ปริมาณวิเคราะห์ของพอลิซอร์เบตโดยมัลดิ-ทอฟแมสสเปกโทรเมทรี

นางสาวรสธร เมืองไพศาล

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QUANTITATIVE ANALYSIS OF POLYSORBATE BY MALDI-TOF MASS SPECTROMETRY

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งานวิจัยนี้เกี่ยวข้องกับวิธีการหาการกระจายน้ำหนักโมเลกุลและการวิเคราะห์เชิงปริมาณของพอลิซอร์เบตซึ่งเป็นสารที่ใช้เป็นอิมัลซิไฟเออร์ โดยใช้เทคนิคเมทริกซ์แอสซิสเทดเลเซอร์ดีซอร์บชันไอออนไนเซชันแมสสเปกโทรเมทรี พบว่าสภาวะต่อไปนี้ให้แมสสเปกตรัมซึ่งแสดงถึงการกระจายน้ำหนักโมเลกุลของพอลิซอร์เบต: การเตรียมสารตัวอย่างด้วยวิธีหยดแห้ง ใช้กรดแอลฟา-ไซยาโน-4-ไฮดรอกซีนามิกในเตตระไฮโดรฟูแลนเป็นเมทริกซ์ อัตราส่วนของสารลดแรงตึงผิวต่อสารละลายเมทริกซ์เท่ากับ 1:75 โดยปริมาตรและพลังงานเลเซอร์ที่ 310 ไมโครจูล จากสเปกตรัมพบสัญญาณของพอลิเอสทิลลีนออกไซด์ ซอร์บิแทน ซอร์ไบต์และไดซอร์บิแทน พบว่าให้การตอบสนองที่เป็นเส้นตรงในช่วงความเข้มข้น 5-50 mg/ml ปริมาณน้อยที่สุดที่สามารถวิเคราะห์เชิงปริมาณได้ที่ 1.03 เฟมโตโมล นำวิธีที่พัฒนาขึ้นนี้มาประยุกต์ใช้วิเคราะห์สารตัวอย่างที่เป็นยาลดไข้สำหรับเด็ก 2 ชนิดและโลชั่นที่จำหน่ายในท้องตลาด 2 ชนิด ผลที่ได้แสดงให้เห็นว่าพอลิซอร์เบตในสารตัวอย่างทั้งหมดมีการกระจายน้ำหนักโมเลกุลในช่วง 1000-2000 Da จากการทำปริมาณวิเคราะห์ของพอลิซอร์เบตในยาลดไข้สำหรับเด็กจากบริษัท A และ B ปริมาณของพอลิซอเบตที่ตรวจวัดได้คือ 0.091 $\pm$ 0.004 และ 0.090 $\pm$ 0.013 เปอร์เซ็นต์โดยน้ำหนัก ตามลำดับ และพอลิซอร์เบตที่พบในโลชั่นจากบริษัท A และ B ที่ตรวจวัดได้คือ 0.83 $\pm$ 0.060 และ 1.32 $\pm$ 0.150 เปอร์เซ็นต์โดยน้ำหนัก ตามลำดับ ดังนั้นเทคนิค MALDI-MS จึงเป็นเทคนิคที่ใช้ในการวิเคราะห์การกระจายน้ำหนักโมเลกุลและการวิเคราะห์เชิงปริมาณของพอลิซอร์เบตในยาและโลชั่นได้อย่างมีประสิทธิภาพ

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต.....
ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEY WORD: MALDI-TOF / POLYSORBATE /QUANTITATIVE ANALYSIS / EMULSIFIERS / POLYMER

ROSSATORN MUANGPAISAL : QUANTITATIVE ANALYSIS OF POLYSORBATE BY MALDI-TOF MASS SPECTROMETRY THESIS ADVISOR : ASSISTANT PROFESSOR POLKIT SANGWANIT, Ph.D., 73 pp. ISBN 974-53-1218-5

This research involves the method for determination of molecular weight distribution and quantitative analysis of polysorbate emulsifier, using matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). The following MALDI-MS conditions were found to provide the good MS spectrum: sample preparation using dried droplet method, α-cyano-4-hydroxycinamic acid in THF as an ideal matrix. The ratio of surfactant: matrix was 1:75 by volume and the laser power was 310 μJ. MALDI spectrum indicated that the presence of residue ethylene oxide polymer, free esterified sorbitan-based, sorbide-based and disorbitan-based species. Linear calibration curves were obtained in the range 5-50 mg/ml. The limit of detection was determined to be 1.03 femtomole. Two commercial pharmaceutical suspension and two commercial lotion were analyzed. The results indicated that all of the samples showed their molecular weight distributions in the range of 1000-2000 Da. Then, the amount of polysorbate in commercial drug A and B were found to be 0.091 ± 0.004 and 0.090 ± 0.006 precent by weight, and commercial lotion A and B were found to be 0.83 ± 0.060 and 1.32 ± 0.150 precent by weight, respectively. Thus, the MALDI-MS technique was an excellent method for determination of the molecular weight distribution and quantitative analysis of polysorbate emulsifier in drugs and cosmetic products.

Field of study: Petrochemistry and Polymer Science Student's signature……
Academic year: 2004 Advisor's signature……..

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LIST OF ABBREVIATIONS

CI	=	Chemical Ionization
cm	=	centimeter
Da	=	Dalton
EI	=	Electron Impact
ESI	=	Electrospray Ionization
HPLC	=	High Performance Liquid Chromatography
IR	=	Infrared
kV	=	kilovolt
LS	=	Light Scattering
MALDI	=	Matrix Assisted Laser Desorption Ionization
mL	=	milliliter
MS	=	Mass Spectrometry
m/z	=	mass to charge ratio
nm	=	nanometer
PS 80	=	polysorbate 80
SEC	=	Size Exclusion Chromatography
TOF	=	Time of Flight
(w/w)	=	weight by weight
μJ	=	microjoule
μL	=	microliter

CHAPTER I

INTRODUCTION

Polysorbates are non-ionic surfactants, consisting of the partial fatty acid esters of sorbitol-derived cyclic ethers (sorbitans and sorbides) condensed with approximately 20 mol of ethylene oxide. Although, sorbitan esters themselves exhibit emulsifying properties, polymerization with ethylene oxide improves their water solubility, thereby expanding their applications.[1] In application, polysorbates are widely used in the pharmaceutical and cosmetic industry as solubilizers for insoluble ingredients.[2] The capacity of polysorbate emulsifiers to perform particular functions is dependent on their chemical nature [3,4], as dictated by the structure of the sorbitol derivative core, the alkyl chain length of the fatty acid, the degree of esterification, and the number of polymerized oxyethylene residues. For example, both the size of the hydrophilic headgroup and the size of the lipophilic tailgroup have been shown to influence the degree of lipid oxidation that occurs in oil-in-water emulsions. Understanding the chemical nature is the key to predicting properties, and by extension, to selecting emulsifiers to suit many different applications.

The molecular heterogeneity of polysorbates presents an analytical challenge. Since the introduction of the hydrophilie-lipophile balance (HLB) system several decades ago, few attempts have been made to further elucidate the nature of polysorbate molecular complexity, although many analytical methods have been developed. Techniques [5] such as colorimetry, infrared spectroscopy and gravimetric determination by precipitation focus on qualitative detection of polysorbates. Quantification characterization have been attempted using techniques such as thin-layer chromatography [5,6], gas chromatography [5,7], high-performance liquid chromatography [7,8] and OH^- negative ion chemical ionization mass spectrometry.[9] Unfortunately, none of these methods allows satisfactory separation or resolution of the individual polymer species, nor identification of the components. Several of these methods require saponification prior to analysis, which simplifies the original composition of the heterogeneous emulsifiers, and all of these methods can be laborious and time-consuming.

The technique known as MALDI-TOF MS [10] was first demonstrated in 1987 and though still in its infancy with regard to polymer analysis. Its advantages over other methodologies include ease of sample preparation, speed of analysis, high sensitivity, and minimal fragmentation allowing direct access to molecular weight. Moreover, few researches have been present about the concept of quantitative analysis of low and high molecular weight compounds by using this technique.[11] Very recently, MALDI-TOF MS has been successfully applied to analyze polysorbate types of surfactant polymers but the spectra were of poor quality and peak identity was not addressed.[12]

In this study, quantitative analysis is demonstrated for the first time for polysorbate types. The method describes a simple way to determine the molecular complexity of polysorbate emulsifiers using MALDI-TOF MS. The objectives of this research were to select a optimize conditions for polysorbate and to develop quantitative analysis of polysorbate for application in analysis sample in drugs and cosmetics products.

Objectives

The objective of this research is to develop mass spectrometric method as a method for determination of molecular weight distribution and quantitative analysis of polysorbate.

Scope of work

In initial work, the MALDI-TOF MS conditions were studied for determination of molecular weight distribution of polysorbate standard. Then, a developed method will be used for determination of polysorbate in drugs and cosmetics products.

CHAPTER II

THEORITICAL AND LITERATURE REVIEWS

Polysorbate 80 as a nonionic surfactant, is widely used in liquid pharmaceutical and household products as a result of its properties of solubilization, and reduction of surface and interfacial tension.

2.1 Synthesis and Properties of Polysorbate [13-15]

Polysorbate 80 or polyoxyethylene (20) sorbitan monooleate; the esters linkage formed by the reaction of the fatty acids with polyhydric alcohols are an interesting group of nonionic emulsifiers, in that, depending on the nature of the alcohol used, they may be predominantly hydrophobic or hydrophilic, and thus suitable as w/o or o/w emulsifiers, respectively.

Extremely useful group of fatty acid esters are those of the products of the dehydration of sorbitol, commonly referred to as *sorbitan* esters. The principal reactions involved in the preparation of these compounds are shown in Figure 2.1. In the presence of the fatty acids, the sorbitol is principally dehydrated to 1,4-sorbitan; this may lose an additional molecule of water to form *isosorbide*. These polyols will then preferentially esterify at the primary hydroxyl of the 1,4-sorbitan.

Small quantities of other dehydration products may be present; esterification may occur at the other than primary hydroxyls as well.

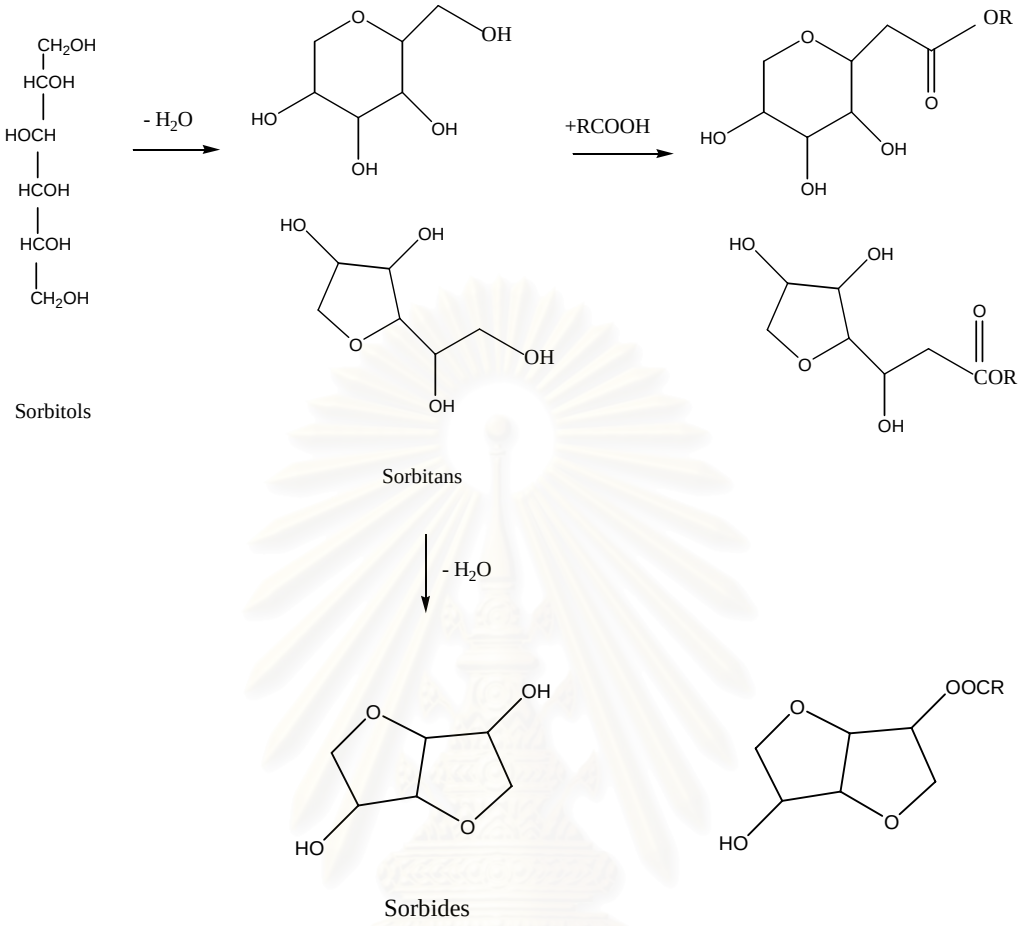


Figure 2.1 Synthesis of polysorbate, where R = $C_{17}H_{33}$

The sorbitan esters can react with ethylene oxide to form ethoxylated products. Because these compounds are partial esters, the unreacted hydroxyls are available for the reaction; however, the primary hydroxyls are still the preferred site, and this reaction proceeds through ester interchange to produce the *polysorbate*-class of non-ionic surface-active agents. A typical member of this class is shown in Figure 2.2.

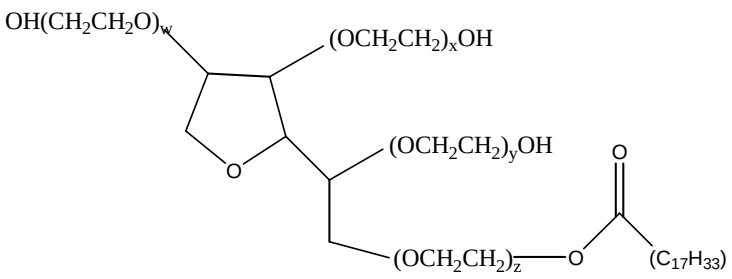


Figure 2.2 A structure of polysorbate 80, where (w + x + y + z) = 20

For application of polysorbate, it is used as emulsifiers and dispersing agents in medicinal products, emulsifiers in foods, and as surfactant in pharmaceutical aid.

2.2 Mass Spectrometry (MS) [16,17]

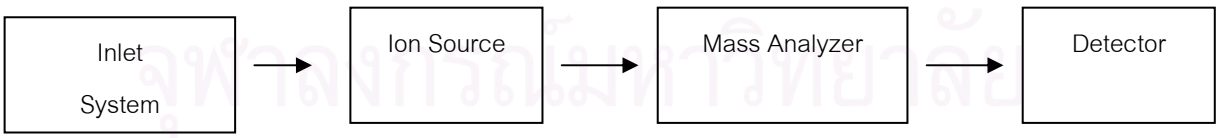
Mass spectrometry is the most versatile and comprehensive analytical technique currently at the disposal of chemists and biochemists. Since the early 1900s, it has enjoyed prominence in several areas science. The ultrahigh detection sensitivity and high molecular specificity are the hallmarks of this technique. Molecular mass determination, structure elucidation, quantification at trace levels, and mixture analysis are some of the major applications of mass spectrometry. In addition, the technique has been used to study ion chemistry and ion-molecule reaction dynamics; to provide data on physical properties such as ionizing energy, appearance energy, enthalpy of a reaction, and proton affinities; and to verify theoretical predictions that are based on molecular orbital calculations. Mass spectrometry is usually performed to determine the molecular weight of compounds. To accomplish this end, one of the several ionization methods for producing intact molecular ion must be used. These methods generate either positive or negative ions related to original molecular by adding or subtracting an electron or by adding or subtracting an anion or cation.

