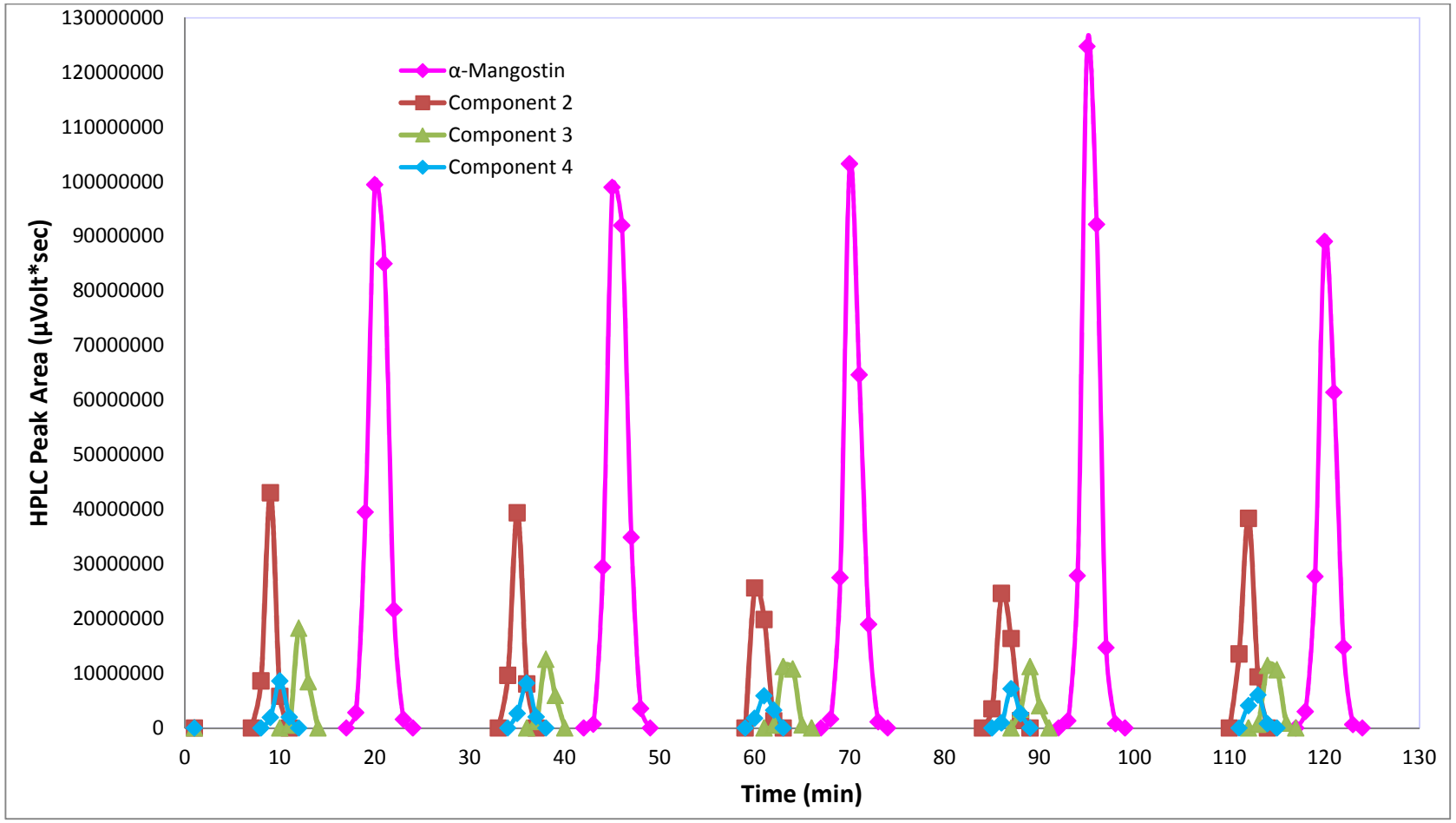

Figure 3.8 Fractogram of SM-17; Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100 rpm; 25°C; 1 mL/min; 5 consecutive injections of the same amount of 13.7 mg ethanol extract in 0.86 mL without any charge of the SP with initial $\%S_F$ 69% and final $\%S_F$ 62%

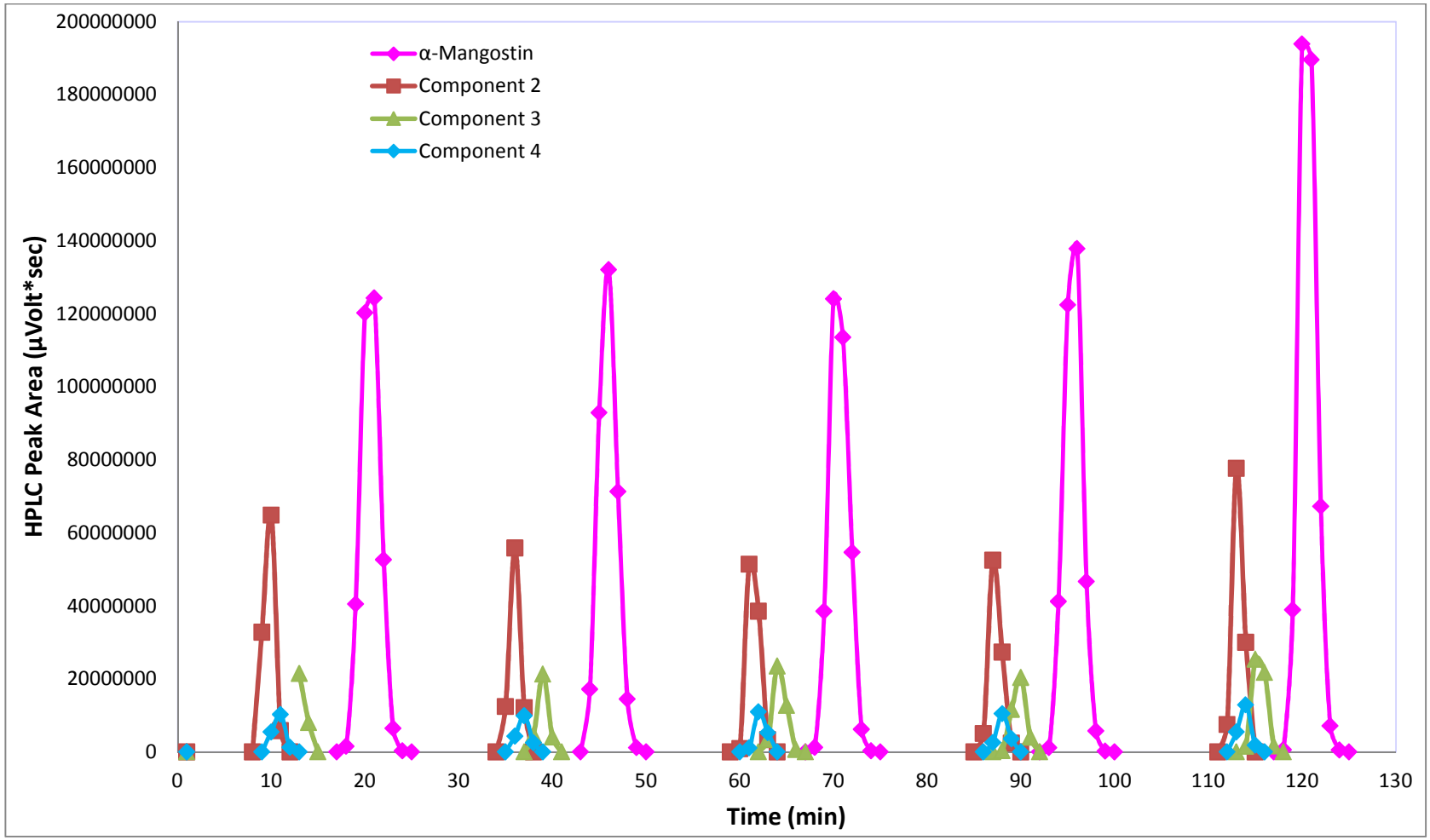

Figure 3.9 Fractogram of SM-18A; Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100 rpm; 25°C; 1 mL/min; 5 consecutive injections of the same amount of 18.2 mg ethanol extract in 0.86 mL without any charge of the SP with initial $\%S_F$=69% and final $\%S_F$=61%

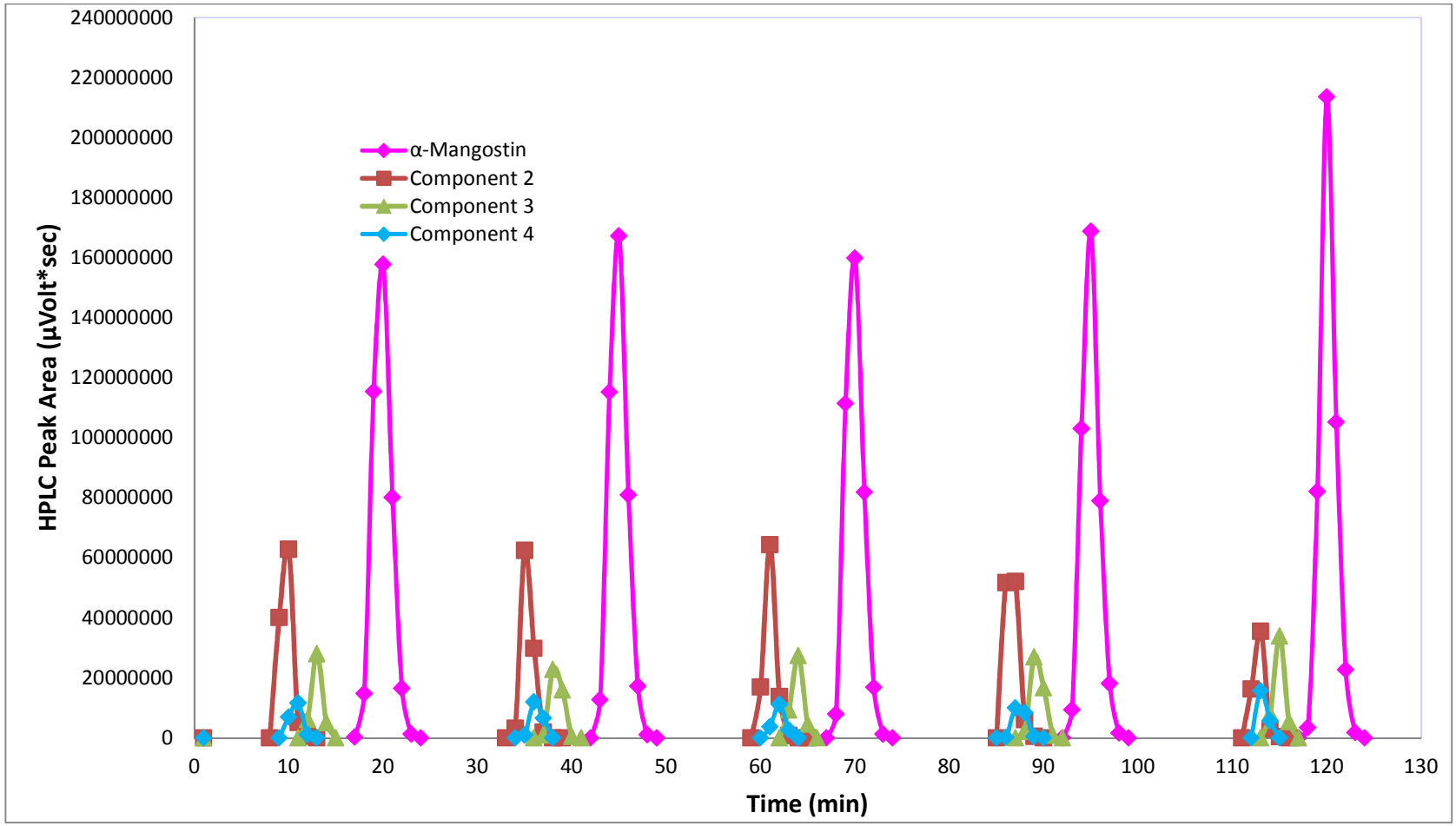

Figure 3.10 Fractogram of SM-19; Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100 rpm; 25°C; 1 mL/min; 5 consecutive injections of the same amount of 22.8 mg ethanol extract in 0.86 mL without any charge of the SP with initial $\%S_F$=69% and final $\%S_F$=61%

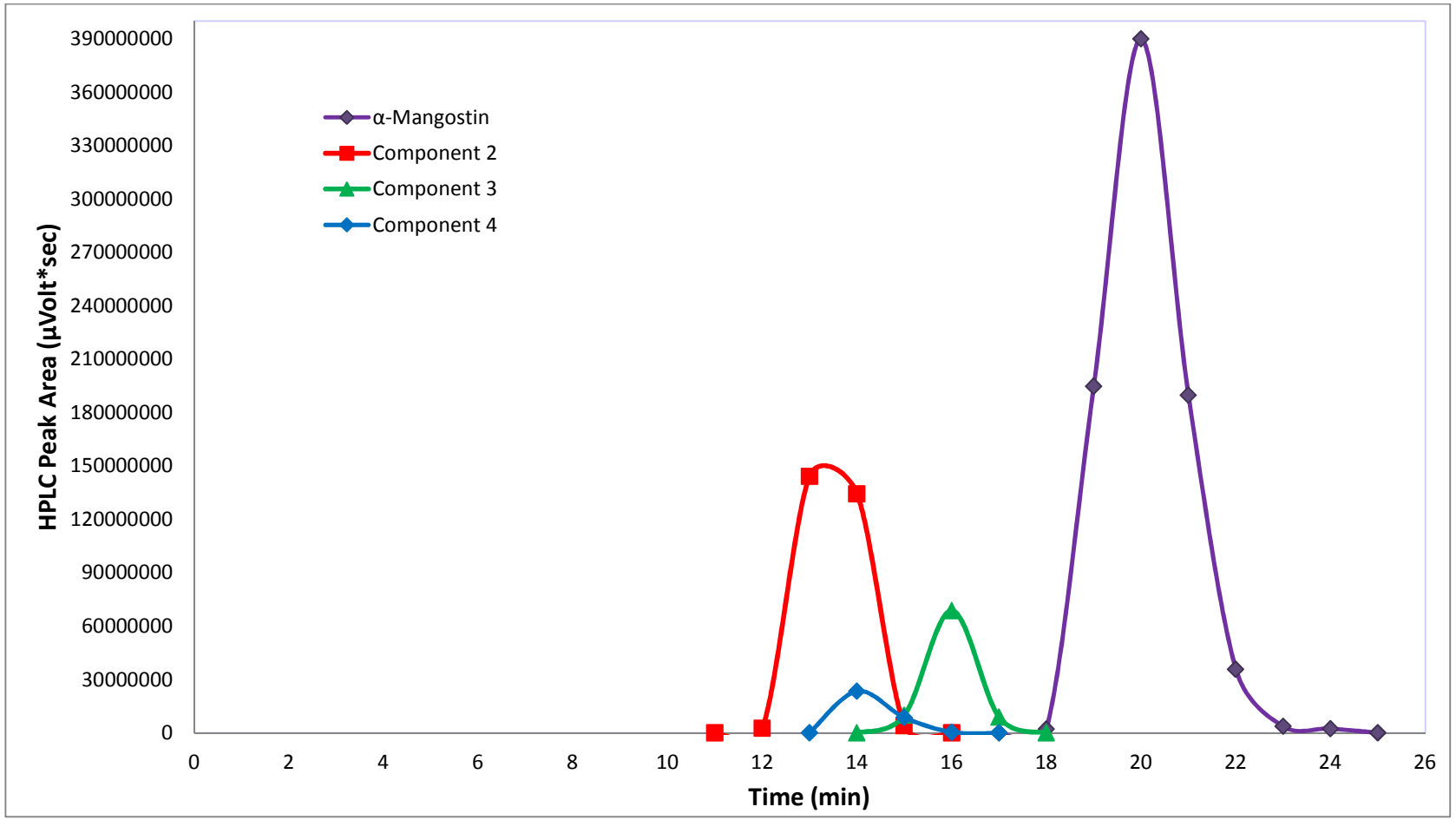

Figure 3.11 Fractogram of SM-22; Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100 rpm; 25 ^{0}C; 1 mL/min; single injection of 45.6 mg ethanol extract in 0.86 mL LP with initial $\%S_F$=72% and final $\%S_F$=56%

