CHEMOMETRIC TOOLS FOR CHROMATOGRAPHIC METHOD DEVELOPMENT AND DATA HANDLING OF CHROMATOGRAPHIC FINGERPRINTS

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ABSTRACT

Nowadays, chemometrics has been introduced as a tool in analytical chemistry. Many analytical chemists apply chemometrics in several domains such as optimization of analytical methods and interpretation of chemical data. This work aimed to use chemometric methods in method development and data analysis of high performance liquid chromatographic (HPLC) fingerprint data.

Firstly, an anion exchang liquid chromatographic method for the separation and determination of heparin and its impurities was developed with the aid of a chemometric approach, including multivariate experimental design and response surface methodology. The separation of heparin and two impurities (i.e. dematan sulfate (DS) and oversulfated chondroitin sulfate (OSCS)) was achieved on an Ion PAC AS22 column with the gradient elution of 10% to 70% of 2.5 M sodium chloride and 20 mM Tris buffer (pH 2.1) at a flow rate of 0.6 mL/min. The detection was performed with a UV detector at 215 nm. The resolutions between DS/heparin and heparin/OSCS were on average, $R_s > 1.8$. Method validation parameters indicate good linearity for heparin (3 – 20 mg/mL), DS (200-400 µg/mL) and OSCS (90-160 µg/mL). The limits of detection were 800, 10.5 and 7.2 µg/mL whereas limits of quantitation were 2,500, 31.5 and 22.0 µg/mL (%RSD 3.22-10.12) for heparin, DS and OSCS, respectively. Moreover, the validated method was applied for the determination of heparin and its impurities in pharmaceutical formulations. They all complied with the USP limit (90.0-110.0% labeled amount for heparin). The % label amounts of heparin in the investigated raw material and products were between 90.3 – 97.8%. DS and OSCS were not detected in all samples.

Secondly, HPLC fingerprints of *Mallotus* and *Phyllanthus* samples were combined with data-handing techniques to model the antioxidant activity and indicate peaks possibly responsible for this activity. In the first step, exploratory analyses, using Principle Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were performed to verify whether the antioxidant samples could be distinguished from the less or non-active samples. The score plot in PCA showed significantly diverging clustering tendencies better than the dendrogram in HCA. Moreover, PCA could distinguish some species of *Mallotus* and *Phyllanthus* samples. Then, the antioxidant activities of the samples were modeled as a function of the fingerprints using Partial Least Squares (PLS) and Orthogonal Projections to Latent Structure (O-PLS). The peaks potentially responsible for the antioxidant activity of the samples were indicated studying the regression coefficients of the models. In this study, comparing the regression coefficients plot of both models, not only showed similar coefficient profiles but also the peaks probably responsible for the antioxidant activity could be indicated at the same positions from both models.

KEY WORDS: CHEMOMETRICS/ HEPARIN/ OVERSULFATED CHONDROITIN SULFATE/ ION EXCHANGE CHROMATOGRAPHY/HPLC FINGERPRINT

117 pages

การประยุกต์ใช้คีโมเมตริกซ์ในงานด้านการพัฒนาวิธีวิเคราะห์ทางโครมาโทกราฟีและการประมวลผลข้อมูลจากลายพิมพ์ทางโครมาโท-กราฟี

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บทคัดย่อ

ปัจจุบันศาสตร์ทางคีโมเมตริกซ์ถูกนำมาใช้ในงานด้านเคมีวิเคราะห์มากขึ้น นักวิเคราะห์นำคีโมเมตริกซ์มาประยุกต์ใช้ในงานหลายแขนง อาทิ การหาสภาวะที่เหมาะสมของวิธีวิเคราะห์ การประมวลผลและการแปรความหมายของข้อมูลทางเคมีที่ซับซ้อน การศึกษานี้มีวัตถุประสงค์เพื่อนำคีโมเมตริกซ์มาประยุกต์ใช้ในงานการพัฒนาวิธีวิเคราะห์และการประมวลผลข้อมูลจากลายพิมพ์ทางโครมาโทกราฟี

งานวิทยานิพนธ์ส่วนที่ หนึ่งได้นำคีโมเมตริกซ์มาประยุกต์ใช้ในการพัฒนาวิธีวิเคราะห์ปริมาณเฮพารินและสารเจือปนในผลิตภัณฑ์ยาในประเทศไทย โดยการวางแผนการทดลองแบบพหุตัวแปรและวิธีของพื้นที่ผิวผลตอบ จนได้สภาวะที่เหมาะสม ดังนี้ คอลัมน์ชนิด ไอออนแพค เอเอส 22 ด้วยการชะตัวทำละลายเคลื่อนที่แบบขั้นบันไดจากร้อยละ 10 - 70 ของ โซเดียมคลอไรด์ความเข้มข้น 2.5 โมลาร์ และ ทริส บัฟเฟอร์ ความเข้มข้น 20 มิลลิโมลาร์ ที่ พีเอช 2.1 อัตรการไหล 0.6 มิลลิลิตรต่อนาทีตรวจวัดที่ความยาวคลื่น 215 นาโนเมตรด้วยแสงอัลตราไวโอเลต สภาวะดังกล่าวสามารถแยก เดอมาแทน ซัลเฟต และ เฮพาริน รวมทั้ง เฮพารินและสารเจือปนโอเอชซีเอส ด้วยค่าการแยกมากกว่า 1.8 วิธีวิเคราะห์ดังกล่าวได้ผ่านการตรวจสอบความถูกต้องของวิธีตามตัวแปรต่างๆดังนี้ ช่วงความเข้มข้นของการตรวจวิเคราะห์ที่มีความเป็นเส้นตรง คือ 3 - 20 มิลลิกรัมต่อมิลลิลิตรสำหรับเฮพาริน 200 - 400 ไมโครกรัมต่อมิลลิลิตร สำหรับเดอมาแทน ซัลเฟต และ 90 – 160 ไมโครกรัมต่อมิลลิลิตร สำหรับโอเอชซีเอส ขีดจำกัดการตรวจวัดอยู่ที่ 800 10.5 และ 7.2 ไมโครกรัมต่อมิลลิลิตร และขีดจำกัดของการตรวจวัดเชิงปริมาณอยู่ที่ 2,500, 31.5 และ 22.0 ไมโครกรัมต่อมิลลิลิตร สำหรับเฮพาริน, เดอมาแทน ซัลเฟต และโอเอชซีเอส ตามลำดับ วิธีวิเคราะห์ที่ผ่านการตรวจสอบความถูกต้องได้ถูกนำมาใช้ในการหาปริมาณเฮพารินและสารเจือปนในผลิตภัณฑ์ยา พบว่า ปริมาณของเฮพารินที่ตรวจพบทั้งในวัตถุดิบและผลิตภัณฑ์ยาอยู่ในช่วงร้อยละ 90.3 - 97.8 และตรวจไม่พบสารเจือปนทั้งสองชนิดในทุกตัวอย่าง ซึ่งเป็นไปตามข้อกำหนดของตำรายา

งานในส่วนที่สองได้นำคีโมเมตริกซ์ไปใช้ในการประมวลผลข้อมูลจากลายพิมพ์ทางโครมาโทกราฟีของพืชสกุล *Mallotus* และ *Phyllanthus* เพื่อทำนายพีคบนโครมาโทแกรมที่แสดงฤทธิ์ต้านอนุมูลอิสระ โดยมีขั้นตอนดังนี้ นำข้อมูลที่ได้จากลายพิมพ์ทางโครมาโทกราฟีของพืชทั้งสองสกุลมาทำการจำแนกตามฤทธิ์ต้านอนุมูลอิสระด้วยเทคนิคการวิเคราะห์องค์ประกอบหลักและเทคนิคการวิเคราะห์องค์ประกอบแบบแผนภูมิ พบว่า เทคนิคการวิเคราะห์องค์ประกอบหลัก ให้ผลการจำแนกที่ดีกว่าเทคนิคการวิเคราะห์องค์ประกอบแบบแผนภูมิ และเทคนิคเทคนิคการวิเคราะห์องค์ประกอบหลัก ยังสามารถจำแนกสายพันธุ์ของพืชสกุล *Mallotus* และ *Phyllanthus* บางสายพันธุ์ ได้อีกด้วย จากนั้น สร้างสมการเพื่อศึกษาความสัมพันธ์ระหว่างข้อมูลจากลายพิมพ์ทางโครมาโทรกราฟีและข้อมูลของฤทธิ์ต้านอนุมูลอิสระด้วยวิธีการถดถอยแบบกำลังสองน้อยที่สุดบางส่วนและวิธีการถดถอยแบบกำลังสองน้อยที่สุดบางส่วนเชิงตั้งฉาก เพื่อทำนายพีคบนโครมาโทแกรมที่มีความสัมพันธ์กับฤทธิ์ต้านอนุมูลอิสระ ด้วยค่าสัมประสิทธิ์ความถดถอย ซึ่งพบว่า ทั้งสองวิธีสามารถแสดงพีคที่มีความสัมพันธ์ที่สามารถบ่งชี้ฤทธิ์ต้านอนุมูลอิสระได้

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LIST OF ABBREVIATIONS

ABTS	2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)
ACN	acetonitrile
ANOVA	analysis of variance
AT	antithrombin
AR	analytical grade
BBD	Box-Behnken design
CCD	central composite design
CCCD	circumscribed central composite design
CE	capillary electrophoresis
CSA	chondroitin sulfate A
Da	dalton
DAD	photodiode array detector
DDTMAHS	dodecyl trimethyl ammonium hydrogen sulfate
DNA	deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
DS	dermatan sulfate
ED	euclidean distance
e.g.	exempli gratia
etc.	etcetera
FCCD	face-centered central composite design
g	gram
GAG	glycosaminoglycan
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometer
HCA	hierarchical cluster analysis

LIST OF ABBREVIATIONS (cont.)

HILIC	hydrophilic-interaction liquid chromatography
HIV	human immunodeficiency virus
HPAE-PAD	high-performance anion-exchange method with pulsed amperometric detection
HPLC	high-performance liquid chromatography
HSV	herpes simplex virus
IC_{50}	half maximal inhibitory concentration
ICH	international conference on harmonization of technical requirements for registration of pharmaceuticals for human use
i.e.	id est
IEC	ion-exchange chromatography
IPR	ion-pairing reagent
k	retention factor
la.	labeled amount
LMWH	low molecular weight heparin
LOD	limit of detection
LOF	lack of fit
LOO-CV	leave-one-out cross-validation procedure
LOQ	limit of quantitation
M	molarity
MB	megabyte
MeOH	methanol
mg	milligram
min	minute
mL	milliliter

LIST OF ABBREVIATIONS (cont.)

MLR	multivariate linear regression
mm	millimeter
mM	milimolar
MS	mass spectrometry
NaCl	sodium chloride
$NaClO_4$	sodium perchlorate
nm	nanometer
NMR	nuclear magnetic resonance
NP-HPLC	normal-phase high performance liquid chromatography
O-PLS	orthogonal projection to latent structure
ORAC	oxygen radical absorbance capacity
OSCS	oversulfated chondroitin sulfate
Pa	Pascal
PCA	principal component analysis
pH	negative logarithm of hydrogen ion concentration
pKa	logarithmic acid dissociation constant
PLS	partial least squares
ppm	part per million
r^2	coefficient of determination
RAM	random access memory
R&D	research and development
RMSECV	root mean squared error of cross-validation
RP-HPLC	reversed-phase high-performance liquid chromatography
RPIP-HPLC	reversed-phase ion-pairing high-performance liquid chromatography

LIST OF ABBREVIATIONS (cont.)

R_s	resolution
RS	reference standard
RSM	response surface methodology
RSD	relative standard deviation
SAX-HPLC	strong-anion-exchange high-performance liquid chromatography
SEC	size-exclusion chromatography
SNV	standard normal variate
TBAHS	tetrabutyl ammonium hydrogen sulfate
TEAC	Trolox equivalent antioxidant capacity
TFA	trifluoroacetic acid
TLC	thin layer chromatography
t_R	retention time
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
UK	United Kingdom
USA	United State of America
USFDA	United State Food and Drug Administration
USP	United State Pharmacopeia
UV	ultraviolet
W	width
WAX-HPLC	weak anion-exchange high-performance liquid chromatography
WHO	world health organization
°C	degree celcius
µg	microgram
µL	microliter

LIST OF ABBREVIATIONS (cont.)

μm	micrometer
μM	micromolar

PART I: DETERMINATION OF HEPARIN AND ITS IMPURITIES IN HEPARIN PRODUCTS FROM THAILAND BY ION EXCHANGE CHROMATOGRAPHY (IEC)

CHAPTER I
INTRODUCTION

Heparin is widely used as an anticoagulant and anti-thrombotic agent (1-2). Moreover, it can also be used to form an inner anticoagulant surface for various experimental and medical devices, such as test tubes and renal dialysis machines.

Pharmaceutical grade heparin is derived from mucosal tissue of slaughtered animals, such as porcine intestine or bovine lung (3). The major supplier of heparin is China, where it is isolated in large amounts from porcine intestines. Because heparin is purified from an animal source, formulations of the processed heparin may contain low levels of several natural contaminants (e.g. chondroitin sulfate A, B, or C) that are not associated with adverse health effects (2). However, problems arose in 2007-2008 when contaminated batches of heparin led to serious allergic reactions. More than 100 patients died from anaphylactic shock after being administered the drug (4). The increased number of reports of adverse events associated with heparin products led to a collaborative study involving researchers from the United States Food and Drug Administration (USFDA), industry and academia, which identified oversulfated chondroitin sulfate (OSCS) as non-native heparin contaminant (5-6). OSCS was found in crude heparin and remained in the active pharmaceutical ingredient and final formulations after the purification processes. In addition, samples of heparin were found to contain more than 1% w/w of the dermatan sulfate (i.e. chondroitin sulfate B), which is a marker of poor-quality purification of the crude material.

Several methods have been developed for the analysis of heparin and its impurities. Among these methods, chromatographic and capillary electrophoresis

(CE) techniques are often used to separate heparin and its impurities (1-2, 7-15). Chemical- or enzyme-induced depolymerization is usually employed to simplify the assay and eliminate interference components of the sample susceptible to acid or enzymatic hydrolysis (9,12). In the standard method (16), the USFDA requires nuclear magnetic resonance (NMR) spectroscopy and CE for qualitative analysis of unfractionated heparin and its impurities in drug products. In addition, the new proposed U.S. Pharmacopeia (USP) assays include expansion of the current NMR technique and a strong-anion-exchange high-performance liquid chromatography (SAX-HPLC) protocol (17). Although, NMR and CE methods are now routinely employed to screen raw materials and to ensure the identity, purity, and safety of these products, simpler analytical approaches that can be used in selected types of production settings, e.g. in hospital, in R&D laboratories and quality assurance, are still necessary. Chromatographic analysis of heparin is a challenge because the drug is a polydisperse mixture, containing chains of alternating uronic acid and amino sugars that vary in molecular weights from 5,000 to 40,000 Da. A modern analytical approach, which enables the reduction of the analytical time without compromising resolution and separation efficiency is hydrophilic interaction liquid chromatography (HILIC) (15). HILIC has been proven convenient for the analysis of polar molecules, charged as well as uncharged, which are not weakly retained in reversed-phase high-performance liquid chromatography (RP-HPLC) (15).

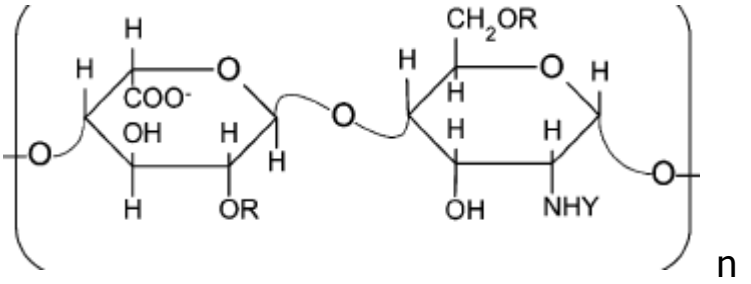
The purpose of this research was to develop an efficient HPLC method for the simultaneous determination of heparin and its two major impurities. Various chemometrics-based techniques, including multivariate experimental design and response surface methodology were utilized to aid in the method optimization. The use of chemometric approaches provides the experimental conditions giving the optimal analytical response within a limited number of experiments. Finally, the developed method was validated, compared with the reported data and applied to the analysis of raw materials and heparin products that are available from different companies in Thailand.

CHAPTER II
LITERATURE REVIEW

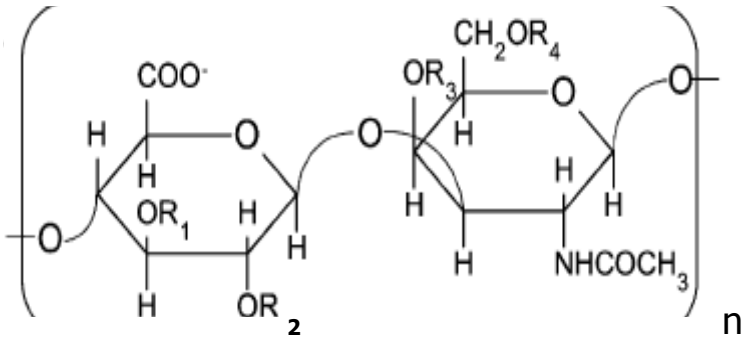
2.1 Heparin

2.1.1 Structure of heparin and its impurities

Heparin is a highly sulfated glycosaminoglycan (GAG) with the highest negative charges density of any known biological molecule (18). The basic structure of heparin comprised of repeated disaccharide subunits of uronic acid- (1 →4)-D-glucosamine, as shown in Figure 2.1A, whereas chondroitin sulfate is a naturally occurring GAG composed of N the repeated disaccharide subunit glucuronic acid-(1 →3)-*N*-acetylgalactosamine as shown in Figure 2.1B. Heparin is modified during biosynthesis and has variability in its structure at the disaccharide level at the positions marked by R (H or SO_3) and Y (H, acetyl, or SO_3). Chondrotin sulfates are modified during biosynthesis to introduce variability at the positions marked by R_2, R_3, or R_4 (H or SO_3); all native chondroitin sulfates have H at the R_1 position. For chondroitin sulfate A (CSA): R_1= H, R_2 = H, R_3 = SO_3, R_4 = H. For dermatan sulfate (DS): R_1 = H, R_2 = SO_3, R_3 = SO_3, R_4 = H. Additionally, the structure of DS differs from that of CSA and OSCS by the epimerization of the glucuronic residue to iduronic acid. In OSCS, which is prepared by chemical sulfonation of CSA, all positions of modification including R_1 are sulfonated (19). Although chondroitin sulfate can be variously sulfonated at the 2-*O*, 4-*O*, and 6-*O* positions, on average it contains one sulfonate for each disaccharide residue. Therefore, it has a lower net negative charge than heparin. In contrast, as a result of chemical sulfonation the OSCS contaminant is indeed “fully sulfonated” at the 2-*O* and 3-*O* positions of the glucuronic acid as well as the 4-*O* and 6-*O* positions of the galactosamine residue (5), as shown in Figure 2.1B. OSCS has a molecular weight similar to that of heparin with an even higher negative charge density (18,20).



(A)



(B)

Figure 2.1 Structures of heparin (A) and chondroitin sulfates (B). For R, Y and R_i see text.

2.1.2 Physicochemical properties of heparin

Comprehensive information of the molecular mass, dispersion in nature, and macromolecular properties of heparin and on the link between its macromolecular characteristics and biological functions has been reported (21-23). Samples of heparin isolated from various sources are different in sizes of molecules and are characterized by molecular weights of fractions from 3,000 to 40,000 (21-23). The longest molecular heparin known in nature (molecular weight over 1,000,000) was isolated by Horner from rat integument (24). The high negative-charge density on the macromolecule makes it possible to consider heparin as a typical polyelectrolyte whose hydrodynamic properties in solution will be sensitive to changes in concentration, ionic strength, and pH. The high content of negatively charged groups does not affect the high anticoagulant activity of the biopolymer. In reality dextrans with the same or higher degrees of sulfonation show appreciably weaker action in

preventing blood clotting in comparison with heparin (21). The anticoagulant activity of heparin reflects a complex of special structural features of the polysaccharide including degrees of sulfonation and dissociation, and the size and shape of the macromolecule.

Because of the complex structure of heparin, containing various functional groups, its pK_a was examined according to the functional groups. The pK_a of O-and N-sulfo groups is not less than 1 to 1.5 while that of the carboxyl group is 3-4 (25).

2.1.2.1 UV absorption

In 1950, Bell and Krantz examined the UV spectrum of heparin sodium and found that heparin exhibited maximum absorption at wavelength 240 nm and 292 nm and minimum absorption between wavelength 245 and 260 nm (26). According to the authors, this absorption behavior is due to its impurities. Further investigations by Awe and Studemann (27) confirmed this hypothesis. A minimum absorbance at wavelength 235 nm and maximum absorbance at wavelength 255 nm were found for heparin raw material. These bands disappear after purification of the product. Ferrari and De Ambrosi examined 43 samples of commercial heparin sodium (28). Different UV spectra were found depending on the sources and activity. A correlation between the UV spectrum and the activity could not be proven. Between 200-230 nm, the UV absorbance is very poor, it is only from this wavelength onwards that the absorbance strongly increases.

2.1.2.2 Solubility

Literatures reported different solubilities of heparin. For example, Merck index (29) indicates that 5% of heparin sodium is soluble in water, whereas Martindale (30) claimed that 1 part of heparin sodium dissolves in 2.5 parts of water. Additionally, saturated solutions of heparin sodium, isolated from porcine mucosa, were dissolved in 4 different solvents at 20°C and their solubility varied as shown in Table 2.1 (31).

Table 2.1 Solubility of heparin sodium (20°C)

Solvent	Solubility (%)
Water	60
Methanol	0.01
Ethanol	< 0.01
Acetone	< 0.01

2.1.2.3 Stability

Lyophilized Heparin sodium and dried heparin sodium can be stored for several years at 30°C in tightly closed containers without loss of activity. No change of efficacy, pH value and appearance of the solution for 36 months in the ampoule solution of heparin sodium ready for administration (31). Experiments carried out by Goodall et al. showed that heparin sodium undergoes no loss in quality in the pH range from 4 to 9 (32). This has been confirmed by other experiments (30). According to other tests, injections of heparin sodium are stable for more than 7 years at room temperature and for 6 – 8 years at 37°C (30).

2.1.3 Mechanism and medical use

Heparin has been employed in a number of important biological processes. It has been widely used as an anticoagulant, preventing the formation of clots and extension of existing clots within the blood. Heparin does not break down clots that have already been formed (unlike tissue plasminogen activator), however, it allows the body's natural clot lysis mechanisms to work normally to break down clots (33). Heparin is used for anticoagulation in several situations, including acute coronary syndrome, atrial fibrillation, deep-vein thrombosis and pulmonary embolism, cardiopulmonary bypass for heart surgery, and in an extracorporeal membrane oxygenation circuit for extracorporeal life support (34).

Heparin binds to the enzyme inhibitor antithrombin (AT) causing a conformational change that results in its activation through an increase in the flexibility of its reactive site loop (35). The activated AT then inactivates thrombin and proteases involved in blood clotting, most notably factor Xa. The rate of

inactivation of these proteases by AT can increase up to 1000-fold due to the binding of heparin (36).

2.2 Determination of heparin and its impurities

Heparin is extremely difficult to be analyzed because of its highly negative charge, polydispersity, and macroheterogeneity. The assay for heparin sodium in USP monograph recommends measurement of heparin by anti-clotting activity (37). However, the assay cannot detect any impurities. Therefore, the USP published a revision of the monograph to address the immediate health issue by identifying OSCS as an important impurity in heparin by CE (38). In August 2009, the USP implemented a new revision which replaced the CE method for identifying OSCS in heparin with an anion exchange chromatographic method. The chromatographic method improves the resolution of heparin from impurities, such as OSCS and DS. In addition, the USP added a high-performance anion-exchange method with pulsed amperometric detection (HPAE-PAD) to quantify the percent of organic impurities (39). Various publications reported the analysis of heparin and its impurities by enzymetric immunoassay, attenuated total reflection infrared spectrometry, nuclear magnetic resonance (NMR) spectroscopy, CE, HPLC as summarized in Table 2.2.