2.2.1 Basic Concept of Mass Spectrometry

Mass spectrometry is an analytical technique that measures the masses of individual molecules and atoms. As conceptualized in Figure 2.3, the first essential step in mass spectrometry analysis is to convert the analyte molecules into gas-phase ionic species because one can experimentally manipulate the motion of ions, and to direct them. The excess energy transferred to the molecule during the ionization event leads to fragmentation. Next, a mass analyzer separates these molecular ions and their charged fragments according to their m/z (mass/charge) ratio. The ion current due to these mass-separated ions is detected by a suitable detector and displayed in the form of a mass spectrum. To enable the ions to move freely in space without colliding or interacting with other species, each of these steps is carried out under high vacuum.

Figure 2.3 Basic concept of mass spectrometry analysis

A mass spectrometer consists of four main functional units; they are depicted in Scheme 2.1 in the form of a block diagram.



Scheme 2.1 Basic component of a mass spectrometer

These units are:

- An inlet system to transfer a sample to the ion source
- An ion source to convert the neutral sample molecule into gas-phase ions
- A mass analyzer to separate and mass-analyze ionic species
- A detector to measure the relative abundance of the mass-resolved ions

The overall analytic capability of a mass spectrometry system depends on the combined performance of these individual units. Several ionization techniques and mass analyzer have emerged, each with special purpose.

Inlet systems

There are several means of introducing samples into the mass spectrometer and the inlet system used will normally depend on the volatility and nature of the sample, the task in hand, and the method of ionization (in particular, the gas pressure in the ion source). The introduction of samples into a mass spectrometer is not a trivial task because, in the most common instruments, the whole system must be maintained at very low pressure (high vacuum) to allow unrestricted movement of ions. The examples of inlet system are

- Batch gas and vapor inlets
- Direct probe inlets
- Membrane interfaces
- Gas or liquid chromatographic inlets

Ionization source

An ion source may be defined simply as the region in which ionization occurs. The region is usually enclosed in a small ion chamber in which the sample is ionized. The ions produced are propelled out of the chamber towards an exit slit by a low positive potential applied to a ‘repeller’ plate. On leaving the ion chamber of magnetic sector mass spectrometer, the ions are accelerated through a high potential of 2-8 kV and passed into the analyzer for separation according to mass-to-charge ratio. The most common ionization sources uses in analysis today are

- Electron Impact (EI)
- Chemical Ionization (CI)
- Fast atom/ion bombardment Ionization (FAB)
- Thermospray Ionization (TSP)
- Electrospray Ionization (ESI)
- Matrix-Assisted Laser Desorption/Ionization (MALDI) etc.

Mass analyzer

Mass analyzers disperse ion space or time according to their mass-to-charge ratios (m/z). Certain analyzers separate the ions simultaneously, while others are scanned to transmit to the detector a narrow m/z range at a given time. The examples of mass analyzer are

- Quadrupolar Analyzers
- The Quadrupole Ion Trap or Quistor
- Time-of-Flight Analyzers
- Magnetic and electromagnetic Analyzers

Detectors

The detector allows a mass spectrometer to generate a signal (current) from incident ions, by generating secondary electrons, which are further amplified, or by inducing a current generated by a moving charge. The most common types of detector are

- Photographic plates and faraday cylinders
- Electron Multipliers
- Array Detectors
- Photon Multipliers

The major ionization methods can be divided in many categories as previous. All of these methods are suitable for the small molecule but they are not suitable for polymer. For this research, the ionization technique which was chosen for analysis of polysorbate is Matrix-Assisted Laser Desorption/Ionization and the mass analyzer is Time-of-flight. The principles of these techniques were explained, respectively.

2.3 Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS)

MALDI-TOF plays an important role in polymer analysis. This method allows desorption and ionization of very large molecule, even the complex mixtures. It gives information on the mass of individual from which repeat units, end groups, the presence of rings, molar mass distributions, and other information can be derived.

2.3.1 Fundamentals of the method

The MALDI Process

MALDI makes use of short, intense pulses of laser light to induce the formation of intact gaseous ions. Analyte molecules are not directly exposed to laser light, but are homogeneously embedded in the large excess of matrix, which consists of small organic molecules. The matrix molecules exhibit a strong absorbtion at the laser wavelength used (usually the 337 nm of the nitrogen laser), and the matrix allows for an efficient and controllable energy transfer. The high energy density obtained in the matrices, induces an instantaneous vaporization of the microvolume, and a mixture of ionized matrix and analyte is released (Figure 2.4). The ions impact onto an ion-detector, and the time interval between the pulse of the laser light and the impact of each ion on the detector is measured. The produces signals whose intensities are proportional to the number of ions arriving at the electron multiplier (molar response). The MALDI-TOF mass spectrum is then obtained by recording the detector signal as a function of time.

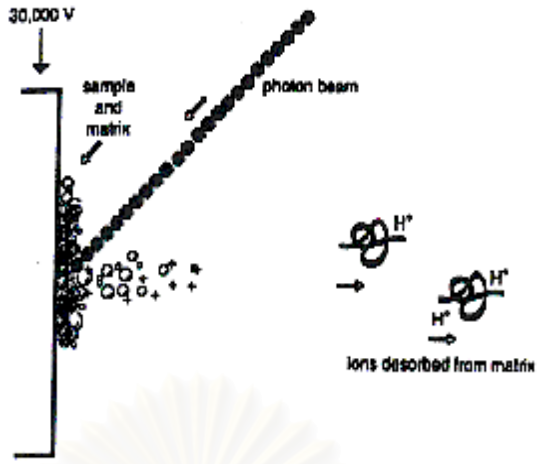


Figure 2.4 Mechanism of ions formation

According to Eq. 2.1, the squre of the fight time is proportional to the m/z ratio.

$$m/z = 2Vt^2/ l^2 \qquad (2.1)$$

where *m* is the mass of the ion, *z* is the number of charges, *V* and *l* is the length of the flight tube. Since the *V* and *l* values are known, the *m/z* ratio can be calculated solely from Eq. 2.1.

In practice, however, exact values for the mass scale are obtained from the empirical Eq. 2.2, because of uncertainties in the determination of the flight time. This uncertainty is due to a short delay in ion formation after the laser pulse, so that the real starting time of the ions is not identical to the time of the laser pulse.

$$m/z = at^2 + b \qquad (2.2)$$

The constants a and b in Eq. 10.2 are measured by the flight times of two ions with known masses, which are used to mass calibrate the MALDI-TOF spectra.

2.3.2 Instrumentation

Time-of-fight (TOF) mass spectrometers are ideally suited for use with the MALDI technique because of their theoretically unlimited mass range, high ion transmission, and for the pulsed nature of the laser used in this method.

Mass resolution is the ability of an instrument to separate the signals from ions of similar mass, expressed as the mass of a given ion divided by the full width at half maximum of the peak.

In MALDI-TOF, the packets of ions produced by laser irradiation of matrix/analyte mixture and accelerated by a fixed electric potential (V) can be detected in two different ways: the linear and reflection (or reflectron) mode. The two detection modes are complementary, since a higher resolution is obtained in the reflection mode and a higher sensitivity at high molar mass is achieved in the linear mode. The sequence of operations in mass spectrometer is shown as same as in Scheme 2.1

Sample Introduction

Direct probe inlets*:* is mainly used in MALDI-TOF system. The multiprobe ion source has a manually operated sample loading facility. The sample selection and movement is totally computer controlled and performed on a circular area, which can be rotated around its own axis. Probe tips are available with 8, 10 or 20 defined sample position.

Ionization

Laser System**:** The laser system provides the pulsed laser light at defined wavelength and intensity on a small spot on the target. In general, the laser system consists of a pulsed standard N_2 laser with 337 nm wavelength and 3 ns pulsed width for use with the matrix components absorbing light of this wavelength, an attenuator which allows fine adjustment of the laser influence, beam splitters to direct a fraction of a laser light to a photodiode starting the time-of-flight measurement, a lens system to focus the laser beam and mirror system to direct the beam into ion source on target.

Ion source: The ion source consist of a positively or negatively charged metal electrode, i.e., the sample probe, and a grounded accelerating grid at the distance of about 1 to 2 cm. Possible accelerating potentials are in the range of +30/-25kV.

The analyte/matrix mixture is deposited on this electrode and exposed to the pulsed laser beam. When the analyte/matrix mixture is hit by the laser beam, gaseous analyte ions are formed which are accelerated by the electrical field, exit the source and press though focusing lenses into the field free drift region.

Detector

Time-of-Flight Mass Spectrometer: A time-of-flight (TOF) mass spectrometer is one of the simplest mass-analyzing devices. A TOF mass spectrometer behaves as a velocity spectrometer, in which ions are separated on the basis of their velocity differences. A short pulse of ions, after exiting the source, is dispersed in time by allowing it to drift in a long flight tube. The principle behind the mass analysis is that after acceleration to a constant kinetic energy (equal to zV, where z is the charge on the ion and V the accelerating potential), ions travel at velocities, v, that are an inverse function of the square root of their m/z values:

$$v = \left(\frac{2zV}{m}\right)^{1/2} \tag{2.3}$$

The lighter ions travel faster and reach the detector placed at the end of the flight tube (of length L) earlier than do the heavier ones. Thus, a short pulse of ions is dispersed into packets of isomass ions (Figure 2.6). Therefore, mass analysis of ions that enter the flight tube can be accomplished by determining their time of arrival given by

$$t = \frac{L}{V} = L\left(\frac{m}{2zV}\right)^{1/2} \tag{2.4}$$

In order to convert the time spectrum into a mass spectrum, the instrument is mass calibrated by measuring the flight times of two different known mass ions.

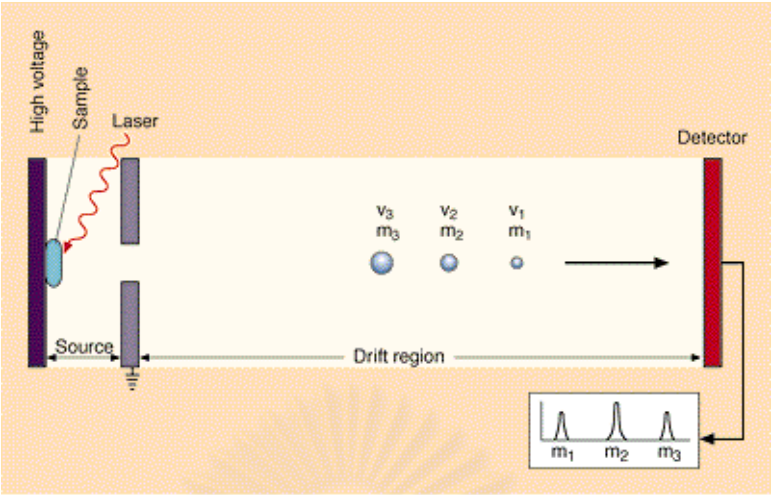


Figure 2.6 Principle of the mass separation by a time-of-flight mass analyzer. Ions are separated on the basic of their size; high-ions (big circles) travel more slowly than the lighter ions

Time-of-flight mass spectrometers have a number of attractive features, such as theoretically unlimited mass range, high ion transmission, very high spectrum acquisition rate, multiplex detection capability, simplicity, in instruments design and operation, reasonable mass resolution, and low cost. The detection sensitivity of TOF instruments is much higher than in scanning instruments because they can record all the ions that reach the detector after each ionization event, and they have a high ion transmission efficiency. A major asset of TOF mass spectrometers is their ability to record a complete mass spectrum in time intervals as short as 25 μs. These attributes make TOF-MS an attractive research instrument as well as a valuable analytical tool.

Sample preparation

Polymer show a distribution of different chain lengths and end-groups. Appropriate methods of sample preparation are required for each class of polymer to avoid selective cationization and discrimination effects, which may affect polymer distributions and cause errors in the calculation of average molar mass values. Homogeneous mixtures of matrix and polymer in the condensed state are a prerequisite to avoid mass distribution.

MALDI Matrices

An ideal matrix should have the following properties: high electronic absorption at the employed laser wavelength, good vacuum stability, low vapor pressure, good solubility in the solvents that can also dissolve the analyte, and good miscibility with the analyte in the solid state.The role of matrix performs two important functions; (1) it absorbs photon energy from the laser beam and transfers it into excitation energy of the solid system; and (2) it serves as a solvent for the analyte, so that the intermolecular forces are reduced and aggregation of the analyte molecules is held to a minimum. Some desirable characteristics of a typical MALDI matrix are

- A strong light absorption property at the wavelength of the laser flux.
- The ability to form microcrystals with the sample.
- A low sublimation temperature, which facilitates the formation of an instantaneous high-pressure plume of matrix-sample material during the laser pulse duration.
- The participation in some kind of a photochemical reaction so that the sample molecules can be ionized with high yields.

Some commonly used matrices, the solvents in which they can be dissolved, and fields of their applications, are listed in Table 2.1.

Table 2.1 The common matrices used in MALDI - MS

Matrix	Mass (Da)	Solvents	Applications
3-Amino-4-hydroxybenzoic acid	153	ACN, water	oligosacharides
2,5-Dihydroxybenzoic acid (DHB)	154	ACN, water, methanol, acetone, chloroform	oligosacharides, peptide, nucleotides, oligosacharides
2[4-hydroxyphenylazo]benzoic acid (HABA)	242	ACN, water, methanol	proteins, lipid
Cinnamic acid	148	ACN, water	general
α-Cyano-4-hydroxycinnamic acid (CCA)	189	ACN, water, ethanol, acetone	peptides, lipids, nucleotides
Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid)	224	ACN, water, acetone, chloroform	lipids, peptides proteins
Dithranol	226	THF	polymers
all-trans retinoic acid (RTA)	300	THF	polymers

2.4 Application of MALDI-TOF-MS to Synthetic Polymers

MALDI is the newest and most promising desorption method for synthetic macromolecules. It is extremely sensitive, with the total amount of sample deposited onto the target being in the pico- to femtomole range. Polymer up to approximately 10^6 Da can be ionization by this method. Up to approximately 50,000 Da, singly charged ions are formed exclusively or predominantly, while at higher molecular weights multiply charged ions are usually coproduced in considerable abundance. MALDI-TOF allows desorption and ionization of very large molecules, even in complex mixtures. It gives information on the mass of individual oligomers from which repeat units, end-groups, the presence of rings, molar mass distributions and other information can be derived.

The most commonly used matrices for synthetic polymers are 2,5-dihydroxybenzoic acid (DHB), 2[4-hydroxyphenylazo]benzoic acid (HABA), 3-β-indoleacrylc acid (IAA), dithranol, all trans-retinoic acid, 3,5-dimethoxy-4-hydroxy cinnamic acid (sinnapic acid) and 5-chlorosalicylic acid. The selection of a good matrix is still a trial and error process in MALDI research.

In principle, in the desorption/ionization process, the amount of pulsed laser energy transferred to the analyte via matrix will depend on the laser power, on the nature of the matrix and sample, and on the dispersion of analyte molecules within the matrix. There exists a threshold irradiance that is matrix-dependent, below which ionization is not observed. Above this level ion production increases nonlinearly.

This research is to study for good MALDI-MS condition, such as types of matrix, analyte to matrix ratio and laser power for analysis polysorbate. After selected the optimized condition for analysis, quantitive analysis polysorbate by using MALDI is demonstrated for the first time.

Determination of Molecular Masses

1. Mass-average molecular weight; $\overline{M}w$

$$\overline{M}w = \frac{\sum\left(N_i M_i^{\,2}\right)}{\sum\left(N_i M_i\right)} \qquad \textbf{(2.5)}$$

2. Number-average molecular weight; $\overline{M}n$

$$\overline{M}n = \frac{\sum\left(N_i M_i\right)}{\sum N_i} \qquad \textbf{(2.6)}$$

Where N_i, M_i represent signal intensity in peak area and mass for the oligomer containing i monomer.

Quantitative Analysis

The quantitative information was given by using standard curve which plot between the concentration of sample and the ratio of signal intensities of analyte to signal of internal stardards.