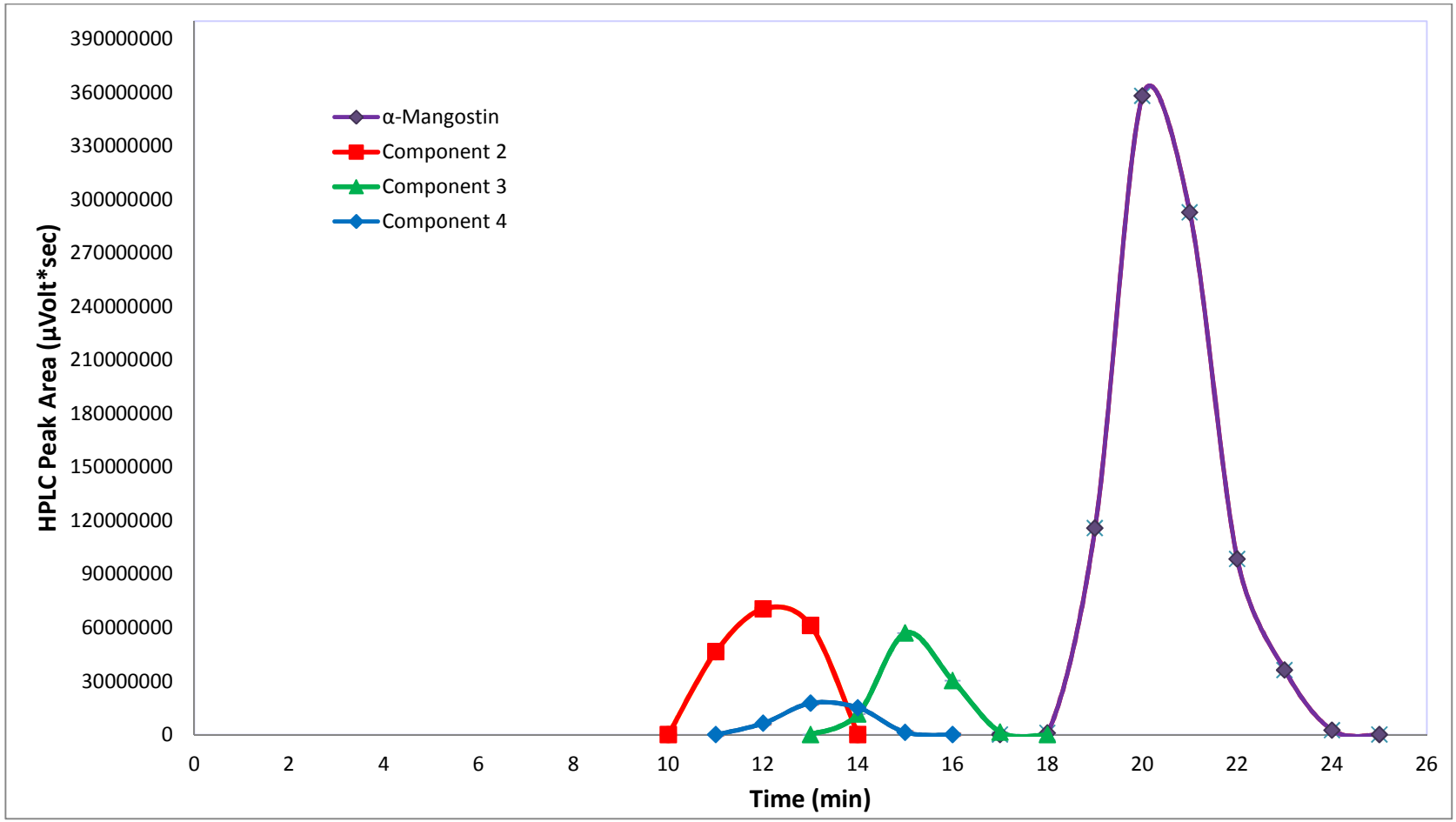

Figure 3.12 SM-23; Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100 rpm; 25 ^{0}C; 1 mL/min; single injection of 47.8 mg ethanol extract in 1.8 mL LP with initial $\%S_F$=72% and final $\%S_F$=52%

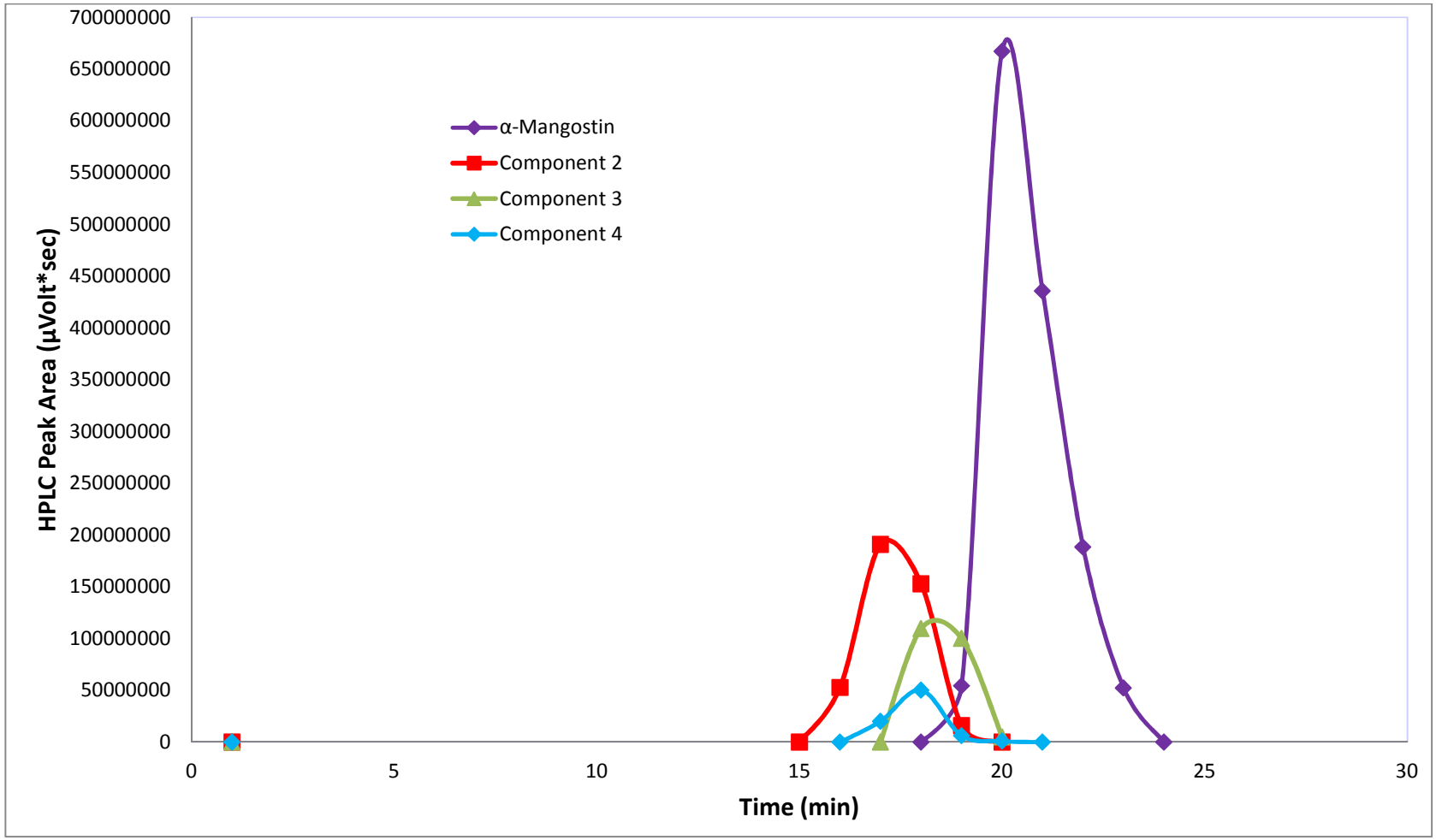

Figure 3.13 Fractogram of SM-21 Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100 rpm; 25 ^{0}C; 1 mL/min; single injection of 91.2 mg ethanol extract in 0.86 mL LP with initial $\%S_F$=72% and final $\%S_F$=40%

3.11 Efficient Method for Production of α-Mangostin

An efficient method for production of purified α-mangostin using liquid flow processing was developed based on the results from the sample loading study on an analytical scale Mini HPCCC instrument that was mentioned above. Using the optimum concentration of 22.8 mg crude extract on Mini; the sample was then scaled up 8 times on semi preparative Spectrum HPCCC instrument and 50 times on preparative Midi HPCCC Instrument for developing an efficient HPCCC method. The running conditions for those instruments are described on table 3.12 below:

Table 3.12 Optimised conditions for development of an efficient method for production of α-mangostin using HPCCC

No	Parameters	Mini HPCCC	Spectrum HPCCC	Midi HPCCC
1	Coil volume (mL)	17.4	143.5	912.5
2	Solvent system	HEMWat (5:5:10:4)	HEMWat (5:5:10:4)	HEMWat (5:5:10:4)
3	Flow rate (mL/min)	1	8	50
4	Running Mode	Reverse phase	Reverse phase	Reverse phase
5	Temperature (°C)	25	25	25
6	Rotational speed (rpm)	2100	1600	1400
7	Elution time /injection (min)	25	25	25
8	Number of injections	5	2-10	5-7
9	Extract amount per injection (mg)	22.8	358.7	1281.1
10	Sample volume per injection (mL)	0.86	7	50
11	Throughput α-mangostin Isolated (g/h)	0.02	0.05	0.88

The method used only one step with multiple injections using the conditions as described above. This multiple injections without the replacement or topping up of the stationary phase that occurs in existing method, could save the use of solvents in particular the volume of stationary phase. It might be simply calculated using the following equation:

$$V_X = V_C(N-1)$$

Where V_X is volume of stationary phase saved; V_C is volume of column; N is number of injections.

In addition, multiple injections also saved the wasted time of pre and post CCC running for each injection i.e. filling the column, equilibration process and emptying the column. A similar equation is suitable to calculate the time saved as follows,

$$t_X = t_E(N-1)$$

Where t_X is time saved for column equilibration/emptying; t_E is time of column equilibration/emptying process; N is number of injections.

Using maximum concentration of sample injected for each instrument, this developed method not only reduced solvent and time consumption of the production process, reducing the cost of production, but also provided a high throughput with high purity and yield of α-mangostin as well as high recovery. The multiple injections method for producing the α-mangostin is efficient with the optimised conditions as shown on Table 3.12.

As in the previous running on Mini, scaling up 8 times on Spectrum and 50 times on Midi resulted in a similar elution time of α-mangostin at approximately 17 to 23 min. The scaling up factor is a simply obtained by dividing the bigger column volume with the smaller one. So the scale up factor is the result of the division between the column volume on Midi or Spectrum with the Mini column. This factor was then used for calculating the amount and the volume of the injection as well as the flow rate. Modification with increasing the concentration or the volume of the sample injected might be possible to obtaining a higher throughput in production process. However, the flow rate should be considered and might be reduced during injection time of a large volume or concentrated sample to avoid losing of stationary phase from the coil, as this leads to loss of resolution.

Table 3.13 Development of an efficient method for production of α-mangostin using liquid flow processing

Running Code	Extract amount per injection (mg)	Instrument	Coil volume (mL)	Number of Injections	Resolution* Mean±SD	Total α-mangostin Isolated (mg)	Throughput Sample Process (g/h)	Throughput α-mangostin Isolated (g/h)	Purity Mean±SD (%)	Yield Mean±SD (%)	Recovery (%)
SM-19	22.8	Mini	17.4	5	1.82±0.18	30.6	0.06	0.02	98.82±0.18	93.68±1.67	> 99
SM-27A	22.0	Mini	17.4	5	1.66±0.20	31.3	0.05	0.02	98.09±0.61	97.86±2.78	> 99
SM-28	21.9	Mini	17.4	5	1.59±0.15	38.0	0.05	0.02	98.13±1.11	94.68±3.62	98.6
SM-29	178.3	Spectrum	143.5	5	2.34±0.10	164.1	0.43	0.08	99.19±0.34	95.43±2.97	91.1
SM-30	178.3	Spectrum	143.5	10	2.06±0.30	343.5	0.43	0.08	99.24±0.26	96.35±1.56	> 99
SM-33	358.7	Spectrum	143.5	2	2.24±0.11	43.4	0.86	0.05	99.34±0.43	99.61±0.55	> 99
SM-26	1281.1	Midi	912.5	5	1.73±0.09	1673.4	3.07	0.80	98.32±0.36	95.56±4.56	98.3
SM-28A	1273.8	Midi	912.5	7	1.63±0.18	2552.8	3.06	0.88	98.24±0.82	94.42±4.64	> 99
SM-34	708.6	Spectrum	143.5	3	1.26±0.06	195.1	1.7	0.16	96.38±1.48	94.97±2.14	43.6
SM-37	496.0	Spectrum	143.5	5	1.46±0.21	424.3	1.20	0.20	98.11±1.71	93.81±3.37	81.2

**Resolution of α-mangostin from component 3*

The fractograms at Figure 3.14 to Figure 3.20 described the CCC running during development of an efficient method for production of α-mangostin using CCC. The fractograms shows that α-mangostin was well separated using any scale of CCC apparatus from analytical to preparative. This can be determined from the values of resolution which are displayed on Table 3.13. All resolutions were acceptable, with value at least 1.59. Having a good resolution allowed increasing either the concentration or the volume of sample injected which means increasing the throughput of sample processing and production of the α-mangostin as well.