Table 2.2 Analytical methods for the determination of heparin and its impurities

Method	Analyte	Sample	Significant result	Ref.
RPIP-HPLC-UV	• Heparin-derived oligosaccharides	• Bovine lung heparin	• 30 components in 30 min	25
RPIP-HPLC-UV	• Low molecular weight heparins	• LMWH pharmaceu-tical preparation	• Linearity range (1-5 mg/mL) • $r^2 > 0.994$ • RSDs = 0.75 – 5.30%	40
RPIP-HPLC-MS	• Heparin-derived oligosaccharides	• MMWH	• 200 components in 60 min	41
CE-UV	• Heparin • DS • OSCS • Hyaluronan	• Raw material	• %RSD(migration time) 0.57% (DS) 1.53%(heaprin) 1.91%(OSCS) 1.53%(hyaluronan) • Resolution 2.67 (Heparin, hyaluronan)	42
CE-UV	• Heparin • OSCS • DS	• Raw material	• LOD < 0.1%OSCS	43

Table 2.2 (continued) Analytical methods for the determination of heparin and its impurities.

Method	Analyte	Sample	Significant result	Ref.
CE-DAD	• Heparin • OSCS • DS	• Raw material	• Range 0.2-5.0 μg/mL (OSCS) • LOD = 0.07% (OSCS) • LOQ = 0.2%(OSCS)	44
CE-UV	• Heparin • OSCS • DS	• Raw material	• Range • LOD = 0.019% (OSCS)	45
CE-UV	• OSCS (in form of glucosamine)	• Pharmaceu-tical product	• LOD = 200 pg	46
CE-UV, SAX-HPLC	• Chondroitin sulfate • DS	• Raw material	• Recovery - 90.2-109.3% (CE) - r^2 = 0.993	47
^{1}H-NMR	• Heparin • OSCS • DS	• Heparin samples	• LOD = 0.1%OSCS	48

Table 2.2 (continued) Analytical methods for the determination of heparin and its impurities.

Method	Analyte	Sample	Significant result	Ref.
^{1}H-NMR, SAX-HPLC-UV, Anticoagulation	• Heparin • OSCS • DS	• Pharmaceutical product	• Range 1.0-10% (^{1}H-NMR) • LOD 0.07% (SAX-HPLC-UV)	49
SAX-HPLC-UV	• Heparin • DS	• Raw material	• Range 5-20 mg/mL (Heparin) • LOD = 0.66 mg/mL (OSCS)	50
WAX-HPLC-UV	• Heparin • OSCS • DS	• Raw material	• LOD = 0.04% (OSCS)	51
HILIC-MS	• Heparin • Heparin sulfate	• Raw material	• GAG-glycomics profiling	52
EIA	• OSCS	• Pharmaceutical product	• LOD = 0.1%	53
Fluorescence assay	• Heparin • OSCS	• Unfractionated heparin	• LOD = 0.5%	54

2.3 HPLC analysis of biomolecules

Biomolecules (for example, nucleic acid, proteins and polysaccharides) are naturally occurring substances of large molecular weight commonly created by polymerization of smaller subunits. The structural and chemical diversity of these compounds usually can complicate their effective characterization. Sample preparation procedures, separation modes, column chemistries, column configurations, and detection techniques have to be carefully optimized. There are several HPLC techniques bioanalytical chemists use to analyze biomolecules (55-75). Typical chromatographic modes (e.g., RP-HPLC, HILIC, size-exclusion, and ion-exchange chromatography) for biomolecule analysis are described.

2.3.1 Reversed-phase chromatography

RP-HPLC is a powerful and widely used technique for analyzing biomolecules. Its primary advantages over other separation modes are high efficiency and ability to distinguish between compounds that are chemically very similar. Properties of the RP-HPLC column that affect the resolution are: particle size, pore size, bonded phase (functionality and chain length), and column length. Each of these parameters influences one or more variables of the chromatographic resolution (R_s) equation:

$$R_s = (1/4)\{(\alpha - 1)/\alpha\} N^{1/2} \{k/(1+k)\}$$

Where α is the chromatographic selectivity, N is the column efficiency, and k is the retention factor, which is a measure of retention. Important system parameters to consider are mobile phase composition (pH, buffer, organic modifier, ion pairing agents, etc.).

Mobile phase for RP-HPLC, most biomolecules exist naturally in highly aqueous environments are stabilized with ions and other water-soluble compounds that help maintain the biological activity of the biomolecules. The ability to use aqueous mobile phases that contain salts and buffers is a main reason why liquid chromatography techniques, including RP-HPLC, HILIC, size-exclusion, and ion-exchange, are so ubiquitous in biomolecular analysis. Rarely does one encounter normal-phase liquid chromatography or gas chromatography, where water and salts are not or less compatible with instruments and highly organic mobile phases (55-58).

Nowadays, reversed-phase ion-pairing high-performance liquid chromatography (RPIP-HPLC) provides promising and increasingly popular methods for the analysis of biomacromolecules. Lipophilic ion-pairing reagents (IPR) act as mobile phase modifiers, which aid in the retention and resolution of charged species on hydrophobic stationary phases (60). The mechanism of retention in RPIP-HPLC is however a matter of some debate. In the classical model of retention in RPIP-HPLC, the hydrophobic IPR and the analyte ion of opposite charge combine in the mobile phase to form a neutral species, which then partitions into the hydrophobic stationary phase (76). The dymanic ion-exchange model, however, suggests that the IPR is first adsorbed onto the surface of the stationary phase creating charged sites, which then act as ion-exchange sites for the oppositely charged analyte (76). The presence of evidence for both theories suggest that RPIP separations occur through a combination of both mechanisms and that the extent to which each contributes to analyte retention may be controlled by the experimental condition. Adding IPR to the mobile phase is to improve retention of biomolecules in RP-HPLC mentioned in various publications (59-61).

2.3.2 Hydrophilic-interaction liquid chromatography

HILIC was defined by Alpert in 1990 as a variant of normal-phase HPLC (NP-HPLC), where a hydrophilic stationary phase is used in combination with a mostly organic aqueous mobile phase, often acetonitrile (sometimes propanol or acetone), and elution is usually performed by increasing the polarity of the mobile phase by increasing the water concentration (77). HILIC has proven quite convenient for the analysis of polar molecules weakly or not retained in conventional RP-HPLC systems (78). In HILIC, the retention depends on the distribution of the analytes between a partially immobilized aqueous layer, bound to the gel matrix surface, and the mobile phase. In addition, hydrogen bonding and electrostatic interactions between the analyte and the stationary phase are also believed to be more important than comparable mixed-mode retention mechanisms in other liquid chromatography techniques, for example RP-HPLC. This is in contrast to conventional NP-HPLC, where mixtures of organic solvents, without water, are used in the mobile phase, and where the separation mainly depends on surface adsorption (77).

Using water in HILIC mobile phases is advantageous because it increases the solubility of polar compounds, and the water content does not need to be completely controlled, as is the case in ordinary NP-HPLC. An increased interest in HILIC has been seen for more than a decade due to the desire to analyze more complex biological samples, such as simple and complex carbohydrates, and in studies of proteomics and metabolomics. During this period, several liquid chromatography column manufacturers have introduced new HILIC columns with different types of ligands (poly-succinimide-derived, amide, and diol, including the zwitterionic type, etc.) as well as bare silica columns, suitable for carbohydrate analysis (77).

2.3.3 Size-exclusion chromatography (SEC)

SEC is a non-interactive technique that separates solutes according to their molecular size in solution. It is often used as the first step in isolation of a protein from a crude sample (66-67). When used with calibration standards it is possible to estimate the molecular mass of compounds with similar molecular shape to the standards, such as proteins and oligosaccharides.

SEC retention is determined by the accessibility of the sample molecule to the pores. If the analyte cannot enter the pores it passes through the column in the channels between the particles. Analytes that can enter the pores, either partially or completely, elute later (79). Maximum retention (maximum elution volume) occurs if the sample can fully access the pores. Minimal retention (minimum elution volume) occurs if the sample is larger than the pores and elutes with the solvent. Hence, samples elute in order of size, with the highest molecular weight (or largest molecule size) samples eluting first. Since, molecules are eluted based on their size in solution, linear or rod-like molecules will elute before globular molecules of the same molecular weight. Elution order is based on whether or not the analyte can enter the pores.

Method development in SEC technique consists of selecting a mobile phase compatible with the sample type, and a column or columns with pore sizes that provide resolution for the molecular weight range of the sample.

2.3.4 Ion-exchange chromatography

In ion-exchange chromatography (IEC), molecules bind by a reversible interaction of electrostatic charges located on the outer surface of the solute molecule, with clusters of groups with an opposite charge on the ion exchangers. To maintain neutrality, the charges on both the molecules of interest and the ion exchangers are associated with ions of opposite charge, termed counter ions. Because a solute must displace the counter ions on the stationary phase to attach to it, the technique is termed "ion exchange" (79).

Ion-exchange mobile-phase conditions are chosen that permit differential migration of sample components through the column. The higher the net charge of the analyte, the higher the ionic strength (salt concentration) needed to bring about elution from the bonded phase. At a certain high level of ionic strength, all the sample components are fully desorbed and move down the column with the same speed as the mobile phase. Conditions that lie somewhere in between total adsorption and total desorption will provide the optimal selectivity at a given pH value of the mobile phase. Because most biomolecules have the ability to exist as a charged species, ion exchange is an excellent choice for biomolecular analysis (73-75).

During method development in the IEC technique, many variables could be considered, including flow rate, temperature, ion-exchange bonded phases, and mobile phase composition (pH and ionic strength). A high column efficiency (number of theoretical plates) is an important parameter to consider when maximizing the resolution of a separation. In IEC the efficiency (sharpness) of a peak is governed, not only by how well the column is packed, but also by the mass transfer characteristics of the ion exchange mechanism (79). The term mass transfer used here refers to the efficient migration of the analyte from the mobile phase to the stationary phase (ion-exchange site) and then back into the mobile phase. If this process is not very fast, broad peaks can result with a possible loss in analyte resolution. The mass transfer can be increased by increasing the column temperature (79).

Most ion-exchange packings fall into two groups, cation exchangers and anion exchangers. Cation exchangers contain acidic groups, such as sulfonic acid or carboxylic acid, and are used to separate cationic (positively-charged) compounds. Strong cation exchangers commonly comprise sulfonate group ($-SO_3^-$). These are

strong acids and their ion exchange character is relatively unaffected by the mobile phase pH. Carboxylate ($-COO^-$) ions are weak cation exchangers. With pK_a values in the 4-6 range, they are ionized and effective as a cation-exchange medium above pH 6. Anion exchangers contain basic groups, such as secondary, tertiary, or quaternary amines, and are used to separate anionic (negatively-charged) compounds. Ammonium ions (NH_4^+) are strong bases and their exchangers is relatively unaffected by mobile phase pH. Secondary and tertiary amines are weak bases with pK_a values between 8 and 11. A weakly basic anion exchanger should be used below pH 8.

The pH of the mobile phase can have a considerable effect on retention and selectivity. A change of pH that causes the analyte to change from its ionized to its neutral state prevents the analyte from taking part in the ion-exchange process and consequently retention is reduced, while a shift in pH that causes the ion-exchange site to change from its ionized to a neutral state essentially eliminates sites available for ion exchange and retention is also lost. Mobile-phase ions compete with the analyte ions of the same charge for adsorption on the stationary phase. Obviously, the more mobile-phase ions (the higher the ionic strength) the better they compete. The result is that ion-exchange retention decreases with increasing ionic strength of ions of the same charge as the analytes (75). The term adsorption used here in a general sense refers to the attraction of an analyte ion with (the ionic sites of) the packing.

The competition between analyte and salt for the ion-exchange site is largely controlled by the concentration and nature of the salt ion (79). The choice of the appropriate counter ion is therefore of significant importance in adjusting retention. One usually employs NH_4^+, H^+, Na^+, K^+, or Ca^{2+} for cation and COO^-, Cl^-, NO_3^-, SO_4^{2-}, or PO_4^{3-} for anion exchangers. The eluting strength of the counter ion correlates directly with its charge. For example, by substituting the eluent ion of a cation exchanger in the series H^+, Na^+, K^+, Ca^{2+} the relative strength between counter ion and fixed ion increases and retention of a given cation (analyte) decreases. When a single ionic strength is insufficient to elute all analytes (both strongly and weakly retained) from the column in a reasonable time, salt or pH gradients can be applied. Salt gradients employ a gradual increase in ionic strength in the mobile phase. This gradient gradually desorbs the sample components in the order of increasing net charge, so that components are desorbed one at a time from the surface to provide

separation of the mixture. Thus the salt gradient compresses a chromatogram so as to elute components with widely different adsorption properties within a reasonable time (79). The gradient profile can be linear or stepwise. pH gradients affect retention in the same manner, by taking advantage of the different pK_a values of analytes and stationary-phase ions. In a typical pH gradient, the mobile phase pH is charged from low pH to high pH for anion exchange or from high to low for cation exchange, bracketing the pK_a range of the analytes.

Table 2.3 shows the HPLC methods that have been reported for the analysis of biomolecules.

Table 2.3 Selected of HPLC analyses of biomolecules

HPLC method	Analyte	Ref.
NP-HPLC	Diacylglyserol in lipid	55
RP-HPLC	Chondroitn sulfate	60
	Heparin oligosaccharide	61
	Polar lipids	62
	Caseinomacropeptides	63
	Wheat flour protein	64
	Buffalo milk protein	65
SEC	Rice storage proteins	66
	Metallothionein	67
	Fatty acid methyl ester	68
	Lipids	69
	Liposomes	70
HILIC	Peptides	71
	Glycans	72
IEC	Egg white proteins	73
	Heparin, OSCS, DS	50,51
	Lipo-proteins	74
	Amino acids	75

2.4 Experimental design methodology

Nowadays, the use of chemometric tools should be the standard way of operating when developing, optimizing and validating a chemical process or methodology. The advantages of experimental designs are well known by chemometricians and are increasingly recognized by the whole scientific community. Their use in the separation science has particularly increased in the last few years (80-85). Through the development of mathematical models, experimental design methodology allows to access the statistical significance of the independent variables effects being investigated in a system, as well as to evaluate their interactions from a limited number of experiments. Moreover, employing multivariate optimization designs in which at least three different points levels of the variables change simultaneously, a second-order polynomial equation may be build to describe the behavior of a particular response as a function of the studied variables and their interactions. Response surface methodology (RSM) is a popular method for optimizing conditions and providing optimal performance characteristics.

The two most common designs generally used for response surface modeling are central composite designs (CCD) and Box-Behnken designs (BBD). In these designs the inputs factors are three or five distinct values (levels), but only a limited number from all combinations of these values appear in the design.

2.4.1 Central composite designs

CCDs are the most frequently applied response surface designs of which the utilities are used to build a full quadratic model. They consist of a two-level full factorial design (2^f experiments), a star design (2f experiments) and a center point. As a consequence the CCD require $N = 2^f + 2f + 1$ experiments to examine f factors. The experiments of the full factorial design are situated at levels -1 and +1, those of the star design at levels $-\alpha$ or $+\alpha$ for one factor and the center point at level 0 (see Figure 2.2). Depending on the α value, two common types of CCD can be distinguished. A face-centered CCD (FCCD) with $|\alpha| = 1$ examines all factors at three levels (-1, 0, +1), while a circumscribed CCD (CCCD) has $|\alpha| > 1$ and evaluates five levels for each factor ($-\alpha$, -1, 0, 1, $+\alpha$). To obtain a so-called rotatable CCCD, the extreme

levels of the star design (-α, +α) should fulfill the requirement; $|\alpha| = (2^f)^{1/4}$. Therefore, $|\alpha|$ is equal to 1.41 and 1.68 for two and three factors, respectively (86). For two and three factors, a CCD requires 9 and 15 experiments, respectively. In Figure 2.2, a CCCD for three factors is presented.

	Factors		
Experiment	A	B	C
1	- 1	- 1	- 1
2	1	- 1	- 1
3	- 1	1	- 1
4	1	1	- 1
5	- 1	- 1	1
6	1	- 1	1
7	- 1	1	1
8	1	1	1
9	- α = - 1.68	0	0
10	+α = +1.68	0	0
11	0	- α = - 1.68	0
12	0	+α = +1.68	0
13	0	0	- α = - 1.68
14	0	0	+α = +1.68
15	0	0	0

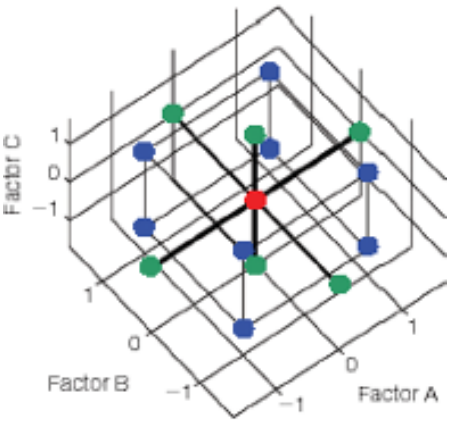


Figure 2.2 Central composite design for three factors (N = 15) (86).

2.4.2 Box-Behnken designs

Like CCD, BBD are response surface designs of which the utilities allow fitting fit a full quadratic model. Unlike most CCD, BBD use only three levels of each factor. This makes them appealing when the set of achievable factor levels is small. FCCD designs also use just three factor levels. However, they are not rotatable as BBD are (86). On the other hand, BBD models can be expected to have poorer prediction ability in the corners of the cube that encloses the design, because unlike FCCD they do not include points at the corners of that cube (Figure 2.3) (87).

A BBD requires N= 2f (f-1)+1 experiments and the factors are examined at three levels (-1, 0, +1). Thus, for three factors, 13 experiments are required. Figure 2.3 presents BBD for three factors.

	Factors		
Experiment	A	B	C
1	1	1	0
2	1	- 1	0
3	- 1	1	0
4	- 1	- 1	0
5	1	0	1
6	1	0	- 1
7	- 1	0	1
8	- 1	0	- 1
9	0	1	1
10	0	1	- 1
11	0	- 1	1
12	0	- 1	- 1
13	0	0	0

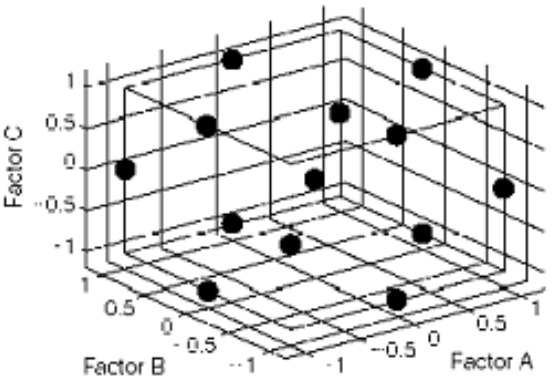


Figure 2.3 Box-Behnken design for three factors (N = 13) (86).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Materials used in this study are listed in Tables 3.1 and 3.2

Table 3.1 List of chemicals and reagents

Name	Grade	Source/Supplier
Chondroitin sulfate A	RS	Sigma-Aldrich (St. Louis Missouri, USA)
Dermatan sulfate	RS	Sigma-Aldrich (St. Louis Missouri, USA)
Heparin sodium salt from porcine intestinal mucosa Grade I-A	RS	Sigma-Aldrich (St. Louis Missouri, USA)
Heparin sodium salt from porcine intestinal mucosa	RS	Sigma-Aldrich (St. Louis Missouri, USA)
Oversulfated chondroitin sulfate	RS	Sigma-Aldrich (St. Louis Missouri, USA)
Heparin LEO	-	LEO Pharma (Ballerup, Danmark)
Heparin Sodium Inj	-	B.Braun Medical Inc. (PA, USA)
DBL Heparin Sodium Injection BP	-	Hospira NZ limited (Wellington, New Zealand)
Acetonitrile (ACN)	HPLC	Fisher Scientific (Leicesterahire, UK)
Ammonium acetate	AR	Riedel-de Haen (Seelze, Germany)
Ammonium dihydrogen phosphate	AR	Fluka (Buchs, Switzerland)

Table 3.1 (continued) List of chemicals and reagents

Name	Grade	Source/Supplier
Ammonium formate	AR	Fluka (Buchs,Switzerland)
Benzyl alcohol		
Dodecyl trimethyl ammonium hydrogen sulfate (DDTMAHS)	AR	Fluka (Buchs, Switzerland)
Formic acid	AR	Sigma-Aldrich (St. Louis Missouri, USA)
Glacial acetic acid	AR	Merck (Darmstadt, Germany)
Lithium perchlorate	AR	ACROS organics (New Jersey, USA)
Methanol (MeOH)	HPLC	Fisher Scientific (Leicesterahire, UK)
Phosphoric acid	AR	Merck (Darmstadt, Germany)
Sodium chloride	AR	Merck (Darmstadt, Germany)
Sodium dihydrogen phosphate Monohydrate	AR	Merck (Darmstadt, Germany)
Sodium hydroxide	AR	Fisher scientific (Leicesterahire, UK)
Tetrabutyl ammonium hydrogen sulfate (TBAHS)	AR	Aldrich (Munich, Germany)
Triethylamine	AR	Fluka (Buchs, Switzerland)
Tris(hydroxymethyl) aminomethane (Tris)	AR	Merck (Darmstadt, Germany)

Table 3.2 List of instruments

Instrument	Source/Supplier
HPLC-DAD system	Hitachi (Tokyo, Japan)
HPLC-UV system	Waters (Massachusetts, USA)
HPLC-DAD system	Waters (Massachusetts,USA)
HILIC amide	Merck (Darmstatdt, Germany)
column i.d. 150 mm x 2.1 mm	
ZIC-HILIC	Merck (Darmstatdt, Germany)
column i.d. 150 mm x 4.6 mm	
Phenyl column	Vertical Chromatography
Vertisep GES ODS	(Nonthaburi, Thailand)
column i.d. 150 mm x 4.6 mm	
RFIC^{TM} Ion PAC AS22	*Thermo Scientific Dionex (Idstein, Germany)*
column i.d. 250 mm x 4.0 mm	
RFIC^{TM} Ion PAC AG22	*Thermo Scientific Dionex (Idstein, Germany)*
guard column i.d. 50 mm x 4.0 mm	
Arium® pro UV Ultrapure water system	Sartorius Stedim Biotech
	(Aubagne, France)
Ultrasonic bath	Branson Ultrasonic Corporation
	(Connecticut, USA)
Water bath	Memmert (Schwabach, Germany)
Analytical balance	Mettler Toledo (Ohio, USA)
UV-vis spectrophotometer	Perkin Elmer (Massachusetts, USA)
Vortex mixer	Scientific Industries, Inc.
	(New York, USA)
Pipette	Duran (Mainz, Germany)
pH meter	Consort (Turnhout, Belgium)

3.2 Method Development

Three chromatographic techniques (e.g., RP-HPLC, HILIC, IEC) were investigated for the analysis of heparin and its impurities. Chemical and instrumental factors in each technique were varied as shown in Table 3.3.

Table 3.3 Chemical and instrumental factors in each selected HPLC technique

	RP-HPLC	HILIC	IEC
Type of column	C18	Amide	SAX
Mobile phase	Buffer:ACN	ACN:water	Buffer:ACN
pH buffer	3.0, 6.5	3.2, 5.8	2.0 – 4.0
Flow rate (mL/min)	1.0	0.4, 0.6, 1.0	0.8, 1.0
Injection volume (μL)	20	10	10
Detection (nm)	215	215	215

3.3 Method Optimization

The anion-exchange chromatographic method was selected from three techniques to optimize condition using the following initial condition;

Column : Anion exchange column AS22 (4.0x250 mm)

Mobile phase : Gradient program

Time (min)	%A	%B
0	5	95
2	5	95
40	60	40
41	60	40
42	5	95
50	5	95

A = 2.5 M NaCl with 20 mM Tris buffer (pH 3.0) in water/ACN 80/20 v/v

B = ACN/water 20/80 v/v

Flow rate : 0.8 mL/min

Detection : UV 215 nm

Injection volume : 40 μL

Run time : 50 min

The initial condition was optimized through the use of experimental design, including BBD and CCD, combined with Multivariate Linear Regression (MLR). The determination of the relationship between the retention factor and the corresponding mobile phase composition, including buffer pH and buffer concentration was studied. Then, the modified simplex approach was utilized to search for optimal chromatographic conditions in the retention map.

3.4 Method Validation

The optimized method was fully validated following the ICH guidelines (88).

3.4.1 Specificity

Placebo and placebo spiked with individual standard solution at middle concentration of calibration curves were prepared. The ability to separate all impurities, and excipients in placebo from heparin in the sample was assessed by the resolution between the peaks. Peak identification was performed by comparing the retention time of major peaks in the chromatogram of the assay solution with those in the chromatogram of the standard solution. Injection order for specificity test of this method is shown in Table 3.4.