2.5 Literature Reviews

Surfactant of the polysorbate type are widely used in the pharmaceutical and cosmetic industry as solubilizers for insoluble ingredients. Many research groups published work on analysis of polysorbate type with other technique; for example, Kato et al.[5] determined polysorbate in food by using colorimetry with confirmation by infrared spectroscopy, thin-layer chromatography and gas chromatography. From the results, detection limit with TLC was 50 mg/kg. Then, Bramley et al [9] was used OH^- negative ion chemical ionization mass spectrometry for characterization of oligomers of polysorbate. From the study, separation of signal from the sorbitan and signal from the polyethylene was found. For quantification characterization, Although, many research have been attempted using several techniques, the successful technique is HPLC which were Tani et al.[8] using the single step method of HPLC to determination concentration of polysorbate. The method consists of size exclusion column and mobile phase containing a surfactant at concentrations above the critical micelle concentration for the surfactant. Polysorbate 80 standards show linearity from 2 to 1000 μg/ml.

Unfortunately, none of these methods allows satisfactory separation or resolution of the individual polymer species, nor identification of the components. Until 1987, The technique known as MALDI-TOF MS [10] was first demonstrated and though still in its infancy with regard to polymer analysis. Its advantages over other methodologies include ease of sample preparation, speed of analysis, high sensitivity, and minimal fragmentation allowing direct access to molecular weight. Thus, in 1997, Cumme et al.[6] used MALDI-TOF of composition analysis of detergents of the polyoxyethylene type include polysorbate, but the spectrum was poor. Then, in 2001 Norrie et al.[12] applied MALDI-TOF for determination of polysorbate in food. From the results, MALDI-TOF is a powerful tool that can provide a polysorbate mass spectrum.

2', 4', 6' – trihydroxyacetophenone monohydrate was chosen to be an ideal matrix by addition of aqueous 0.01 M potassium chloride.

In 1994, the concept of quantitative analysis of low and high molecular weight compounds by using MALDI-TOF has been reported by Nelson et al..[19] In the study, protein and peptides were analyzed and the intensities of single peaks were considered. The quantitative information was calculated by comparing the signal intensities of analyte to internal stardards.

In this study, quantitative analysis is demonstrated for the first time for polysorbate types. The method describes a simple way to determine, the molecular complexity of polysorbate emulsifiers using MALDI-TOF MS. The objectives of this research were to select a optimize conditions for polysorbate and to develop quantitative analysis of polysorbate for application to analysis in drugs and cosmetics products.

CHAPTER III

EXPERIMENTAL

3.1 Materials

Sorbitan-20EO-monooleate standard (polysorbate 80, Disponil SMO 120 spez.) was from Cognis Co.,Ltd. The sample raw materials, pharmaceutical suspensions were from Nurofen for children (Boot Co.,Ltd) and Brusil syrup (Silom medical Co.,Ltd.), and the commercial-lotion were from Revlon dry skin relief moisture lotion (Revlon, NY) and Dove body silk moisturising cream (Lever Faberge, USA). Acetone, acetonitrile and ethyl alcohol were solvent grade (J.T. Baker). Tetrahydrofuran (HPLC grade) was purchased from Labscan. The matrices: 2,5-dihydroxybenzoic acid, dithranol and 2-(4-hydroxyphenylazo)-benzoic acid were purchased from Aldrich, and α-cyano-4-hydroxycinamic acid were purchased from Sigma. Standards used for calibration was Ubiquitin (Fluka).

3.2 Apparatus and Instruments

All MALDI spectra were acquired using a MALDI-TOF MS mass spectrometer from BIFLEX (BRUKER, Germany). The instrument is equipped with a nitrogen laser (λ = 337 nm). Laser desorbed positive ions were analyzed in the linear mode. External calibration was performed using the ion CCA and Ubiquitin standards. Spectra were acquired as the sum of 200 laser shot. Apparatus used were vortex mixer (vorttex-genie No.2, Scientific Industries), multichannel probe (BRUKER, Germany), autopipettp (Pipetman, Gilson), pipette tip and eppendorpf.

3.3 Procedure

3.3.1 Sample and reagent preparation for MALDI analysis

Polysorbate 80 (standard; 100 mg) was dissolved in 1 mL of tetrahydrofuran. The matrix solutions applied were separately used: dithranol, 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinamic acid and 2-(4-hydroxyphenylazo)-benzoic acid.

- 10 mg/ml dithranol was prepared in THF.
- 10 mg/ml DHB was prepared in acetone.
- CCA was prepared in a variety of ways to investigate sample effect
    a) at 10 mg/ml in water/acetonitrile (70:30, 0.1% (v/v) TFA)
    b) at 10 mg/ml in ethanol/acetonitrile (50:50, 0.1% (v/v) TFA)
    c) at 10 mg/ml CCA in THF
- 10 mg/ml HABA was prepared in THF.

Mass calibration was performed using CCA and Ubiquitin standard. To prepare the internal standard, Ubiquitin standard was dissolved in deionized water (1mg/ml).

3.3.2 Condition for the analysis of polysorbate 80

For MALDI-TOF mass spectrometry analysis the following conditions and parameters were studied:

- Target preparation
- Types of matrix
- Analyte-to-matrix ratio(A/M)
- Laser power
- Solvent effect

Target preparation

Two methods for sample preparation were carried out.

1) Dried droplet method – sample and matrix were mixed and vortexed. Then 0.5 μL of mixture was deposited on target and allowed to dry.

2) Thin layer method – first, the matrix solution was dropped on a target and allowed to dry which form a microcrystal layer. Then, an analyte solution was added to the top of the matrix layer and dried. Finally, the last matrix layer was droped on the top of sample layer and allowed to dry.

Types of matrix

Samples for MALDI analysis required a suitable matrix to obtain good spectra. Sample solution (10% w/w) and matrices were mixed with various saturated solutions of the matrix (ratio 1:1, v/v). The matrix solutions applied for this experiment were dithranol, 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinamic acid (CCA) and 2-(4-hydroxyphenylazo)-benzoic acid (HABA).

Analyte-to-matrix ratio(A/M)

The amount ratio of analyte to matrix were varied in 1:1, 1:25, 1:50, 1:75, 1:100, 1:125 and 1:150.

Laser power

The values of laser power 280, 290, 300, 310 and 320 μJ were used for this experiment.

Solvent effect

The types of solvents are important for obtaining good spectra. CCA is a good matrix for polymer wherever solvents may affect the MS spectrum. Thus, various solutions were used.

1) CCA in water/acetonitrile (70:30, 0.1% (v/v) TFA)
2) CCA in ethanol/acetonitrile (50:50, 0.1% (v/v) TFA)
3) CCA in THF

3.3.3 Determination of molecular weight distribution

The molecular weight distribution of polysorbate emulsifiers was analysis at the optimum condition for identification of polysorbate molecular components.

Precision

Sample solution (100 mg/ml) and matrix were mixed in a 1:75 ratio and vortexed. Then a mixture of 0.5 μL was deposited on a target and allowed to dry. Finally, the target was taken into MALDI-MS and bombarded with laser power. The peaks series were calculated and then given a precision of the method.

Detection limit

Polysorbate stock solution 100 mg/ml was diluted in THF at concentration ratio 1:20, 1:40, 1:60, 1:80 and 1:100, respectively. All of solutions were mixed with CCA matrix with a ratio of 1:75. For limitation of detection, the amount of polysorbate was more than three times ratio of sample and noise intensities.

3.3.4 Quantification of sorbitan-20EO-monooleate (Polysorbate 80)

MALDI was used for quantitative analysis of polysorbate 80 , using ubiquitin as an internal standard. The threshold of analyte/matrix molar ratio was given the best linearity of the standard curve and the accuracy of the quantitative analysis.

1. Standard preparation

Standard (polysorbate 80) 100 mg/ml in THF was a stock solution and dilute stock solution to concentration 50, 25, 20, 15, 10 and 5 mg/ml, respectively. Three concentrations are prepared for accuracy test: 35, 17 and 12.5 mg/ml, respectively. Then standard solution and CCA as a matrix solution were mixed in the ratio of analyte to matrix 1:75. Finally, mixture of standard and 1.0 μL of ubiquitin as an internal standard solution were mixed. To prepare the target, 0.5 μL of the mixture of standard and internal standard was applied and allowed to air-dry. The polyoxyethylene sorbitan esters were ionized by a nitrogen laser pulse (337 nm) before entering the time-of-flight mass analyser. Spectra were acquired as the sum of 200 laser shots.

2. Sample preparation

In pharmaceutical suspensions

Each polysorbate sample (1g) was dissolved in 0.1 mL of deionized water. Then sample solution and CCA were mixed in the ratio of analyte to matrix 1:75. Finally, mixture of standard and 1.0 μL of ubiquitin were added and spotted 0.5 μL of the mixture of standard and internal standard in the target and allowed to air-dry.

In commercial lotions

Each polysorbate sample (100 mg) was dissolved in 0.1 mL of THF and then vortexed. Waiting for five minutes before vortexed the sample again. Allowed the sample to separate into two phase. Then pipette 1.0 μL of the top layer of sample mixed with CCA (1:75) and deposited the mixture on the probe before bombarded with laser power for checking the efficiency of the residue polysorbate. For quantitative analysis, polysorbate 80, which retained in the bottom layer, was used as a sample. Then sample solution and CCA were mixed in the ratio of analyte to matrix 1:75. Finally, mixture of standard and 1.0 μL of ubiquitin was added and spotted 0.5 μL of the mixture in the target and allowed to air-dry.

CHAPTER IV

RESULTS AND DISCUSSION

This research was related to the development of mass spectrometric method for quantitative determination of sorbitan-20EO-monooleate (polysorbate80). As proposed in previous chapter, the results will be shown and discussed in each part, respectively.

4.1 The optimized conditions and parameters for the analysis of polysorbate 80.

4.1.1 Target preparation

Several options are available for transferring the mixture onto the MALDI target. For MALDI-TOF analysis, a comparison of MALDI spectra of sample from a dried droplet and thin layer method was investigated. The results in Figure A1 showed that the dried droplet method gave higher intensity of sample peaks in mass spectrum than the thin layer method. In the dried droplet method, sample and matrix are mixed, and 0.5-1.0 μl of the mixture is applied to the target and air-dried at room temperature. Under these conditions crystallization is relatively slow, thereby homogeneous cocrystallization of analyte and matrix occurred. Unlike the dried droplet method, the thin layer method, where a matrix solution is prepared and allowed to crystallize and next the sample is added and dried. As a result, the risk of segregation between sample and matrix may occur.

4.1.2 Types of matrices

Efficiency of MALDI analysis is highly dependent upon matrix selection and laser power. An ideal matrix should show that the highest absorption at the employed laser wavelength property. Four common matrixes were tested for desorption and ionization with the present polymer sample: dithranol, 2,5-dihydroxybenzoic acid (DHB), 2-(4-hydroxyphenylazo)-benzoic acid (HABA) and α-cyano-4-hydroxy cinamic acid

(CCA). Each type of matrices yield a difference of m/z range of the molecular weight distribution. The spectra were shown in Figure A2 and summarized in Table 4.1.

Table 4.1 Mass to charge molecular weight distribution and laser power of matrices.

Type of matrix	*Laser power (μJ)*	*m/z range of molecular weight distribution*
dithranol	320	700 - 3000
2,5-dihydroxybenzoic acid	320	1000 - 2500
2-(4-hydroxyphenylazo)-benzoic acid	320	600 - 2200
α-cyano-4-hydroxycinamic acid	310	600 - 2200

All of matrices were found to provide m/z values ranging approximately from 600 to 3000 Da. Peaks in any given series are separated by a mass of 44 Da which corresponds to the molecular weight of ethylene oxide. The polydispersity of the ethylene oxide polymer is responsible for the observed Gaussian distributions. Of these matrices, polysorbate 80 using dithranol produced high quality spectrum, but it produced wide m/z values ranging and high intensity of noise. Thus, this matrix is not suitable for determination of molecular weight distribution of polysorbate. When DHB was used as a matrix, the result suggested that it was not suitable as response was low and the intensity of noise is high in m/z of 600-800. The sample using CCA and HABA were found to provide the highest quality spectra but the sample using CCA gave more resolution than HABA; therefore, this matrix was used for the analysis of the samples examined in this study.

4.1.3 Analyte to matrix ratio (A/M)

The polymer and matrix ratio was optimized for the highest intensity. Polysorbate was mixed with the matrix solution (CCA) with a various ratios of 1:1, 1:25, 1:50, 1:75, 1:100, 1:125 and 1:150. The spectra are shown in Figure A3 and the average intensity from each spectrum was shown in Table 4.2.

Table 4.2 Average intensity in various ratio of analyte using CCA

Ratios of analyte to matrix	*Average intensity*
1:1	361
1:25	8168
1:50	14336
1:75	17521
1:100	6943
1:125	6889
1:150	5467

From Table 4.2, the average intensity is higher when polymer-to-matrix ratio is increased. When the amount of matrix is increased, the laser has more chanced to hit the matrix molecule. Consequently, the energy transfer from matrix to polymer is easier to happen. When the polymer-to-matrix ratio is above 1:75, the intensity is decreased due to the amount of polymer is decrease.

4.1.4 Laser power

In principle, in the desorption/ionization process, the amount of pulsed laser energy transferred to the analyte via matrix will depend on the laser power, and on the nature of the matrix and sample. There exists a threshold irradiance that is matrix-dependent, below which ionization is not observed. Above this level ion productions were increasing. From the result, the energy laser more than 310 μJ does not show the increase of the intensity of polysorbate, so the energy laser at 310 μJ was suitable for polysorbate with CCA because it gave the highest intensity of the sample.

4.1.5 Solvent effect

The types of the solvents also have an effect on the intensity and resolution of the spectrum. It is significant factor for MALDI analysis because it dissolves the sample in homogeneous phase. The small crystals with the little segregation are obtained from the fast crystallization solvent. In Figure A4, the same concentration of polysorbate was mixed with various solvents. From the results, THF was selected because it gave the highest quality spectra.

4.2 Determination of polyoxyethylene (20) sorbitan monooleate (polysorbate 80)

4.2.1. Molecular weight distribution

The MALDI-TOF mass spectra were obtained with CCA at the optimum condition to which K^+ was added to promote ion formation. Most ions belonged to the $[M + K]^+$ series. The mass spectra can indeed provide molecular weight averages as well as the molecular weight distribution in a single experiment. Complete polysorbate analysis presents a challenge, as there are several factors contributing to molecular heterogeneity. From Norris et al. report [12], polysorbate was identified by using MALDI-TOF and GC analysis. From the result, the spectrum is similar to my study, so identification peaks are the same. The result showed a complex mixture of esters and partial esters of sorbitans, sorbides and disorbitan-based species with varying degrees of esterification, and varying alkyl chain lengths of fatty acids. The spectrum was shown in Figure 4.1. It showed the complexity introduced by the varying degrees of esterification and different types of fatty acids.

The peaks were identified as belonging to the following four series:
1. polyoxyethylenes (simple linear chains not bound to sorbitol-derived ethers),
2. isosorbide carbowaxes, 3. disorbitan carbowaxes, and 4. sorbitan carbowax.

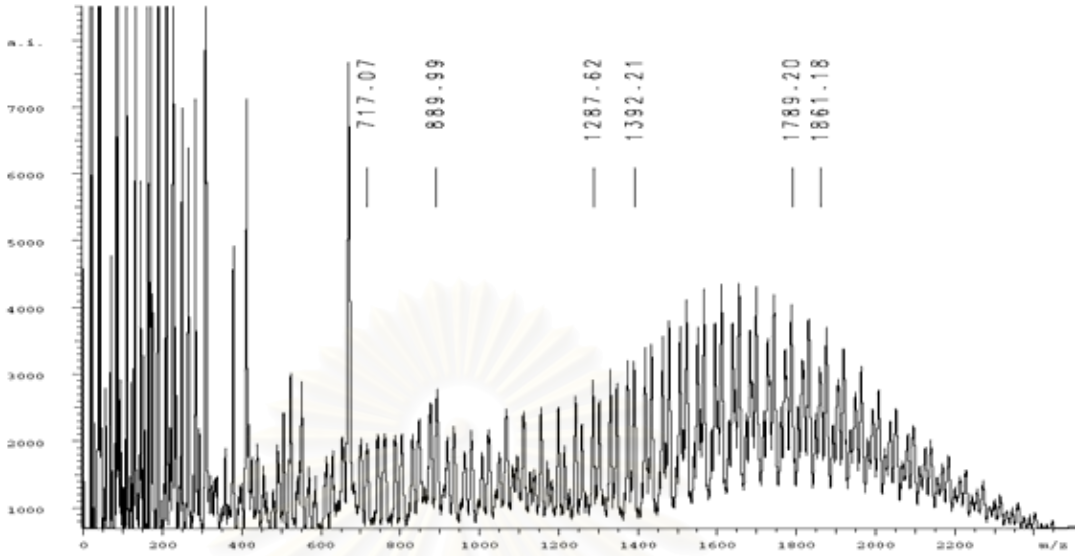


Figure 4.1. MALDI-TOF MS positive ion spectrum of PS 80 (standard). Unlabeled peaks below m/z 500 result from fragmentation of the matrix.