Figure 3.16 to Figure 3.18 shows that the peaks of α-mangostin looked different with the α-mangostin peaks obtained from samples stored in methanol. Usually α-mangostin peak has the highest peak in the fractogram as shown on Figure 3.3. However in Figs 3.16-3.18, the mangostin peak was smaller than target no 2 especially for the run SM-33 (figure 3.18) where the sample was the most unfresh one, having been stored for more than a week in LP. These mean that the ethanol extract of mangosteen was more unstable in LP than in methanol.

Table 3.13 also presents the purities of α-mangostin produced in one step using any scale of CCC machine. In general, the Spectrum HPCCC produced the highest purity of α-mangostin at >99% compared to those on Mini and Midi, which produced a bit lower roughly 98%. Although the separation was conducted with 10 injections on SM-30, the resolution was still very good. It can be seen on Figure 3.17. The α-mangostin was well separated from the other compounds with resolution value of 2.06 ± 0.30; and obtained very high purity at 99.24 ± 0.26 % with 96.35 ± 1.56% yield and nearly 100% recovery. This pure α-mangostin had purity acceptable for use as a reference standard for quality control of mangosteen based products and also for marker quantitative analysis and standardization of the raw materials and preparations from mangosteen plant (Pothitirat and Gritsanapan, 2009). This is very important since mangosteen is one of the medicinal plants where components often depend on the place where it grew.

Based on the results of Spectrum HPCCC on SM-30, the concentration of sample injected was increased 2 times to 358.7 mg (SM-33). Two injections with this twice higher concentration obtained an equal percentage of purity at 99.34 ± 0.43 % with a higher yield

at 99.34 ± 0.43%. So, the sample injected was then increased again with a larger amount of 496 mg extract in 7 mL with 5 injections (SM-37) and 708.6 mg extract in 10 mL with 3 injections (SM-34). The experiments resulted α-mangostin isolated with the purity 98.11% and 96.38% by HPLC peak area and the yield 93.81% and 94.97% respectively. However the recovery decreased to 81.2% on SM-37 and 48.6% on SM-34. It can be concluded that the production of α-mangostin in preparative scale using Spectrum HPCCC with all conditions as mentioned above was optimum and α-mangostin was obtained with high purity, yield and recovery when injecting a sample with the concentration approximately 450 mg / 7 mL (65 mg/mL) aqueous ethanol extract of the fruit rind of *Garcinia mangostana* L in LP with maximum volume of 10 mL. α-Mangostin with the purity at minimum 98% is acceptable to be used as a reference standard for quality control of mangosteen based products. This method was also efficient because up to 5 injections were possible without replacement or topping up the stationary phase, the separation was kept in stable with acceptable resolution value at 1.46±0.21.

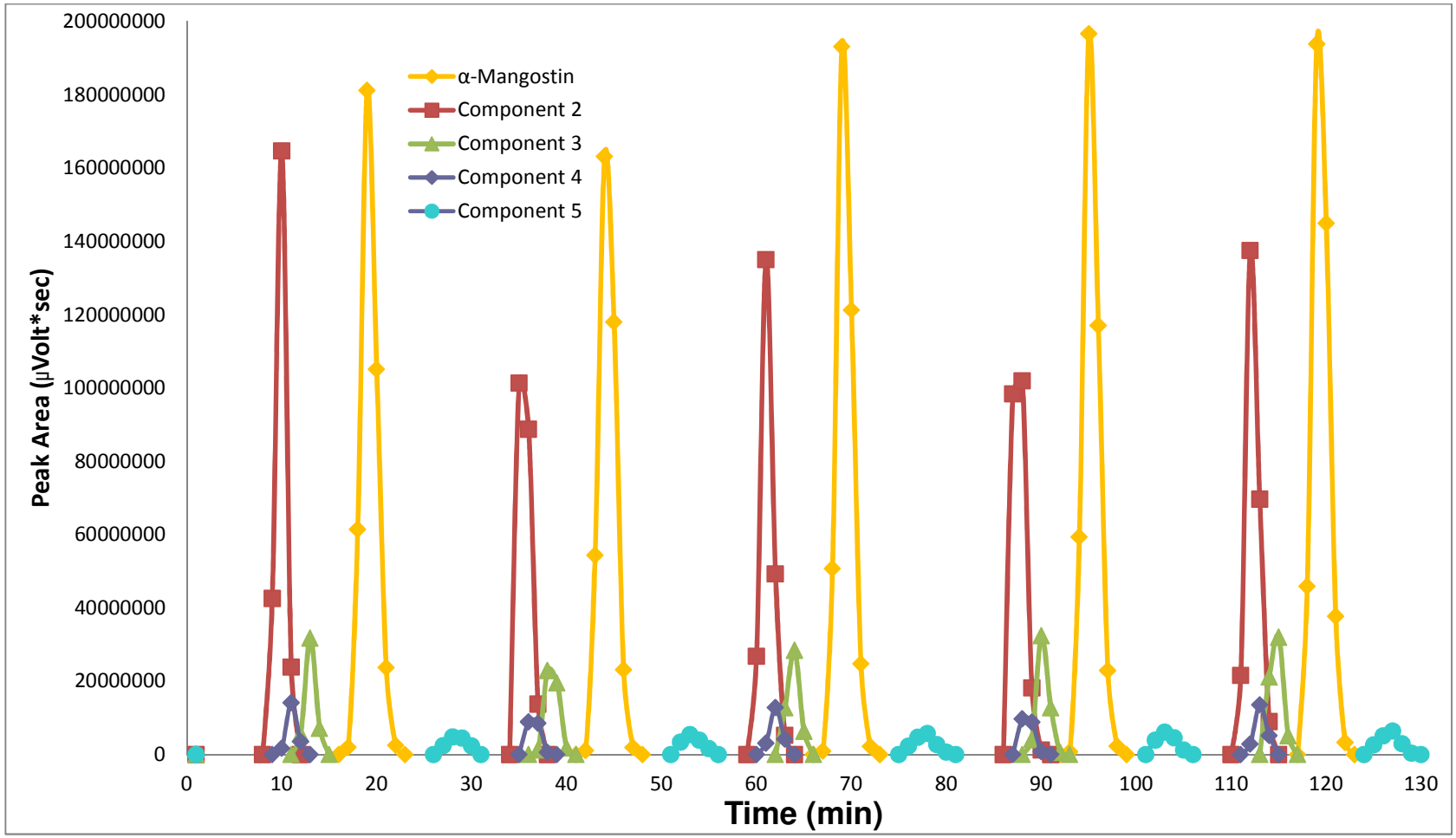

Figure 3.14 Fractogram of SM-27A; Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100 rpm; 25°C; 1 mL/min; 5 consecutive injections of the same amount of 22.0 mg ethanol extract in 0.86 mL without any charge of the SP with initial $\%S_F$=66% and final $\%S_F$=60%

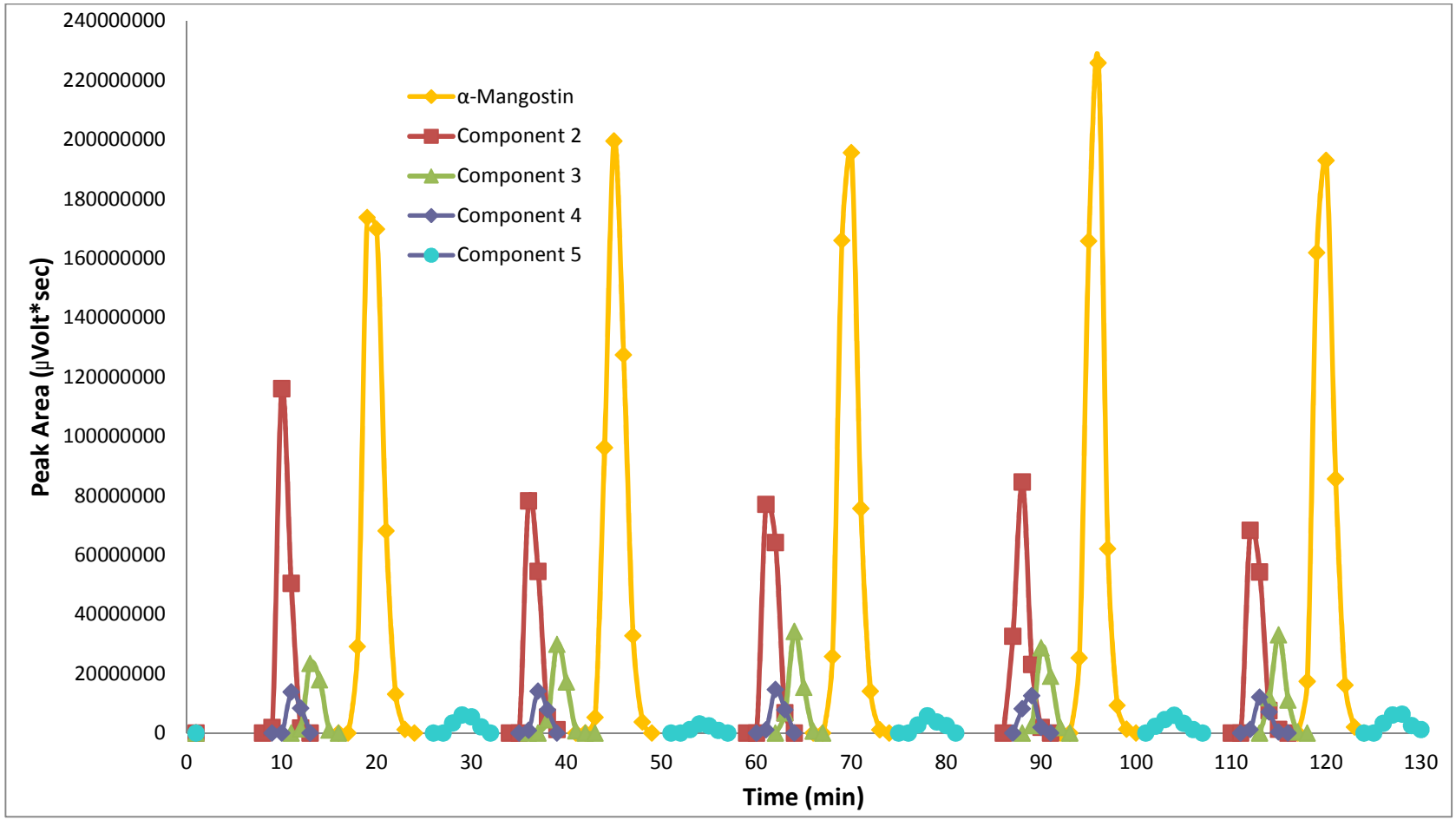

Figure 3.15 Fractogram of SM-28; Mini-17.4 mL; HEMWat (5:5:10:4 v/v); RP; 2100rpm; 25°C; 1 mL/min; 5 consecutive injections of the same amount of 21.9 mg ethanol extract in 0.86 mL LP without any charge of the SP with initial $\%S_F$=69% and final $\%S_F$=61%

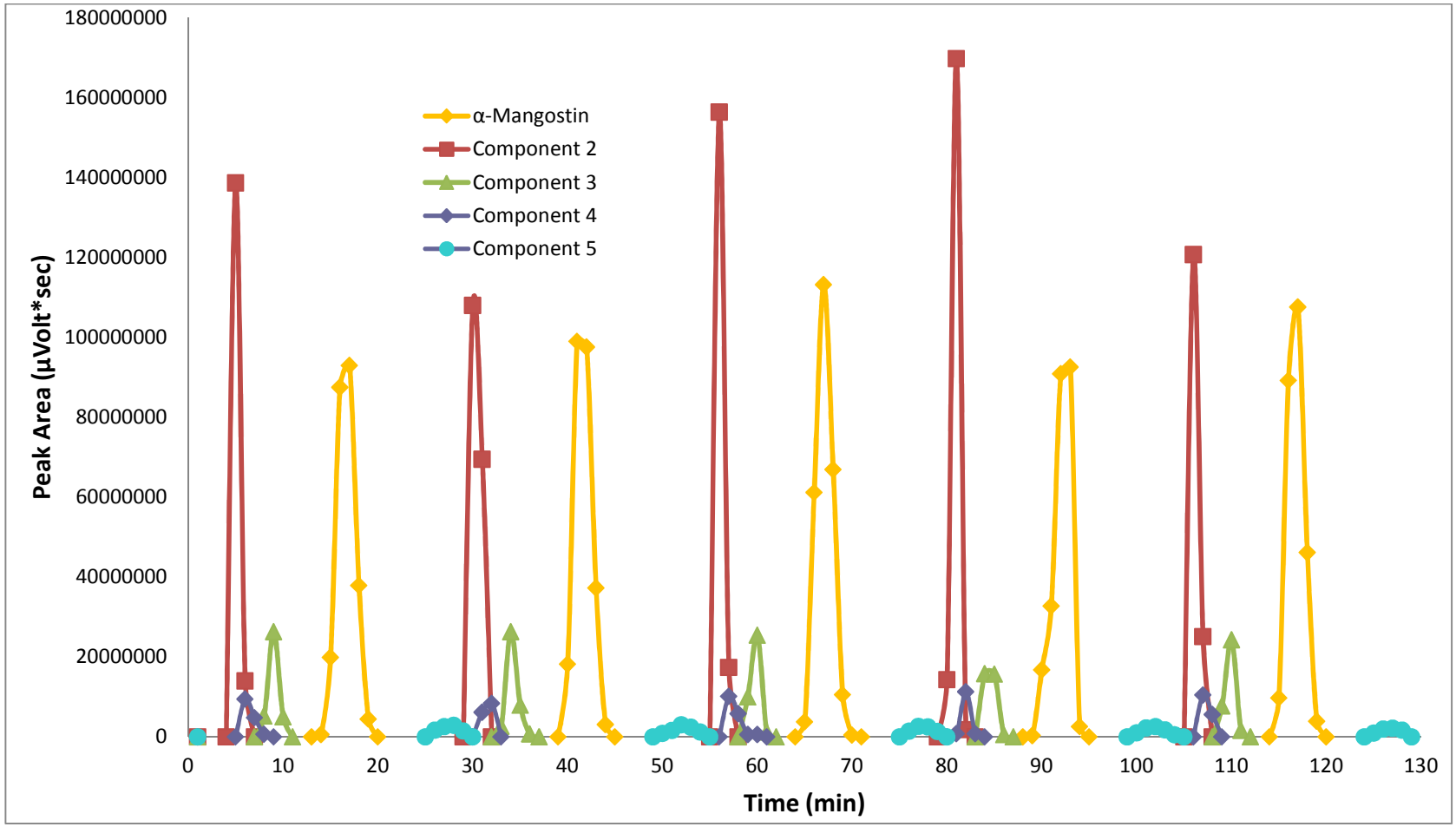

Figure 3.16 Fractogram of SM-29; Spectrum-143.5 mL; HEMWat (5:5:10:4 v/v); RP-1600 rpm; 25°C; 8 mL/min; 5 consecutive injections of the same amount of 178.3 mg ethanol extract in 7 mL LP without any charge of the SP with initial $\%S_F$=93% and final $\%S_F$=79%