Table 3.4 Injection order for specificity test

Injection No.	Name
1	Mobile phase (starting composition)
2	Heparin solution (10 mg/mL)
3	Water + 5% benzyl alcohol (placebo)
4	Dermatan sulfate (0.30 mg/mL)
5	Dermatan sulfate (0.30 mg/mL) + OSCS (0.12 mg/mL)
6	Mixture of heparin solution (10 mg/mL), Dermatan sulfate (0.30 mg/mL) and OSCS (0.12 mg/mL)
7	Heparin Injection (sample)

3.4.2 Linearity and range

Linearity of the optimized method was determined from two sets of five concentrations of standards. The concentrations were in the ranges of 3 to 20 mg/mL for heparin, 90 to 160 μg/mL for OSCS and 200-400 μg/mL for DS. Each concentration was prepared in triplicate (Tables 3.5 to 3.6).

Linear regression was performed and the coefficient of determination (r^2), the standard deviation of the slope and the intercept were calculated. The linearity was evaluated visually from the plotted calibration data, and statistically by performing for lack-of-fit (LOF) test.

3.4.3 Trueness

Trueness of the method was evaluated by the standard addition method and expresses as percent recovery. Standard addition solutions were prepared by spiking the placebo with heparin and its impurities at the same concentrations as in the calibration curves. The solutions were prepared in triplicate (Tables 3.7 to 3.8).

3.4.4 Precision

Precision of the method was expressed as the percent relative standard derivation of repeatability and time-different intermediate precision. Repeatability and time-different intermediate precision were determined simultaneously. Three concentrations (lowest, middle and highest concentrations of the calibration curves) of each compound were spiked into the placebo (Tables 3.7 to 3.8). The analysis was daily performed in triplicate and repeated for five days. The %RSDs of the peak area was calculated.

The repeatability (s_r^2) and the time-different intermediate precision ($s_{i(t)}^2$) were then estimated at each concentration level from an ANOVA table and the equation below;

$$s_{i(t)}^2 = s_r^2 + s_b^2$$

where s_b^2represents the between-days variance.

$$s_b^2 = (MS_{between} - MS_{within})/n$$

where $MS_{between}$ and MS_{within} are between-days and within-day mean squares, respectively, obtained from the ANOVA table and n is number of replicates within one day.

3.4.5 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were estimated based on the standard deviation of the response and the slope of the calibration curve as follow;

$$LOD = \frac{3.3\ \sigma}{S}$$

$$LOQ = \frac{10\ \sigma}{S}$$

where σ is the standard deviation of the peak area and S is the slope of the calibration curve. The slope S was calculated from the calibration curves. The standard deviation σ was obtained from the standard deviation of y-intercepts of regression lines.

3.5 Standard solutions preparation

3.5.1 Stock standard solutions

5.1.1 Dermatan sulfate stock solution (1,000 μg/mL)

Twenty milligrams of dermatan sulfate was dissolved in twenty millilites of water in a volumetric flask.

5.1.2 OSCS stock solution (2,000 μg/mL)

Two milligrams of OSCS was dissolved in one milliliter of water in a vial.

5.1.3 Benzyl alcohol stock solution (36 mg/mL) for placebo preparation

Appropiate amounts of benzyl alcohol (360 μL) was diluted with ten milliliters of water in a volumetric flask.

3.5.2 Working standard solutions

Working standard solutions of heparin, DS and OSCS for validation studies were prepared according to Tables 3.5 to 3.8.

Table 3.5 Heparin standard solutions in water, for linearity

Concentration (mg/mL)	Weight (mg)	Volume of water (μL)
3	3	1,000
5	5	1,000
10	10	1,000
15	15	1,000
20	20	1,000

Table 3.6 Standard mixture solutions in water, for linearity

Concentration heparin,DS,OSCS (μg/mL)	Volume DS stock (μL)	Volume OSCS stock (μL)	Mass heparin (mg)	Volume water (μL)
10,000, 200, 90	200	45	20	755
10,000, 250, 100	250	50	20	700
10,000, 300, 120	300	60	20	640
10,000, 350, 140	350	70	20	580
10,000, 400, 160	400	80	20	520

Table 3.7 Heparin standard solutions in water, for recovery

Concentration (mg/mL)	Mass heparin (mg)	Volume benzyl alcohol stock (μL)	Volume water (μL)
3 (low)	3	100	900
5	5	100	900
10 (middle)	10	100	900
15	15	100	900
20 (high)	20	100	900

Table 3.8 Standard mixture solutions in water, for recovery

Concentration heparin,DS,OSCS (μg/mL)	Volume DS stock (μL)	Volume OSCS stock (μL)	Mass heparin (mg)	Volume benzyl alc. stock (μL)	Volume water (μL)
10,000, 200, 90 (low)	200	45	10	100	655
10,000, 250, 100	250	50	10	100	600
10,000, 300, 120 (middle)	300	60	10	100	540
10,000, 350, 140	350	70	10	100	480
10,000, 400, 160 (high)	400	80	10	100	420

3.6 Sample preparation

720 µL of Heparin Na Injection (5,000 IU/mL) was transfered into a 1 mL vial and diluted to volume with water. The final concentration was 10 mg/mL.
(180 IU = 1 mg)

3.7 Mobile phase preparation

The mobile phase consisted of 2.5 M NaCl and 20 mM Tris buffer (pH 2.1). The mobile phase was prepared by dissolving 438.3 g NaCl and 7.3 g Tris in three liters of water and adjusting the pH to 2.1 with 85% phosphoric acid.

3.8 Applications

One lot of heparin raw material and six lots of heparin products available in Thailand were purchased from local manufacturers and drug stores and were analyzed by the developed method. Results from the developed method were compared with the reported data.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Method development

The aim of this study was to develop a simple HPLC method for the simultaneous determination of heparin and its impurities (i.e. DS and OSCS) in pharmaceutical products. Due to its extremely complex, polydisperse and heterogeneous structure, heparin belongs to one of the most challenge in pharmaceutical analysis. Moreover, its impurities usually have properties that resemble heparin, which make it difficult to be distinguished. In this study, the potential chromatographic techniques, including RP-HPLC, HILIC, and IEC, were studied to reach this purpose.

4.1.1 Reversed-phase chromatography

RP-HPLC is the most widely used method for analytical method development because of its simplicity and availability. To develop a method, a common reversed-phase column, ODS, was selected. The compositions of the mobile phase, including pH of buffer (3.0 and 6.5), type and amounts of organic modifiers (methanol and acetonitrile) and percent of organic modifier (10% and 20%) were varied during development. Results showed that heparin and its impurities were not retained on the ODS column resulting in overlapping peaks at the solvent front at 1.5 min (Figure 4.1). Because of the presence of multiple negative charges associated with N- and O- sulfo groups, heparin and its impurities were not able to retain on a reversed phase column.

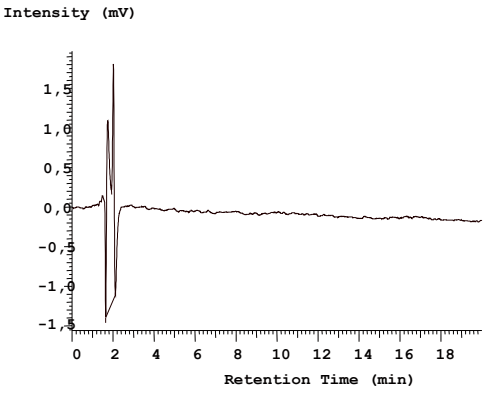
Two ion-pairing reagents, tetrabutyl ammonium hydrogen sulfate (TBAHS) and dodecyl trimethyl ammonium hydrogen sulfate (DDTMAHS), a long chain ion-paring reagent, were investigated to retain heparin and its impurities on the stationary phase. Effects of ion-pairing reagent with a larger alkyl group showed more

retention time for heparin but it could not be used for quantitative analysis because of poor resolution and peak shape (Figure 4.2).

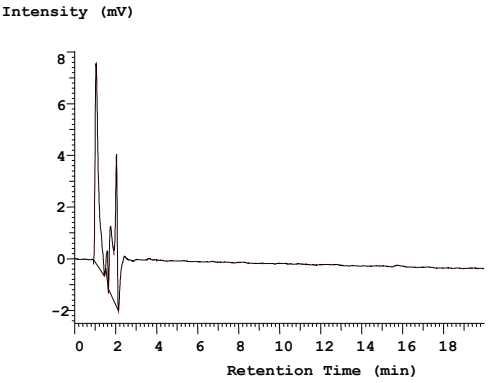
4.1.2 Hydrophilic-interaction liquid chromatography

HILIC was introduced as an alternative method for separating and analyzing highly polar compounds. HILIC gives retention of compounds that are weakly or not at all bound in RP-HPLC. For HILIC method development, many variables were studied, including the composition of the mobile phase (pH of buffer, type and amounts of organic modifiers (methanol and acetonitrile) and percent of organic modifier), the column temperature, and the flow rate. Many reports suggested that an amide column is more stable than other HILIC columns and has been frequently used to separate polar compounds, especially carbohydrates (77). Thus, an amide column was applied for this study.

Effects of the percent of acetonitrile (80% and 90% in water), type of buffer solutions (50 mM ammonium formate and 50 mM ammonium acetate) and buffer pH (3.2 and 5.8) were investigated. Results showed that neither percent of acetonitrile, type of buffer solutions nor pH have significant effects on retention and separation (Figure 4.3). In summary, the HILIC conditions were not able to retain and separate heparin and its impurities. Because HILIC interaction between the analytes and the particles occurred at the surface of the particles was not strong enough to retain the analytes.



(A)



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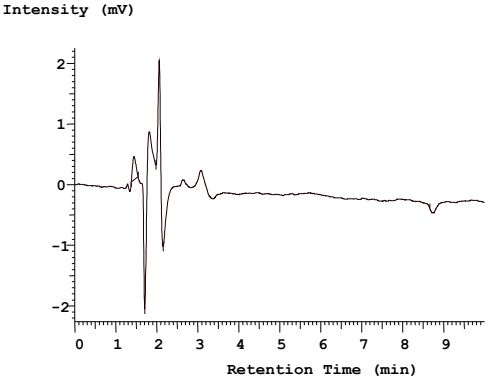
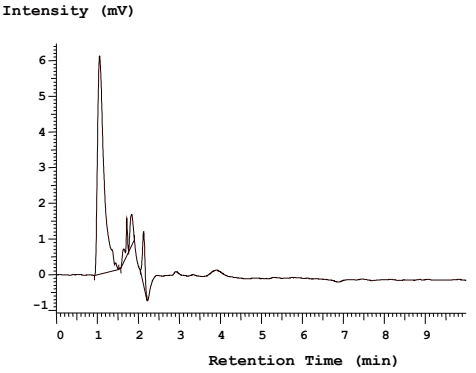
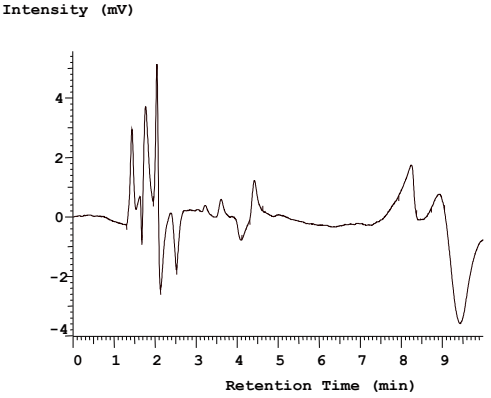


(C)



(D)



(E)

Figure 4.1 The RP-HPLC chromatograms of heparin solution (8 mg/mL) on C18 column at flow rate 1.0 mL/min, UV detection at 215 nm and injection volume at 20 µL with various mobile phase compositions containing (A) water and ACN (80:20), (B) water and MeOH (80:20), (C) phosphate buffer (pH 3.0) and ACN (80:20), (D) phosphate buffer (pH 3.0) and ACN (90:10), and (E) phosphate buffer (pH 6.5) and ACN (80:20).

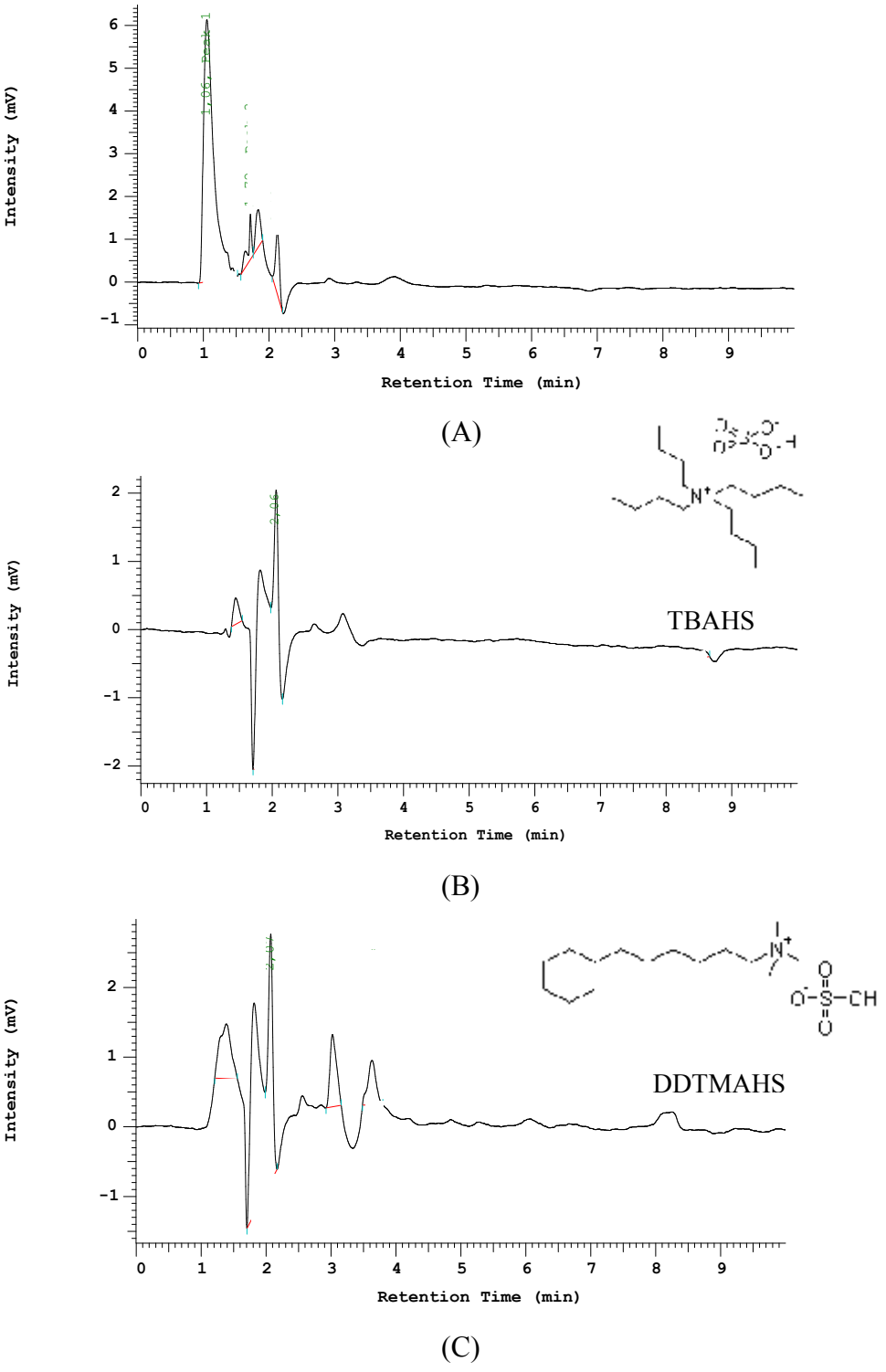


Figure 4.2 The effect of ion-pairing reagents containing in mobile phase (A) no ion pairing reagent, (B) TBAHS, and (C) DDTMAHS. HPLC conditions see Figure 4.1 D.

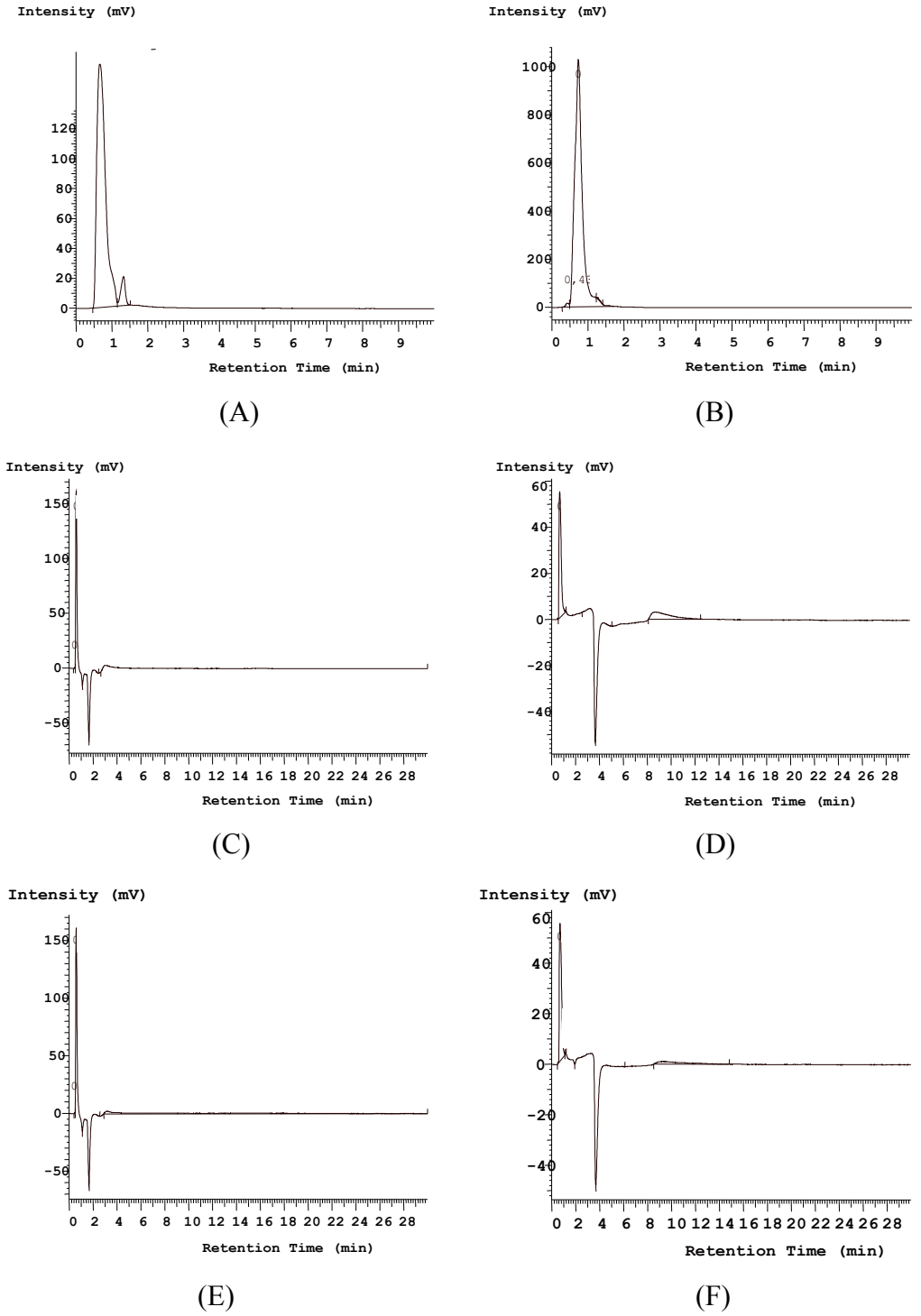


Figure 4.3 The HILIC chromatograms of heparin solution (5 mg/mL) on HILIC amide column at flow rate 0.6 mL/min, UV detection at 215 nm and injection volume at 10 µL with various mobile phase compositions containing (A) water and ACN (20:80), (B) water and ACN (10:90), (C) formate buffer (pH 3.2) and ACN (20:80), (D) acetate buffer (pH 5.8) and ACN (20:80), (E) formate buffer (pH 3.2) and ACN (10:90), and (F) acetate buffer (pH 5.8) and ACN (10:90).

4.1.3 Ion-exchange chromatography

IEC techniques, especially SAX-HPLC method have been reported to allow separating heparin and its impurities (73-75). The published methods were used only for qualification and identification. In this study, a rapid, robust and accurate method for the quantitative analysis of heparin and its impurities was developed. The mobile phase compositions, including types of salts (i.e. $NaClO_4$, NaCl and LiCl), salt concentration (2.0 - 2.5 M), pH (2.0 – 4.0) and organic modifier (methanol and acetonitrile) and the flow rate (0.6 – 1.0 mL/min) were varied.

Following the conditions listed in the USP heparin sodium monograph, the retention and separation of heparin and OSCS was demonstrated in Figure 4.4. Two peaks were identified as heparin and OSCS with a resolution (R_s) of 2.5. The condition consisted of a 30 min gradient elution from 2.8% to 12.6% of 2.5 M $NaClO_4$ in 20 mM NaH_2PO_4 buffer (pH 3.0). Because sodium perchlorate is hygroscopic and used in pyrotechnics, it is not allowed for trading in Thailand. Thus, sodium perchlorate was replaced by sodium chloride with a gradient elution from 5% to 60% of 2.5 M NaCl in 20 mM Tris buffer (pH 3.0). The chromatograms which resulted from mobile phase containing sodium perchlorate and sodium chloride are shown in Figure 4.5. Although the R_s was less than that of the USP condition, (R_s 1.8 vs 2.5), better peak shapes were obtained. Furthermore, effects of organic modifier types (e.g. methanol and acetonitrile), flow rates (0.6, 0.8, 1.0 mL/min), and lithium chloride were studied. The results indicated that adding low amounts of acetonitrile (not more than 20%) into the mobile phase improved the signal of the analytes, while adding methanol resulted in high back pressure of the system. Even a mobile phase containing a low amount of acetonitrile was able to increase signal, it could not be used because of unknown peak interference problems. The flow rates had no significant effects on the separation, but the signals increased at lower flow rates (Figure 4.6). Replacement of sodium chloride with lithium chloride did not affect the separation, but caused a baseline drift (Figure 4.7).

In summary, final condition in the development phase was a 30 min gradient elution of 2.5 M sodium chloride and Tris buffer (pH 3.0) at a flow rate of 0.6 mL/min. The detection was performed with a UV detector at wavelength of 215 nm. Injection volume was set at 20 µL. In order to obtain an optimal response and improve

the method performance, the experimental design and response surface methodology were used to assist in the method optimization.

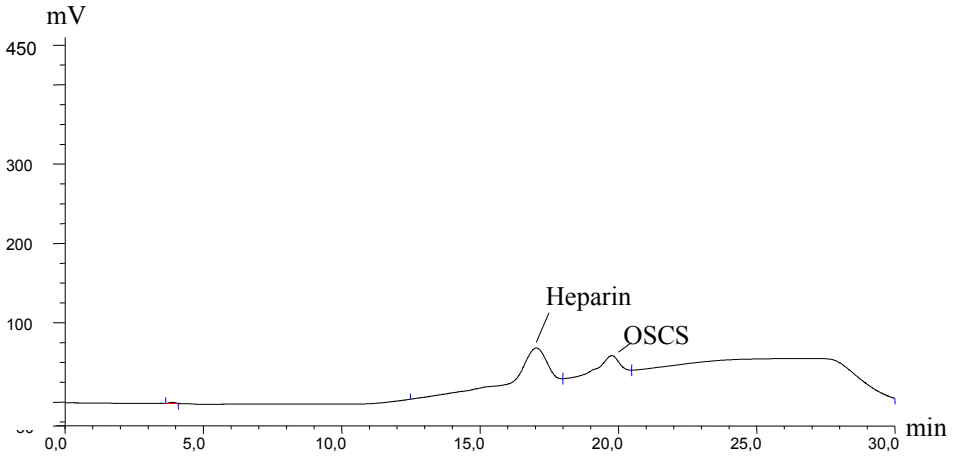


Figure 4.4 SAX-HPLC chromatogram of standard solution containing 9 mg/mL heparin and 3 mg/mL OSCS with elution gradient from 2.8% to 12.6% of 2.5 M sodium perchlorate and 20 mM sodium dihydrogen phosphate buffer (pH 3.0) on SAX column at flow rate 0.8 mL/min, UV detection at 215 nm and injection volume at 10 µL.