Table 4.3 Identification of polysorbate peaks labled in Figure 4.1

Observed MW	*Possible identities*	*Theoretical MW*
717.07	polyoxyethylene (14)	717.1
889.99	Polyoxyethylene (16) isosorbide	890.0
1287.62	polyoxyethylene (18) disorbitan	1287.5
1392.21	Polyoxyethylene (21) sorbitan monooleate	1392.7
1789.20	polyoxyethylene (30) sorbitan monooleate	1789.2
1861.18	Polyoxyethylene (32) disorbitan	1861.4

The number-average molecular weight M_n (eq 4.1) and the weight-average molecular weight M_w (eq 4.2) were readily calculated from the masses of the observed ion peaks and their intensities.

Number-average molecular weight; M_n

$$M_n = \frac{\Sigma(N_i M_i)}{\Sigma N_i} \qquad (4.1)$$

Mass-average molecular weight; M_w

$$M_w = \frac{\Sigma(N_i M_i^2)}{\Sigma(N_i M_i)} \qquad (4.2)$$

Where, M_i is the mass of a molecular ion with a degree of polymerization i and N_i is the number of molecules of mass M_i. For simplicity, N_i is assumed to be proportional to the signal intensity in the MALDI mass spectrum.

The ratio of the two averages denotes the polydispersity index PDI (eq 4.3).

$$PDI = \frac{M_w}{M_n} \qquad (4.3)$$

The calculation of M_n, M_w and PDI were shown in Table 4.3 and the example of calculation were shown in example 4.1.

Example 4.1: Calculation of M_n, M_w and PDI (polyoxyethylene sorbitan monooleate series)

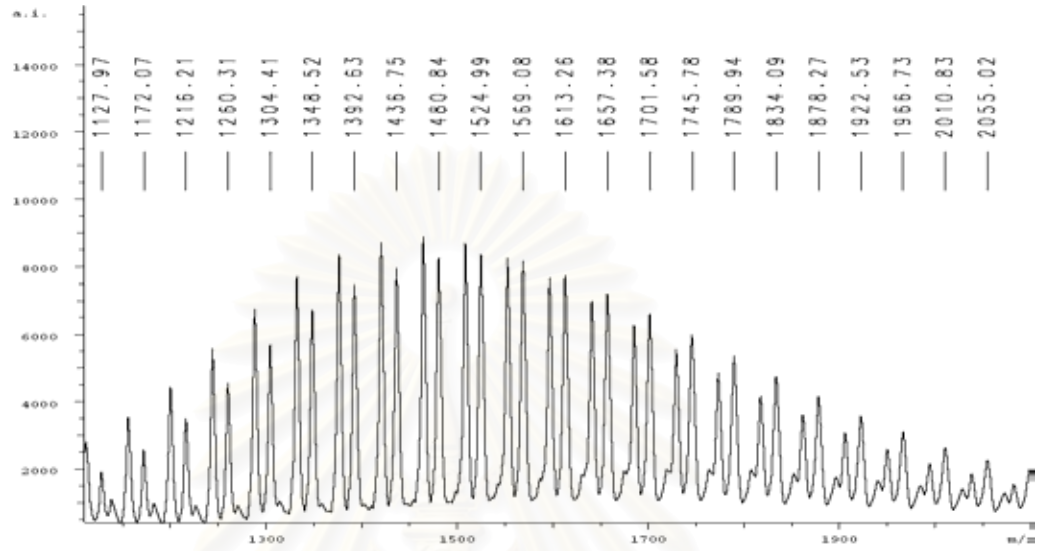


Figure 4.2. Mass spectrum of polysorbate 80

$$M_n = \frac{\Sigma(N_i M_i)}{\Sigma N_i}$$

= [(0.1764x1172.07) + (0.2414x1216.21) + (0.3173x1260.31) + (0.3967x1304.41) + (0.4728x1348.52) + (0.5381x1392.63) + (0.5913x1436.75) + (0.6266x1480.84) + (0.6451x1569.08) + (0.6295x1613.26) + (0.6098x1657.38) + (0.5694x1701.58) + (0.5284x1745.78) + (0.4822x1789.94) + (0.4382x1834.09) + (0.3842x1878.27) + (0.3388x1922.53) + (0.2978x1966.73) + (0.2568x2010.83)] / (0.1764 + 0.2414 + 0.3173 + 0.3967 + 0.4728 + 0.5381 + 0.5913 + 0.6266 + 0.6465 + 0.6451 + 0.6295 + 0.6098 + 0.5694 + 0.5248 + 0.4822 + 0.4328 + 0.3842 + 0.3388 + 0.2978 + 0.2568)

= 1593.5342

$$M_w = \frac{\Sigma(N_i M_i^2)}{\Sigma(N_i M_i)}$$

= [(0.1764x1172.072) + (0.2414x1216.212) + (0.3173x1260.312) + (0.3967x1304.412) + (0.4728x1348.522) + (0.5381x1392.632) + (0.5913x1436.752) + (0.6266x1480.842) + (0.6465x1524.992) + (0.6451x1569.082) + (0.6295x1613.262) + (0.6098x1657.382) + (0.5694x1701.582) + (0.5284x1745.782) + (0.4822x1789.942) + (0.4382x1834.092) + (0.3842x1878.272) + (0.3388x1922.532) + (0.2978x1966.732) + (0.2568x2010.832)]/ [(0.1764x1172.07) + (0.2414x1216.21) + (0.3173x1260.31) + (0.3967x1304.41) + (0.4728x1348.52) + (0.5381x1392.63) + (0.5913x1436.75) + (0.6266x1480.84) + (0.6465x1524.99) + (0.6451x1569.08) + (0.6295x1613.26) + (0.6098x1657.38) + (0.5694x1701.58) + (0.5284x1745.78) + (0.4822x1789.94) + (0.4382x1834.09) + (0.3842x1878.27) + (0.3388x1922.53) + (0.2978x1966.73) + (0.2568x2010.83)]

= 1622.8749

$$PDI = \frac{M_w}{M_n}$$

= 1622.8749/1593.5342 = 1.0184

The calculations of M_n, M_w and *PDI* were shown in Table 4.4 .

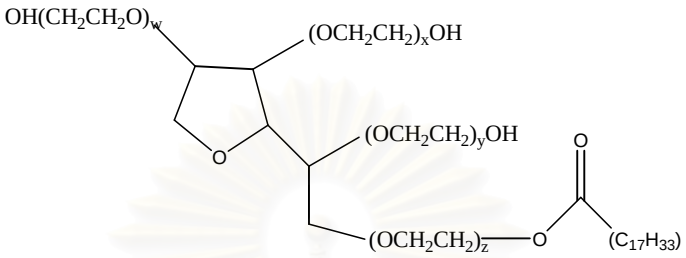
Table 4.4 Calculation of M_n, M_w and *PDI* of polysorbate standard

Polysorbate standard (polysorbate 80)					
polyoxyethylene isosorbide			polyoxyethylenes		
M_w	M_n	PDI	M_w	M_n	PDI
806.65	786.76	1.03	830.46	806.80	1.03
812.72	795.13	1.02	822.30	804.77	1.02
813.31	792.33	1.03	824.25	804.74	1.02
815.66	794.28	1.03	823.15	802.71	1.03
806.14	783.04	1.03	836.43	811.80	1.03
812.48	791.49	1.03	820.69	803.07	1.02
816.41	795.43	1.03	832.00	807.84	1.03
Average					
811.91	791.21	1.029	827.04	805.96	1.026
Standard deviation					
4.05	4.66	0.004	5.92	3.17	0.005

Polysorbate standard (polysorbate 80)					
polyoxyethylene disorbitan			polyoxyethylene sorbitan monooleate		
M_w	M_n	PDI	M_w	M_n	PDI
1548.63	1515.89	1.02	1622.87	1593.53	1.02
1550.49	1518.72	1.02	1612.08	1580.54	1.02
1542.60	1507.18	1.02	1616.37	1585.08	1.02
1554.65	1518.05	1.02	1613.28	1585.13	1.02
1551.42	1518.40	1.02	1626.11	1595.82	1.02
1556.05	1520.69	1.02	1619.78	1580.50	1.02
1542.55	1507.66	1.02	1619.88	1585.82	1.02
Average					
1549.48	1515.23	1.02	1618.62	1586.63	1.02
Standard deviation					
5.33	5.12	0.000	5.94	5.05	0.00

4.4.2. Degree of ethylene oxide polymerization and end groups analysis

Polysorbate consisted of the combination of sorbitan esters which bonded with a repeating unit (44 Da) of ethylene oxide ($-CH_2\ CH_2O-$)



$$R + 44\,(w+x+y+z) + 191 = M \qquad (4.4)$$

Where R is the alkyl group such as $C_{17}H_{33}$ and w+x+y+z is the number of repeating unit and M is the molecular weight.

Example 4.2: Calculation of degree of ethylene oxide polymerization and end groups

From eq.4.3 $M + 39 = 1348.52$

$M = 1309.52$

$R + 44\,(w+x+y+z) + 191 = M$

If $R = C_{17}H_{33}$ $44\,(w+x+y+z) = 881.52$

Thus $w+x+y+z = 20.03$

~ 20

From the results, the calculation of end group and repeating unit showed C_{17}-series as a monooleate type and approximate repeating unit showed 20 units of polyethylene oxide.

4.3. Quantification of sorbitan-20EO-monooleate (Polysorbate 80)

4.3.1 Standard curve

An important aspect of quantitative analysis using the MALDI-TOF technique is sample preparation. To prepare the standard, 100 mg/ml of polysorbate 80 was dissolved in THF to be stock solution. The stock solution was diluted to concentration 50, 25, 20, 15, 10 and 5 mg/ml, respectively. Then, three concentrations were prepared 35, 17 and 12.5 mg/ml to compare with concentration from calibration curve. Then, all of solutions and CCA as a matrix solution were mixed in the ratio of analyte to matrix 1:75. Finally, mixture of standard and 1.0 μL of ubiquitin as an internal standard solution were mixed. To prepare the target, 0.5 μL of the mixture of standard and internal standard was applied and allowed to air-dry. The calibration curve was plotted of concentration of polysorbate 80 and the ratio of the average intensity of polysorbate 80 and ubiquitin. The results were shown in Table 4.5 and the spectra were shown in Figure A5-A6.

Table 4.5 Average intensity of polysorbate 80 and ubiquitin for accuracy test

Concentration of polysorbate 80 (mg/ml)	*Average intensity of polysorbate 80 (a)*	*Intensity of ubiquitin (n)*	*a/n*
50	6671.09	709.00	9.41
25	3764.73	812.02	4.64
20	1189.16	339.61	3.50
15	5811.66	1848.80	3.14
10	4998.50	2450.70	2.04
5	2234.89	1883.30	1.19
Accuracy test			
35	5067.36	789.74	6.42
17	2387.52	709.03	3.37
12.5	5953.66	2368.10	2.51

Then, plot of concentration of polysorbate 80 and the ratio of the average intensity of polysorbate 80 and ubiquitin (internal standard) were shown in Figure 4.3

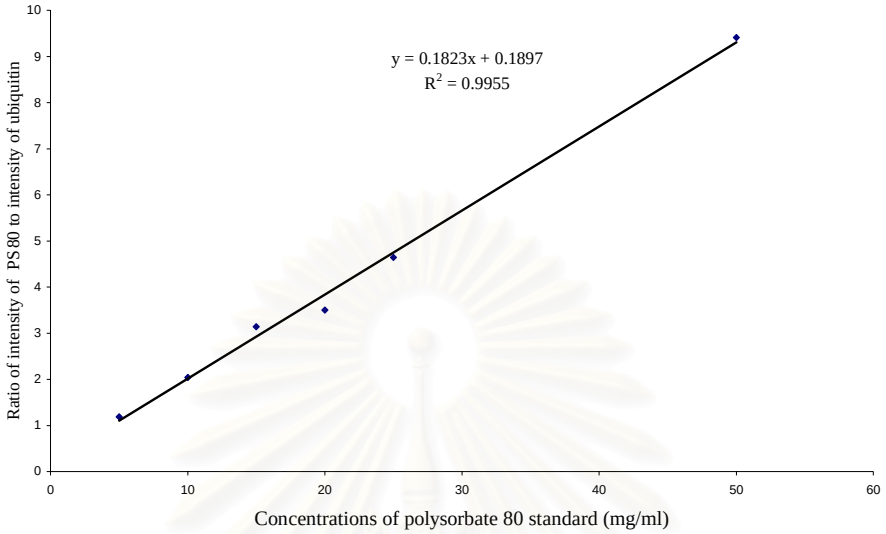


Figure 4.3 Plot of concentration of polysorbate 80 standard *versus* ratio of the average intensity of polysorbate 80 to ubiquitin from Table 4.5.

From the standard calibration curve (Figure 4.3), a linear response was obtained over the concentration range 5-50 mg/ml. The equation of the line is $y = 0.1823x + 0.1897$ (eq 4.5) with a correlation coefficient (r^2) = 0.9955, where y is the intensity ratio and x is the concentration of polysorbate.

The accuracy was calculated in an example 4.3

Example 4.3: Accuracy test

$$y = 0.1823x + 0.1897 \qquad \text{(eq 4.5)}$$

When y = 6.42 then, x = 34.18

Real concentration = 34.97 mg/ml and calculated concentration = 34.18 mg/ml, so percent error is [(34.97-34.18)/ 34.97] x 100 = 2.24 %

According to, y = 3.36 and y = 2.51 from the Figure A6 (b and c), percent error was 2.25 and 1.79 % , respectively. From the results of the accuracy test, the calibration curve has efficiency.

4.3.2 Precision

The precision of the proposed method to determine polysorbate was tested by loading sample to the target with the same amount in various positions. The results were shown in Figure A7 and average intensity were shown in Table 4.6. The precision of the proposed method, expressed as relative standard deviation (%SD), was 1.96 % (n = 20) at a polysorbate 80 concentration of 100 mg/ml.

Table 4.6 Precision Test

Time	*Intensity at n = 20*
1	1672.3
2	1605.0
3	1686.0
4	1688.5
5	1624.3
6	1664.0
7	1635.0
Standard deviation	32.38

4.3.3 Detection limit

Polysorbate stock solution 100 mg/ml was diluted in THF at concentration ratio 1:20, 1:40, 1:60 and 1:80, respectively. All of solutions were mixed CCA matrix with a ratio 1:75. The limit of detection was calculated from the signal-to-noise ration (S/N). These limit was defined as three times the S/N ratio. The limit for polysorbate 80 was determined to be 1.03 femtomole. The spectra were shown in Figure A8.