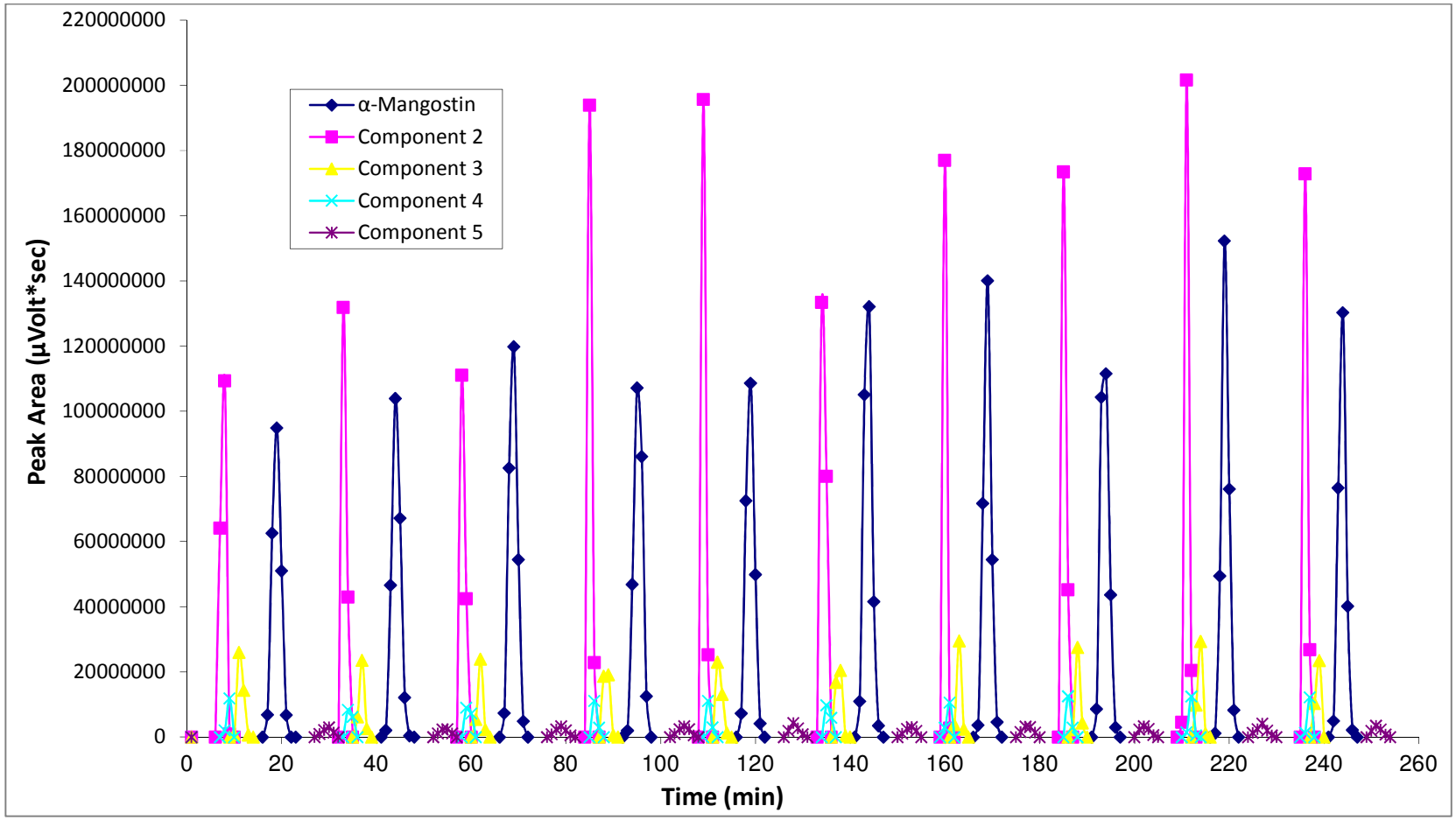

Figure 3.17 Fractogram of SM-30; Spectrum-143.5 mL; HEMWat (5:5:10:4 v/v); RP-1600 rpm; 25^{o}C; 8 mL/min; 10 consecutive injections of the same amount of 178.3 mg ethanol extract in 7 mL LP without any charge of the SP with initial $\%S_F$=77% and final $\%S_F$=63%

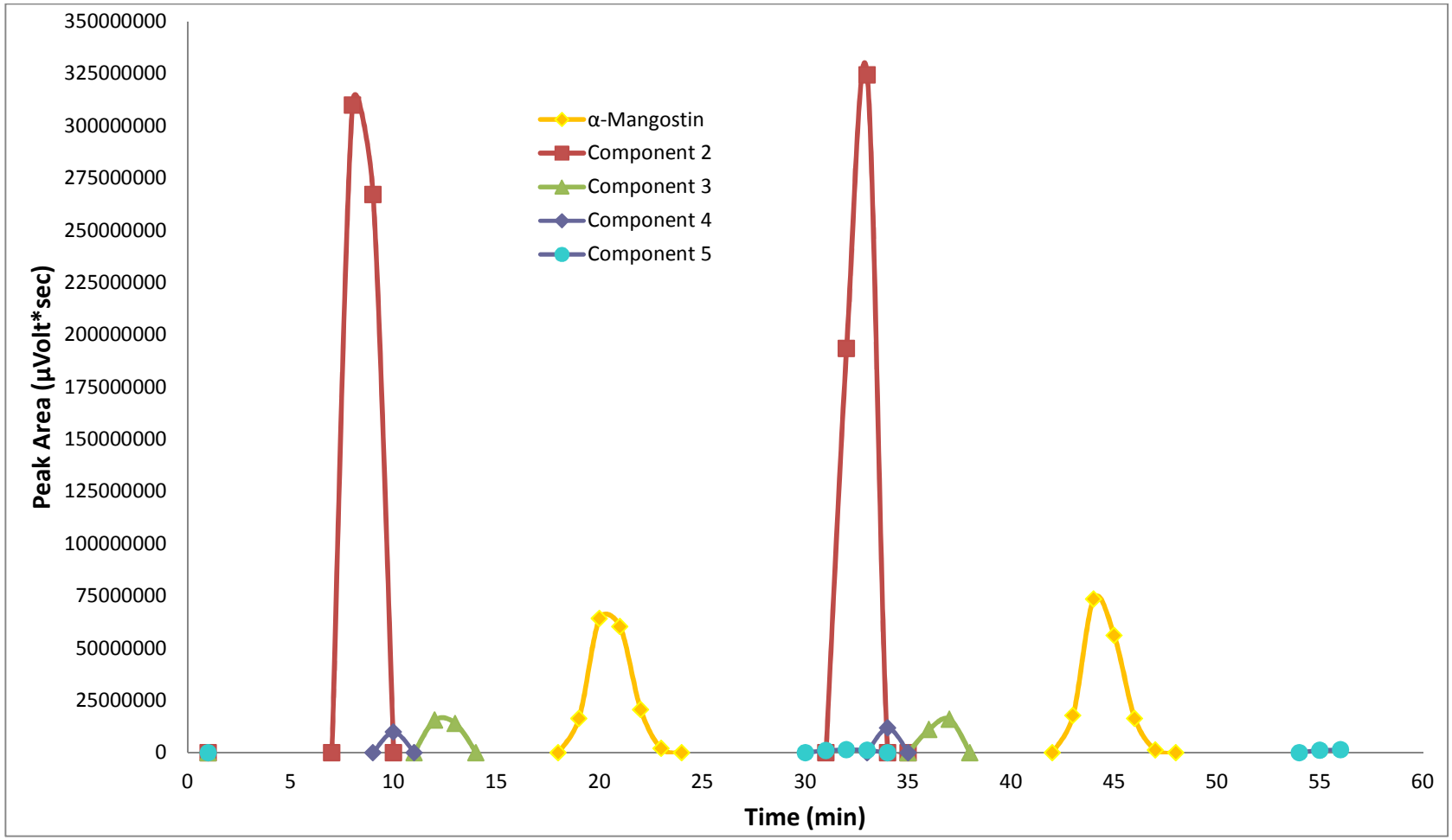

Figure 3.18 Fractogram of SM-33; Spectrum-143.5 mL; HEMWat (5:5:10:4 v/v); RP; 1600 rpm; 25°C; 8 mL/min; 2 consecutive injections of the same amount of 358.7 mg ethanol extract in 7 mL LP without any charge of the SP with initial $\%S_F$=81% and final $\%S_F$=68%

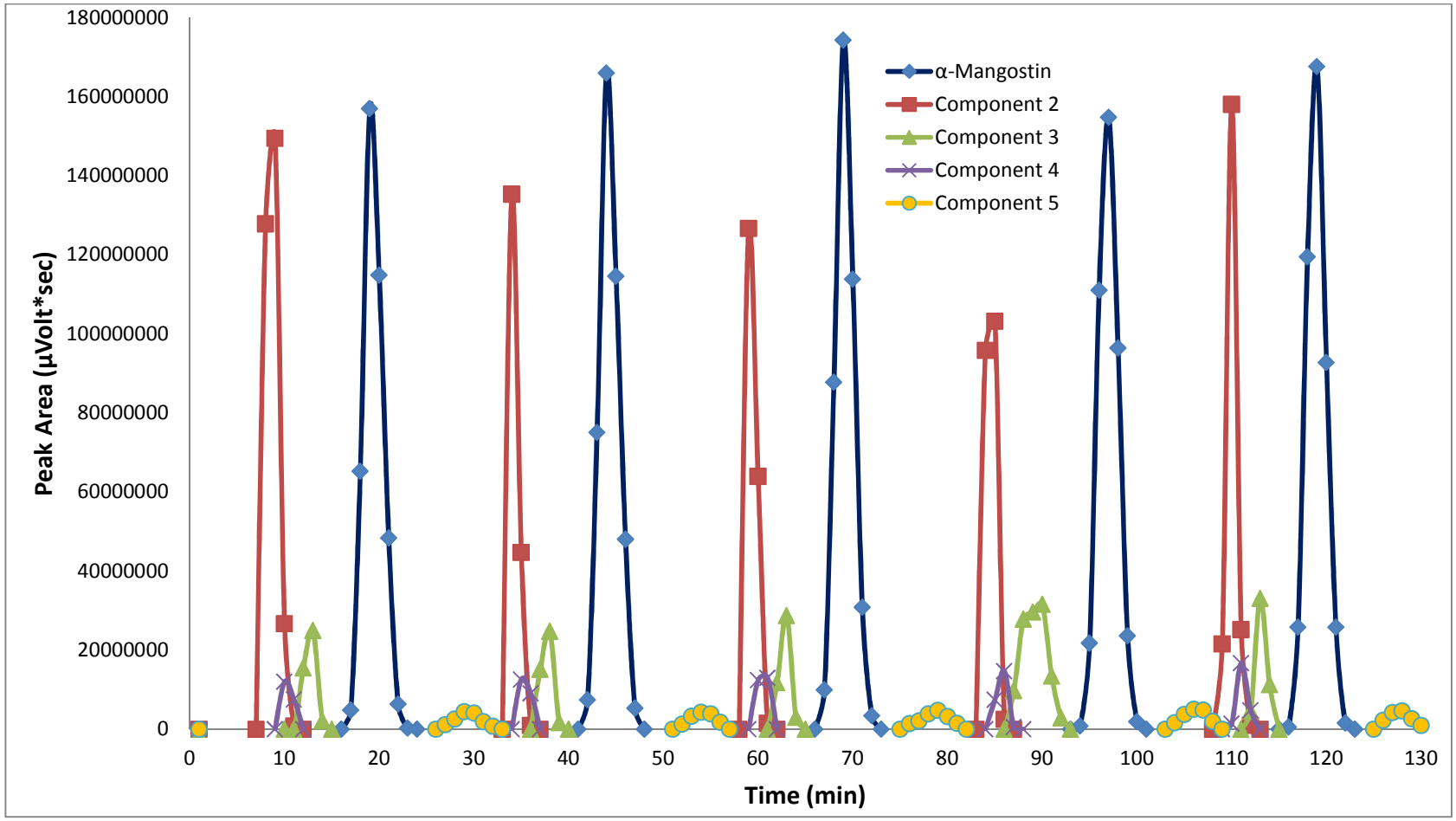

Figure 3.19 Fractogram of SM-26; Midi-912.5 mL; HEMWat (5:5:10:4 v/v); RP; 1400 rpm; 25°C; 50 mL/min; 5 consecutive injections of the same amount of 1281.1 mg ethanol extract in 50 mL LP without any charge of the SP with initial $\%S_F$=85% and final $\%S_F$=57%