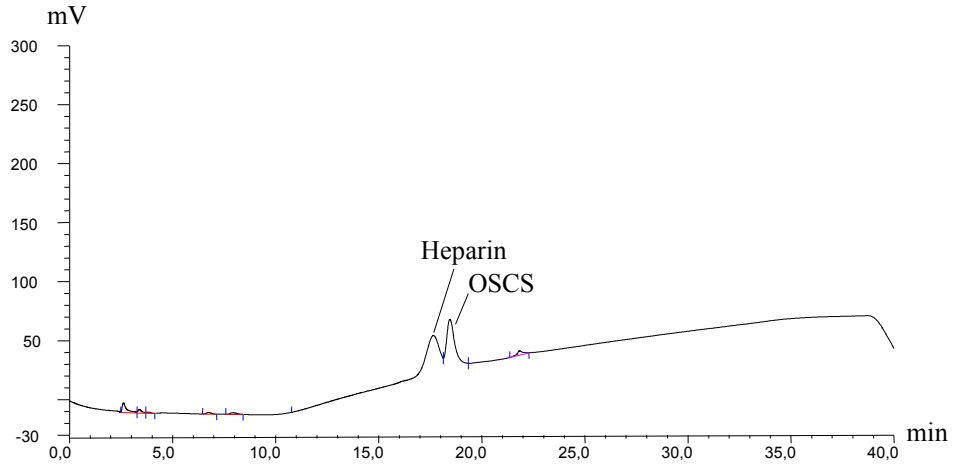


Figure 4.5 SAX-HPLC chromatogram of standard solution containing 9 mg/mL heparin and 3 mg/mL OSCS with elution gradient from 5% to 60% of 2.5 M sodium chloride and 20 mM Tris buffer (pH 3.0) on SAX column at flow rate 0.8 mL/min, UV detection at 215 nm and injection volume at 10 µL.

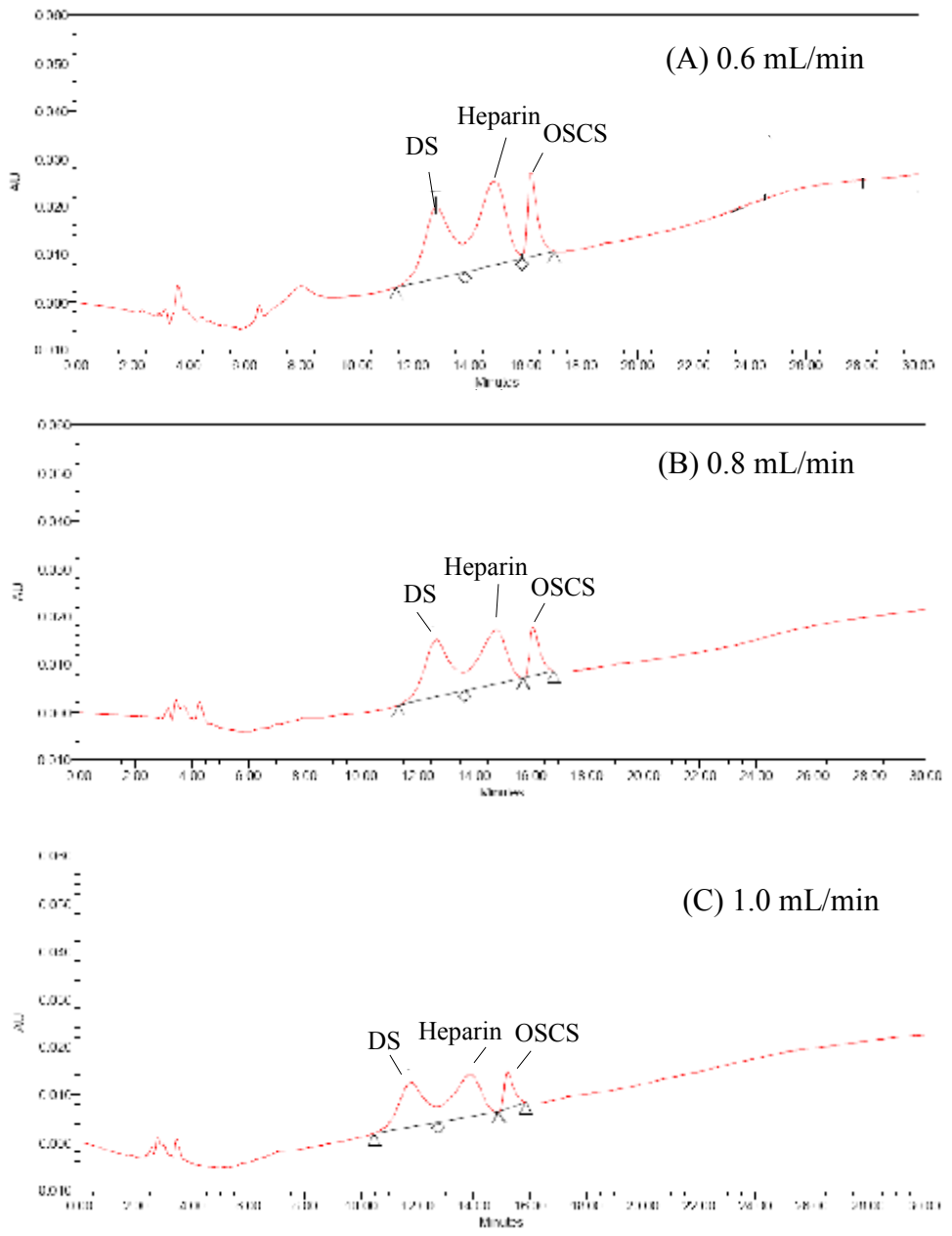
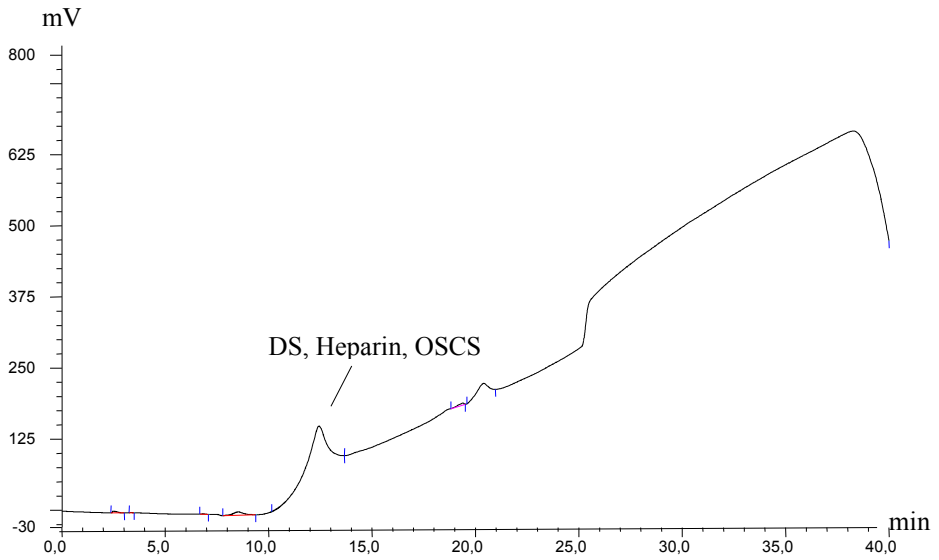
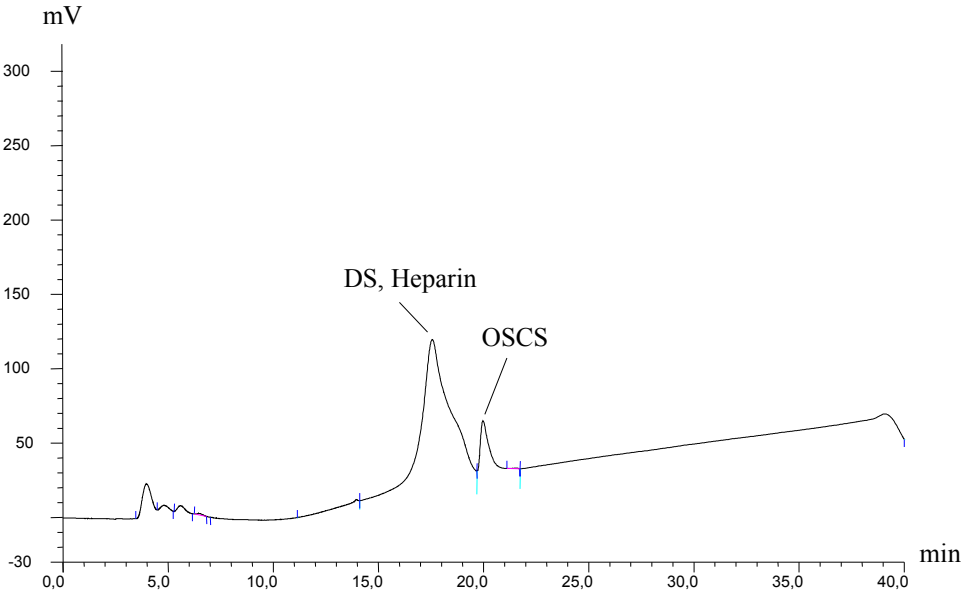


Figure 4.6 The effect of flow rate (A) 0.6 mL/min, (B) 0.8 mL/min and (C) 1.0 mL/min with elution gradient from 5% to 60% of 2.5 M sodium chloride in 20mM Tris pH 3.0 on SAX column at flow rate 0.8 mL/min, UV detection at 215 nm and injection volume at 10 µL.



(A)



(B)

Figure 4.7 Comparison of chromatograms of mixture (heparin 9 mg/mL, DS 3 mg/mL, OSCS 3 mg/mL) obtained from mobile phase containing (A) lithium chloride and (B) sodium chloride. HPLC conditions see in Figure 4.6 B.

4.2 Method optimization

In this study, a Box-Behnken design (BBD) and a central composite design (CCD) were compared and used to study the effects of two important factors, i.e. pH and concentrations of sodium chloride in the mobile phase and afterwards to define the optimal chromatographic conditions for the separation. The experimental plans for BBD and CCD are displayed in Tables 4.1 and 4.2, respectively. Each factor is examinated at three levels (-1, 0, +1) in the BBD and at five levels ($-\sqrt{2}$, -1, 0, +1, $+\sqrt{2}$) in the CCD. The concentration of sodium chloride was set in the range of 0.8 to 2.5 M, and the pH of mobile-phase buffer was adjusted in the range of 2.0 to 4.0, while the flow rate was held at a constant level (0.6 mL/min) as well the closen detection wavelength (215 nm) during all experiments. Nine experiments varying two factors in each experimental design, were performed in randomized sequence to minimize effects of uncontrolled factors that may introduce a bias on the response. The investigated variables and their values, for each design, are reported in Tables 4.3 and 4.4. The retention time (t_R), resolution (R_s) and peak width (W) of the compounds were determined as responses.

Table 4.1 A two-factor Box-Behnken design

Experiment No.	pH of bufer	Conc. of NaCl
1	-1	-1
2	-1	+1
3	+1	-1
4	+1	+1
5	-1	0
6	+1	0
7	0	-1
8	0	+1
9	0	0

Table 4.2 A two-factor central composite design

Experiment No.	pH of buffer	Conc. of NaCl
1	-1	-1
2	-1	+1
3	+1	-1
4	+1	+1
5	-√2	0
6	+√2	0
7	0	-1
8	0	+√2
9	0	0

Table 4.3 Investigated variables and their values for a 2-factor Box-Behnken design

Experiment No.	pH of buffer	Conc.of NaCl
1	2.0	1.5
2	2.0	2.5
3	3.0	1.5
4	3.0	2.5
5	2.0	2.0
6	3.0	2.0
7	2.5	1.5
8	2.5	2.5
9	2.5	2.0

Table 4.4 Investigated variables and their values for a 2-factor central composite design

Experiment No.	pH of buffer	Conc.of NaCl
1	2.3	0.8
2	2.3	2.2
3	3.7	0.8
4	3.7	2.2
5	2.0	1.5
6	4.0	1.5
7	3.0	0.8
8	3.0	2.5
9	3.0	1.5

Results from the nine experiments of each design were reported in Tables 4.5 and 4.6. Coefficients of the second-order polynomial models of the three responses were estimated by least squares regression. Coefficients from the polynomial models were shown in Tables 4.7 and 4.8. From the polynomial models, three-dimensional response surfaces of each response were plotted in order to easily and more precisely define the chromatographic behaviors of the investigated variables (Figures 4.8 and 4.9). The response surfaces from CCD showed clearly influences of variables on responses more than BBD. An increase in the concentrations of sodium chloride in the mobile phase led to an increase of resolution between both pairs but shortens the retention and reduces the peak widths of all investigated compounds. Influence of pH value was not the same for all analytes. The resolution between heparin and OSCS slightly increased, whereas resolution of dermatan sulfate and heparin increased with the decrease in pH. Moreover, the increase of pH value prolonged the retention of all investigated compounds.

Table 4.5 Experimental results for the 2-factor Box-Behnken design

	Factors		Responses							
Experiment No.	pH of buffer	Conc. of NaCl	R_s DS/Hep	R_s Hep/OSCS	t_R (min) DS	t_R (min) Hep	t_R (min) OSCS	W (min) DS	W (min) Hep	W (min) OSCS
1	2.0	1.5	1.64	1.05	17.47	21.78	23.75	1.44	1.62	0.65
2	2.0	2.5	1.61	1.09	12.74	15.08	16.27	0.73	0.89	0.36
3	3.0	1.5	1.34	1.12	17.43	19.97	22.13	1.05	1.23	1.15
4	3.0	2.5	1.06	1.64	12.57	13.47	14.71	0.48	0.67	0.30
5	2.0	2.0	1.56	1.06	14.57	17.59	19.14	1.04	1.17	0.53
6	3.0	2.0	1.26	1.35	14.32	15.97	17.56	0.72	0.89	0.53
7	2.5	1.5	1.49	1.18	17.40	20.97	23.23	1.32	1.43	0.64
8	2.5	2.5	1.30	1.43	12.42	13.86	15.16	0.56	0.74	0.30
9	2.5	2.0	1.39	1.32	14.32	16.73	18.35	0.92	1.04	0.46

R_s = resolution, t_R = retention time, W = peak width

Table 4.6 Experimental results for the 2-factor central composite design

	Factors				Responses					
Experiment No.	pH of buffer	Conc. of NaCl	R_s DS/Hep	R_s Hep/OSCS	t_R (min) DS	t_R (min) Hep	t_R (min) OSCS	W (min) DS	W (min) Hep	W (min) OSCS
1	2.3	0.8	0.00	1.12	27.41	27.41	29.83	1.80	1.80	1.06
2	2.3	2.2	1.45	1.08	13.57	16.04	17.48	0.94	1.02	0.52
3	3.7	0.8	0.92	0.00	27.57	29.88	29.88	0.60	1.44	1.44
4	3.7	2.2	0.00	1.27	13.53	13.53	14.78	0.87	0.87	0.48
5	2.0	1.5	1.54	1.042	17.35	21.52	23.57	1.41	1.57	0.72
6	4.0	1.5	0.00	1.37	17.47	17.47	19.38	1.24	1.24	0.46
7	3.0	0.8	0.00	1.00	27.42	27.42	30.04	2.10	2.10	0.69
8	3.0	2.5	0.82	1.03	12.50	13.67	14.85	0.30	0.73	0.26
9	3.0	1.5	1.28	1.37	17.45	19.96	22.17	1.01	1.25	0.89

R_s = resolution, t_R = retention time, W = peak width

Table 4.7 The estimates of the coefficients for regression models from the BBD results

Model Coefficents	R_s DS/Hep	R_s Hep/OSCS	t_R DS	t_R Hep	t_R OSCS	W DS	W Hep	W OSCS
b0	1.3950	1.3033	14.3086	16.6787	18.3398	0.9100	1.0251	0.4281
b1	-0.1908	0.1518	-0.0763	-0.8400	-0.7925	-0.1610	-0.1467	0.0745
b2	-0.0842	0.1350	-2.4293	-3.3865	-3.8310	-0.3398	-0.3305	-0.2482
b11	0.0165	-0.0925	0.1377	0.1250	0.0158	-0.0270	0.0123	0.1198
b22	0.0005	0.0080	0.6037	0.7585	0.8563	0.0375	0.0638	0.0588
b12	-0.0623	0.1203	-0.0350	0.0470	0.0155	0.0353	0.0443	-0.1428

R_s = resolution, t_R = retention time, W = peak width

Table 4.8 The estimates of the coefficients for regression models from the CCD results

Model Coefficents	R_s DS/Hep	R_s Hep/OSCS	t_R DS	t_R Hep	t_R OSCS	W DS	W Hep	W OSCS
b0	1.0755	1.4637	17.4711	19.5751	22.1194	1.3122	1.4024	0.7094
b1	-0.3383	-0.0587	0.0364	-0.7200	-1.0705	-0.1882	-0.1212	-0.0032
b2	0.2794	0.2590	-7.1317	-6.7576	-6.8301	-0.3569	-0.4210	-0.3106
b11	-0.1340	-0.1527	0.0713	0.0690	-0.3169	-0.0254	-0.0301	0.0067
b22	-0.3891	-0.3992	2.7721	1.8523	1.1817	-0.1693	-0.0262	0.0309
b12	-0.5913	0.3263	-0.0483	-1.2458	-0.6868	0.2838	0.0543	-0.1063

R_s = resolution, t_R = retention time, W = peak width

In order to achieve the best separation performances and reasonable retention of all compounds in the HPLC system, data analysis led to the conclusion that the final composition of the mobile phase in the first step of the gradient program should contain 2.5 M NaCl with 20 mM Tris buffer in water (pH 2.1). This condition conforms to the optimal chromatographic condition predicted from the polynomial model of the CCD. The chromatogram obtained from the optimal condition is shown in Figure 4.10. The optimal condition is described as below;

Column : Anion exchange column AS22 (4.0x250 mm)

Mobile phase : Gradient program

Time	%A	%B
0	10	90
2	10	90
33	70	30
34	70	30
35	10	90
40	10	90

A = 2.5 M NaCl with 20mM Tris buffer (pH 2.1) in water

B = water

Flow rate : 0.6 mL/min

Detection : UV 215 nm

Injection volume : 200 µL

Run time : 40 min

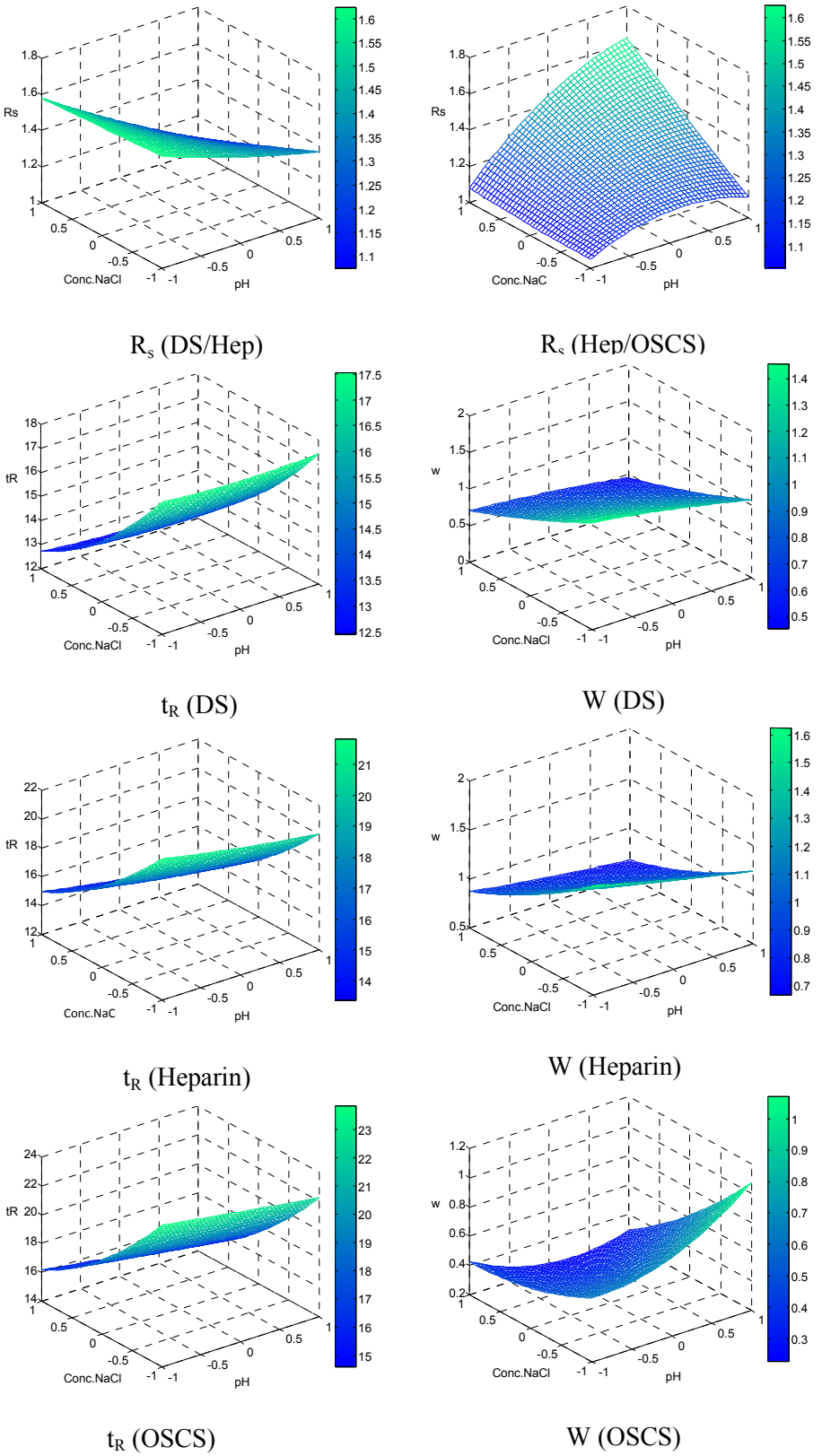
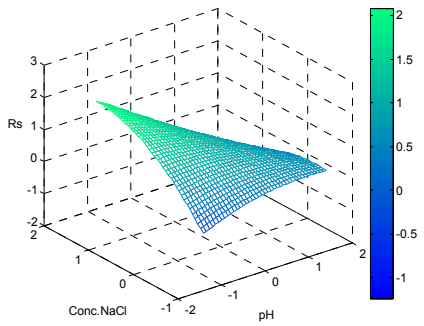
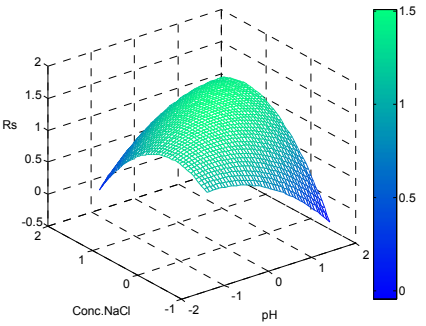


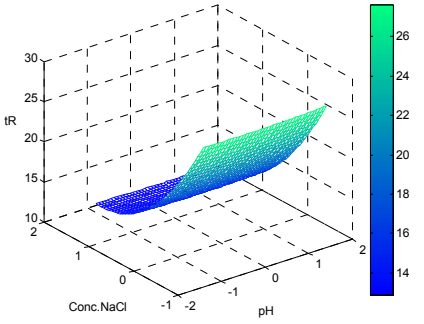
Figure 4.8 3D plots of the response surfaces for R_s, t_R, and W from the BBD experiments.



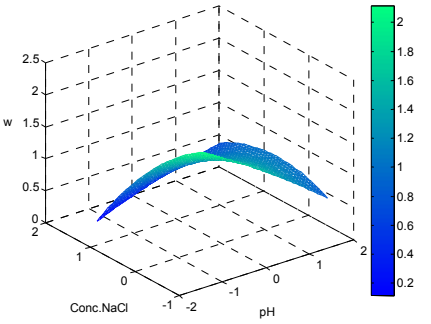
R_s (DS/Hep)



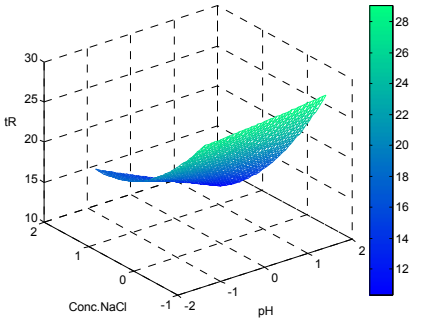
R_s (Hep/OSCS)



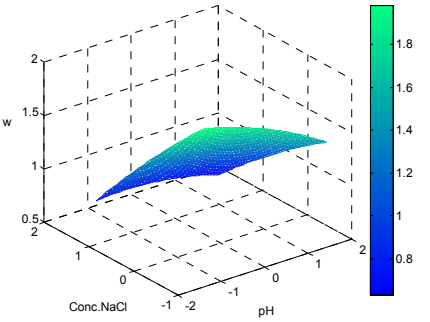
t_R (DS)



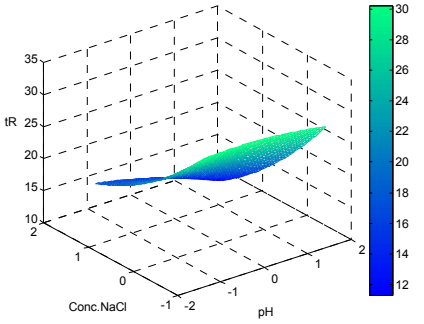
W (DS)



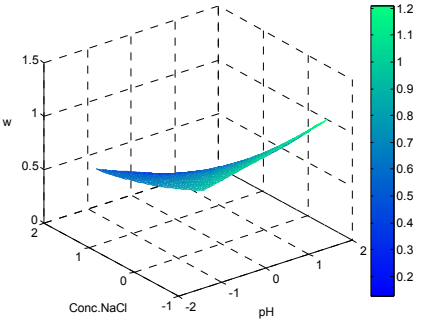
t_R (Heparin)



W (Heparin)



t_R (OSCS)



W (OSCS)

Figure 4.9 3D plots of the response surface for Rs, t_R, and W from the CCD experiments.