Example 4.4: Calculation of detection limit of polysorbate 80

Calculating

$0.1\ \mu g \times 1/20 \times 1/75 = 6.67 \times 10^{-5}\ \mu g = 6.67 \times 10^{-11}\ g$

$6.67 \times 10^{-11} / 1618.62 = 41.19$ femtomole

1.0 μg of 1: 40 polysorbate 80 and 1:75 CCA = 20.59 femtomole
1.0 μg of 1: 60 polysorbate 80 and 1:75 CCA = 1.37 femtomole
1.0 μg of 1: 80 polysorbate 80 and 1:75 CCA = 1.03 femtomole

4.4 Application of MALDI-MS for analysis of polysorbate in commercial products

4.4.1 Determination and quantitative analysis of polysorbate in commercial drug A and B

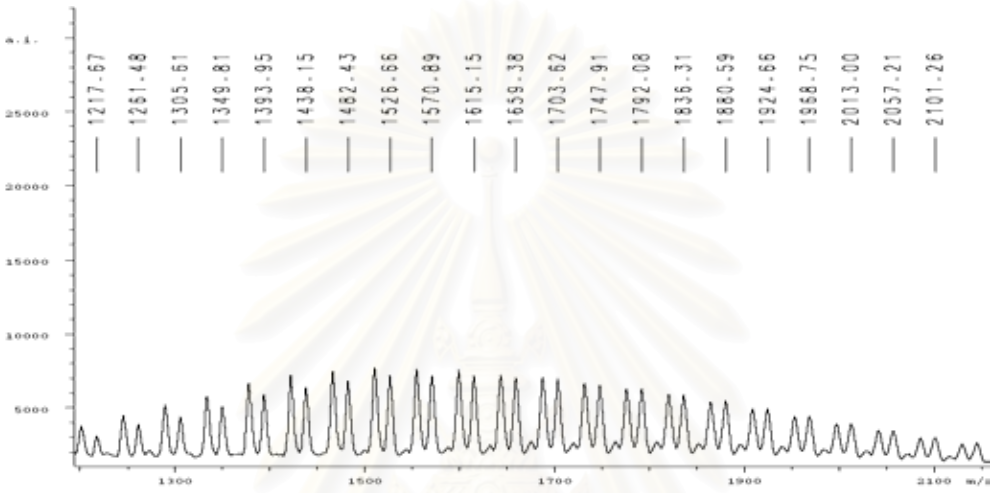


Figure 4.4 MALDI-MS spectrum of polysorbate in commercial drug A

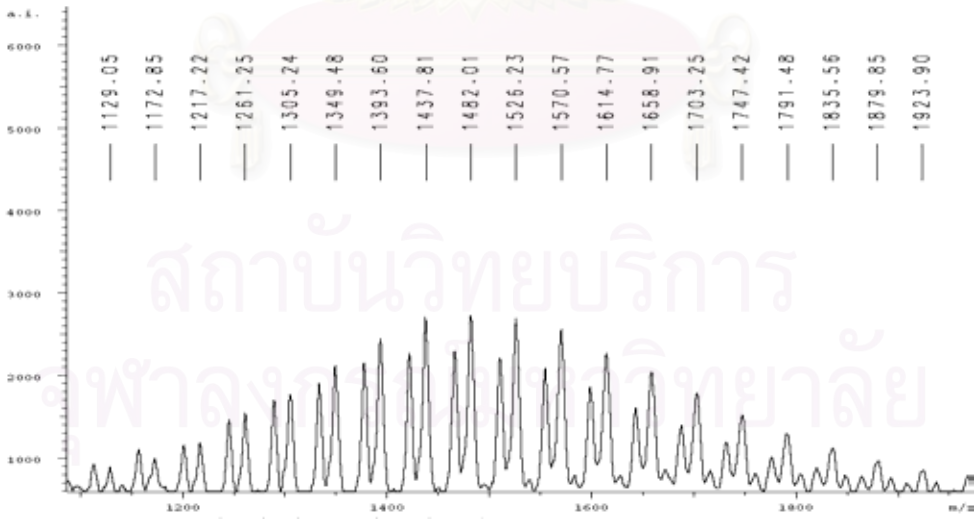


Figure 4.5 MALDI-MS spectrum of polysorbate in commercial drug B

The interpretation of Figures 4.4 and 4.5 uses the same calculation as the previous example. The peaks showed m/z of polyoxyethylene sorbitan monooleate series. The mass spectrum showed C_{17}- series like the mass spectrum of polysorbate standards.

All of them were found to provide m/z values ranging approximately from 1000 to 2000. Peaks in any given series were separated by a mass of 44 Da which corresponded to the molecular weight of ethylene oxide. The calculation of M_n, M_w and *PDI* were shown in Table 4.7

Table 4.7. The calculation of M_n, M_w and *PDI* of polysorbate in commercial drug A and B

Polysorbate 80 in commercial drug A			*Polysorbate 80 in commercial drug B*		
M_w	M_n	PDI	M_w	M_n	PDI
1738.77	1703.71	1.02	1556.99	1533.45	1.01
1750.72	1719.05	1.02	1559.52	1538.65	1.01
1744.75	1711.62	1.02	1558.82	1533.78	1.02
1764.21	1732.56	1.02	1558.79	1534.77	1.02
1744.34	1710.13	1.02	1533.61	1515.46	1.01
1747.12	1713.04	1.02	1549.85	1529.00	1.01
1761.52	1724.88	1.02	1551.58	1527.26	1.02
Average					
1750.20	1716.42	1.02	1552.66	1530.25	1.014
Standard deviation					
9.39	9.79	0.00	9.84	7.44	0.005

To compare of sample A and B (Table 4.7), commercial drug A showed the mass spectrum in the region of high masses than commercial drug B. Thus, commercial drug A reflected the higher degree of polymerization than commercial drug B.

Standard curve for analysis polysorbate 80 in commercial drugs.

Standard curves were prepared as same as the concentration of polysorbate 80 standard curve (real concentration of stock solution = 99.98 mg/ml) . To prepare the drug sample, 1 g of commercial drug A and B was measured three times (Table 4.8) and dissolved in 0.1 ml of deionized water.

Table 4.8 Weight of commercial drug sample

Commercial drug A		*Commercial drug B*	
Time	Weight (g)	Time	Weight (g)
1	1.0004	1	1.0006
2	1.0001	2	1.0002
3	1.0003	3	1.0009

Then sample solution and CCA were mixed in the ratio of analyte to matrix 1:75. Finally, mixture of standard and 1.0 μl of ubiquitin were added before spotted 0.5 μL of the mixture of standard and internal standard in the target and allowed to air-dry. The spectra of polysorbate standard and commercial drug A were shown in Figure A9-A10. The average intensities of polysorbate 80 and ubiquitin were shown in Table 4.9.

Table 4.9 Average intensity of polysorbate 80 and ubiquitin for quantitative analysis polysorbate in commercial drug A

Concentration of polysorbate 80 (mg/ml)	*Average intensity of polysorbate 80 (a)*	*Intensity of ubiquitin (n)*	*a/n*
50	2926.00	247.00	11.85
25	2069.48	310.77	6.66
20	5194.23	1138.20	4.56
15	1063.85	330.83	3.22
10	2201.80	900.55	2.44
5	2360.73	1917.00	1.23
Commercial A			
1	1128.82	503.00	2.24
2	1504.55	719.00	2.09
3	1119.27	503.00	2.23

Then, plot of concentration of polysorbate 80 and the ratio of the average intensity of polysorbate 80 and ubiquitin (internal standard) were shown in Figure 4.6

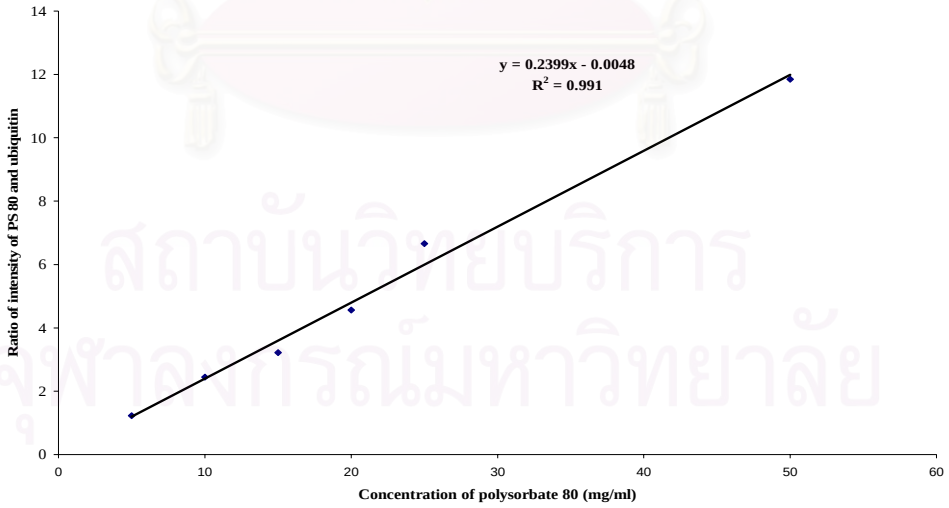


Figure 4.6 Plot of concentration of polysorbate 80 standard *versus* ratio of the average intensity of polysorbate 80 to ubiquitin from Table 4.9.

The spectra of polysorbate standard and commercial drug B were shown in Figure A11-A12. The average intensities of polysorbate 80 and ubiquitin were shown in Table 4.10

Table 4.10 Average intensity of polysorbate 80 and ubiquitin for quantitative analysis polysorbate in commercial drug B

Concentration of polysorbate 80 (mg/ml)	*Average intensity of polysorbate 80 (a)*	*Intensity of ubiquitin (n)*	*a/n*
50	4618.64	255.00	18.11
25	5675.35	624.44	9.09
20	7396.35	936.40	7.90
15	4019.45	747.00	5.38
10	1962.82	509.00	3.86
5	1443.18	748.00	1.93
Commercial B			
1	1895.26	355.49	5.33
2	1007.19	199.68	5.04
3	1125.61	207.06	5.44

Then, plot of concentration of polysorbate 80 and the ratio of the average intensity of polysorbate 80 and ubiquitin were plotted in Figure 4.7

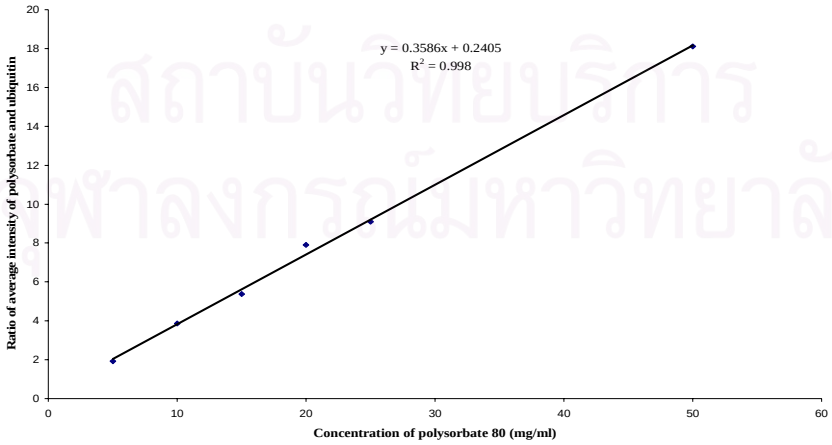


Figure 4.7 Plot of concentration of polysorbate 80 standard *versus* ratio of the average intensity of polysorbate 80 to ubiquitin from Table 4.10.

From the standard calibration curve (Figure 4.6), the equation of the line is $y = 0.2399x - 0.0048$ (eq 4.6) with a correlation coefficient (r^2) = 0.9910, where y is the intensity ratio and x is the concentration of polysorbate.

The concentration of sample drugs were calculated in an example 4.5

Example 4.5 Calculated concentration of polysorbate in commercial drug A

$$y = 0.2399x - 0.0048 \qquad \text{(eq 4.6)}$$

When, y = 2.24 then, x = 9.36

Thus, the concentration in commercial drugs A1 = 9.36 mg/ml

So, sample A 10.004 g has got a polysorbate 9.36×10^{-3} g
If, sample A 100 g has got a polysorbate 0.094 g

There was a polysorbate 0.09 %w/w in sample A1. The concentration of polysorbate in commercial drug B was calculated the same as commercial A, but the equation of the line is $y = 0.3586x + 0.2405$ (eq 4.7) with a correlation coefficient (r^2) = 0.9980. The concentration of commercial drugs A and B were shown in Table 4.11.

Table 4.11 Concentration of polysorbate in commercial drugs A and B

Commercial drug A				Commercial drug B			
Time	y	x	%w/w	Time	y	x	%w/w
1	2.24	9.36	0.094	1	5.33	14.19	0.142
2	2.09	8.73	0.087	2	5.04	13.38	0.134
3	2.23	9.32	0.093	3	5.44	14.49	0.145

The average concentrations of polysorbate in commercial drugs A and B were 0.091 and 0.140 %w/w and standard deviation was 0.004 and 0.006, respectively.

4.4.2. Determination and quantitative analysis of polysorbate in commercial lotion A and B.

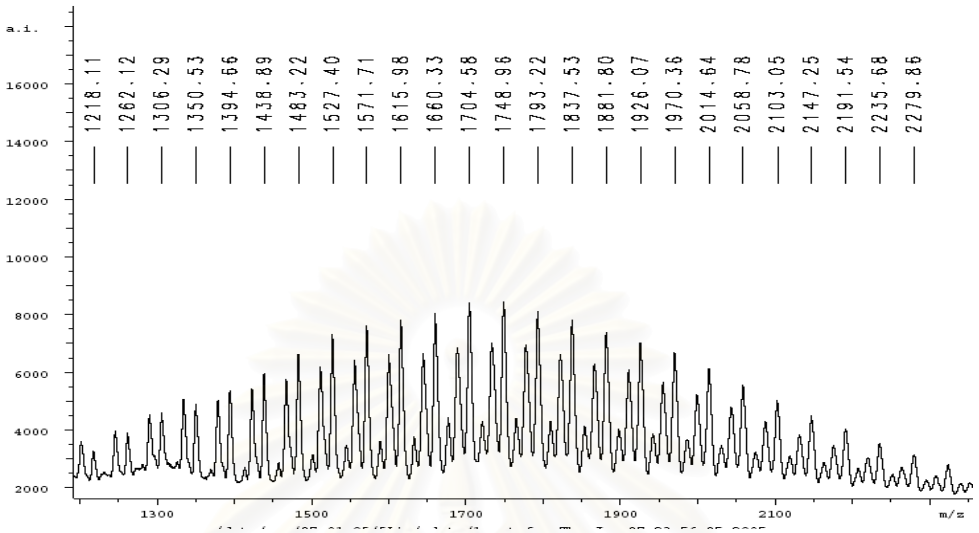


Figure 4.8 MALDI-MS spectrum of polysorbate in commercial lotion A

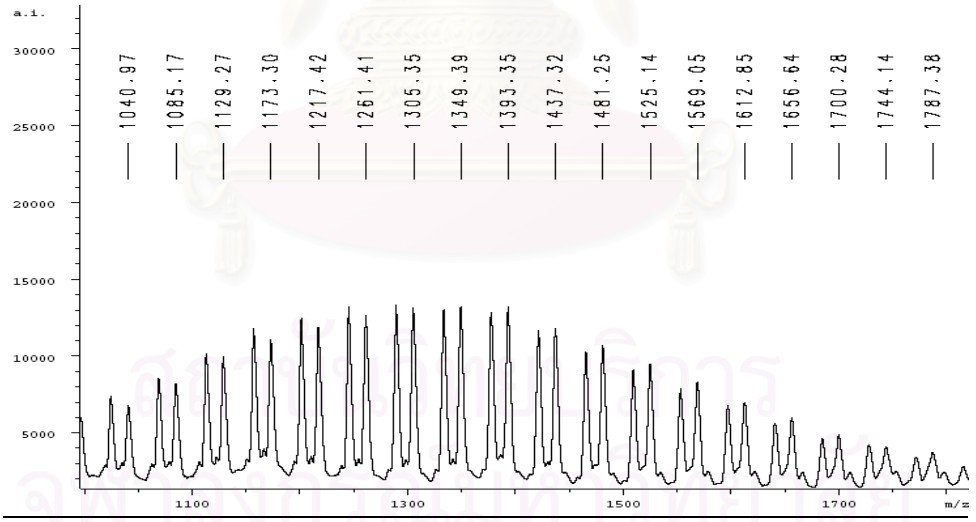


Figure 4.9 MALDI-MS spectrum of polysorbate in commercial lotion B

The interpretation of Figure 4.8 and 4.9 uses the same calculation as previous example. The peaks showed m/z of polyoxyethylene sorbitan monooleate series like the mass spectrum of polysorbate standards. The calculation of M_n, M_w and *PDI* of polysorbate in commercial lotion were shown in Table 4.12.