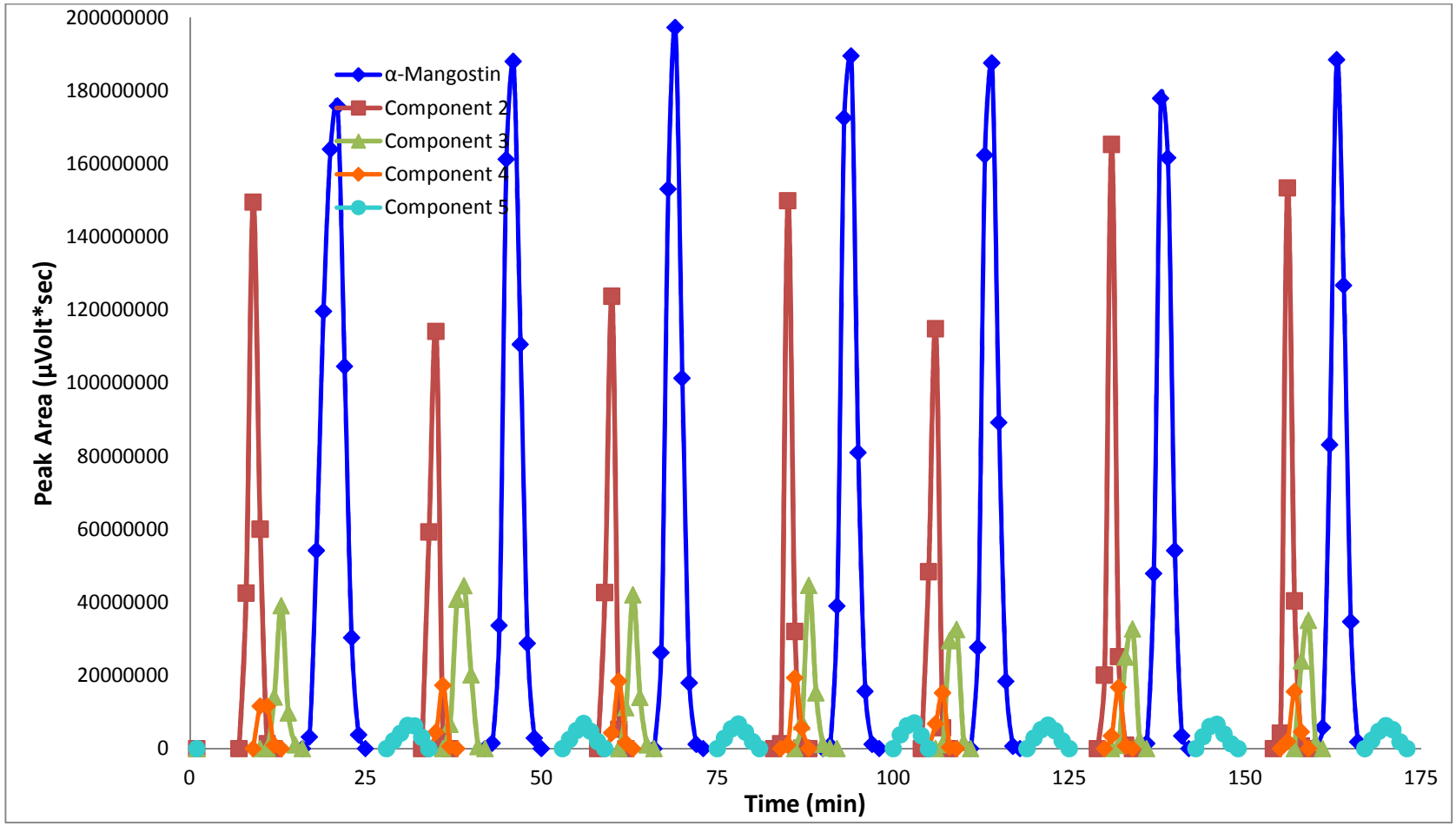

Figure 3.20 Fractogram of SM-28A; Midi-912.5 mL; HEMWat (5:5:10:4 v/v); RP; 1400 rpm; 25°C; 50 mL/min; 7 consecutive injections of the same amount of 1273.8 mg ethanol extract in 50 mL LP without any charge of the SP with initial $\%S_F$=82% and final $\%S_F$=58%

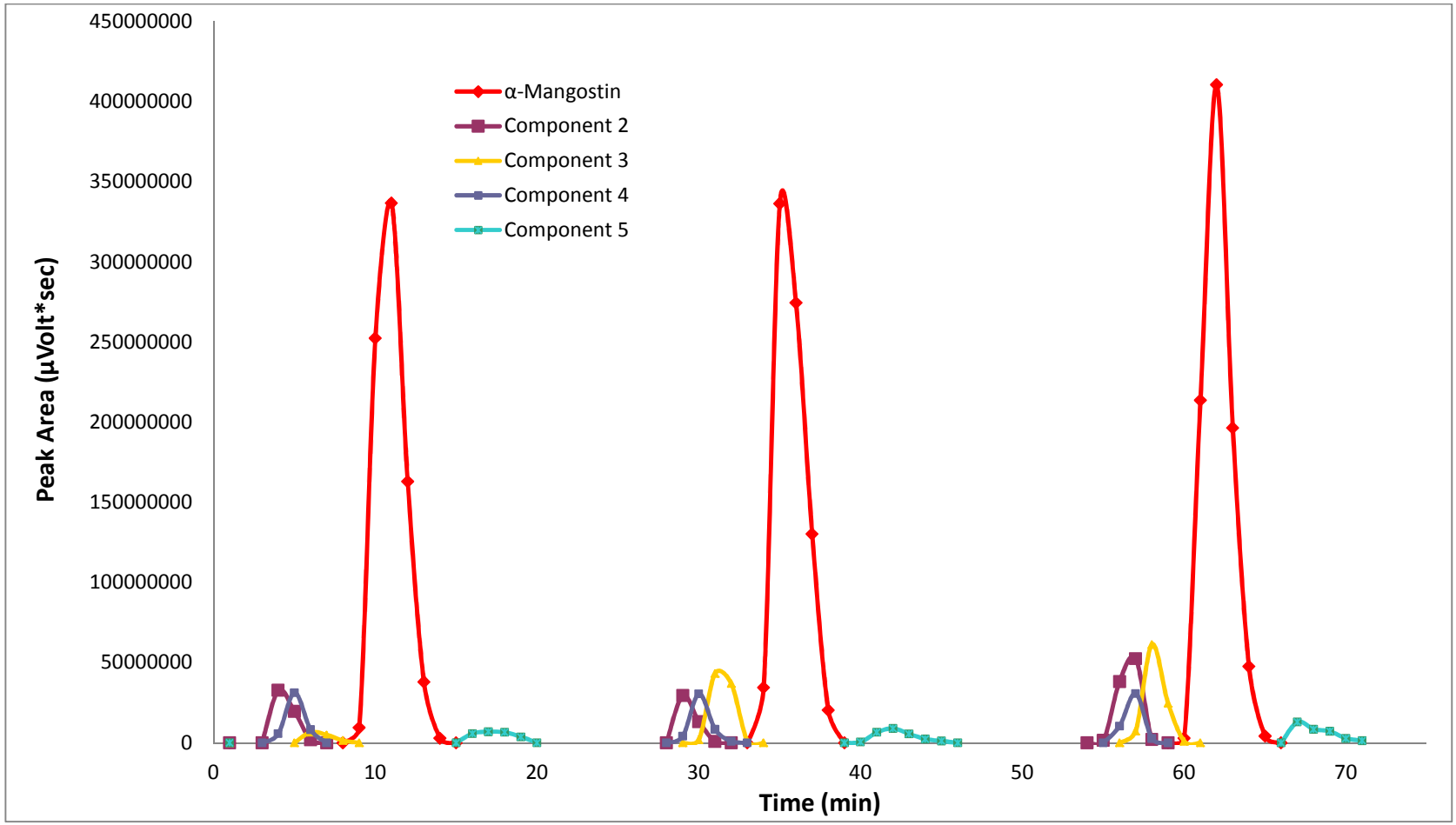

Figure 3.21 Fractogram of SM-34; Mini-143.5 mL; HEMWat (5:5:10:4); RP; 1600 rpm; 25°C-8mL/min; 3 consecutive injections of the same amount of 708.6 mg ethanol extract in 10mL LP without any charge of the SP with initial $\%S_F$=76% and final $\%S_F$=38%

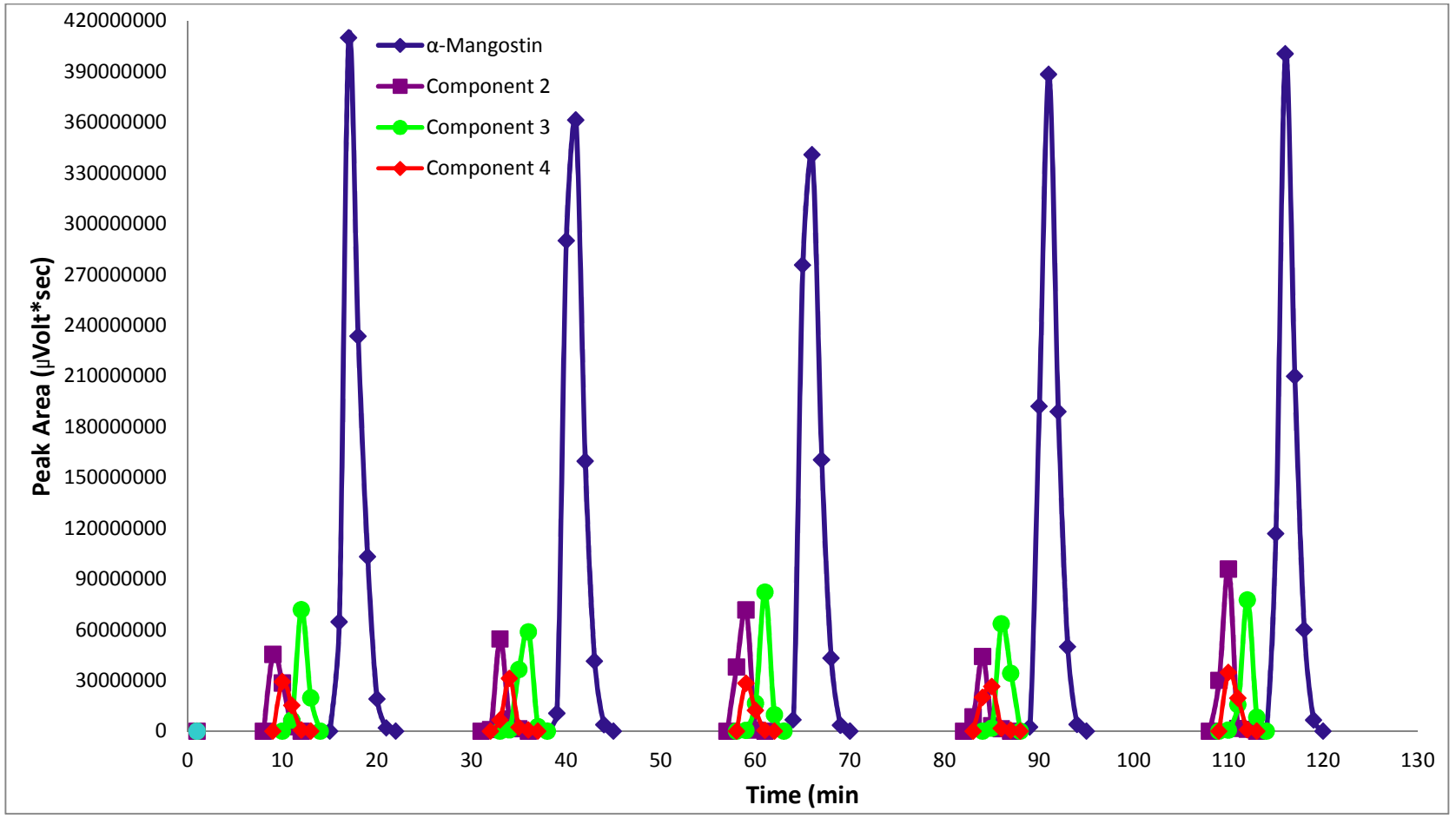

Figure 3.22 Fractogram of SM-37; Mini-143.5 mL; HEMWat (5:5:10:4); RP; 1600 rpm; 25°C-8mL/min; 5 consecutive injections of the same amount 496 mg ethanol extract in 7mL LP without any charge of the SP with initial $\%S_F$=90% and final $\%S_F$=61%

Identification and characterisation of isolated α-mangostin by NMR was provided and interpreted by Kingston University, London. The identified sample was from SM 23 which was run with a single injection on Mini. It was a combined pure fractions (F20 and F21) which had a single peak on analytical HPLC. (Figure 3.23 and 3. 24) ^{13}C-NMR and ^{1}H-NMR on fractions 20-21 which ran similarly to an α-mangostin commercial reference standard, indicated α-mangostin. There was a ketone region at the spectral line of 183.1690 ppm, the six spectral lines in the 15 – 30 ppm. range suggesting –CH_2 or –CH_3 carbons. The spectral line at 61.9 ppm indicated the presence of a –CH_2 or –CH_3 attached to an oxygen while spectral lines in the 90 – 155 ppm range indicated the presence of alkenes, benzenes or other hetero aromatics. The spectral lines that show up in the 160 – 170 ppm range indicated the presence of an anhydride of some description or a carbon directly bonded to oxygen. This spectrum also showed a 24 carbon structure, which fits with α-mangostin.

The spectral lines in the 1.6 – 1.8 ranges from proton spectrum suggested the presence –CH_3 protons. The three spectral lines in the 3.0 – 4.2 ppm range suggested the presence of carbons bonded to nitrogen, oxygen or a halogen; although, in this situation, it was likely to represent a carbon bonded to oxygen. The large multiplet at 5.2 ppm could represent a –CH-O or –CH-Halogen pairing, however it could also represent a non-conjugated alkene. The spectral lines between 6 – 8 ppm suggested protons in the aromatic region while the spectral line at 13.5 ppm suggested the presence of aldehydes.