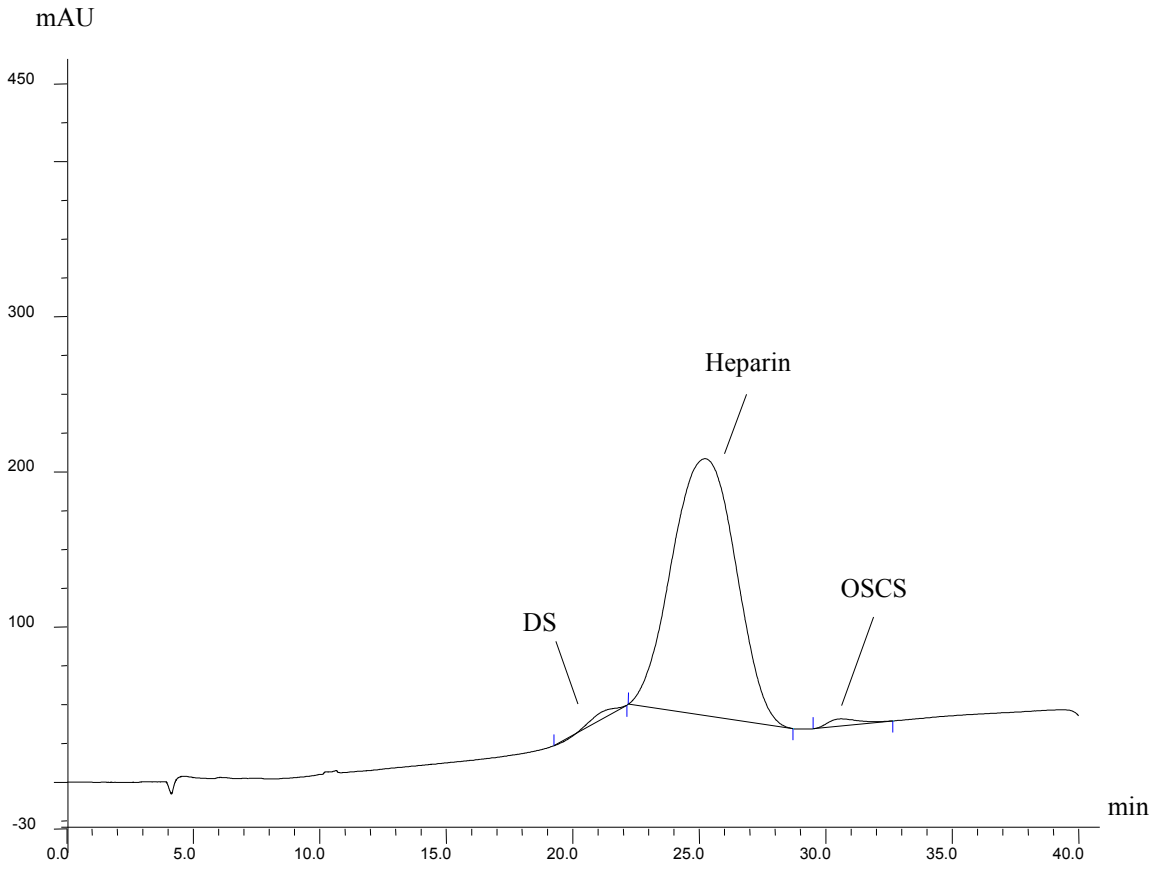


Figure 4.10 SAX-HPLC chromatogram of mixture containing 20 mg/mL heparin, 0.3 mg/mL DS, and 0.12 mg/mL OSCS with elution gradient from 10% to 70% of 2.5 M sodium chloride and 20 mM Tris buffer (pH 2.1) on a SAX column at the flow rate of 0.6 mL/min, UV detection at 215 nm and injection volume at 200 µL.

4.3 Method validation

The optimized condition was validated by a standard procedure to ensure adequate specificity, linearity, precision, accuracy, limit of detection, and limit of quantitation. The obtained data fully met the criteria of the ICH regulations (88) for method validation.

4.3.1 Chromatographic separation and specificity

The specificity of method was investigated with placebo to observe interferences with heparin and its impurities. No interfering peak was observed in the chromatograms (Figure 4.11). Therefore, the specificity of the method was found to be appropiate. The optimal conditions provided retention times of heparin, DS, and OSCS of 25.24, 21.13 and 30.65 minutes, respectively (Figure 4.10). The resolution between DS and heparin was 2.05 and between heparin and OSCS was 2.70. The analysis was completed in 40 minutes.

4.3.2 Linearity

The calibration curves of heparin, DS, and OSCS were established by triplicate injections of five different concentrations of working standard solutions. From Table 4.9, the calibration curves were plotted from the concentrations versus peak area response. The obtained r^2 were 0.9998 for heparin (3-20 mg/mL), 0.9994 for OSCS (90-160 μg/mL) and 0.9980 for DS (200-400 μg/mL).

Visual observation of the calibration curves showed that the lines were straight. The lack-of-fit (F_{cal}) test results for the heparin calibration data was = 0.18, for DS was 0.02 and for OCSC was 0.24. These values were smaller than the critical value, $F_{tab(\alpha=0.05;\ df1=3,\ df2=20)} = 3.10$ for heparin and for dermatan sulfate and $F_{tab(\alpha=0.05;\ df1=3,\ df2=10)} = 3.71$ for OSCS. This means that the straight lines were considered adequate to describe the relationships between the responses and the concentrations for each compound.

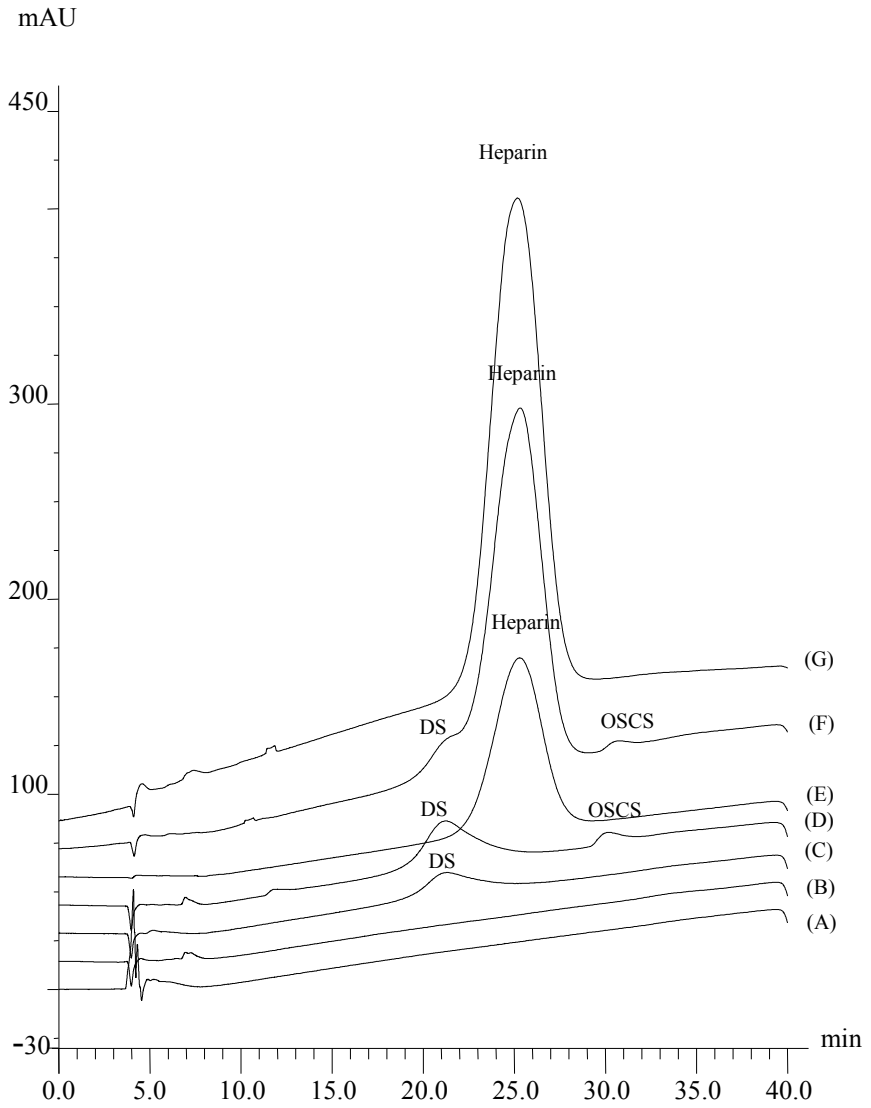


Figure 4.11 Specificity of the SAX-HPLC method at the optimal condition (see condition at Figure 4.10) : (A) mobile phase (staring composition), (B) Placebo (5% benzyl alcohol in water), (C) DS solution (3 mg/mL), (D) mixture of DS (5 mg/mL) and OSCS (5 mg/mL), (E) heparin solution (8 mg/mL), (F) mixture of DS (0.3 mg/mL), heparin (10 mg/mL), and OSCS 0.12 mg/mL), and (G) sample solution (heparin sodium injection : 10 mg/mL heparin).

Table 4.9 Linear regression analysis for heparin and its impurities

Analyte	Range (μg/mL)	Slope	Intercept	Standard deviation		r^2	F-Value
				Slope	Intercept		
Heparin	3,000-25,000	1,319.2	-3,660.7	404.9	3,284.03	0.9998	0.18
DS	200-400	18.6	-2,879.0	0.9	59.40	0.9980	0.02
OSCS	90-160	78.9	-5,844.9	9.0	796.19	0.9994	0.24

r^2 = coefficient of determination

4.3.3 Trueness

The results from examining the accuracy of the method are tabulated in Table 4.10. The mean percentage recovery for each compound was calculated at each concentration level and reported with its standard deviation. The range of % recovery for heparin was 95.5-103.6, while the mean recovery was 99.4 ± 2.6 at all concentration levels. For DS, the range of % recovery values was 92.3-103.9% and the mean value covering all concentration levels was 98.4 ± 3.2. The range of % recovery values for OSCS was 92.6-102.0% and the overall mean was found to be 98.9 ± 2.2. In conclusion, the method was considered to have an acceptable recovery.

4.3.4 Precision

The method precision was perceived as the percent relative standard deviation of repeatability (within-days precision) and of time-different intermediate precision (between-days precision). Table 4.11 shows the precision of the method. The %RSD of repeatability for heparin at the lowest, middle and highest concentrations of the calibration levels (3, 10, and 20 mg/mL) were 1.46, 0.55 and 0.80, while %RSD of the time-different intermediate precision at the same levels were 3.37, 3.59 and 3.20, respectively.

For dermatan sulfate, the %RSD were 4.61, 2.09 and 3.56 for the repeatability and 6.38, 5.95 and 3.35 for the time-different intermediate precision, at the lowest, middle and highest dermatan sulfate concentrations of calibration levels 200, 300, and 400 μg/mL, respectively. The %RSD values for the OSCS concentrations at 90, 120 and 160 μg/mL were 3.38, 2.53 and 2.32, respectively, for

repeatability, and 11.42, 5.80 and 4.21, respectively, for the time-different intermediate precision. In conclusion, the precision values obtained in this method are considered acceptance.

Table 4.10 Investigation of the accuracy of the method

Analyte	Concentration (μg/mL)	% RSD (n = 3)	% Recovery
Heparin	3,000	3.55	96.2-103.6
	5,000	1.60	98.3-102.3
	10,000	2.62	97.6-103.3
	15,000	2.01	95.2-100.6
	20,000	2.16	95.3-100.7
DS	200	5.32	93.6-103.9
	250	2.73	97.0-102.0
	300	1.65	96.3-99.4
	350	3.21	95.1-101.0
	400	4.60	92.3-101.1
OSCS	90	0.70	99.1-100.5
	100	1.11	99.9-102.0
	120	1.36	98.8-101.1
	140	1.54	97.2-100.1
	160	3.13	92.6-98.1

% RSD = percent relative standard deviation

Table 4.11 Precision of heparin and its impurities presented as %RSD

Analyte	Concentration (μg/mL)	Repeatability	Time-different intermediate precision
Heparin	3,000	1.46	3.37
	10,000	0.55	3.59
	20,000	0.80	3.20
DS	200	4.61	6.38
	300	2.09	5.95
	400	3.56	3.35
OSCS	90	3.38	11.42
	120	2.53	5.80
	160	2.32	4.21

%RSD = percent of relative standard deviation

4.3.5 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of the compounds were the concentrations that can determined with acceptable precision and accuracy at the signal to noise ratio of 3.3 and 10, respectively. Table 4.12 shows LODs and LOQs of heparin, dermatan sulfate and OSCS. The LOQs of heparin, dermatan sulfate and OSCS were 2.5 mg/mL, 31.5 and 22.0 μg/mL, respectively. The %RSDs at the LOQ of the compounds were 3.22, 6.37 and 10.12, respectively.

Table 4.12 LODs and LOQs of heparin and its impurities

Analyte	LOD (μg/mL)	LOQ (μg/mL)
Heparin	800	2,500 (3.22)
Dermatan sulfate	10.5	31.5 (6.37)
OSCS	7.2	22.0 (10.12)

Number in parentheses represent %RSDs (percent relative standard deviation), LOD = limit of detection, LOQ = limit of quantitation.

4.4 Applications

The developed and validated method was applied for the determination of heparin and its impurities in one lot of heparin raw material and six lots of heparin injection products available in Thailand**.** Results are shown in Table 4.13, which reveals that percent label amounts of heparin in the six different lots in the three brands ranged from 90.3% to 97.8%, while that of the raw material was 95.8%. These values comply with the assay specifications for active drugs in the USP pharmacopeia (90.0-110.0% la.). The impurities dermatan sulfate and OSCS were not detected in neither the raw material nor the heparin injection products. The chromatograms of samples are shown in Figure 4.12.

Table 4.13 Results of analysis of marketed raw material and formulations

Product name	Brand	Lot No.	% Labeled amount (n=3)		
			Heparin	DS	OSCS
Heparin sodium Inj. (5,000 IU/mL)	A	I	97.8 (2.09)	ND	ND
		II	95.8 (1.36)	ND	ND
Heparin sodium inj. (5,000 IU/mL)	B	I	90.9 (1.99)	ND	ND
		II	94.6 (1.50)	ND	ND
Heparin sodium Inj. (5,000 IU/mL)	C	I	95.2 (3.40)	ND	ND
		II	90.3 (0.82)	ND	ND
Heparin sodium salt (raw material)	D	I	95.8 (0.98)	ND	ND

ND = not detectable, numbers in parentheses represent %RSDs (percent relative standard deviation).

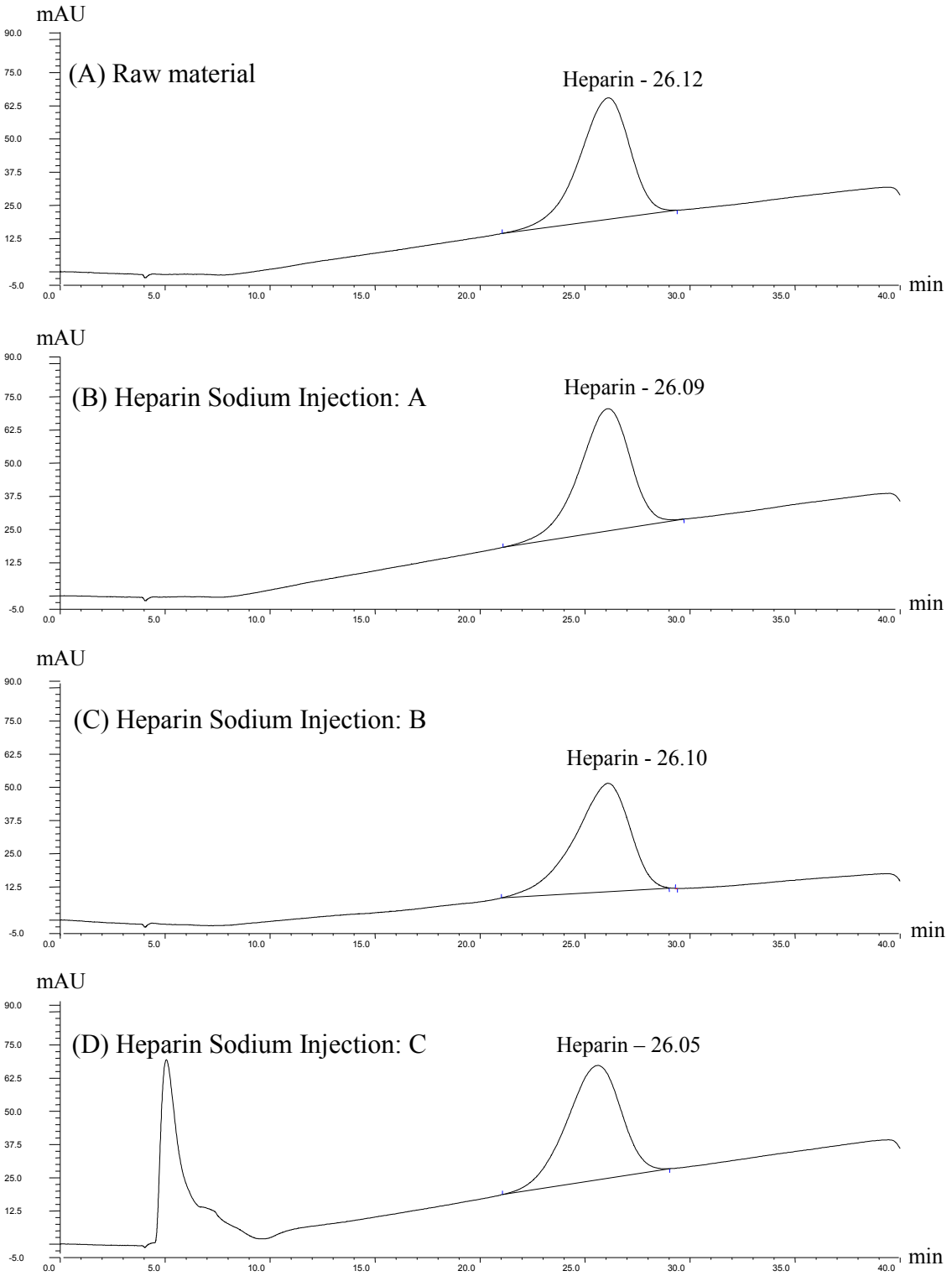


Figure 4.12 HPLC chromatograms of (A) raw material, (B) Heparin sodium injection brand A , (C) Heparin sodium injection brand B, and (D) Heparin sodium injection brand C. HPLC conditions see Figure 4.10.

CHAPTER V
CONCLUSION

RP-HPLC, HILIC and IEC techniques were investigated for development of an analytical method for the simultaneous determination of heparin and its impurities (i.e. OSCS, DS) in heparin products. Different types of HPLC columns, i.e. ODS, HILIC amide and SAX columns were studied in the different techniques. Composition of mobile phases, including types and pH of buffer, and type and amounts of organic modifiers (methanol and acetonitrile) were varied. The examined RP-HPLC and HILIC conditions did not retained heparin and its impurities, resulting in overlapping peaks at the solvent front. SAX-HPLC (gradient elution of sodium chloride and Tris pH 3.0) showed some retention of the analytes. Thus, IEC seemed the most suitable technique among the three selected techniques for the analysis of heparin and its impurities. Response surface methodology based on central composite design (CCD) was used to study the effects of the important factors, pH and concentration of sodium chloride in the mobile phase, and to determine the optimal chromatographic conditions. Lowering mobile-phase pH and increasing the concentration of sodium chloride enhanced the separation and optimal conditions were a 30 min gradient elution of 5% to 60% of 2.5 M sodium chloride and 20 mM Tris buffer (pH 2.1) at a flow rate of 0.6 mL/min. The detection was performed with UV detector at 215 nm. Injection volume was set of 200 μL.

The results at the optimal conditions were validated in term of linearity, precision, accuracy, LOD and LOQ. Calibration curves showed good linearity with r^2 between 0.9980-0.9998 for heparin (3 – 20 mg/mL), DS (200-400 μg/mL) and OSCS (90-160 μg/mL). F values for each compound showed no significant lack of fit in the chosen concentration ranges and models are recognized to be adequate. Precision of the method was performed by the repeatability and the time-different intermediate precision. In both cases, the percentage relative standard deviations for each compound are well below 12%. The range of recovery values covering all

concentration levels for each compound were 95.5-103.6% for heparin, 92.3-103.9% for DS and 92.6-102.0% for OSCS. Thus, the methods were considered to have an acceptable precision and recovery. The LODs were 800, 10.5 and 7.2 μg/mL whereas LOQs were 2,500, 31.5 and 22.0 μg/mL (%RSD 3.22-10.12) for heparin, DS and OSCS, respectively. Finally, the validated method was applied for the determination of heparin and its impurities in pharmaceutical formulations. The % label amounts of heparin in the investigated raw material and products (n = 7) were between 90.3 – 97.8%. DS and OSCS were not detected in all samples. They all complied with the USP limit (90.0-110.0% la. for heparin). In conclusion, this method is economical, fast, simple and reliable for the identification and quantification of heparin and its impurities for the quality control of both heparin raw material and formulations.

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PART II: Data Handling of HPLC Fingerprints from *Mallotus* and *Phyllanthus* Samples

CHAPTER I
INTRODUCTION

Herbal medicines have been used by many people around the world for thousands of years. Unfortunately, their quality, safety, and efficacy are not always sufficiently evaluated (1,2). Because of their complex composition, the development of suitable analytical procedures to separate all or as many compounds as possible are not always established. Furthermore, concentrations of herbal components can vary significantly depending on cultivation conditions of plants, drying processes and harvest seasons (2). Some researchers only use information from a limited number of compounds, e.g. the so-called biomarkers, to evaluate the quality of herbal medicines. However, there are doubts that only certain compounds are sufficient to describe and evaluate the complexity of herbal samples.

World Health Organization (WHO) has introduced and accepted chromatographic fingerprint analysis as a strategy for assessments of herbal medicines (3). Chromatographic fingerprints are usually obtained from reversed-phase high-performance liquid chromatography (RPLC) characterized the complete composition of the herbal medicine. Fingerprints represent chromatographic profiles, in which all detectable chemical constituents are separated as many as possible. The obtained fingerprints can be used as a unique identification tool to evaluate the authenticity of a herbal sample, or to the quality, assurance of the consistency, and stability of a herbal medicine. Nowadays, the combination of (hyphenated) chromatographic instruments and chemometrical approaches for data (pre-) treatment allows a fast investigation of herbal samples (4-11). Moreover, chemometric treatment of the chromatographic fingerprints also allows modeling and predicting pharmacological activities (e.g. antioxidant and cytotoxic activities) and/or indicating peaks potentially responsible for the modeled activities (2,4,12-15).

Mallotus and *Phyllanthus* genera, belonging to *Euphorbiaceae* family, are widely distributed in most tropical and sub-tropical countries. They have long been extensively used as folk medicine in India, China, Vietnam and other countries for thousands of years for treatments of different diseases. For instance, the genus *Mallotus* has been used for treatments of chronic hepatitis and enteritis (16-17), while the genus *Phyllanthus* has been used for disturbances of kidney and urinary bladder, intestinal infections, and diabetes (18,19). Recently, many researchers investigated chemical components of *Mallutus* and *Phyllanthus* species and identified several pharmacologically active constituents (12, 20-24), especially those with antioxidant activity.

The goal of this study was to model the antioxidant activity of *Mallotus* and *Phyllantus* samples from different species, origins and/or collection times as a function of their chromatographic fingerprints, and to indicate peaks potentially responsible for the antioxidant activity. The antioxidant activity of the herbal extracts was determined with a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test and expressed in term of IC_{50}. First, an unsupervised data analysis using Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) was performed to verify whether the antioxidant samples could be distinguished from the less or inactive samples. Then, the antioxidant activity was modeled as a function of the earlier developed HPLC fingerprints (25) using the multivariate calibration techniques i.e., Partial Least Squares (PLS) (26-28) and Orthogonal Projection to Latent Structure (O-PLS) (27,29). The regression coefficients of the resulting models were evaluated to indicate the peaks possibly responsible for the antioxidant activity.