Table 4.12. The calculation of M_n, M_w and *PDI* of polysorbate in commercial lotion A and B

Polysorbate 80 in commercial lotion A			*Polysorbate 80 in commercial lotion B*		
M_w	M_n	PDI	M_w	M_n	PDI
1767.04	1737.52	1.02	1359.01	1335.43	1.02
1792.77	1753.94	1.02	1346.35	1327.00	1.01
1784.36	1740.30	1.03	1358.82	1326.78	1.02
1762.45	1728.75	1.02	1345.85	1319.87	1.02
1764.31	1726.51	1.02	1335.70	1309.50	1.02
1764.71	1722.39	1.02	1344.77	1318.99	1.02
1757.21	1732.39	1.02	1350.70	1324.08	1.02
Average					
1770.41	1734.53	1.021	1348.74	1323.09	1.019
Standard deviation					
12.99	10.56	0.004	8.27	8.10	0.004

To compare of sample A and B (Table 4.12), commercial lotion A showed in the region of high masses than commercial lotion B, by means commercial lotion A reflected the higher degree of polymerization than commercial lotion B.

Standard curve for analysis polysorbate 80 in commercial lotions.

Standard curves were prepared as same as the concentration of polysorbate 80 standard curve (real concentration of stock solution = 10.03 mg/ml) . To prepare the lotion sample, 0.1 g of commercial lotion A and B was measured three times (Table 4.13) and dissolved in 0.1 ml of THF.

Table 4.13 Weight of commercial lotion sample

Commercial lotion A		*Commercial lotion B*	
Time	Weight (g)	Time	Weight (g)
1	0.1005	1	0.1006
2	0.1003	2	0.1009
3	0.1008	3	0.1003

Then sample solution and CCA were mixed in the ratio of analyte to matrix 1:75. Finally, mixture of standard and 1.0 μl of ubiquitin were added before spotted 0.5 μL of the mixture of standard and internal standard in the target and allowed to air-dry. The spectra of polysorbate standard and commercial lotion A were shown in Figure A13-A14. The average intensities of polysorbate 80 and ubiquitin for quantitative analysis polysorbate in commercial lotion A were shown in Table 4.14.

Table 4.14 Average intensity of polysorbate 80 and ubiquitin to quantitative analysis of polysorbate in commercial lotion A.

Concentration of polysorbate 80 (mg/ml)	*Average intensity of polysorbate 80 (a)*	*Intensity of ubiquitin (n)*	*a/n*
50	5314.20	255.00	20.84
25	2027.99	200.00	10.14
20	5099.83	602.01	8.47
15	3993.02	577.10	6.92
10	988.06	216.07	4.57
5	7178.91	3443.00	2.09
Commercial A			
1	2086.45	547.00	3.81
2	859.09	248.00	3.46
3	386.55	98.72	3.92

Then, concentration of polysorbate 80 and the ratio of the average intensity of polysorbate 80 and ubiquitin were plotted in Figure 4.10.

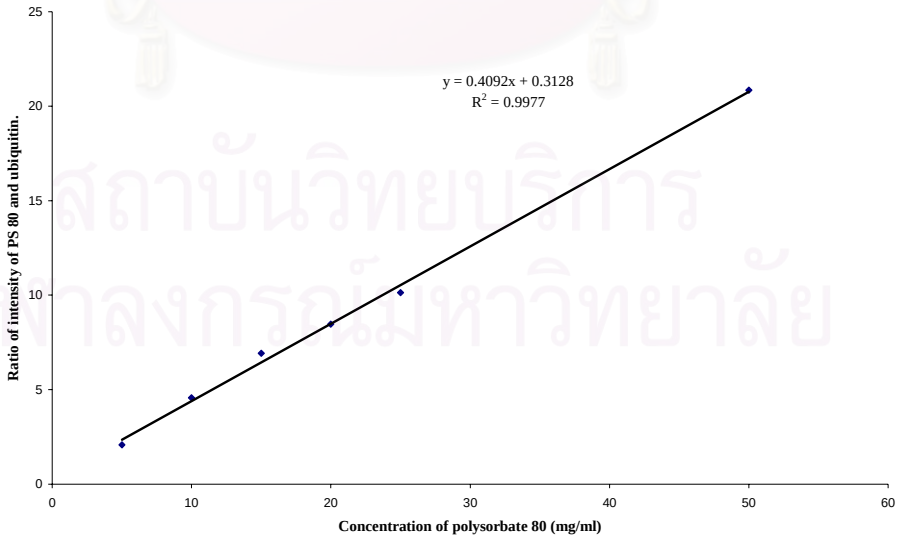


Figure 4.10 Plot of concentration of polysorbate 80 standard *versus* ratio of the average intensity of polysorbate 80 to ubiquitin from Table 4.14.

The spectra of polysorbate standard and commercial lotion B were shown in Figure A15-A16. The average intensities of polysorbate 80 and ubiquitin were shown in Table 4.15.

Table 4.15 Average intensity of polysorbate 80 and ubiquitin to quantitative analysis of polysorbate in commercial lotion B.

Concentration of polysorbate 80 (mg/ml)	*Average intensity of polysorbate 80 (a)*	*Intensity of ubiquitin (n)*	*a/n*
50	4751.26	221.09	21.49
25	6623.85	695.25	9.53
20	2555.73	334.00	7.65
15	6183.55	1193.00	5.18
10	6991.19	1642.30	4.26
5	4912.39	2450.70	2.00
Commercial B			
1	227.85	44.49	5.12
2	2192.18	383.00	5.72
3	737.36	168.00	4.38

Then, plot of concentration of polysorbate 80 and the ratio of the average intensity of polysorbate 80 and ubiquitin were plotted in Figure 4.11.

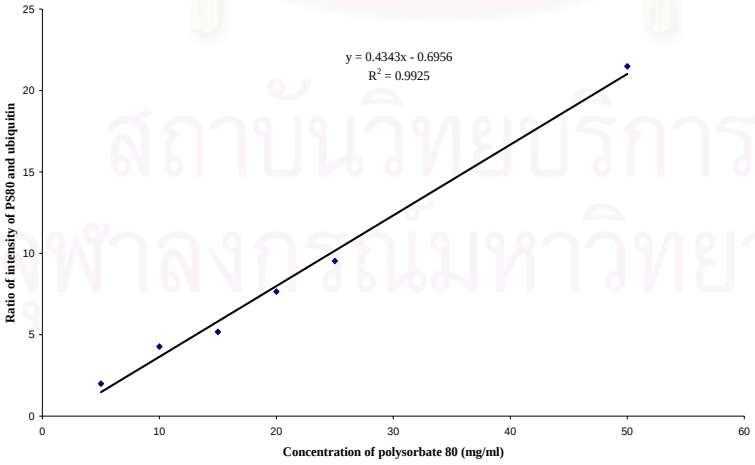


Figure 4.11 Plot of concentration of polysorbate 80 standard *versus* ratio of the average intensity of polysorbate 80 to ubiquitin from Table 4.15.

From the standard calibration curve (Figure 4.10), the equation of the line is $y = 0.4092x + 0.3128$ (eq 4.8) with a correlation coefficient (r^2) = 0.9977, where y is the intensity ratio and x is the concentration of polysorbate.

The concentrations of sample lotions were calculated in an example 4.5

Example 4.6 Calculated concentration of polysorbate in commercial lotion A.

$$y = 0.4092x + 0.3128 \quad \text{(eq 4.8)}$$

When, y = 3.81 then, x = 8.55

Thus, the concentration in commercial lotion A1 = 8.55 mg/ml

So, sample A 1.005 g has got a polysorbate 8.55×10^{-3} g
If, sample A 100 g has got a polysorbate 0.851 g

There was a polysorbate 0.85 %w/w in sample A1. The concentration of polysorbate in commercial lotion B was calculated the same as commercial A, but the equation of the line is $y = 0.4343x - 0.6956$ (eq 4.9) with a correlation coefficient (r^2) = 0.9927. The concentrations of commercial lotion A and B were shown in Table 4.16.

Table 4.16 Concentration of polysorbate in commercial lotion A and B

Commercial lotion A				Commercial lotion B			
Time	y	x	%w/w	Time	y	x	%w/w
1	3.81	8.55	0.85	1	5.12	13.39	1.33
2	3.46	7.69	0.76	2	5.72	14.77	1.46
3	3.92	8.82	0.88	3	4.38	11.68	1.16

The average concentrations of polysorbate in commercial lotion A and B were 0.83 and 1.32 %w/w and standard deviation was 0.06 and 0.15, respectively.

CHAPTER V

CONCLUSION

In this research, MALDI-MS was developed for determination of molecular weight distribution and quantitative analysis of polysorbate 80. The optimum conditions and parameter were studied.

The results show that a dried droplet method is suitable for sample preparation of MALDI-MS analysis, in comparison with a thin layer method. From the results, the optimal condition for this analysis: CCA in THF was chosen as ideal matrix and laser power at 310 (J. The best analyte and matrix ratio was found to be 1:75. MALDI-TOF MS provides the most complete and detailed account to the molecular composition of polysorbate emulsifier. It confirms the polydispersity of the ethylene oxide chain, and offers insight into the degree of esterification and the relative distribution of sorbitan- and sorbide-based species.

The method for quantitative analysis has been developed. For quantification of polysorbate, linear relationships were observed using the plot of concentration of polysorbate 80 and the ratio of the average intensity of polysorbate 80 and ubiquitin (internal standard). The polysorbate molecular ion signal increased significantly, relative to the ubiquitin signal, when the solution contained a higher concentration of polysorbate. The calibration curves prepared by this method was accurate and percent error less than three percent.

The method was applied for determination and quantitative analysis two classes of commercial products. Firstly, two pharmaceutical suspension were analyzed. From the results, there is polysorbate in commercial drug A and B. The average concentration of polysorbate 80 in commercial drug A and B are 0.091 (0.004 and 0.090 (0.013 %w/w, respectively. Moreover, two commercial lotions were analyzed. From the MS-spectra, it showed that there is polysorbate in commercial lotions. The average concentrations of polysorbate 80 in commercial lotion A and B are 0.83 (0.06 and 1.32 (0.15 %w/w, respectively. A detection limit of this method for polysorbate is 1.03 femtomole.

It is concluded that MALDI-MS is an excellent method for determination of molecular weight distribution and quantitative analysis of polysorbate. Futhermore, this technique is applied to determination and quantitative analysis of polysorbate in complex mixture as well as polysorbate standard without separation. Consequencely, MALDI-MS has a potential application for quality control during manufacture.

The suggestion for the future work :

1. Studying the effect of cationizing agent for ion formation of the polysorbate sample by varying the cation e.g. Na+, K+ and Ag+.

2. Application of this method of determination and quantification of other polysorbate-types.

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APPENDICES

APPENDIX A

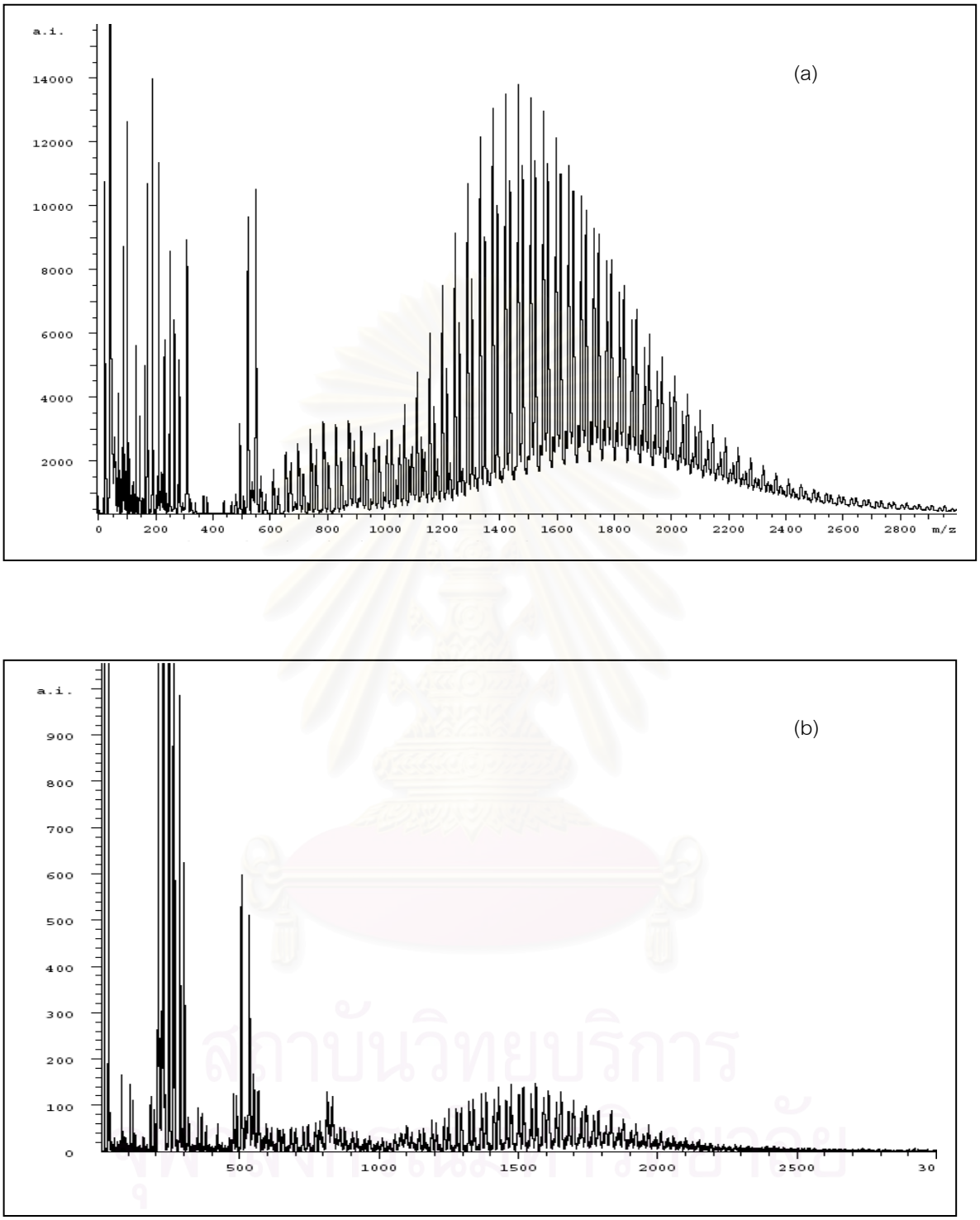


Figure A1. MALDI-MS mass spectra of polysorbate using a dried droplet method (a) and thin layer method (b).