Looking at the COSY spectrum and HSQC spectrum for EKSM23F2021 and compared to the standard, it was finally concluded that the sample had chemically structure of α-mangostin as follows:

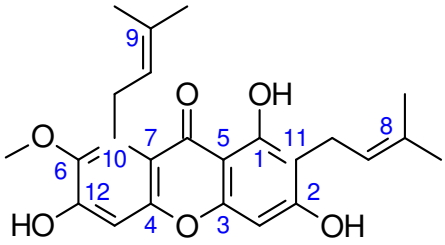

Figure 3.23 Chemical structure of EKSM23F2021 on NMR

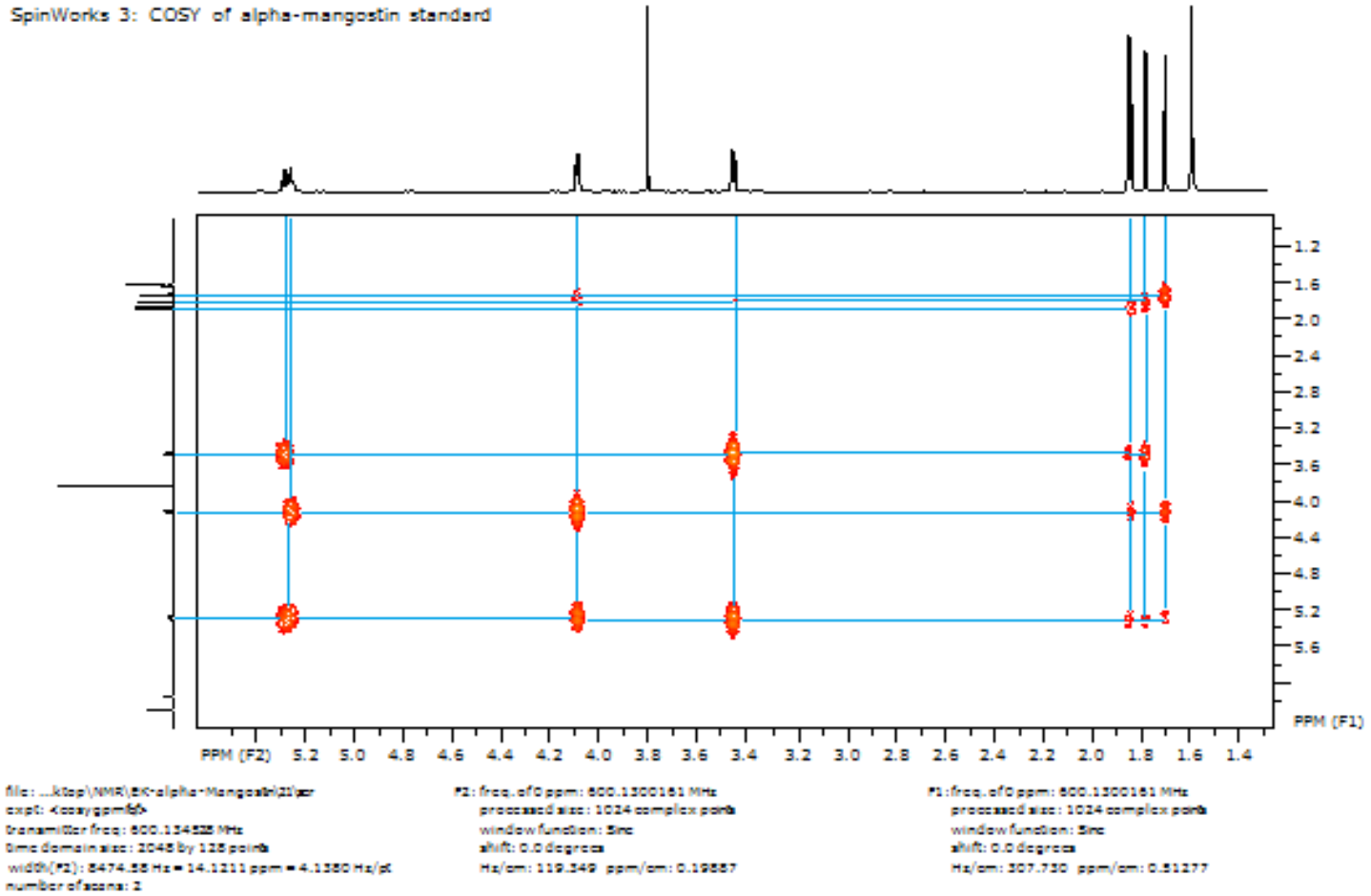

Figure 1 COSY spectrum of a-mangostin standard in chloroform

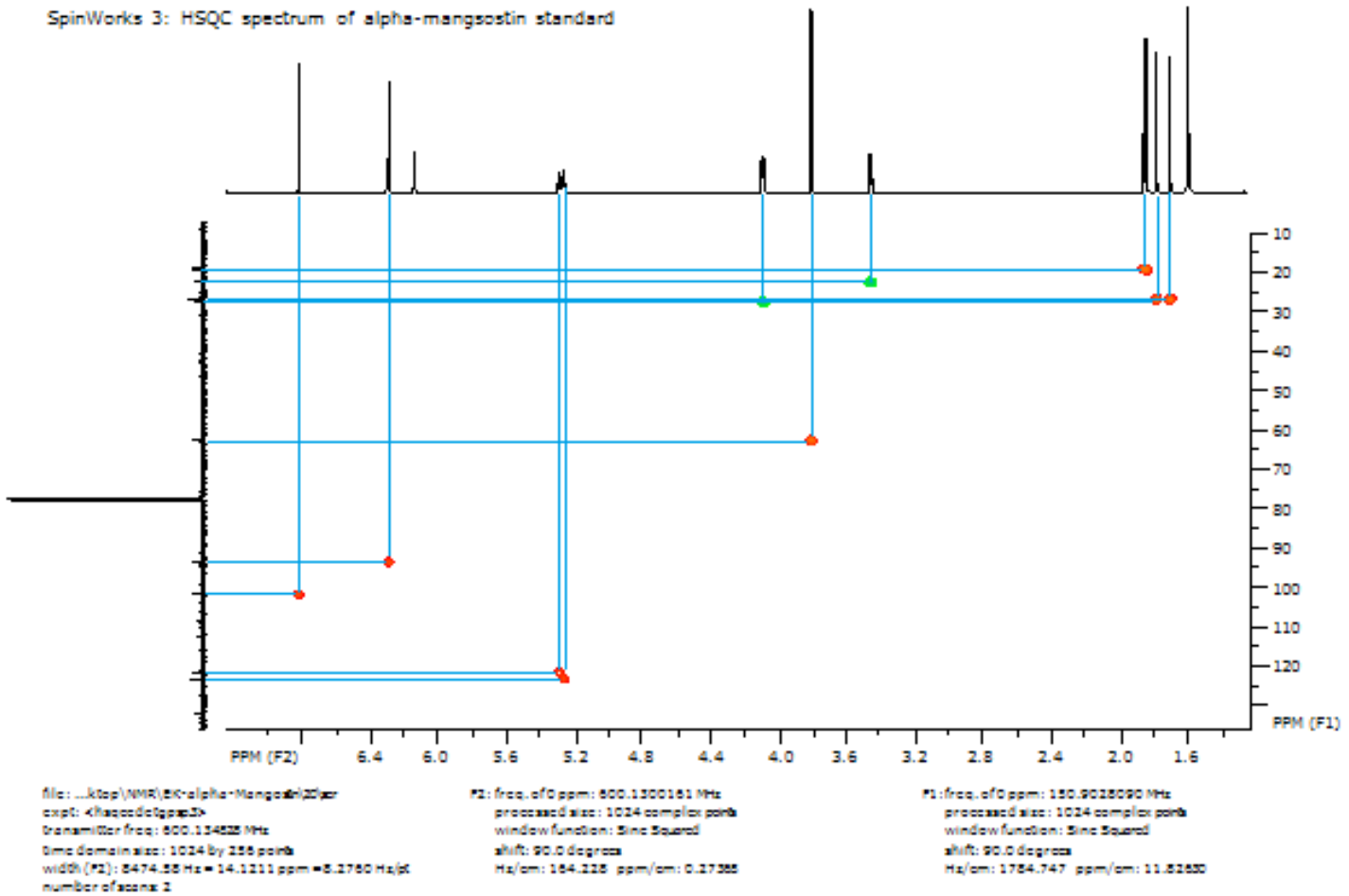

Figure 3.25 HSQC spectrum of a-mangostin standard in chloroform

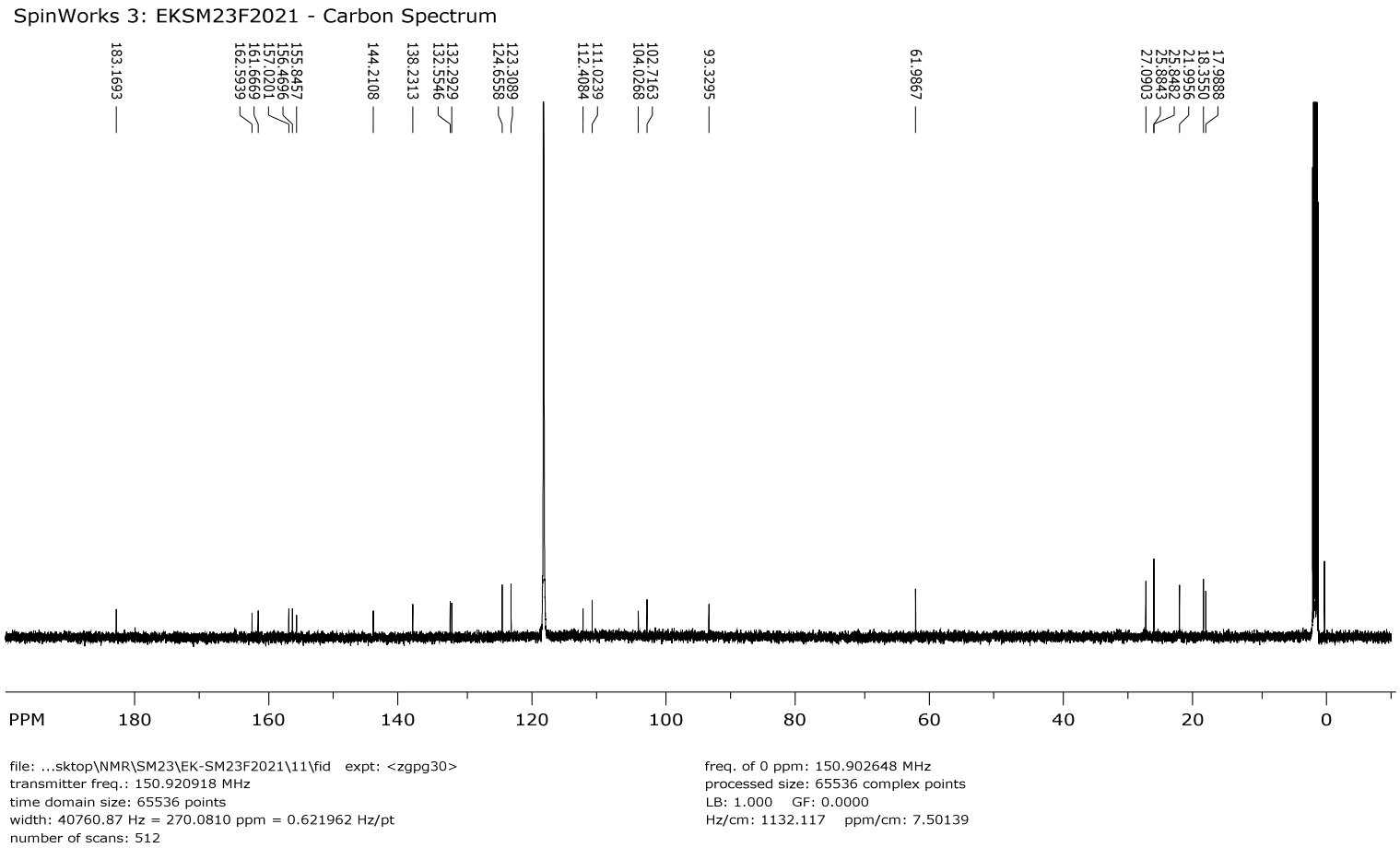

Figure 3.26 Carbon spectrum of EKSM23F2021 which had a single peak on HPLC was identified as α-mangostin on NMR

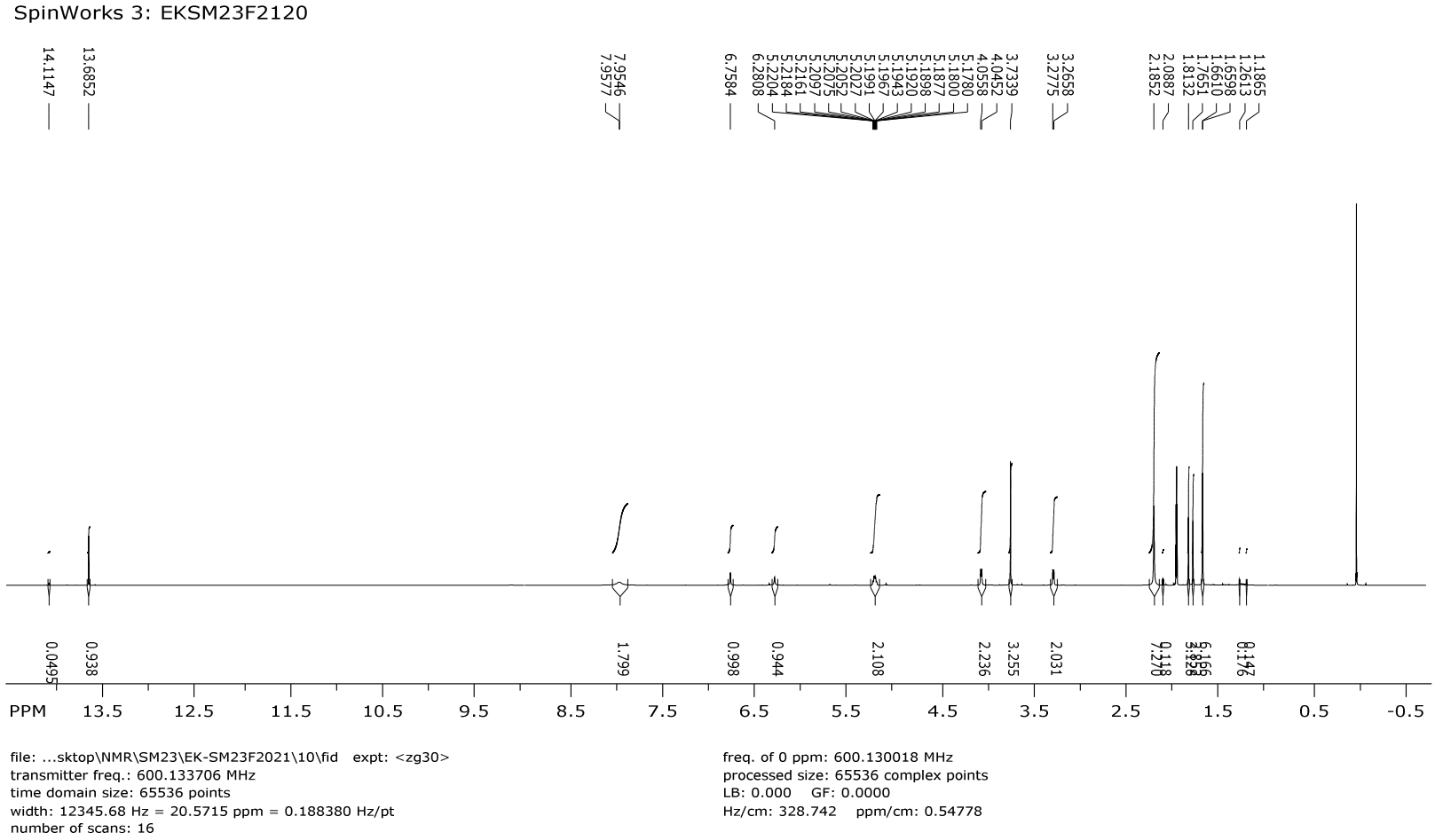

Figure 3.27 Proton spectrum of EKSM23F2021 which had a single peak on HPLC was identified as α-mangostin on NMR