Results reveal that exploratory analyses using PCA show significantly diverging clustering tendencies between *Mallotus* and *Phyllanthus* samples and could classify some species better than HCA. For multivariate calibration techniques, PLS and O-PLS models demonstate good predictive ability. By comparing the regression coefficients with the chromatographic fingerprints, peaks that were protentially responsible for the antioxidant activity of the extracts could be confirmed.

CHAPTER II
LITERATURE REVIEW

2.1 *Mallotus* and *Phyllanthus* species

2.1.1 *Mallotus*

Mallotus genus is one of the most diverse and richest genus of Euphorbiaceae family. This genus includes approximately 150 species distributed in tropical and sub-tropical regions in Asia (Cambodia, China, India, Laos, Malaysia, Thailand, and Vietnam). A few species are found in the North and the East of Australia and Africa. The plants are usually shrub or small trees and grow in rainy season (21). Naturally, they are propagated from seeds and concentrated in mountainous areas with an altitude below 1,000 meter (21). Some of them are used as medicinal plants in traditional medicines in several countries for treatments of various ailments ranging from minor infections such as gastrointestinal disorders to dysentery, hepatic diseases, cutaneous diseases, fever and malaria, and a series of other indications (17,21,30,31). Additionally, some *Mallotus* species are known to contain different natural compounds, mainly terpenoids, polyphenols and benzopyrans (17,21,30,31-35). The chemical compounds isolated from the *Mallotus* genus show many different biological activities including antibiotic, antiviral, antimicrobial, anti-inflammatory, antioxidant and cytotoxic (17,21,30-38). In this review paper, the data of literature concerning the traditional uses, phytochemistry, and the pharmacological activities of *Mallotus* species, described over the past few decades, are shown in Table 2.1.

Table 2.1 Summarizes active substances and pharmacological activities of selected *Mallotus* species

Species	Part used	Active constituents	Pharmacological activities	Traditional uses	References
Mallotus apelta	Leaves	Triterpenoids, steroids, alkaloid	Antibiotic	Chronic hepatitis, white blood and enteritis	17, 30
Mallotus barbatus	Bark, leaves,roots	Polyphenols	ND	Gastrointestinal disorders, acne, hemostatic, oedema, scabies, analgesic, fever, diuretic, cholera, headache	21
Mallotus chryscocarpus	ND	ND	Antiviral HIV	ND	21
Mallotus floribundus	Roots, whole plant	ND	ND	Diarrhea, fever, gynecological infection, scabies	21
Mallotus glabriusculus	Roots	ND	ND	Cough	21
Mallotus hookerianus	Leaves	Pentacyclic triterpenoids, casbane-type diterpenoid lactone	ND	ND	21

ND = no data

Table 2.1 (continued) Summarizes active substances and pharmacological activities of selected *Mallotus* species

Species	Part used	Active constituents	Pharmacological activities	Traditional uses	References
Mallotus japonicas	Leaves	Mallotinrilagin, acid, mallotusinic acid, corilagin, geraniin, rutin, ellagic acid	Antioxidant	Stomach disorders, gastic ulcers	31
Mallotus macrostachyus	Leaves	ND	ND	Acne, hemostatic, wounds	21
Mallotus metcalfianus	Whole plant	Polyphenols	Antiradical, antimicrobial	ND	32
Mallotus oblongifolius	Roots, whole plant	ND	ND	Malaria, diarrhea, gastrointestinal disorders	21
Mallotus oppositifolium	Leaves	ND	Antibacterial, antidiarrheal	Diarrhea	36
Mallotus pallidus	Leaves	Phloroglucinol derivative	Antiviral HIV-1, HSV-1, HSV-2	ND	21
Mallotus paniculatus	Fruits, roots, whole plant	Pentacyclic triterpenoids, steroids, cardenolides fatty acid	ND	Contusions, oedema, gynecological infection, fever, headache, wounds	21

ND = no data

Table 2.1 (continued) Summarizes active substances and pharmacological activities of selected *Mallotus* species

Species	Part used	Active constituents	Pharmacological activities	Traditional uses	References
Mallotus peltatus	Leaves	Tannin, triterpenoid, flavonoid, sterol, alkaloid, steroids, saponins	Antimicrobial, anti-inflammatory, antipyretic, Neuropharmacological	Intertinal ailments, skin infections, stomachache	21,33,34,39
Mallotus philippinensis	Bark, Fruits,	Cardenolides, kamalins, Phloroglucinol derivatives, phenolics, tannin,	Antioxidant, Antibacterial, anti-allergic, anti-tumor, HIV-reverse transcriptase inhibitory	Kamala dye, a purgative agent for animals, anthelminticum	37,40-42
Mallotus repandus	Leaves, roots, whole plant	Iso-coumarin, cyano-γ-pyridone, triterpenoids, bergenin, diterpenic lactone, coumarin	Antiviral HIV-1, antiradical, anti-tumor, antihepatotoxic, antiulcergenic, uterus muscle stimulant	Scabies, fever, influenza, contusions, sedative	21
Mallotus resinosus	Roots	Coumarin (Scopoletin)	DNA cleavage	ND	21
Mallotus roxburghianus	Leaves	β-sitosterol, stigmasterol, betulinic acid, 4-hydroxybenzoic acid, *β*-sitosterol -*β*-D-glucoside, bergenin,	Antioxidant	Splint, snake bites	35

ND = no data

2.1.2 *Phyllanthus*

Phyllanthus genus (Euphorbiaceae) is widely distributed in most tropical and subtropical countries including Brazil, China, Cuba, India, Indonesia, the Philippines, Thailand, and Vietnam. It is a very large genus consisting of approximately 750 species. *Phyllanthus* has a remarkable diversity of growth forms including annual and perennial herbs, shrubs, climbers, floating aquatics, and pachycaulous succulents. Some have flattened stems resembling and functioning as leaves called cladodes. *Phyllanthus* species have been traditionally used as folk medicines to treat a broad spectrum of diseases, such as disturbances of the kidney and urinary bladder, intestinal infections, diabetes, hepatitis B virus, and painful disorders (19,43-55). Many plants in great variety of species of plants belonging to the *Phyllanthus* genus have been phytochemically and pharmacologically investigated and many active constituents have been isolated and identified. Over the years, different chemical compounds of medicinal interest have been reported, including alkaloids, flavonoids, lactones, steroids, terpenoids, lignans, tannins, and so forth (19,20,23,43-49,54-64). The reported activities include antidiabetic, anti-inflammatory, antimicrobial, antipyretic, antioxidant, antiviral and cytotoxic (19,20,23,43-65). The data of traditional uses, phytochemistry and the pharmacological activities of *Phyllanthus* species are summarized and listed in Table 2.2.

Table 2.2 Summarizes active substances and pharmacological activities of selected *Phyllanthus* species

Species	Part used	Active constituents	Pharmacological activities	Traditional uses	References
Phyllanthus emblica	Leaves, fruits	Alkaloids, Benzenoids, Furanolactone, Diterpene, Triterpene, Flavonoids, sterol, carbohydrate	Anti-inflammatory, antipyretic, Antimicrobial, antioxidant	Jaundice, diarrhea	20,23,56-59
Phyllanthus urinaria	Whole plant	Ellagitannins, flavonoids, hydroxylated aromatic acids, geraniin	Anti-tumor, antiviral, antioxidant, anti-hypertensive, anti-inflammatory	Liver damage, hepatitis, jaundice, enteritis, diarrhea, dropsy	43-45
Phyllanthus amarus	Leaves, seeds	Lignans, geraniin, flavonoids, alkaloids	Antioxidant, anti-inflammatory, Antinociceptive, Antihepatotoxic, immunostimulant, anti-tumor, antiviral, antibacterial, hepatoprotective, anti hyperglycemic	Dropsy, jaundice, diarrhea, dysentery, fever, scabies, ulcers, wounds, Chronic viral hepatitis B	46-49
Phyllanthus reticulatus	Whole plant	ND	Antidiabetic, antihyperlipidemic, antioxidant	astringent, skin eruption, diuretic, constipating,, syphilis, asthma, diarrhea , obesity	50

ND = no data

Table 2.2 (continued) Summarizes active substances and pharmacological activities of selected *Phyllanthus* species

Species	Part used	Active constituents	Pharmacological activities	Traditional uses	References
Phyllanthus niruri	Whole plant	Flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannin, coumarins, saponins	Antihyperuricemic, anti-tumor, antioxidant, antiviral	Jaundice, asthma, hepatitis, malaria	60-64
Phyllanthus debilis	Whole plant	ND	Antimicrobial, Anti-inflammatory	swelling, intestinal worms, cold, sedative	51
Phyllanthus kozhikodianus	Whole plant	ND	Antimicrobial	Anticonvulascent, antidysentery, jaundice, ulcer, itchin	51
Phyllanthus maderaspetensis	Whole plant	ND	antimicrobial, antihepatotoxic	Anti-edematic, antidysentery, Immunomodulatory, fever, ulcer, burn, jaundice, cold	51-52
Phyllanthus simplex	Whole plant	ND	Antidiabetic, antioxidant	Gonorrhea, jaundice, hyperglycemia, liver disease, mammary abscess, pruritis, itching	53
Phyllanthus polyphyllus	Whole plant, Leaves	Benzenoid, lignans, triterpene	Anti-inflammatory, cytotoxic	Liver protection, intestinal infection, antihepatitis B, diabetes, astringent, diuretic, cathartic	54-55

ND = no data

2.2 Chromatography and chemical fingerprints in quality control of herbal medicines

Traditional herbal medicines and their preparations have significant roles in the primary healthcare in many oriental countries. During the past decades, they became more important in western countries for preventive and therapeutic purposes. Uses of traditional medicines become increasingly popular, thus their identity and quality are concerned issues to ensure the patient's safety (3).

In the conventional approach, just one or two marker constituents are considered as indicators for the quality of herbal medicines. Identification of a few compounds hardly describes the complex nature of herbal medicines and ignores synergic interactions between the compounds and does not always allow assessing the total intrinsic quality of the herb. Concentrations of herbal constituents may vary significantly depending on the harvest season, plant origins, drying processes and other factors, making it difficult to determine and isolate compounds of interest. Therefore, in general, quality control of herbal medicines by assaying only a few compounds might be unreliable.

Recently, the chromatographic fingerprint technique was introduced as a powerful tool to evaluate the quality of herbal samples or their derived products (2,4,12-15). This technique has been accepted by the WHO as a strategy for the quality assessment of herbal medicines (3). It has recently gained popularity because it emphases on the characterization of the complete sample composition. Moreover, it can also be used to evaluate the authenticity and stability of herbal samples (66-69). Several chromatographic techniques, for instance, high performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE) and thin layer chromatography (TLC), can provide the chromatographic fingerprints of medicinal plants (1,4,66,70). Furthermore, the recent approaches of hyphenated techniques such as high performance liquid chromatography-diode array detection (HPLC-DAD), gas chromatography-mass spectroscopy (GC-MS), capillary electrophoresis-diode array detection (CE-DAD), HPLC-MS and HPLC-NMR, offer additional spectral information, which is valuable for qualitative analysis and even for the on-line structural elucidation (1,4,6,66,70,71).

2.3 Antioxidant activity methods

Numerous methods are used to evaluate antioxidant activities of natural compounds (72-74). Two free radicals that are commonly used to assess the antioxidant activity, in vitro, are 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$). The ABTS assay measures the relative ability of an antioxidant to scavenge $ABTS^{\bullet+}$ generated in aqueous phase, as compared with Trolox (a water soluble vitamin E analogue). $ABTS^{\bullet+}$ is generated by reaction of a strong oxidizing agent (e.g. potassium permanganate or potassium persulfate) with an ABTS salt. Reductions of blue-green $ABTS^{\bullet+}$ radical by hydrogen-donating antioxidants are measured by the suppression of its characteristic long wave absorption spectra (72). The method is usually expressed as Trolox equivalent antioxidant capacity (TEAC). The method is simple, rapid and shows good repeatability (72). It can be performed in both aqueous and organic solvents. However, correlations of results with *in vivo* antioxidant efficacy are ambiguous.

$DPPH^{\bullet}$ is a stable free radical with a maxima absorption wavelength at 515 nm. It loses this absorption when it is reduced by an antioxidant or a free radical species. The $DPPH^{\bullet}$ method is widely used to determine antiradical/antioxidant activity of purified phenolic compounds as well as herbal extracts (75-76). Bondet et al. (76) found that most phenolic antioxidants react slowly with $DPPH^{\bullet}$, reaching a steady state in 1-6 h or longer. The method is frequently used since it offered good repeatability. Similar to the ABTS method, the DPPH method shows limited relevance in biological systems. Additionally, color interference from samples that contain anthocyanins can lead to underestimation of the antioxidant activity.

The oxygen radical absorbance capacity (ORAC) method developed by Cao et al.(77) measures the ability of antioxidants to protect protein from damage by free radicals. In this assay, different generators are used to produce different radicals. Usually, three radicals are generated as follows: peroxyl radical ($ROO^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), and Cu^{2+}, a transition metal. This is important since measured antioxidant activity of biological samples depends on which free radical or oxidant is used in the assay (78). The results with this method shows poor repeatability, which is attributed to the protein interacting with sample polyphenols, among other factors. A

major advantage of ORAC is that the method is automated and largely standardized, thus values can be easily compared across laboratories. The ORAC method is reported to imitate antioxidant activity of phenols in biological systems better than other methods, since it uses biologically relevant free radicals and integrates both time and degree of activity of antioxidants (78). However, the method often requires the use of expensive equipment.

2.4 Assessment of herbal medicines by chemometrics

Chemometrics is a statistical approach for interpretations of patterns in multivariate data. When used to analyze instrument data, chemometrics often results in a faster and more precise assessment of composition of a product or even physical or sensory properties. Two general applications of chemometrics technology are 1) to predict properties of interest, for example, modeling and predicting pharmacological activities and 2) to classify samples into one of several categories (e.g., good versus bad, type A versus type B versus type C etc.) (1). Chemometrics software is designed to recognize patterns in virtually any type of multidimensional analytical data. Many mathematical algorithms are used for data processing in chemometric approaches (26-29). Basic principles for this approach are variation determination of common peaks and similarity comparison with similarity indices and linear correlation coefficients. Similarity indices and linear correlation coefficients can be used to compare common pattern of the chromatographic fingerprints obtained. In general, proper chemometric treatments of analytical data is comprised of the following steps: (a) appropriate pretreatment or preprocessing of the raw data by removing experimental variations and transforming the data, improving the quality of the data set for further analyses, (b) exploratory analyses of the data providing additional insights in the data and revealing its underlying structures, and (c) applications of data analytical tools depending on the purpose of the study (4). The discrimination and classification of herbal samples are performed by clustering and pattern recognition tools, while the prediction of pharmacological activities is done by multivariate calibration techniques. Data handling of herbal fingerprints using chemometric tools are reviewed and discussed.

2.4.1 Data pretreatment

Prior to data analysis, the chromatographic fingerprints are used to form the *n* x *p* data matrix **X**, where the *n* objects (herbal samples) constitute the rows and the *p* variables (time points) the columns. In each cell of the matrix, the signal intensity at a given time point is presented. Data handling techniques require the same variables to contain equivalent information for every fingerprint. However, due to experimental variations and column aging, shifts in retention times between fingerprints occur. As a consequence, a column of data matrix X does not always contain the equivalent information, which affects the outcome of the data analysis. Therefore, the adjustment and transformation of data are commonly applied. Many methods to adjust and transform the data, i.e. column centering, normalization and standard normal variate (SNV), have been suggested (4,26-28).

One of the standard procedures involves column centering. The useful information resides in the between-sample variation of the variables, not in their absolute levels. By removing the column mean from each corresponding value, every centered variable has a mean of zero. Other commonly applied preprocessing techniques include normalization to correct for unequal amounts of injected sample, SNV transformation corresponds to row centering, followed by row scaling, where row centering removes the row mean from each corresponding row element and row scaling divides each row element by its corresponding row standard deviation.

2.4.2 Unsupervised exploratory data analysis

A common way to gain insight in the structure of a multivariate data table is the application of exploratory data analysis tools such as PCA and HCA. These techniques are base solely on the information residing within the fingerprints.

2.4.2.1 Principal component analysis

PCA reduces the number of variables and visualizes the information included in the *n* x *p* data matrix **X** (26, 28). PCA makes linear combinations of the original variables, thus creating the so-called latent variables or principal components (PC's), in such a way that they describe the largest possible variation in **X** and are orthogonal to each other. The projection of an object on a PC is called a score on this PC, while the projection of each original variable to the PC is

called a loading. A score plot represents the scores on two PCs and gives information regarding the (dis)similarity of the objects, while a loading plot provides information on the contribution of the original variables to the considered PCs.

2.4.2.2 Hierarchical cluster analysis

HCA is a clustering method applied to find the underlying structure of objects through an iterative process that associates (agglomerative method) or dissociates (division method) object by object, and that is halted when all objects have been processed (28,79).

A division method starts with all objects in one cluster divides them into two or more subsets, and continues to make smaller clusters until all objects are completely separated (28,80). On the other hand, an agglomerative procedure starts with each object in a separate cluster combines the clusters sequentially, reducing the number of clusters at each step until all objects belong to only one cluster (28). The hierarchical clustering process can be represented as a tree or dendrogram, where each step in the clustering process is illustrated by a joint of clusters.

In this study, an agglomerative HCA was selected to visualize the data contained in the fingerprint matrix **X** and give insight in the clustering tendency of the data. Several measures for the (dis)similarity between objects can be used, e.g. Euclidean distance, standardized Euclidean distance, Mahalanobis distance, Pearson correlation coefficient, Spearman's rank correlation coefficient (28).

In this work, the Euclidean distance and the Pearson correlation distance, which is calculated as "1-Pearson correlation coefficient r", were evaluated as distance measures (28). Consider x (x_1, x_2, … x_n) and z (z_1, z_2, … z_n) as two sets of n measurements/variables (fingerprints) with means (averages) $\bar{x}$ and $\bar{z}$. The Euclidean Distance (ED) between **x** and **z** is calculated as follows

$$ED = \sqrt{\sum_{i=1}^{n}(x_i - z_i)^2} \qquad (1)$$

where x_i and z_i are the i^{th} elements of **x** and **z**.

The Pearson correlation coefficient r is estimated as

$$r(x,z) = \frac{cov(x,z)}{s_x\ s_z} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(z_i - \bar{z})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2 \sum_{i=1}^{n}(z_i - \bar{z})^2}} \qquad (2)$$

where cov(x,z) is the covariance of the variables **x** and **z**, and s_x and s_z are the standard deviations of **x** and **z**.

Secondly, there are several linkage methods using different criteria to decide which individual objects should be merged together, i.e. single linkage, complete linkage, average linkage, weighted average linkage, centroid's method, median's method, and Ward's method. Single linkage, also called nearest neighbor, uses the smallest distance between objects in the two clusters. Complete linkage follows the opposite approach of single linkage. It uses the largest distance between objects in the two clusters. In average linkage, the average distances between all pairs of objects in any two clusters are obtained. This mode, one may introduce a weighting of the objects when clusters of unequal size are linked. Both weighted and unweighted methods exist. Another method which gives good results is known as Ward's method. It is based on a heterogeneity criterion. This is defined as the sum of the squared distances of each member of a cluster to the centroid of that cluster. Clusters are joined with as criterion that the sum of heterogeneities of all clusters should increase as little as possible. Last group, centroid's method and median's method use the Euclidian distance between the centroids and weighted centroids of the two clusters, respectively.

The hierarchical cluster process can be delineated as a tree or dendrogram, where each step in the clustering process is illustrated by a joint of the tree.

2.4.3 Supervised data analysis: Multivariate calibration techniques

In herbal analysis, multivariate calibration techniques model a continuous property (e.g. total flavonoid content, antioxidant activity…) as a function of the

recorded fingerprints. Linear multivariate calibration techniques try to relate the information contained in the *n* x *p* data matrix **X** to an *n* x *1* response vector **y**, with **y** being a continuous response. In general, supervised techniques make use of a calibration or training set with a priori known information to build a model. The model is constructed based on a calibration set and the model's optimal complexity and predictive ability can be evaluated by prediction of a test set or by cross-validation of the calibration set. The model can then be applied to unknown sample to predict their value for this property (28,29,81). For this purpose, two techniques have been proposed including Partial Least Squares (PLS) and Orthogonal Projections to Latent Structure (O-PLS).

Generally, the relationship between **X** and **y** can be described as follows:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{e} \quad (3)$$

where **b** represents the *p* x *1* vector of regression coefficients and **e** the *n* x *1* residual vector.

2.4.3.1 Partial least squares

PLS (26,28) is a latent-variable technique that maximizes the covariance between **X** and **y**. The PLS model can be presented as follows;

$$\mathbf{X} = \mathbf{TP}^{\mathbf{T}} + \mathbf{E} \quad (4)$$

$$\mathbf{y} = \mathbf{TP}^{\mathbf{T}}\mathbf{b} + \mathbf{f} = \mathbf{Tq} + \mathbf{f} \quad (5)$$

$$\mathbf{b} = \mathbf{Pq} \quad (6)$$

where **T** represents the *n* x *n* score matrix for **X** and **y**, **P** the *p* x *n* loading matrix representing the regression coefficients of **X** on **T**, **E** the *n* x *p* residual matrix of **X**, **b** the *p* x *1* vector of PLS regression coefficients, **q** the *n* x *1* loading vector representing the regression coefficients of **y** on **T**, and **f** the *n* x *1* residual vector of **y**. The regression coefficients **b** can be used to evaluate the contribution of the original variables to the final model (2,4,13-15).

The optimal model complexity was determined by a leave-one-out cross-validation procedure (LOO-CV). During LOO-CV each object is left out once and the model is built using the remaining objects. The root mean squared error of cross-validation (RMSECV) (Eq.(7)) is then calculated for models with different complexities (28).

$$\mathrm{RMSECV(f)} = \sqrt{\sum_{i=1}^{N} \frac{(\hat{y}_{cv,i} - y_i)^2}{N}} \qquad (7)$$

where f is the model complexity, N the number of calibration samples, y_i the measured response of the i^{th} sample, and $\hat{y}_{cv,i}$ the response for the i^{th} sample predicted from the calibration model obtained without the i^{th} sample. The optimal model complexity corresponds to the number of latent factors resulting in the (nearly) lowest RMSECV.

2.4.3.2 Orthogonal projections to latent structures

O-PLS (29) removes the variation in **X** that is not correlated to **y**. This is done by subtracting PLS components, orthogonal to **y**, from the original data matrix **X**. Thus, the original data is split into two data sets, i.e. one that containing the information relevant to **y** and another with the information orthogonal to **y**.

An O-PLS model can be written as follows

$$\mathbf{X} = \mathbf{T}\mathbf{P}^{\mathbf{T}} + \mathbf{T}_{\mathbf{orth}}\mathbf{P}^{\mathbf{T}}_{\mathbf{orth}} + \mathbf{E} \qquad (8)$$

where **T** represents the orthonormal *n* x *n* score matrix for **X** and **y**, **P** the orthonormal *p* x *n* loading matrix representing the regression coefficients of **X** on **T**, $\mathbf{T}_{\mathbf{orth}}$ the orthogonal *n* x *n* score matrix for **X** and **y**, and $\mathbf{P}_{\mathbf{orth}}$ its corresponding orthogonal *p* x *n* loading matrix**,** and **E** the *n* x *p* residual matrix of **X**, **y** and **b** are calculated according to Eq (5) and Eq (6), respectively. Again, the regression coefficients **b** (Eq (6)) can be used to evaluate the contribution of the original variables to the final model (2,4,12-13).