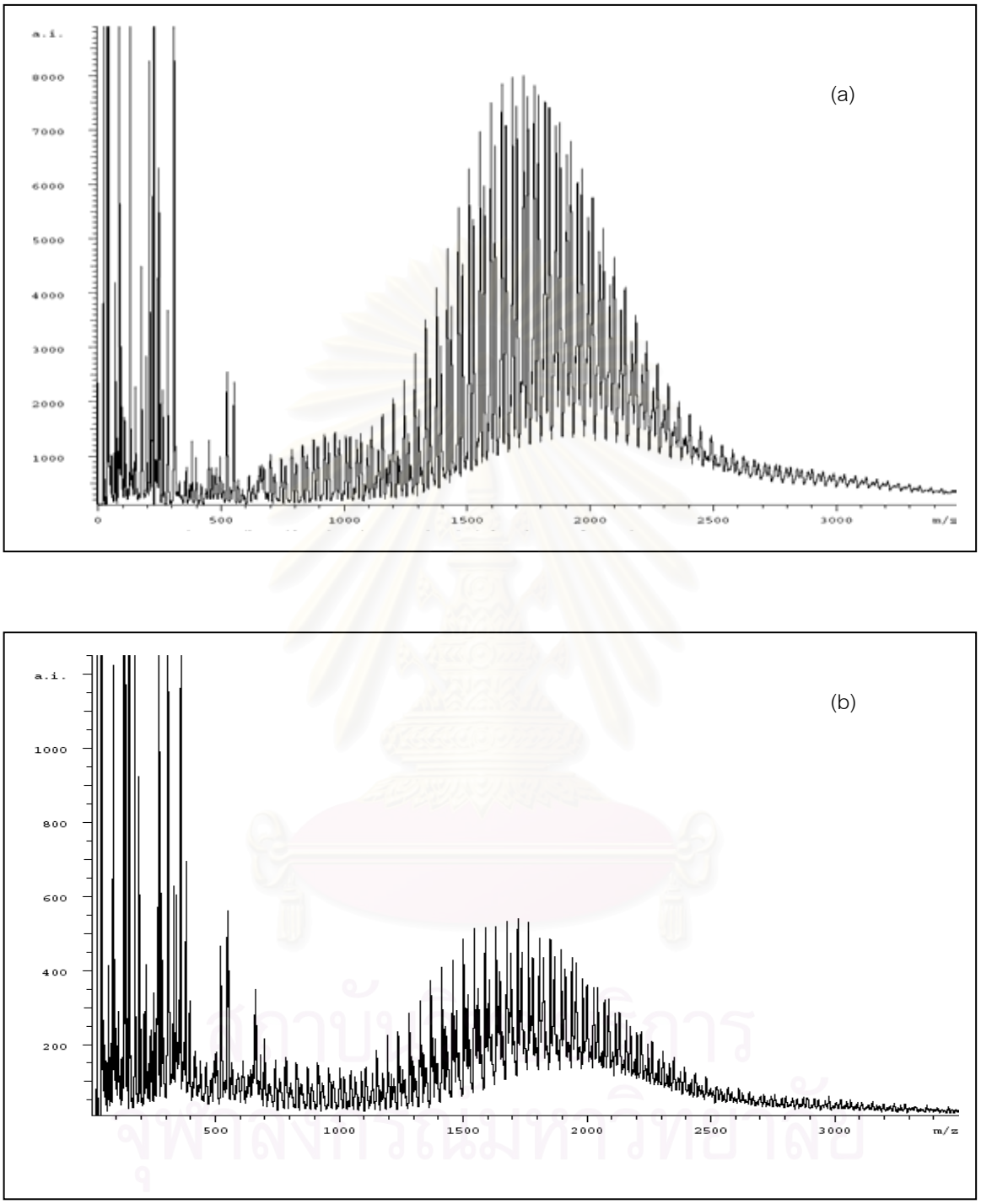
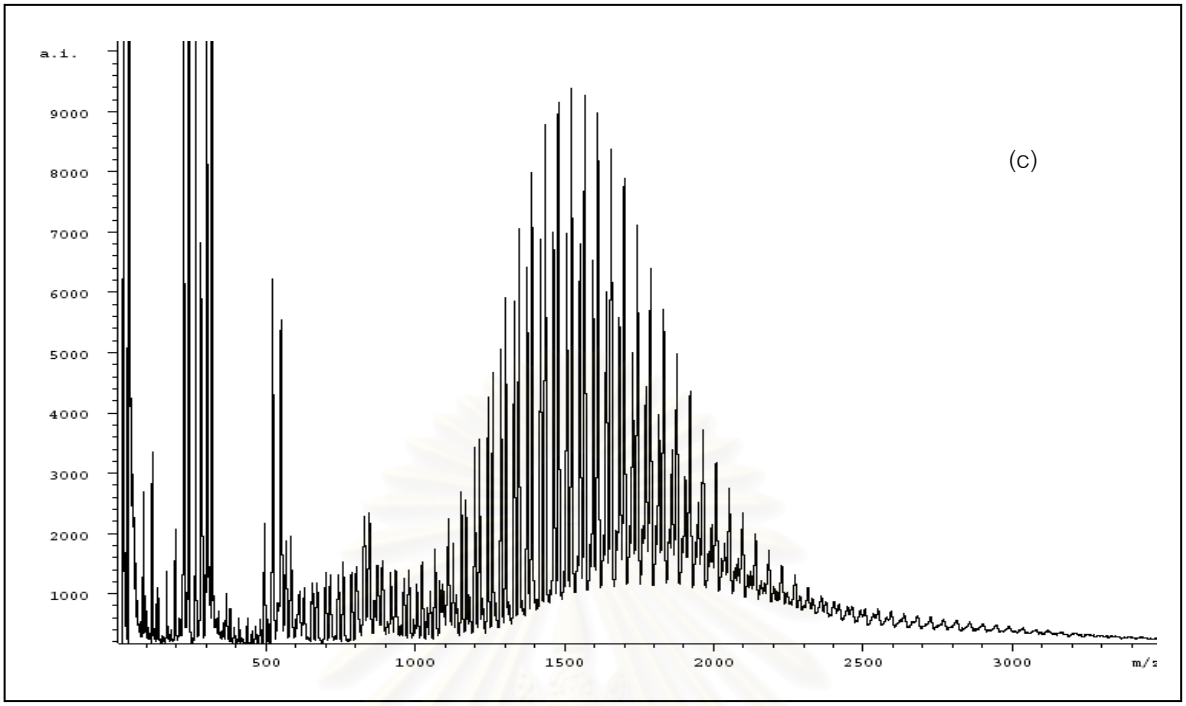


Figure A2. MALDI-MS mass spectra of polysorbate using various matrix:
(a) dithranol, (b) 2,5-dihydroxybenzoic acid.



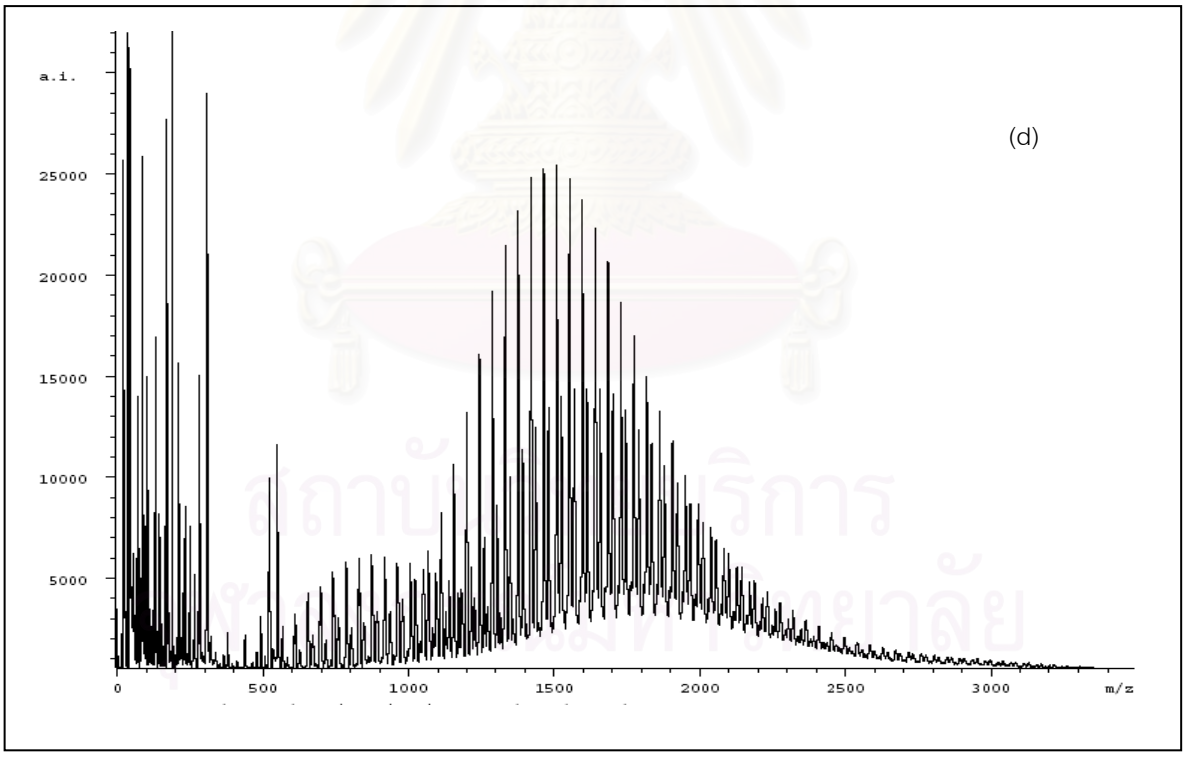


Figure A2 (continued) MALDI-MS mass spectra of polysorbate using various matrix: (c) 2-(4-hydroxyphenylazo)-benzoic acid, (d) α-cyano-4-hydroxycinamic acid.

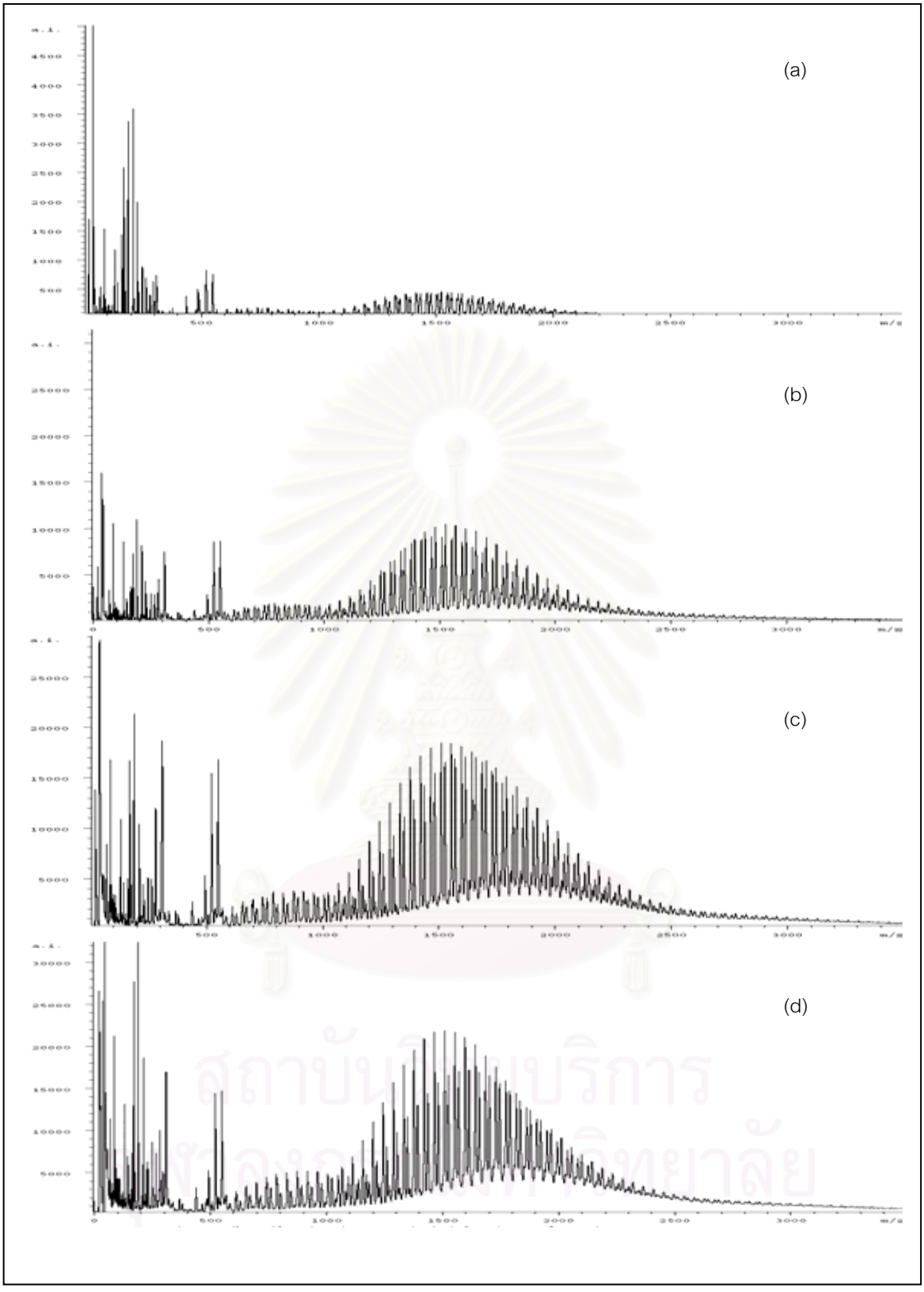


Figure A3. MALDI-MS mass spectra of polysorbate mixed with CCA with various ratios: (a) 1:1, (b) 1:25, (c) 1:50 and (d) 1:75.

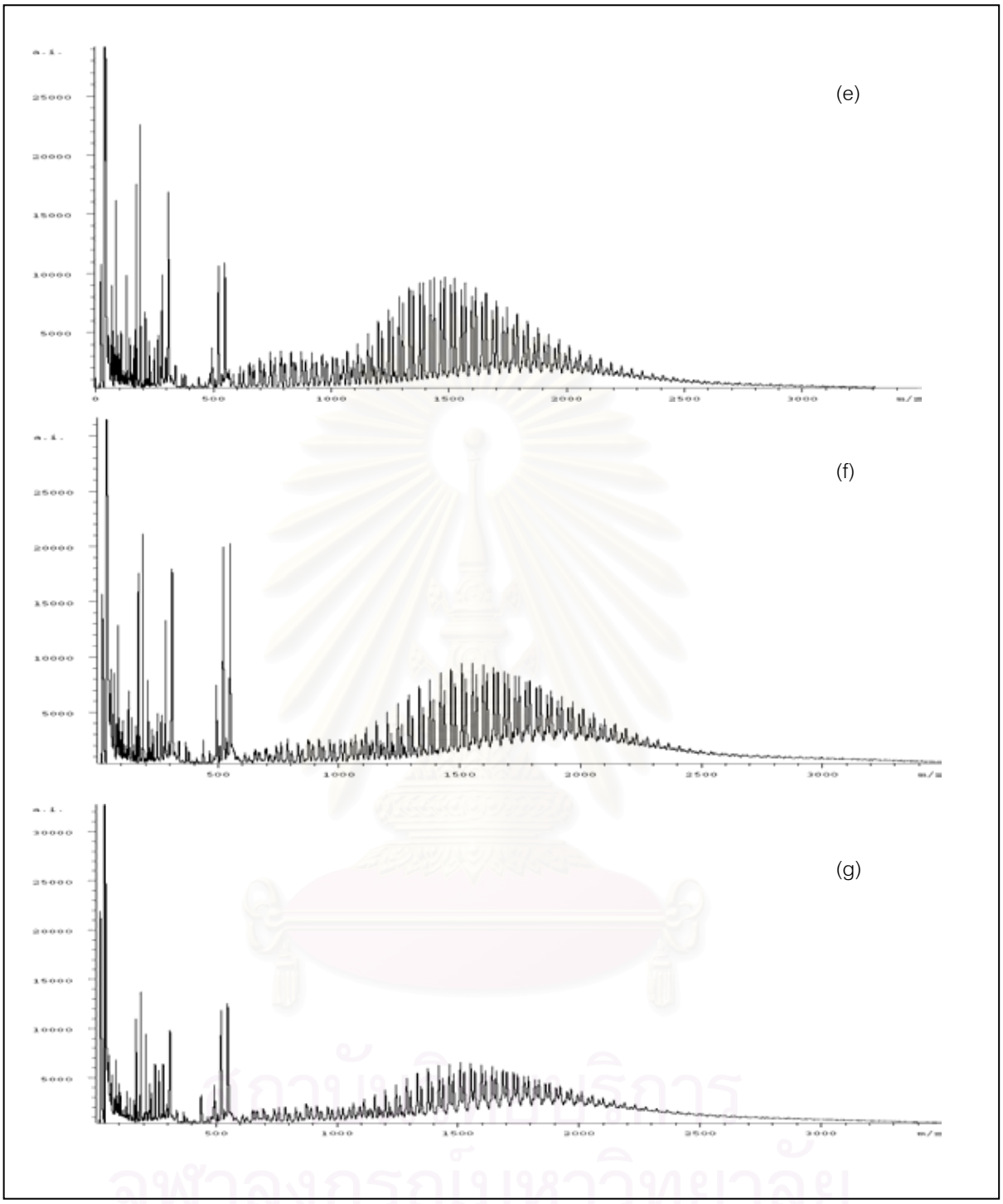


Figure A3 (continued). MALDI-MS mass spectra of polysorbate mixed with CCA with various ratios: (e) 1:100, (f) 1:125 and (g) 1:150.