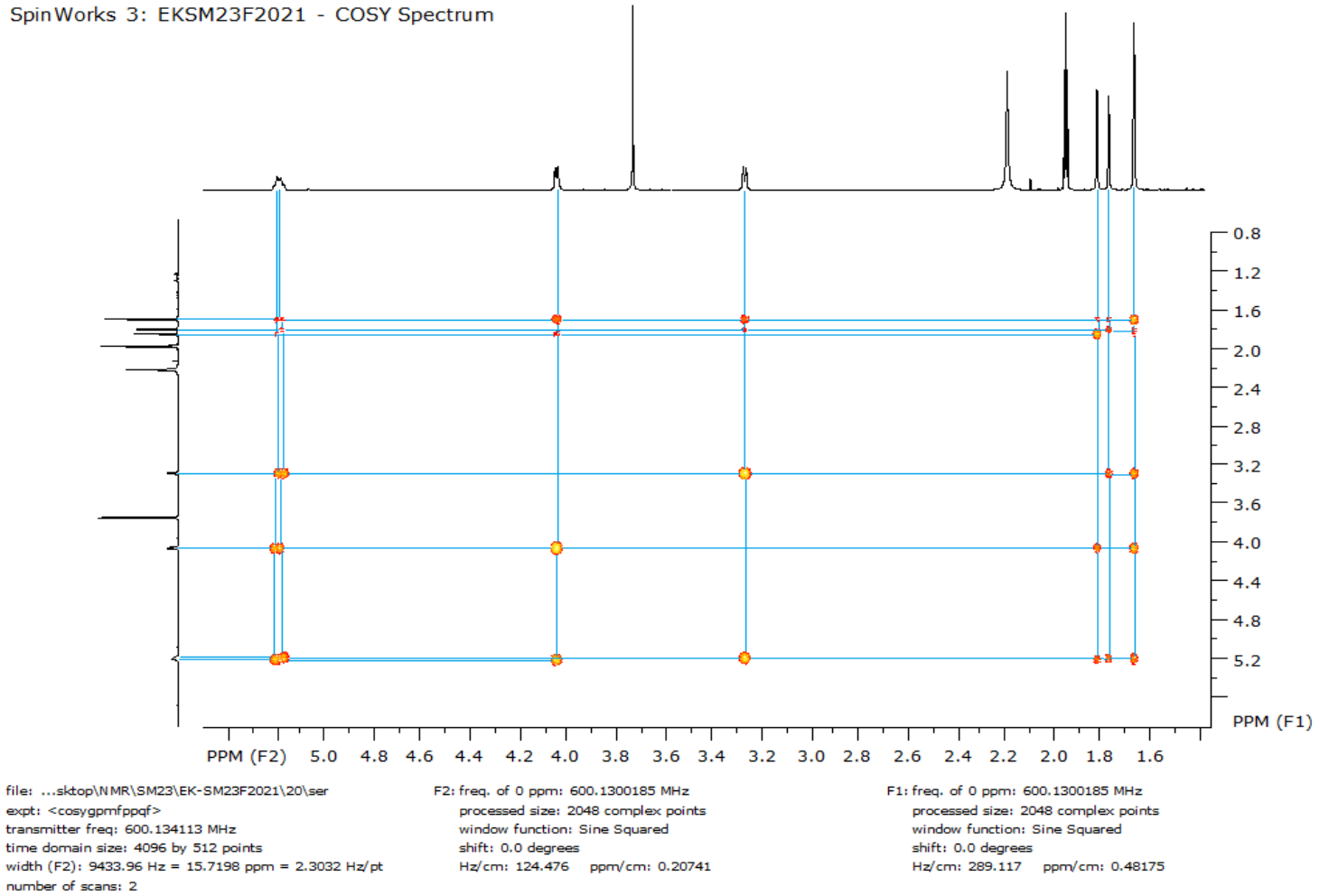

Figure 3.28 COSY spectrum of EKSM23F2021

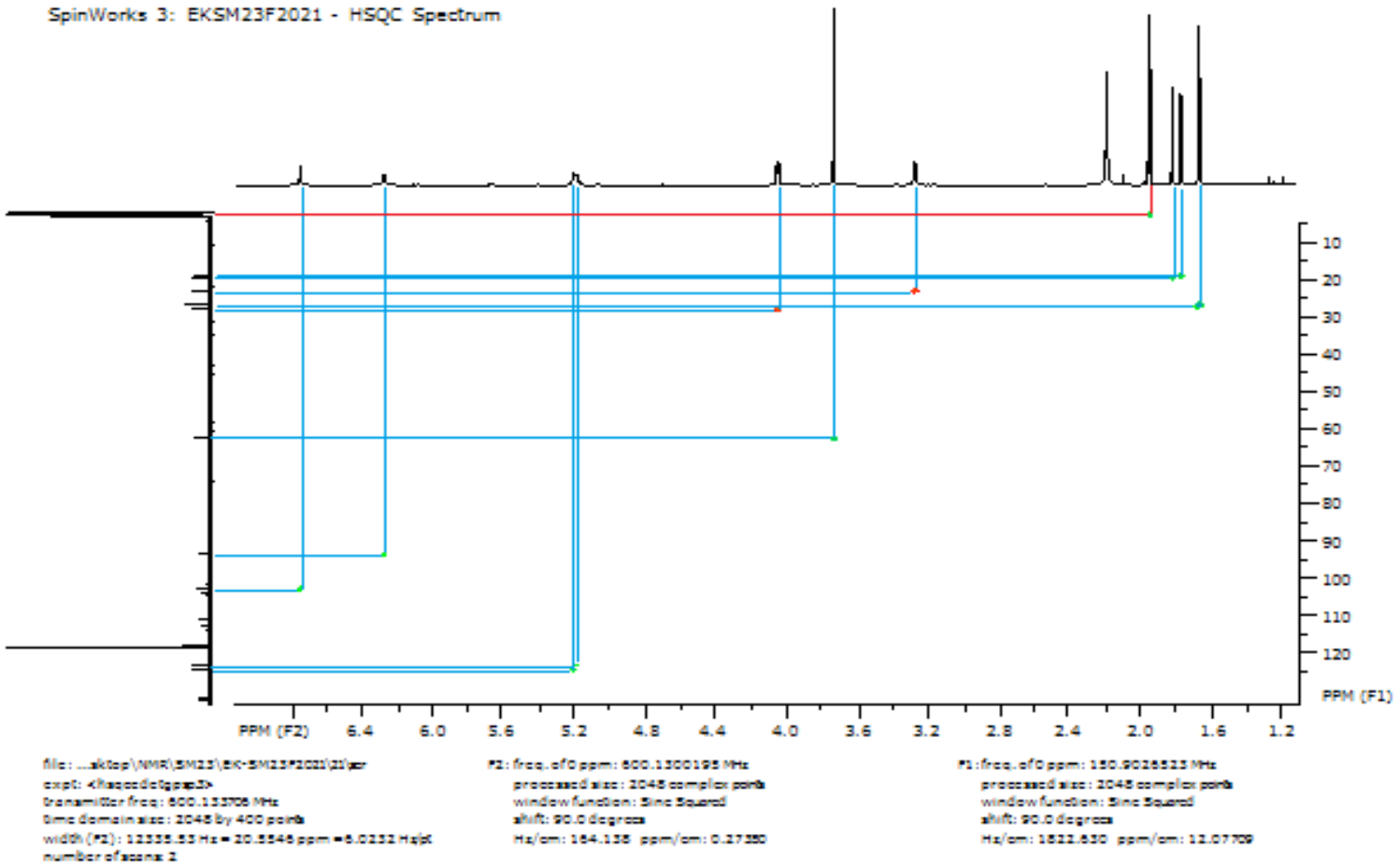

Figure 3.29 HSQC spectrum of EKSM23F2021

Chapter 4 SUMMARY AND CONCLUSIONS

4.1 SUMMARY

Table 4.1.1 Optimum Conditions for Analysis of α-mangostin on HPLC

No	HPLC Parameters	Optimum Conditions of HPLC
1	Column	Agilent Zorbax Bonus RP C_{18} with dimension (3.5um; 4.6x150mm)
2	Temperature	40 °C
3	Flow rate	0.5 mL/min
4	Mobile phase	Methanol with 0.05% TFA (C) Water with 0.05% TFA (D)
5	Elution program	Gradient system: 0-3 min: 95-100% C; 3-8 min: 100% C;
6	Run time	12 min
7	Delay time between injection	2 min
8	Collecting data	12 min
9	Wave length detection	PDA Max Plot 210 nm to 400 nm
10	Low limit of detection (LLOD)	0.000008 µg/µL (8 ppm)
11	Retention time of α-mangostin (min)	6.00 - 6.50

Table 4.1.2 Optimum conditions of sample loading study

No	Parameters	Optimum Conditions
1	Instrument	Analytical Mini HPCCC
2	Column volume	17.4 mL
3	Bore	0.8 mm
4	Solvent system	HEMWat (5:5:10:4 v/v)*
5	Stationary phase	Upper phase
6	Mobile phase	Lower phase

7	Elution mode	Reverse phase
8	Rotational speed	2100 rpm
9	Flow rate	1 mL/min
10	Temperature	25 ºC
11	Run time per injection	25 min
12	Optimum amount of sample injected (mg)	22.8
13	Volume of sample (mL)	0.86
14	Number of injections	Up to 10 times

*H stands for hexane

Table 4.1.3 Optimum Conditions for Production of α-mangostin using HPCCC

No	Parameters	Spectrum HPCCC	Midi HPCCC
1	Coil volume (mL)	143.5	912.5
2	Solvent system	HEMWat (5:5:10:4)	HEMWat (5:5:10:4)
3	Flow rate (mL/min)	8	50
4	Running Mode	Reverse phase	Reverse phase
5	Temperature (ºC)	25	25
6	Rotational speed (rpm)	1600	1400
7	Elution time /injection (min)	25	25
8	Number of injections	2-10	5-7
9	Extract amount per injection (mg)	358.7	1281.1
10	Sample volume per injection (mL)	7	50
11	Throughput α-mangostin Isolated (g/h)	0.05	0.88

4.2 CONCLUSIONS

It can be concluded that an efficient method for production of α-mangostin using liquid flow processing on HPCCC with multiple injections using optimum condition as mentioned above was as follows:

1. Multiple injections without replacement or topping up the stationary phase. This could save solvent use especially on stationary phase equal to the volume of stationary phase used for the dynamic equilibration multiplied by the following injections number. This means a lower cost of production.
2. Reduce time consuming compared to single injection as existing method since any number of injections only needs once column equilibration and once column empty/washing. So, it should be faster to isolate the same amount of α-mangostin with the developed method.
3. In this research, the most efficient method for production of α-mangostin using liquid flow processing was on semipreparative HPCCC with the optimum concentration of sample injected approximately 65 mg/mL with maximum volume of 10 mL per injection.

The prediction of solvent system and time consumed on various HPCCC instruments can be seen on the Table 4.1.4 and Table 4.1.5 below:

Table 4.1.4 Prediction of solvent system consumed on various HPCCC instruments

No	Parameters	10 continous injections		
		Mini	Spectrum	Midi
1	Coil volume (mL)	20	22 and 132	1000
2	Stationary Phase saving for column equilibration (mL)	9x20=180	9x154=1,386	9x1000=9,000
3	Mobile Phase saving for column equilibration (mL)	9x20=180	9x100=900	9x700=6,300
	Total solvent saving (mL)	360	2,286	15,300

Table 4.1.5 Prediction of time consumed on various HPCCC instruments

No	Parameters	10 continous injections		
		Mini	Spectrum	Midi
1	Time saving for column equilibration (min)	9x15=135	9x15= 135	9x15=135
2	Time saving for column empty (min)	9x10=90	9x30=270	9x30=270
3	Time saving for column washing (min)	9x5=45	9x10=90	9x10=90
4	Total time saving (hours)	4.5	8.25	8.25

4.3 FUTURE WORK

The future work that can be recommended regarding this project includes:

1. Scale up for production of α-mangostin on preparative and industrial scale, based on the optimum concentration of injected sample on Spectrum HPCCC.

2. Separation and isolation of component 4, which had the same UV spectrum but different retention time to α-mangostin. The isolated compound can then be assayed for the bioactivities as for α-mangostin because having the same UV active component may have similar bioactivities. It might be an isomer of mangostin.

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APPENDIX

Appendix 1

Authenthication certificate for fruit rinds of *Garcinia mangostana* L.

UNIVERSITAS GADJAH MADA
FAKULTAS BIOLOGI
LABORATORIUM TAKSONOMI TUMBUHAN
Jl. Teknika Selatan Sekip Utara Yogyakarta 55281, Telp. (0274) 6492272/6492262; Fax: (0274) 580839

SURAT KETERANGAN
Nomor : 0300 /T.Tb. /I/ 2012

Yang bertanda tangan dibawah ini, Kepala Laboratorium Taksonomi Tumbuhan Fakultas Biologi UGM, menerangkan dengan sesungguhnya bahwa :

Nama : Muhammad Bachroni
Alamat : Jl. Bantul N0.391 Dongkelan Yogyakarta

telah melakukan identifikasi tumbuhan dengan hasil sebagai berikut :

NO.	FAMILIA	GENUS	SPECIES	NAMA DAERAH
1.	Clusiaceae	Garcinia	*Garcinia mangostana* L.	Manggis

Identifikasi tersebut dibantu oleh Drs. Heri Sujadmiko, M.Si.
Demikian surat keterangan ini diberikan untuk dapat dipergunakan seperlunya.

Yogyakarta, 9 Januari 2012

Mengetahui,
Dekan Fakultas Biologi
Universitas Gadjah Mada

Dr. Retno Peni Sancayaningsih, M.Sc.
NIP. 195509291982032002

Kepala Laboratorium
Taksonomi Tumbuhan
Fakultas Biologi UGM

Drs. Heri Sujadmiko, M.Si.
NIP. 19640209 199103 1001

Appendix 2

Product specification of α-mangostin reference standard purchased from Sigma Aldrich, UK.