Removing the orthogonal information of the original data set leads to a reduction of the number of PLS components in the O-PLS model, i.e. only

one PLS component is used. This allows an improved interpretability of the regression coefficients (29).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Materials used in this study are listed in Table 3.1 and 3.2

Table 3.1 List of chemicals and reagents

Name	Grade	Source/Supplier
Acetonitrile (ACN)	HPLC	Fisher Scientific (Leicesterahire, UK)
Methanol (MeOH)	HPLC	Fisher Scientific (Leicesterahire, UK)
(±)-α-Tocopherol	RS	Sigma-Aldrich (Steinheim, Germany)
Trifluoroacetic acid (TFA)	HPLC	Sigma-Aldrich (Steinheim, Germany)

Table 3.2 List of instruments

Instrument	Source/Supplier
HPLC-DAD system	Shimadzu (Tokyo, Japan)
Chromolith™ Performance RP-18e column i.d. 100 mm x 4.6 mm	Merck (Darmstatdt, Germany)
Chromolith™ Performance RP-18e guard column i.d. 5 mm x 4.6 mm	Merck (Darmstatdt, Germany)
Arium® pro UV Ultrapure water system	Sartorius Stedim Biotech (Aubagne, France)

Table 3.2 (continued) List of instruments

Instrument	Source/Supplier
Ultrasonic bath	Branson Ultrasonic Corporation (Connecticut, USA)
Shaking bath	Edmund Bühler (Hechingen, Germany)
Filter paper pore size 240 nm	Whatman (Hanoi, Vietnam)
125-mm syringe filters 2.0 μm	Schleicher & Schuell (Dassel, Germany)
25-mm syringe filters with polypropylene membrane 0.2 μm	VWR International (Leuven, Belgium)
Polystyrene flat-bottomed 96-well plates (Nunc MicroWell Scientifics)	Thermo Fisher Scientific (Massachusetts, USA)
Absorbance microplate reader (SpectroMax 190)	Molecular Devices (California, USA)

3.2 Herbs and preparation of the extracts

Ten *Mallotus* and twenty-six *Phyllanthus* sp.samples, from 6 different species, were collected from various Vietnamese regions (Table 3.3). Leaf samples of the plants were collected and authenticated by Professor Nguyen Nghia Thin (Hanoi National University, Vietnam).

Ten grams of the leaf samples were extracted with 100 mL methanol in an ultrasonic bath at a temperature between 30 to 45°C for 1 h. This residue was repeated three times and the extract was filtered through a 240 nm pore size filter paper and evaporated at reduced pressure (60 Pa) and elevated temperature (40°C). The obtained crude extract was divided into 3 fractions, i.e. one for the DPPH radical scavenging assay at the UCL, one for the HPLC analysis at the VUB, and one as a library sample for reference purposes in Vietnam.

Table 3.3 The *Mallotu*s and *Phyllanthus* samples with their species, origin, and collection time

Sample	Species	Collection time	Origin
1	*Mallotus apelta*	August, 2009	Van Ban-Lao Cai
2	*Phyllanthus emblica*	August, 2009	Van Ban-Lao Cai
3	*Phyllanthus emblica*	November, 2009	Dong Dang-Lang Son
4	*Mallotus apelta*	November, 2009	Tam Dao-Vinh Phuc
5	*Mallotus apelta*	November, 2009	Dong Dang-Lang Son
6	*Mallotus paniculatus*	December, 2009	Dong Van-Ha Giang
7	*Phyllanthus emblica*	December, 2009	Dong Van-Ha Giang
8	*Mallotus apelta*	December, 2009	Ham Yen-Tuyen Quang
9	*Phyllanthus reticulatus*	February, 2010	Nghia Trai-Hung Yen
10	*Phyllanthus urinaria L.*	February, 2010	Nghia Trai-Hung Yen
11	*Phyllanthus amarus*	February, 2010	Nghia Trai-Hung Yen
12	*Phyllanthus amarus*	March, 2010	Van Dien-Hanoi
13	*Mallotus paniculatus*	March, 2010	Huong Hoa-Quang Tri
14	*Phyllanthus reticulatus*	March, 2010	Van Dien-Hanoi
15	*Phyllanthus emblica*	March, 2010	Huong Hoa-Quang Tri
16	*Phyllanthus urinaria L.*	March, 2010	Van Dien-Hanoi
17	*Mallotus paniculatus*	April, 2010	Me Linh-Vinh Phuc
18	*Phyllanthus reticulatus*	April, 2010	Me Linh-Vinh Phuc
19	*Phyllanthus amarus*	April, 2010	Me Linh-Vinh Phuc
20	*Phyllanthus reticulatus*	April, 2010	Lan Ong-Hanoi
21	*Phyllanthus emblica*	April, 2010	Me Linh-Vinh Phuc
22	*Phyllanthus amarus*	April, 2010	Lan Ong-Hanoi
23	*Phyllanthus urinaria L.*	April, 2010	Me Linh-Vinh Phuc
24	*Phyllanthus urinaria L.*	April, 2010	Lan Ong-Hanoi
25	*Phyllanthus reticulatus*	May, 2010	Ninh Hiep-Hanoi
26	*Phyllanthus reticulatus*	May, 2010	Dong Anh-Hanoi
27	*Phyllanthus amarus*	May, 2010	Que Vo-Bac Ninh
28	*Phyllanthus emblica*	May, 2010	Dong Anh-Hanoi
29	*Phyllanthus amarus*	May, 2010	Dong Anh-Hanoi
30	*Phyllanthus urinaria L.*	May, 2010	Que Vo-Bac Ninh
31	*Phyllanthus urinaria L.*	May, 2010	Ninh Hiep-Hanoi
32	*Phyllanthus amarus*	May, 2010	Ninh Hiep-Hanoi
33	*Phyllanthus urinaria L.*	May, 2010	Dong Anh-Hanoi
34	*Mallotus apelta*	June, 2010	Pa Co-Hoa Binh
35	*Mallotus paniculatus*	June, 2010	VQG-Pumat
36	*Mallotus paniculatus*	July, 2010	Cuc phuong-Ninh Binh

3.3 HPLC

3.3.1 Sample preparation

To prepare the samples for HPLC analysis, 50 mg of the crude extract was weighed, diluted to volume with methanol in a 2.0-mL volumetric flask and mixed on a shaking bath for 15 min at 400 rpm. The extract was then filtered through a filter with a diameter of 125 mm and a pore size smaller than 2 μm and consecutively by filtration through a 25-mm syringe filter having a polypropylene membrane with 0.2 μm pore size.

3.3.2 Chromatographic conditions

Mobile phase consisted of (A) 0.05%TFA in ACN, and (B) 0.05%TFA in ultra pure water and run by gradient elution for 60 min. The gradient was 5-20% A in 0-20 min, 20-95% A in 20-50 min and 95% A during 50-60 min. Column temperature of 25°C, a flow rate of 1.0 mL/min and injection volume 10 μL were carried out and detection wavelength was at 254 nm.

3.4 DPPH radical scavenging assay

Stock solutions of samples were prepared from the crude extract at a concentration of 2 mg/mL in HPLC grade methanol. Polystyrene flat-bottomed 96-well plates were filled with 100 μL of serial 2 fold dilutions of sample stock solutions to final concentrations ranging from 1 to 500 μg/mL. 100 μL of a freshly prepared solution of DPPH in methanol (50 μg/mL) was added to each well giving a final DPPH concentration of 25 μg/mL (63.4 μM). (±)-α-Tocopherol was used as an antiradical control. Blank controls (MeOH only) were also introduced. Plates were kept in the dark at room temperature for 20 min. After 20 min, absorbances at 515 nm were measured using a SpectraMax 190 Microplate Reader equipped with the SoftMax Pro software (Molecular Devices). A DPPH calibration curve was obtained between 1 and 50 μg/mL to confirm linearity between DPPH concentrations and

measured absorbances. Percentages of remaining DPPH (% $DPPH_{remaining}$) were therefore calculated as follows:

$$\%DPPH_{remaining} = \frac{Abs_{sample}}{Abs_{MeOH\ blank}} \times 100 \qquad (9)$$

Two independent assays were carried out in duplicate to assess intra and inter-day repeatability. For each assay; percentages of remaining DPPH were reported as mean ± SD of duplicate values. Using the software GraphPad Prism 4, percentages of remaining DPPH were plotted against log_{10} of corresponding concentrations. A sigmoidal curve was then fitted on experimental points allowing determination of IC_{50} values that was necessary to scavenge half of the DPPH initially introduced. IC_{50} values were reported with their 95% confidence intervals (82).

Note: sections 2,4 were performed by Mr. N. Nguyen Hoai and Mr. M. Chau Van, Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST), Vietnam, whereas section 3 was performed by Mr. Mohammad Goodarzi, Department of Analytical Chemistry and Pharmaceutical Technology, Center for Pharmaceutical Research (CePhaR), Vrije Universiteit Brussel (VUB), Belgium.

3.5 Data analysis

3.5.1 Data preprocessing

Prior to data analysis, chromatographic fingerprints were used to form the *n* x *p* data matrix **X**, where the *n* objects (herbal samples) constituted the rows and the *p* variables (time points) consisted of the columns and IC_{50} values were organized in the vector. To remove level differences and adjust several peaks, different preprocessing methods, i.e. column centering, normalization followed by column centering and SNV followed by column centering, were applied and compared.

3.5.2 Unsupervised exploratory data analysis

Unsupervised data analysis uses information containing only matrix **X**, i.e. only fingerprints. Pre-treatment data sets were analyzed and evaluated with two unsupervised methods, i.e. PCA and HCA to explore and distinguish samples.

3.5.3 Supervised data analysis: Multivariate calibration techniques

Combining the IC_{50} values (vector **y**) with chromatographic fingerprints (matrix **X**) from the corresponding sample allows constructing a multivariate regression model. Two linear multivariate calibration techniques, i.e. PLS and O-PLS were used for modeling and their regression coefficient were studied in order to indicate peaks possibly responsible for the antioxidant activity.

Computation was performed on a PC with an Intel 2.8 GHz Pentium-IV processor, 512 MB RAM running on Microsoft Windows XP and Matlab^{TM} 7.1 (The Mathworks, Natick, MA). All data (pre)processing methods were performed using m-files written for Matlab^{TM} 7.1.

CHAPTER IV
RESULTS & DISCUSSION

4.1 DPPH radical scavenging test

Table 4.1 presents the 36 *Mallotus* and *Phyllanthus* samples with their species, and the DPPH scavenging activity results, given as average IC_{50} values with their 95% confidence interval. The lower the average IC_{50} value is, the higher the antioxidant activity of the sample is.

Except *Phyllanthus amarus* (sample 11), all *Phyllanthus* samples were considered to have a high antioxidant activity (average IC_{50} < 30 μg/mL). All five *Mallotus paniculatus* were considered to have an intermediate antioxidant activity (30 μg/mL < average IC_{50} < 50 μg/mL). On the other hand, all five *Mallotus* samples did not possess any antioxidant activity (average IC_{50} > 70 μg/mL). Range of IC_{50} values between *Mallotus* samples and *Phyllanthus* samples were 31.47 – 110.00 μg/mL and 8.45 – 99.32 μg/mL respectively.

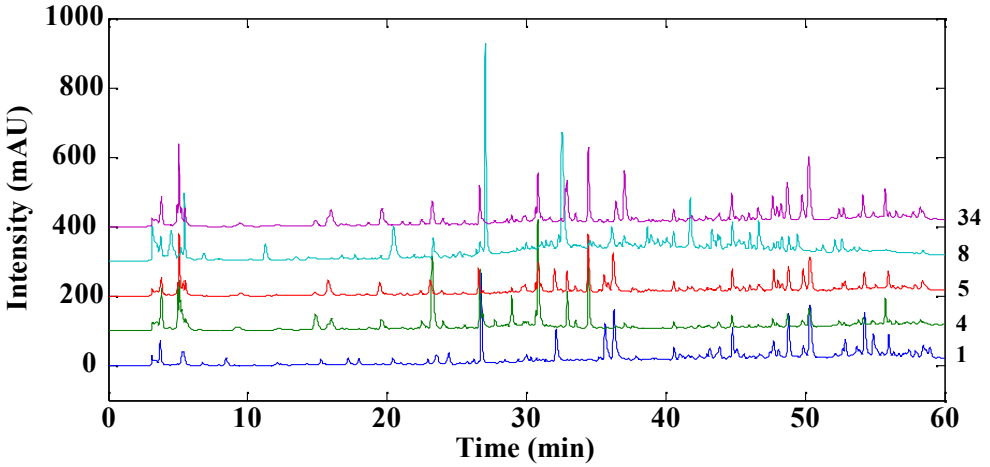
4.2 HPLC fingerprints

HPLC fingerprints of *Mallotus* and *Phyllanthus* extracts have been developed. Six different species for both genera, of which the fingerprints are shown in Figure 4.1a-f. Except *Phyllanthus amarus* (Sample 11), similar fingerprints were observed within the same species whereas there were differences among species, probably due to differences in chemical constituents. Therefore, possibly different pharmaceutical activities can be attributed to different species.

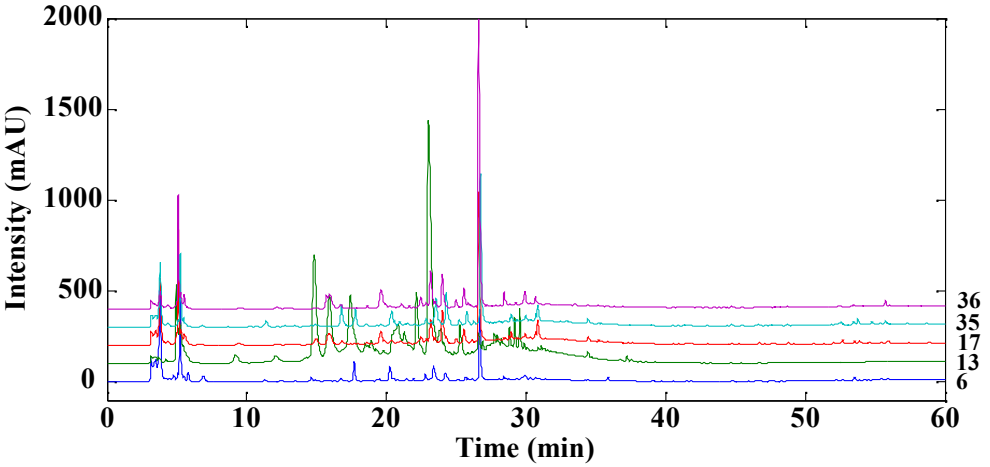
Table 4.1 DPPH scavenging activity of the investigated *Mallotu*s and *Phyllanthus*

Sample	Species	Average IC_{50} (95%CI)	Category*
1	*Mallotus apelta*	72.66 (51.36-101.20)	Low
2	*Phyllanthus emblica*	9.90 (7.61-13.89)	High
3	*Phyllanthus emblica*	9.55 (7.54-12.58)	High
4	*Mallotus apelta*	74.59 (54.91-102.40)	Low
5	*Mallotus apelta*	98.42 (65.89-137.60)	Low
6	*Mallotus paniculatus*	37.31 (27.84-55.08)	Moderate
7	*Phyllanthus emblica*	8.57 (6.95-10.57)	High
8	*Mallotus apelta*	110.00 (/)	Low
9	*Phyllanthus reticulatus*	28.06 (19.25-38.18)	High
10	*Phyllanthus urinaria L.*	9.75 (7.36-12.43)	High
11	*Phyllanthus amarus*	99.32 (29.23-142.50)	Low
12	*Phyllanthus amarus*	13.74 (9.64-19.08)	High
13	*Mallotus paniculatus*	38.87 (31.07-52.68)	Moderate
14	*Phyllanthus reticulatus*	29.70 (21.74-45.66)	High
15	*Phyllanthus emblica*	10.92 (7.61-14.79)	High
16	*Phyllanthus urinaria L.*	8.45 (6.03-11.46)	High
17	*Mallotus paniculatus*	35.76 (27.30-48.59)	Moderate
18	*Phyllanthus reticulatus*	13.42 (10.51-17.28)	High
19	*Phyllanthus amarus*	13.12 (10.77-15.87)	High
20	*Phyllanthus reticulatus*	22.18 (13.47-33.60)	High
21	*Phyllanthus emblica*	8.75 (6.99-10.85)	High
22	*Phyllanthus amarus*	14.33 (9.90-20.69)	High
23	*Phyllanthus urinaria L.*	8.83 (7.25-10.70)	High
24	*Phyllanthus urinaria L.*	8.56 (6.95-10.49	High
25	*Phyllanthus reticulatus*	23.49 (15.23-33.48)	High
26	*Phyllanthus reticulatus*	27.31 (13.42-42.45)	High
27	*Phyllanthus amarus*	15.51 (11.14-21.22)	High
28	*Phyllanthus emblica*	10.26 (8.32-12.73)	High
29	*Phyllanthus amarus*	10.15 (6.91-14.95)	High
30	*Phyllanthus urinaria L.*	17.99 (11.95-19.88)	High
31	*Phyllanthus urinaria L.*	21.13 (13.71-28.78)	High
32	*Phyllanthus amarus*	16.12 (11.62-21.39)	High
33	*Phyllanthus urinaria L.*	13.60 (8.35-19.86)	High
34	*Mallotus apelta*	105.56 (70.08-151.70)	Low
35	*Mallotus paniculatus*	31.47 (23.72-42.63)	Moderate
36	*Mallotus paniculatus*	36.70 (28.85-49.01)	Moderate

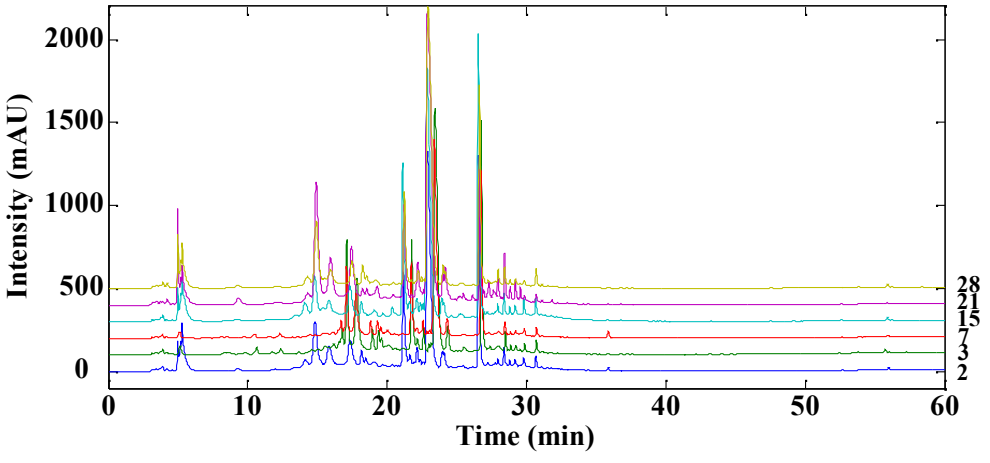
*High (average IC_{50} < 30 μg/mL), moderate (average IC_{50} between 30 to 50 μg/mL), and low (average IC_{50} > 70 μg/mL)



(A) *Mallotus apelta*

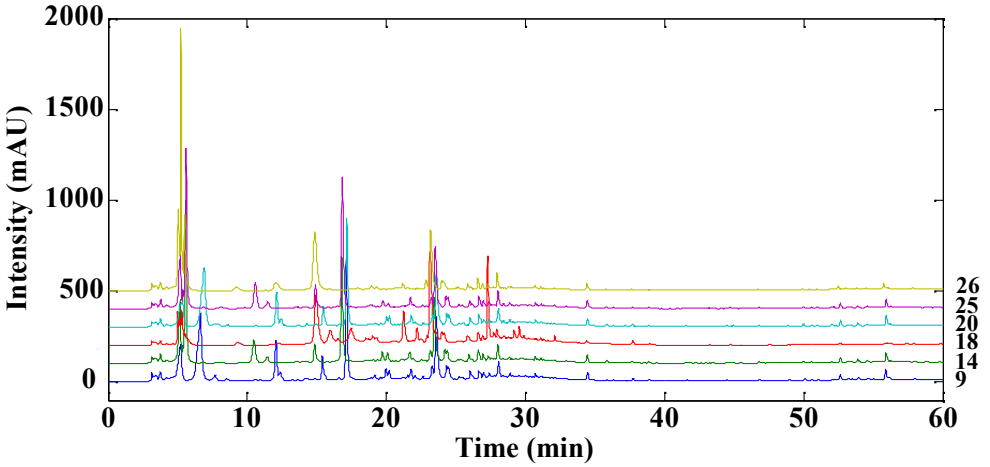


(B) *Mallotus paniculatus*

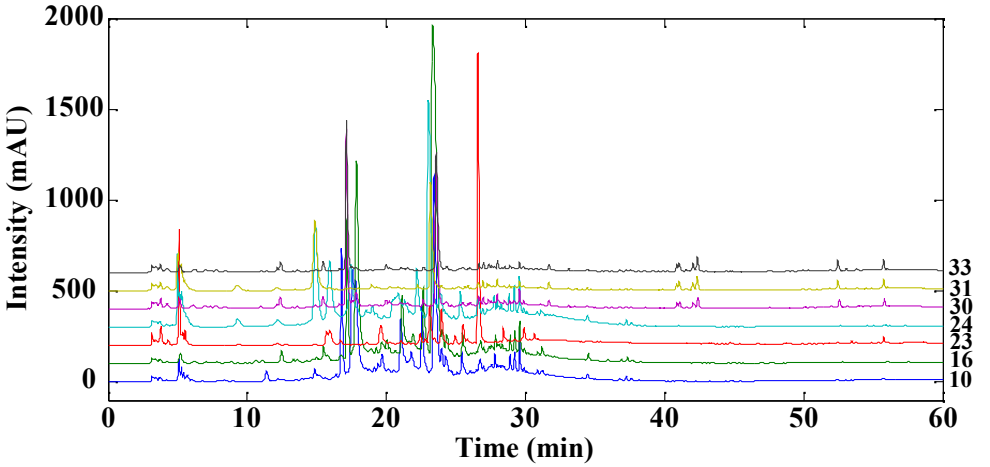


(C) *Phyllanthus emblica*

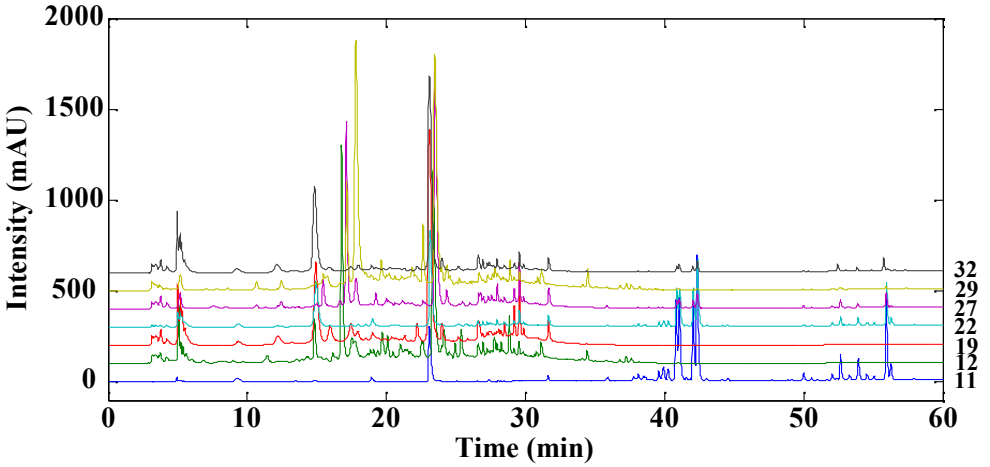
Figure 4.1 Chromatographic fingerprints of the investigated plants. Chromatographic condition as described in section 2. Numbers in the second y-axis represent sample no. in Table 4.1.



(D) *Phyllanthus reticulatus*



(E) *Phyllanthus urinaria* L.



(F) *Phyllanthus amarus*

Figure 4.1 (continued) Chromatographic fingerprints of the investigated plants. Chromatographic condition as described in section 2. Numbers in the second y-axis represent sample no. in Table 4.1.

4.3 Evaluation of antioxidant activity

4.3.1 Data preprocessing

Prior to data analysis with chemometric techniques, the fingerprint data was preprocessed. Different preprocessing methods were evaluated, i.e. column centering, normalization followed by column centering, and standard normal variate (SNV) transformation followed by column centering. It was found that for PLS and O-PLS techniques, normalization followed by column centering gave the best results for this specific data set while for PCA and HCA, standard normal variate followed by column centering was required. All further discussion results are acquired by data preprocessing as indicated above.

4.3.2 Exploratory analysis

PCA has been applied to verify whether groups of samples could be distinguished according to, for instance, the antioxidant activity or species. The score plot (Figure 4.2) of PC1 (21.59% explained variation) versus PC2 (16.32% explained variation) was examined after different preprocessing techniques. Standard normal variate and column centering was considered to be the best preprocessing approach. All samples with high antioxidant activity (marked in bold) are clustered, i.e. can be distinguished along PC2. Combining the proximity of the samples on the score plot and a previous knowledge of their fingerprint profiles and species resulted in the distinction of two groups of the genera. Samples belonging to the genus *Mallotus* (samples 1,4,6,17,34,36) are distinguished from those from the genus *Phyllanthus* (samples 2,3,7,9,12,14,16,18,22,24,33), except for *Mallotus paniculatus* (sample 13). The above-mention distinction between antioxidant and non-antioxidant samples along PC2 is the same as that between *Mallotus* and *Phyllanthus* samples. Three groups of species, i.e. (a) *M. apelta* (sample 1,4,5,8,34), (b) *M. paniculatus* (sample 6,17,35,36) and (c) *P. reticulatus* (sample 9,14,20,25,26) were clustered nicely. Obviously, samples 13 seem to be outlier, its (dis)similarity to the other *M. paniculatus* samples or to fingerprints from other species can be evaluated in a similarity analysis. The fact that the different species are not separated in largely distinct groups in both an

indication and a consequence of the fact that the fingerprints are not extremely different.