SIGMA-ALDRICH

sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Product Specification

Product Name:
α-Mangostin – ≥98% (HPLC)

Product Number:	**M3824**
CAS Number:	6147-11-1
Formula:	C24H26O6
Formula Weight:	410.46 g/mol
Storage Temperature:	2 - 8 °C

TEST	Specification
Appearance (Color)	Faint Yellow to Yellow
Appearance (Form)	Solid
Solubility (Color)	Colorless to Very Light Yellow
Solubility (Turbidity) 1 mg/mL, MeOH	Clear
Proton NMR spectrum	Conforms to Structure
Purity (HPLC)	≥ 98 %

Specification: PRD.0.ZQ5.10000011193

Appendix 3

Abstract accepted for Research Student Poster Conference 2012, 13-14 March 2012, Brunel University London, UK

ABSTRACT

Reference Standards for Indonesian Herbal Medicines: Isolation and Purification of α-Mangostin from *Garcinia mangostana* L. Rinds using Liquid Flow Processing

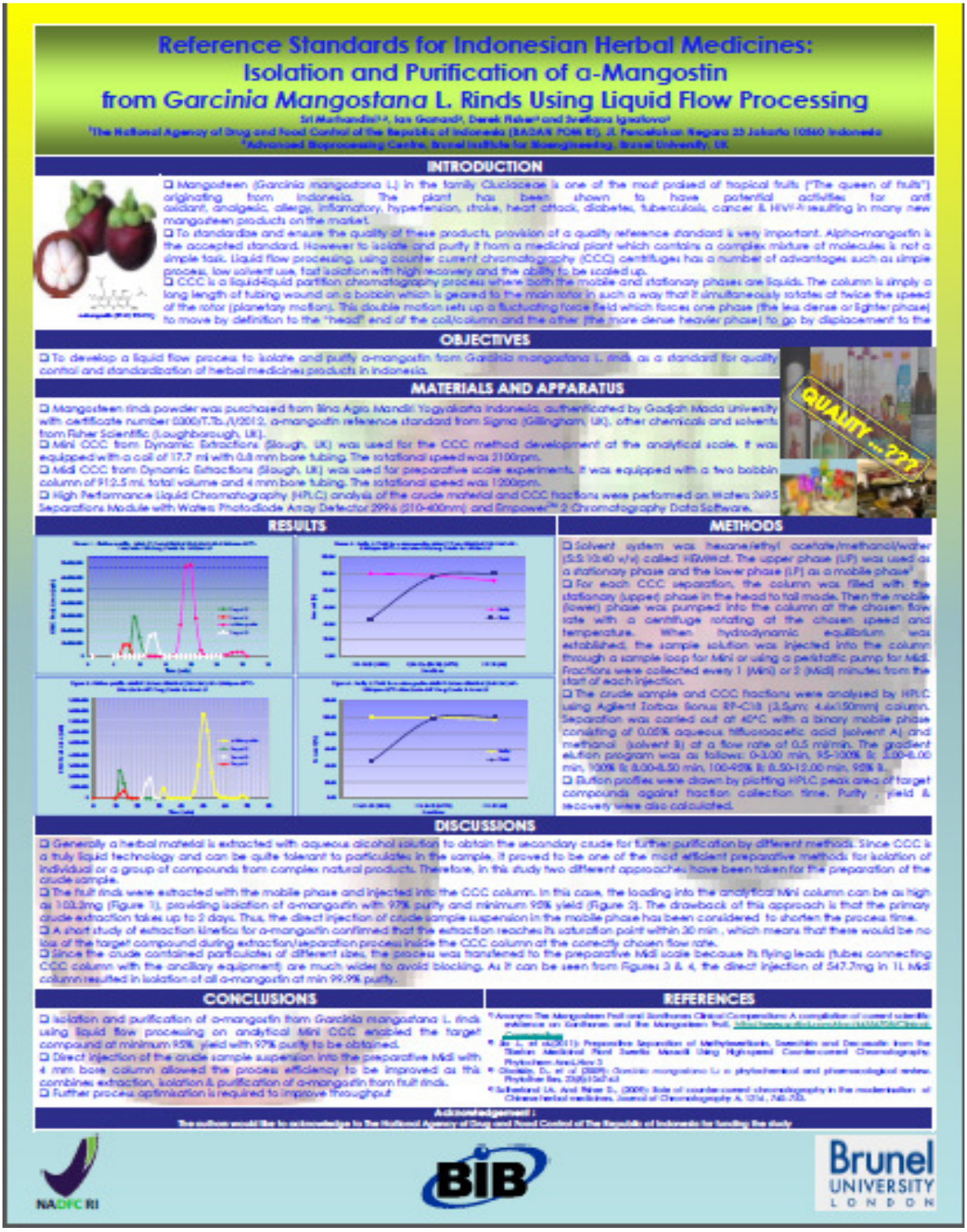
Sri Murhandini[1,2], Ian Garrard[2], Derek Fisher[2], Svetlana Ignatova[2]
1. The National Agency of Drug and Food Control of the Republic of Indonesia
2. Advanced Bioprocessing Centre, Brunel Institute for Bioengineering, Brunel University, UK

Garcinia mangostana L., a medicinal plant originated from Indonesia, has been shown to have potential activities for anti bacterial, inflammation, diabetes, allergy, cancer and HIV, resulting in many new mangosteen products appearing on the market. To standardize and ensure the quality of these products, provision of a quality reference standard is very important. This study aimed to isolate and purify α-mangostin from *Garcinia mangostana* L. Rinds using liquid flow processing known as counter current chromatography (CCC). The methodology was first developed on an analytical scale Mini instrument (17.7ml) using primary and pre-treated crude material. Further scale up to preparative Midi scale (1L) allowed direct injection of crude particulate matter into the CCC column. The results showed that the α-mangostin can be isolated at minimum 95% yield with 95% purity.

Key words:
α- mangostin, reference standards, isolation, purification, CCC

Appendix 4

Poster presented in Research Student Poster Conference 2012, 13-14 March 2012, Brunel University London, UK



Appendix 5

Abstract accepted for International Conference on Counter Current Chromatography 2012, 6-8 August 2012, Hangzhou, China

METHOD OPTIMISATION FOR ISOLATING PURIFIED α-MANGOSTIN FROM *GARCINIA MANGOSTANA* L. RINDS USING HPCCC

POSTER

Sri Murhandini[1,2*], Emily Keaveney[2], Stephen J Barton[2], James Barker[3], Ian Garrard[2], Derek Fisher[2] and Svetlana Ignatova[2]

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2. Advanced Bioprocessing Centre, Brunel Institute for Bioengineering, Brunel University London, UK
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Keywords: α-mangostin, production, multiple injections, HPCCC

Separation and purification techniques using liquid flow processing by Counter Current Chromatography (CCC) are widely applied particularly for the separation of components from plant extracts. However, generally only a single injection of the sample into a CCC apparatus is used due to sample complexity. Multiple injections to increase the overall yield and scale of the purification are seldom used. This has the advantage of reducing solvent consumption and run turnover time, both of which reduce the cost of the purification process. We report a method for the production of α-mangostin from *Garcinia mangostana* L. rinds with high purity and yield as reference material for quality control and standardisation of mangosteen based products. Separations were conducted at an analytical scale on a Mini High Performance Counter Current Chromatography (HPCCC) instrument with column volume 17.7 mL; using a hexane/ethyl acetate/methanol/water (5:5:10:4 v/v) solvent system in reverse phase at 2100 rpm; 25°C; and flow rate of 1 mL/min. The extract was prepared by overnight maceration of mangosteen rinds powder in 80% aqueous ethanol at 30°C, with a yield of 10.6% (w/w) of crude mangosteen extract from the rinds. The extract was injected up to 10 times with 25 min interval time between injections without any replacement or topping up the stationary phase. There was little loss of stationary phase after first injection. α-Mangostin was well separated after 10 injections of 9.1 mg extract in 0.86 mL lower phase (~100 mg/mL crude powder) and 5 injections of 22.8 mg extract in 0.86 mL lower phase (~250 mg/mL crude powder), although the resolution of the peaks of the α-mangostin and other target compounds reduced with each injection. The α-mangostin was produced with average 98% purity and 96% yield for the 10 injections and 99% purity and 93% yield for the 5 injections based on quantitative HPLC analysis. Characterisation of the 4 peaks obtained in HPCCC by LC-MS and NMR was also attempted. This optimised method for production of α-mangostin gave high purity and yield with reduced processing time and less solvent use. Although this approach may have general applicability to other extracts, variations in the stability of the selected phases system to the extracts can be expected and this may limit the number and concentration of the samples injected.

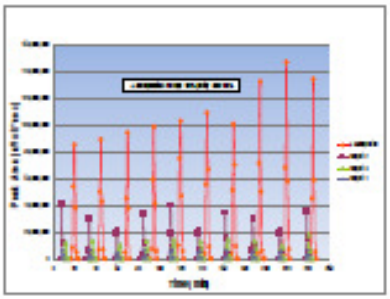


Figure 1. HPCCC Fractogram vs. Time; 10 injections of 9.1 mg extract in 0.86 mL LP

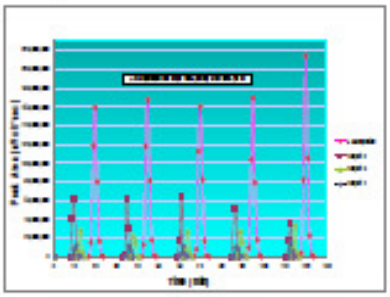
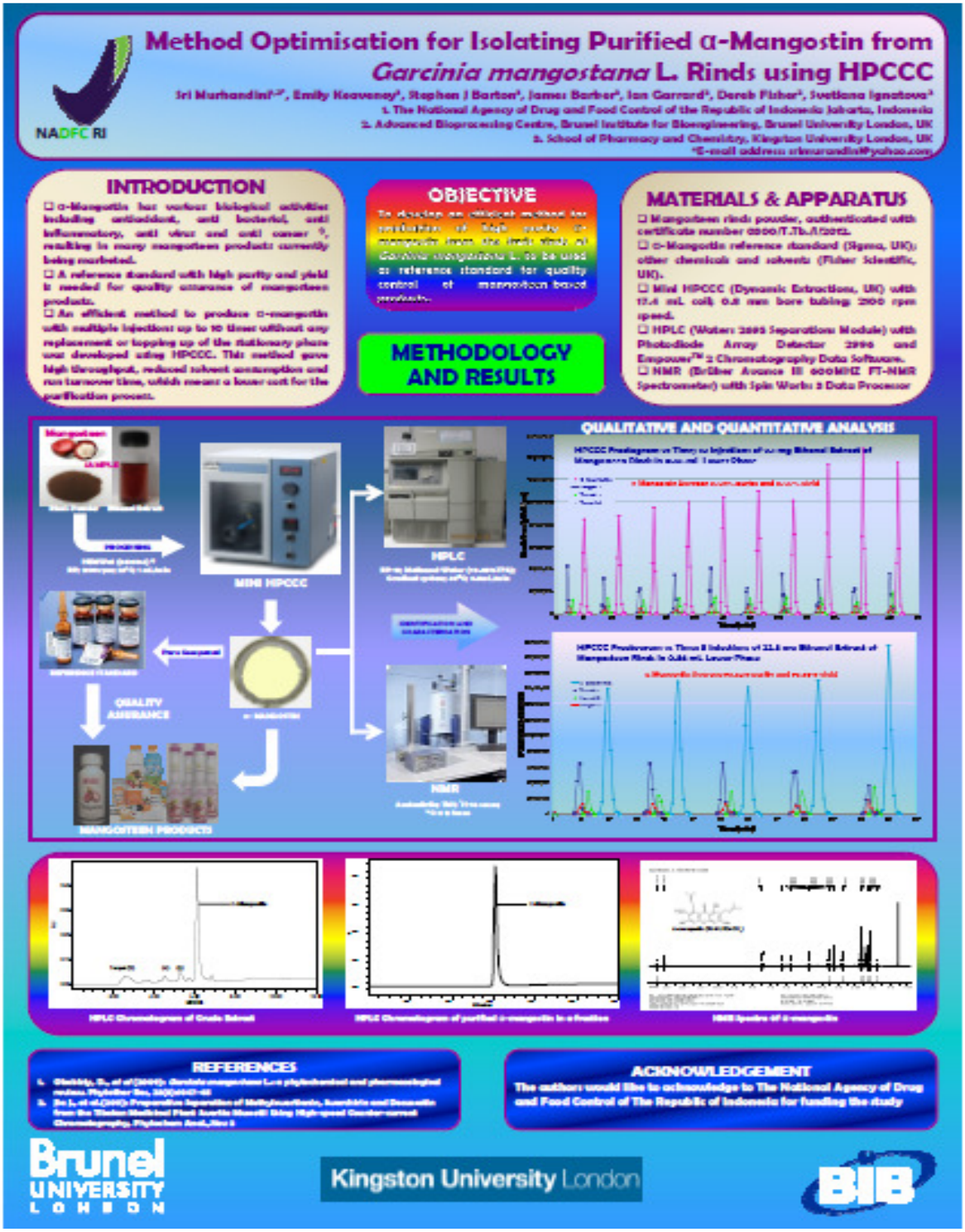


Figure 2. HPCCC Fractogram vs. Time; 5 injections of 22.8 mg extract in 0.86 mL LP

Appendix 6

Poster presented for International Conference on Counter Current Chromatography 2012, 6-8 August 2012, Hangzhou, China


Method Optimisation for Isolating Purified α-Mangostin from Garcinia mangostana L. Rinds using HPCCC
NADFC RI
INTRODUCTION
OBJECTIVE
METHODOLOGY AND RESULTS
MATERIALS & APPARATUS
QUALITATIVE AND QUANTITATIVE ANALYSIS
MINI HPCCC
HPLC
NMR
QUALITY ASSURANCE
MANGOSTEEN PRODUCTS
REFERENCES
ACKNOWLEDGEMENT
Brunel UNIVERSITY LONDON
Kingston University London

Appendix 7

Abstract accepted for The 14th International Symposium on Preparative and Industrial Chromatography and Allied Techniques (SPICA) on 30th September 2012 – 3rd October 2012 in Brussels, Belgium

ABSTRACT

Development of an Efficient Method for Production of α-Mangostin Reference Standard from *Garcinia Mangostana* L. Rinds Using Liquid Flow Processing

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A reference standard for quality control of any product requires a high purity; however this is not a simple task when the pure compound needs to be isolated from natural materials such as medicinal plants which contain very complex molecules. Using a classical method like column chromatography is not always satisfactory due to its column blockage with particulates from the crude sample, column properties changing with time and the expense of replacing them. There will be occasions when an alternative and efficient method therefore needs to be developed, and liquid flow processing known as Counter-current Chromatography (CCC) seems to be suitable for this purpose. CCC is a chromatography technique that uses two immiscible liquid phases without any solid support. This method has total recovery of injected sample, no irreversible adsorption, low solvent consumption and easily scaled up. Our research has been aimed at developing an efficient method for the production of purified α-mangostin for reference standard from *Garcinia mangostana* L. rinds using High Performance CCC. The method development was conducted initially on an analytical scale Mini HPCCC instrument (17.4 mL coil; 0.8 mm bore) using hexane/ethyl acetate/methanol/water (5:5:10:4 v/v) as solvent system. A 22 mg ethanol extract was injected 5 times with 25 min interval time between injections without any replacement or topping up the stationary phase. α-Mangostin was obtained at 98.09±0.61% purity and 97.86±2.78% yield. The method was then scaled up on the semi-preparative Spectrum instrument (143.5 mL coil; 1.6mm bore) and on the preparative Midi instrument (912.5 mL coil; 4 mm bore). Seven injections of 1273.8 mg extract each on Midi produced α-mangostin at 98.24±0.82% purity and 94.42±4.64% yield. Further increase in loading on the Spectrum allowed 2 injections of 358.7 mg extract each with producing α-mangostin at 99.34±0.43% purity and 99.61±0.55% yield. The developed method was able to reduce the purification steps and obtain the required reference standard at a higher throughput and reduced solvent consumption.

Key words: α- mangostin, reference standard, production, HPCCC

Appendix 8

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