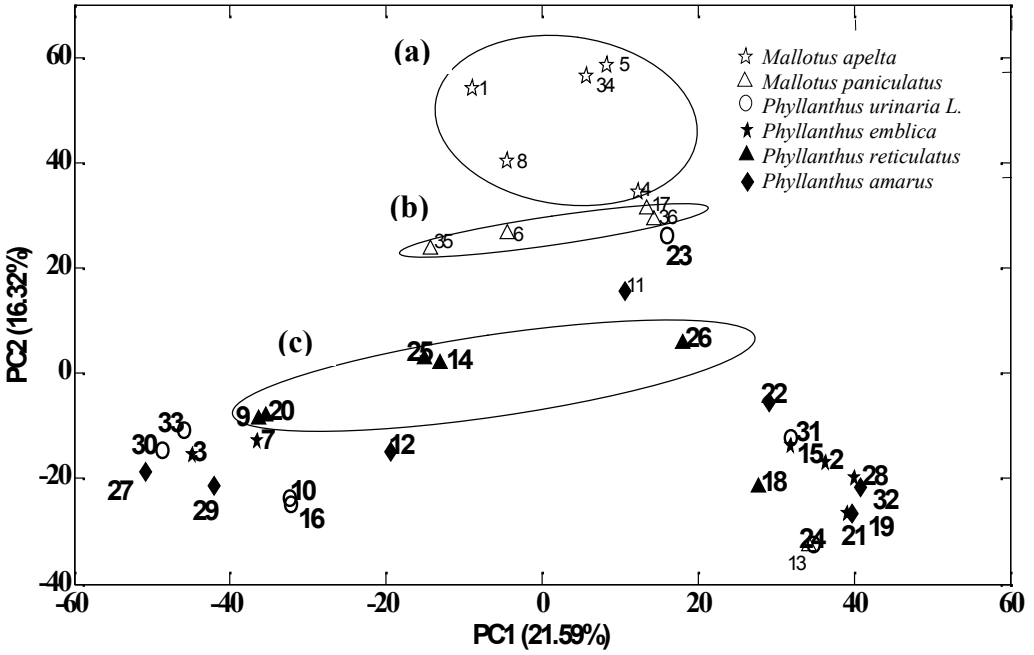


Figure 4.2 PC1-PC2 score plot for fingerprints of 36 *Mallotus* and *Phyllanthus* samples, standard normal variate and column centered. The highly active antioxidant samples are marked in bold.

In order to confirm the clustering results of PCA, HCA clustering technique has been utilized. Several distance measures (i.e. Euclidean distance and the Pearson correlation coefficient) and linkage methods (i.e. single linkage, complete linkage, average linkage, weighted average linkage, centroid's method, median's method and Ward's method) were used to find the best clustering. When using the Euclidean distance measure and the Median linkage method after SNV and column centering as preprocessing, the dendrogram given in Figure 4.3 was generated. This dendrogram revealed the relationships amongst the studied samples. It is clear that samples with antioxidant activity could be distinguished from other samples. Except for sample 23, *Mallotus urinaria* L., it was clustered together with the medium active *Mallotus paniculatus* samples. Secondly, sample 13, which is a medium active *Mallotus paniculatus* sample, clustered together with the highly active *Phyllanthus*

samples. These results agreed with those obtained with PCA. On the other hand, *Mallotus* and *Phyllanthus* samples could be more or less distinguished from each other, except for *Mallotus paniculatus* (sample 13), *Phyllanthus urinaria* L. (sample 23), and *Phyllanthus amarus* (sample 11). For sample 13 and 23, this was also clearly seen from the PC1-PC2 score plot of Figure 2. From the PC1-PC2 score plot, sample 11 was situated at the border between the *Mallotus* and *Phyllanthus* species samples. Moreover, sample 11 had a low antioxidant activity comparing to other *Phyllanthus* samples, which was rather situated in the range of IC_{50} values for the *Mallotus apelta* samples. This also reasoned why sample 11 clustered with the *Mallotus apelta* samples in HCA.

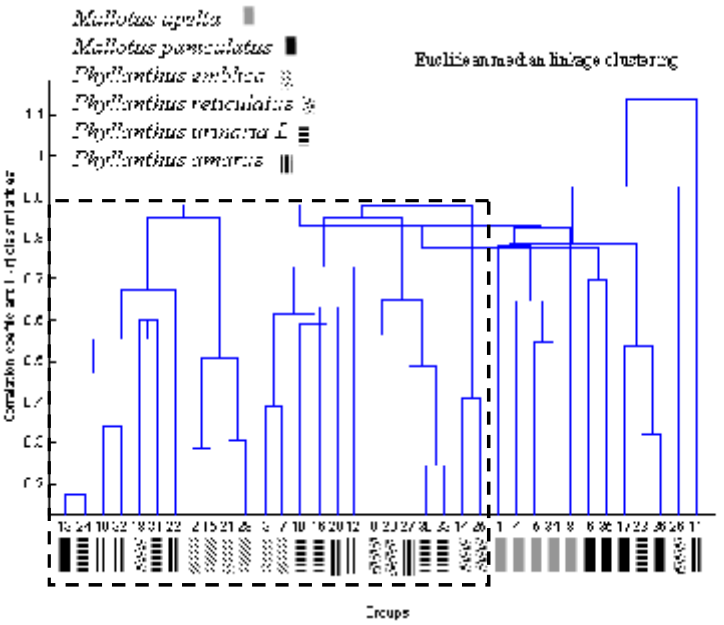


Figure 4.3 HCA dendrogram obtained using Euclidean distance as distance measure and Median's method as linkage method (pretreatment: SNV and column centering).

4.3.3 Linear multivariate calibration techniques

Multivariate calibration models for antioxidant activity were established using two techniques i.e. PLS and O-PLS, from the data matrix **X** consisting of 36 fingerprints and response vector **y**, representing the IC_{50} test results. No division of the data into a calibration set and a test set was made because the number of samples is rather small and prediction of the antioxidant activity of new samples was not the primary goal of this study, but the indication of peaks potentially responsible for the antioxidant activity is. For PLS, the optimal model complexity (A_{opt}) was chosen from a LOO-CV procedure and the simplest model with (nearly) the lowest RMSECV was selected. For O-PLS, a one-component PLS model with A_{opt}-1 orthogonal components was built. In Table 4.2, the number of model components and the RMSECV are presented for both calibration techniques, applying three preprocessing approaches. The O-PLS model after normalization or SNV followed by column centering were found to be most simple model, i.e. they contain the lowest number of components. For both model, normalization followed by column centering lead to the model with

the best predictive ability, i.e. the lowest RMSECV for the whole data set (16.5 and 16.1, respectively).

Table 4.2 Number of components and RMSECV for calibration models built with the fingerprints and three preprocessing approaches

Calibration technique	Preprocessing	No. of component	RMSECV
PLS	Column centering	4	20.1
	Normalization and column centering	2	16.5
	SNV and column centering	2	16.8
O-PLS	Column centering	1(3*)	19.7
	Normalization and column centering	1(1*)	16.1
	SNV and column centering	1(1*)	16.4

*For O-PLS, the number of removed orthogonal components is given between brackets.

To evaluate the model's ability to predict the antioxidant activity of the samples, the prediction of the antioxidant activity for the samples was taken into account. Table 4.3 shows the results of the 25 highest antioxidant samples (IC_{50} < 30 μg/mL) for the DPPH radical scavenging assay and predictions from the models after application of normalization and column centering as preprocessing approach. For these highly antioxidant samples, none of the two models predicted any as being non-active (IC_{50} > 70 μg/mL). The PLS model predicted 24 out of 25 highly active samples correctly as being highly active antioxidant. Only sample 22 was predicted as being intermediately active (30 μg/mL < IC_{50} < 50 μg/mL). The mean bias of these highly antioxidant samples was found to be 4.49 μg/mL. The O-PLS model predicted 21 highly active samples correctly and four (22,23,25, and 26) incorrectly as intermediately active. For O-PLS, the mean bias of these highly active antioxidant samples was found to be 5.95 μg/mL. None of the models predicted inactive samples to have an intermediate or highly antioxidant activity.

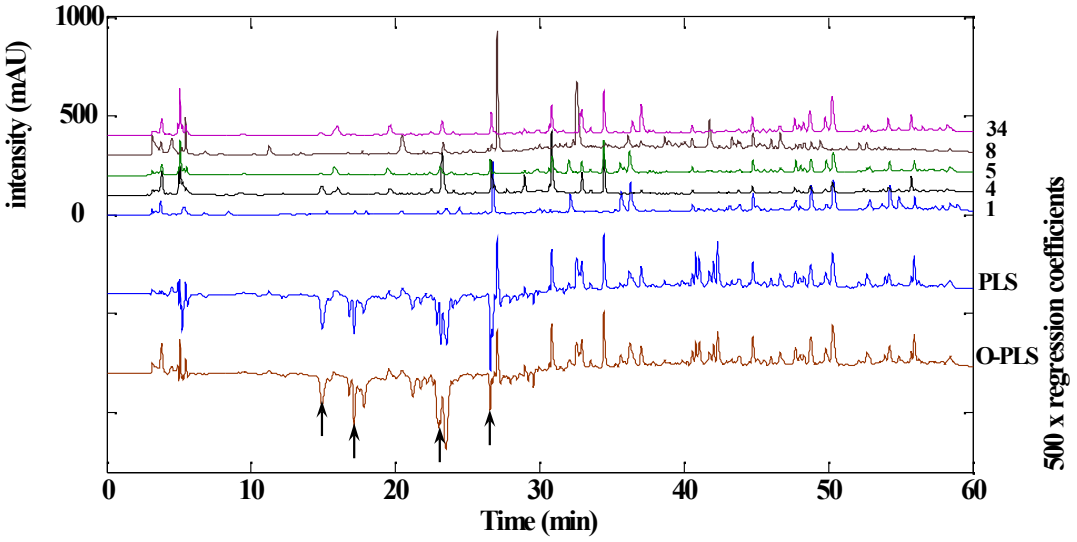
Table 4.3 Results from the DPPH radical scavenging assay (IC_{50}) and predictions from the models built

Sample No.	IC_{50} (μg/mL)	Predicted IC_{50} (μg/mL)	
		PLS	O-PLS
2	9.90	6.09	11.48
3	9.55	6.94	7.55
7	8.57	8.16	9.91
9	28.06	26.53	26.05
10	9.75	12.73	8.53
12	13.74	15.82	16.14
14	29.70	25.82	29.26
15	10.92	7.82	13.86
16	8.45	9.23	4.31
18	13.42	15.39	20.45
19	13.12	12.02	12.94
20	22.18	26.76	28.03
21	8.75	6.92	7.11
22	14.33	45.36	45.67
23	8.83	18.56	43.20
24	8.56	15.94	12.58
25	23.49	25.35	31.27
26	27.31	24.45	35.49
27	15.51	13.64	9.54
28	10.26	6.52	9.55
29	10.15	14.35	9.52
30	17.99	19.83	17.29
31	21.13	23.66	28.96
32	16.12	17.88	18.93
33	13.60	26.34	25.21
Mean bias		4.49	5.95

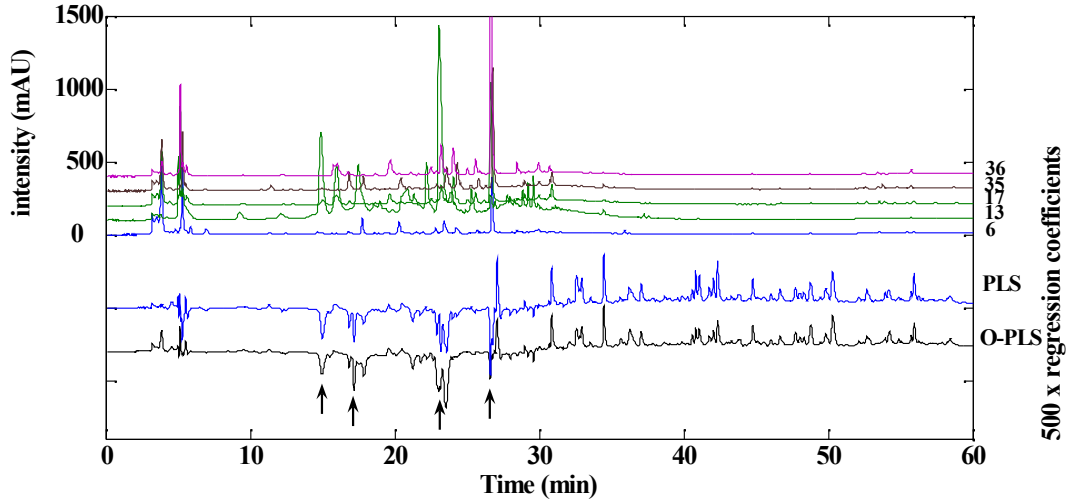
4.3.4 Regression coefficients

In order to indicate peaks in the fingerprints potentially responsible for the antioxidant activity of the measured samples, the regression coefficients of the models were examined. Peaks with negative regression coefficients (indicated with an arrow in Figure 4.4) corresponding to the chromatographic peaks showed trends of potential antioxidant compounds. The lower IC_{50} values represent the higher antioxidant activity. In this study, the fingerprints of each species on both models i.e., PLS and O-PLS were compared with the obtained regression coefficients (Figure 4.4).

The major negative coefficient peaks corresponding to the potentially antioxidant compounds are found in four peaks (at retention times of about 15.0, 17.0, 23.0, and 27.0 min). The antioxidant samples have no major peaks present at retention times corresponding to the positive coefficient peaks in the regression plots. In Figure 4.4 b (*Mallotus paniculatus* samples), none of the negative coefficient peaks match major compounds present in these fingerprints, except for sample 13. This sample has an antioxidant activity and was already found atypical from the PCA score plot and the HCA dendrogram. In Figure 4.4 c-f, all samples have a high antioxidant activity and the major negative coefficients correspond to substance peaks seen in the fingerprints. These peaks are probably responsible for most antioxidant activity. In a next step, the indicated relevant peaks should be isolated, identified, and investigated using structure-elucidation techniques.

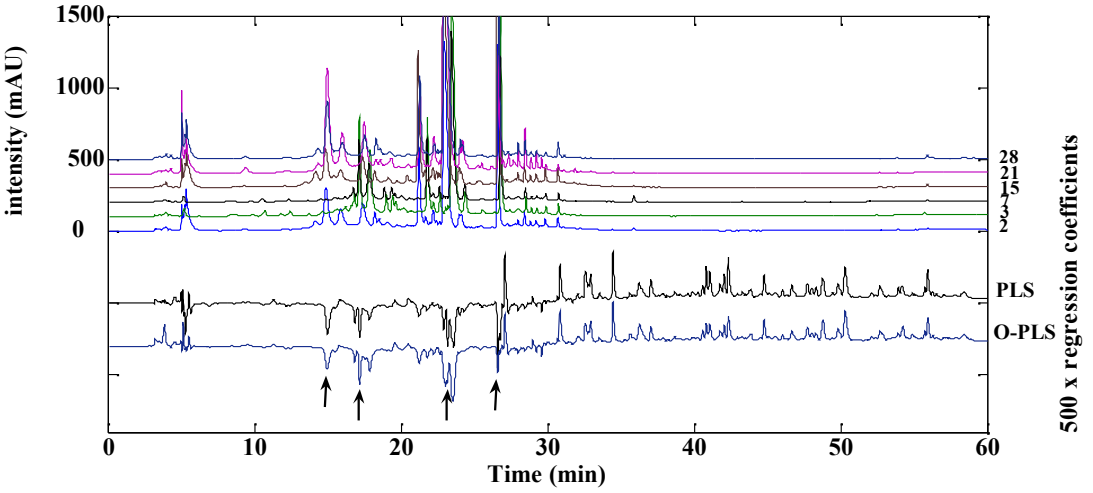


(A) *Mallotus apelta*

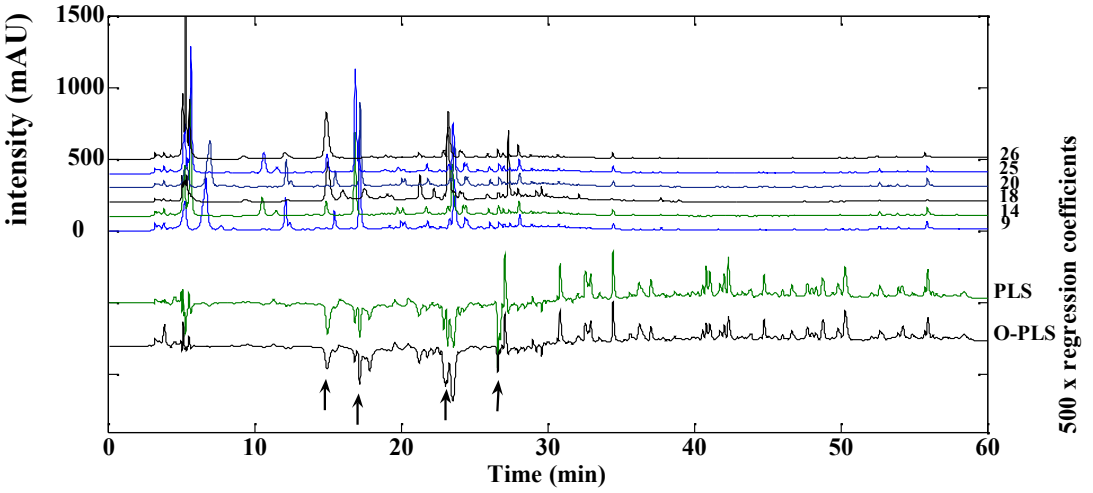


(B) *Mallotus paniculatus*

Figure 4.4 Chromatographic fingerprints of the investigated plants with their regression coefficients from PLS and O-PLS, preprocessed with normalization and column centering. Numbers in the second y-axis represent sample no. in Table 4.1.

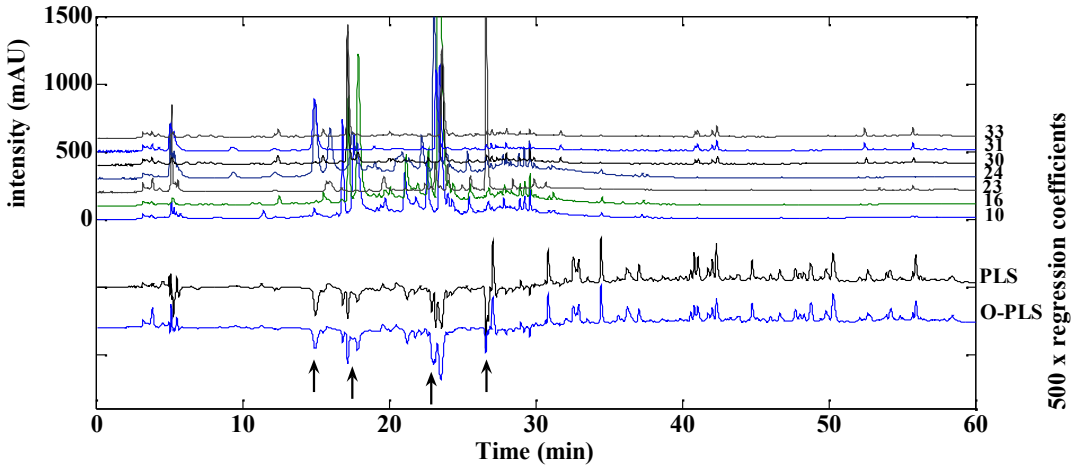


(C) *Phyllanthus emblica*

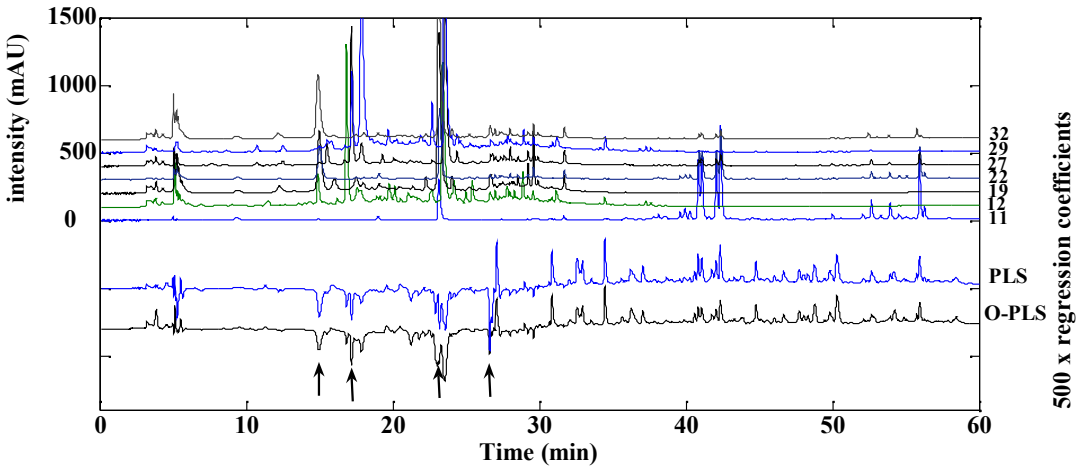


(D) *Phyllanthus reticulatus*

Figure 4.4 (continued) Chromatographic fingerprints of the investigated plants with their regression coefficients from PLS and O-PLS, preprocessed with normalization and column centering. Numbers in the second y-axis represent sample no. in Table 4.1.



(E) *Phyllanthus urinaria* L.



(F) *Phyllanthus amarus*

Figure 4.4 (continued) Chromatographic fingerprints of the investigated plants with their regression coefficients from PLS and O-PLS, preprocessed with normalization and column centering. Numbers in the second y-axis represent sample no. in Table 4.1.

CHAPTER V
CONCLUSION

HPLC fingerprints of *Mallotus* and *Phyllanthus* samples were combined with data-handing techniques to model the antioxidant activity and indicate peaks possibly responsible for this activity.

In the first step, exploratory analyses using PCA and HCA were performed to verify whether the antioxidant samples could be distinguished from the less or non-active samples. The score plot in PCA showed significantly diverging clustering tendencies between them better than the dendrogram in HCA. Moreover, PCA could distinguish some species of *Mallotus* and *Phyllanthus* samples.

Then, the antioxidant activities of the samples were modeled as a function of the fingerprints using PLS and O-PLS. The peaks potentially responsible for the antioxidant activity of the samples were indicated studying the regression coefficients of the models. In this study, comparing the regression coefficients plot of both models, not only showed similar coefficient profiles but also the peaks probably responsible for the antioxidant activity from both models could be indicated at the same positions. In the next step, the substances from these relevant peaks should be identified, isolated, and further examined.

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PUBLICATION/PRESENTATION

Publication :

1. Thiangthum S, Dejaegher, B, Goodarzi M, Tistaert C, Gordien AY, Nguyen Hoai N, Chau Van M, Quetin-Leclercq J, Suntornsuk L, Vander Heyden Y, Potentially antioxidant compounds indicated from Mallotus and Phyllanthus species fingerprints. J Chromatogr B. 2012;910:114-21.

Presentation :

1. Thiangthum S, Dejaegher B, Goodarzi M, Tistaert C, Suntornsuk L, Vander Heyden Y, Data analysis of HPLC fingerprints from *Mallotus* and *Phyllanthus* samples, The 15th Forum of Pharmaceutical Sciences, Spa, Belgium, 12 - 13 May 2011, oral presentation.

2. Thiangthum S, Dejaegher B, Goodarzi M, Tistaert C, Suntornsuk L, Vander Heyden Y, Chemometric tools for ata analysis of HPLC fingerprints from *Mallotus* and *Phyllanthus* samples, The 85th RGJ Seminar Series, Faculty of Science, Mahidol University, Bangkok, Thailand, 9 September 2011, oral presentation.

PUBLICATION/PRESENTATION

3. Thiangthum S, Suntornsuk L, Method Development of High Performance Liquid Chromatography for the Determination of Heparin and Its Impurities in Pharmaceutical Products, RGJ-Ph.D. Congress, XIII, Jomtien Palm Beach Hotel & Resort, Pattaya, Chonburi, Thailand, 6 - 8 April 2012, oral presentation.