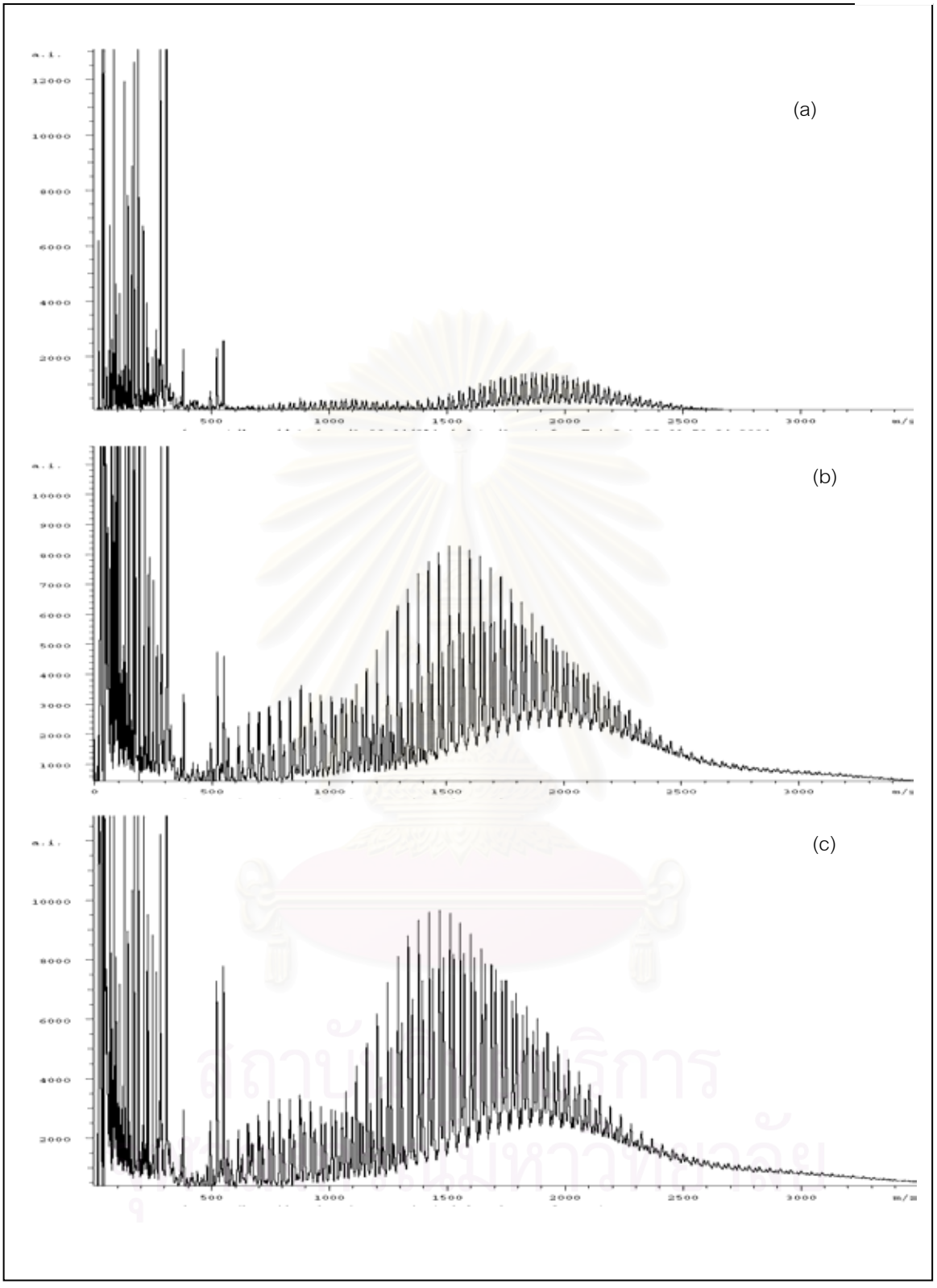


Figure A4 MALDI-MS mass spectra of polysorbate mixture in various solvents: (a) water:acetonitrile (70:30), (b) ethanol:acetonitrile (50:50) and (c) THF.

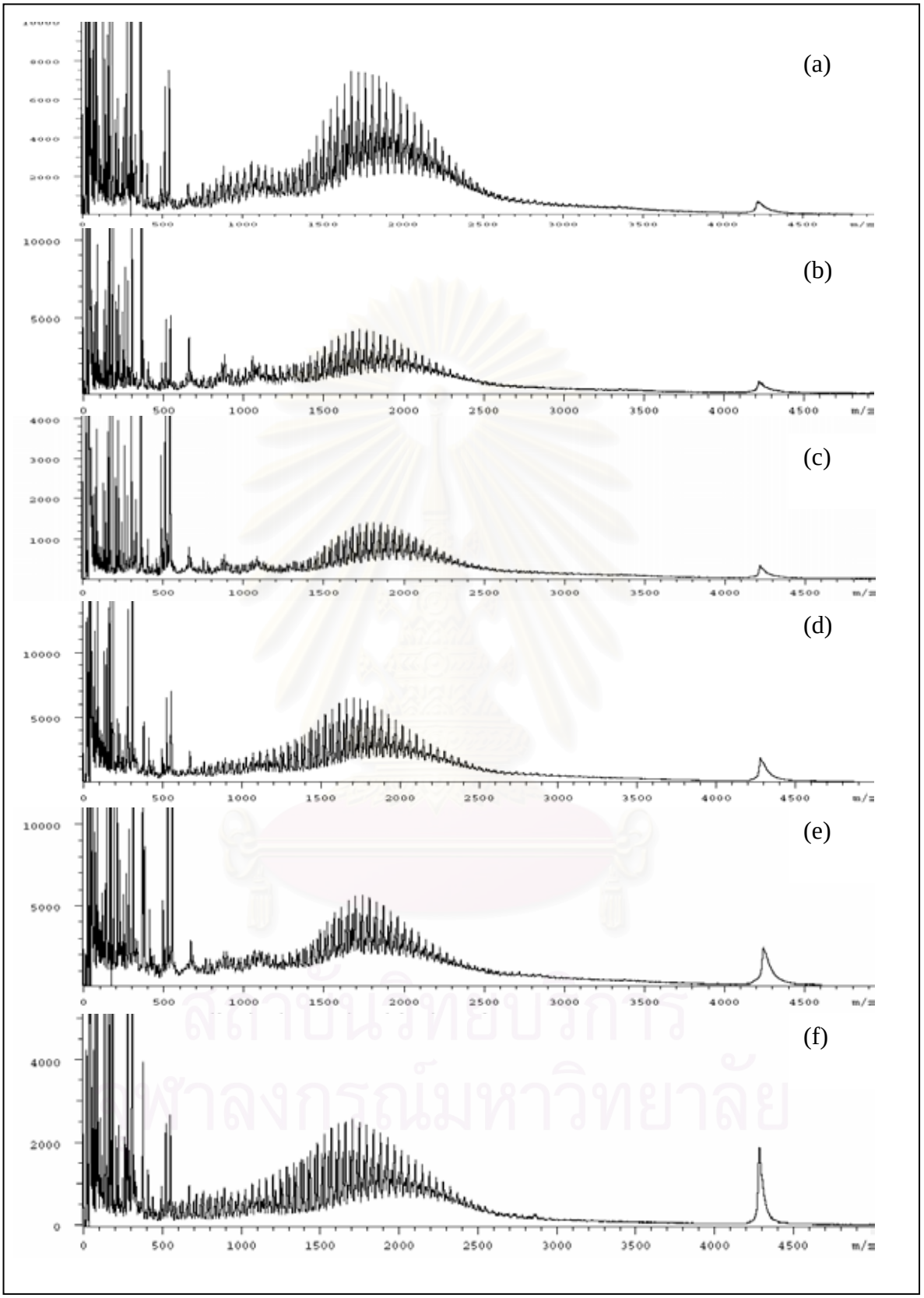


Figure A5 MALDI-MS mass spectra of various concentration of polysorbate mixtures: (a) 50.00, (b) 25.00, (c) 20.00, (d) 15.00, (e) 10.00 and (f) 5.00 mg/ml.

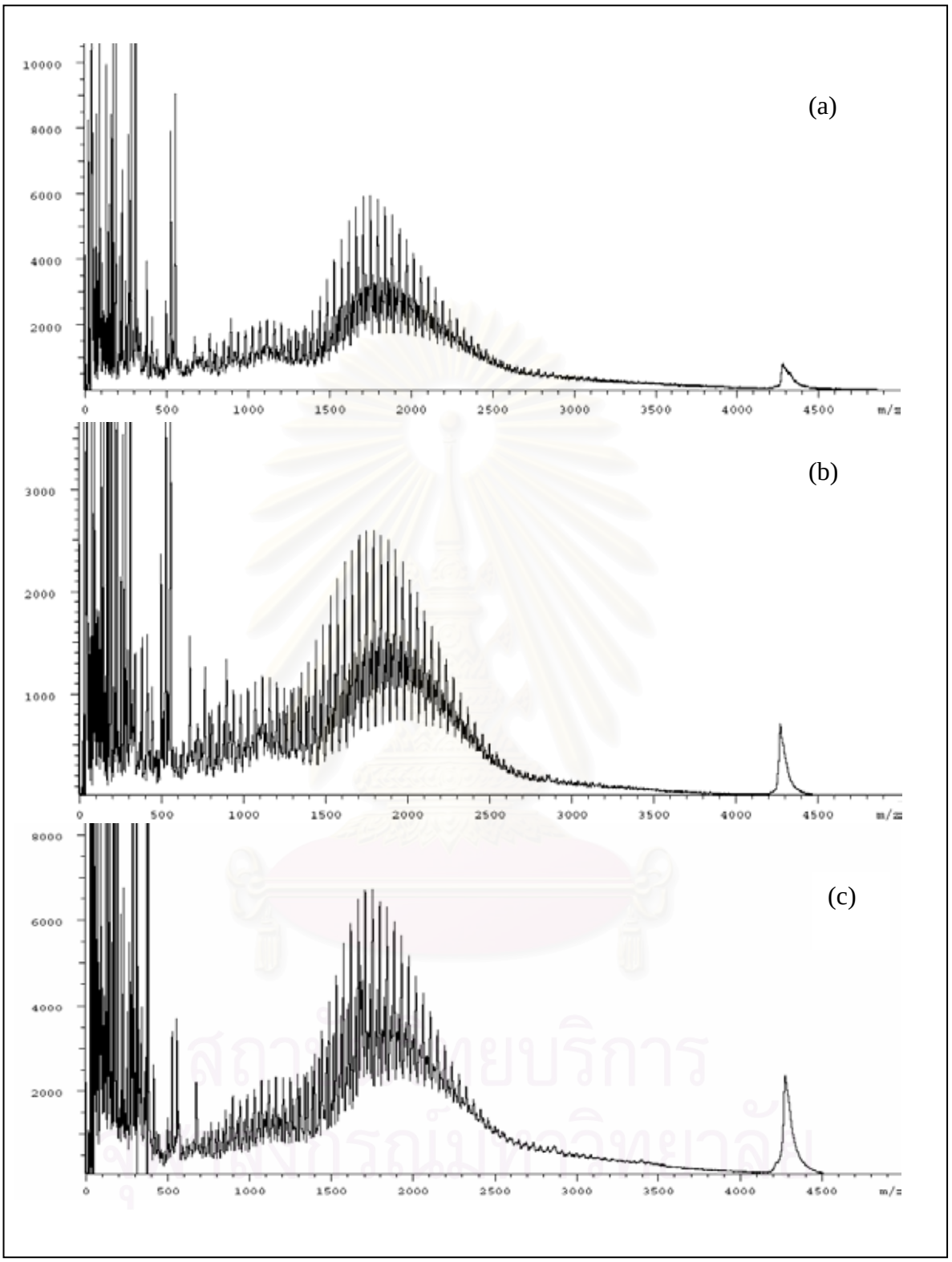


Figure A6 MALDI-MS mass spectra of various concentration of polysorbate mixtures: (a) 35.00, (b) 17.00 and (c) 12.50 mg/ml

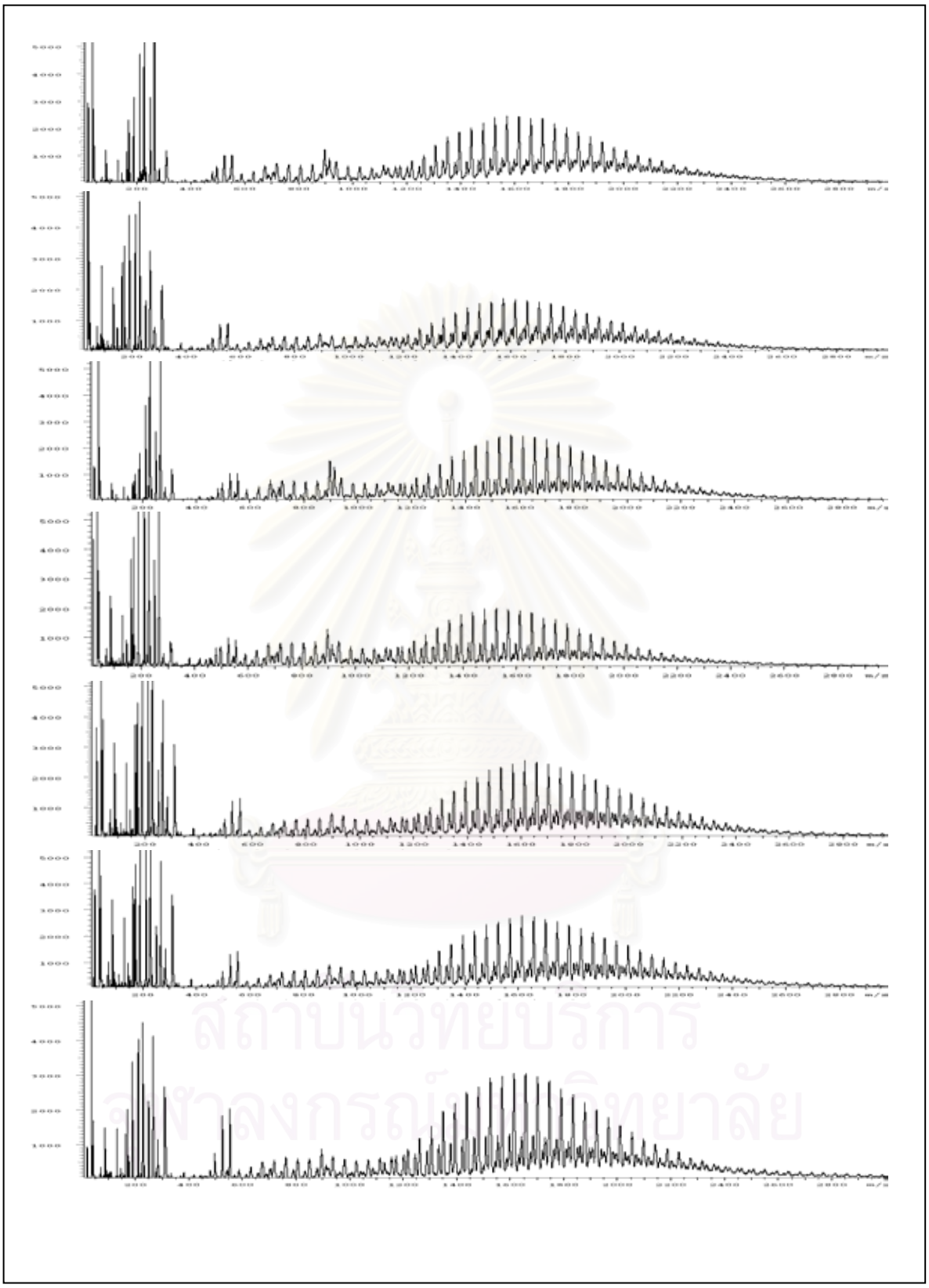


Figure A7 MALDI-MS mass spectra of polysorbate mixture when used 0.5 μL by load sample to the target with the same amount repeat in various positions.

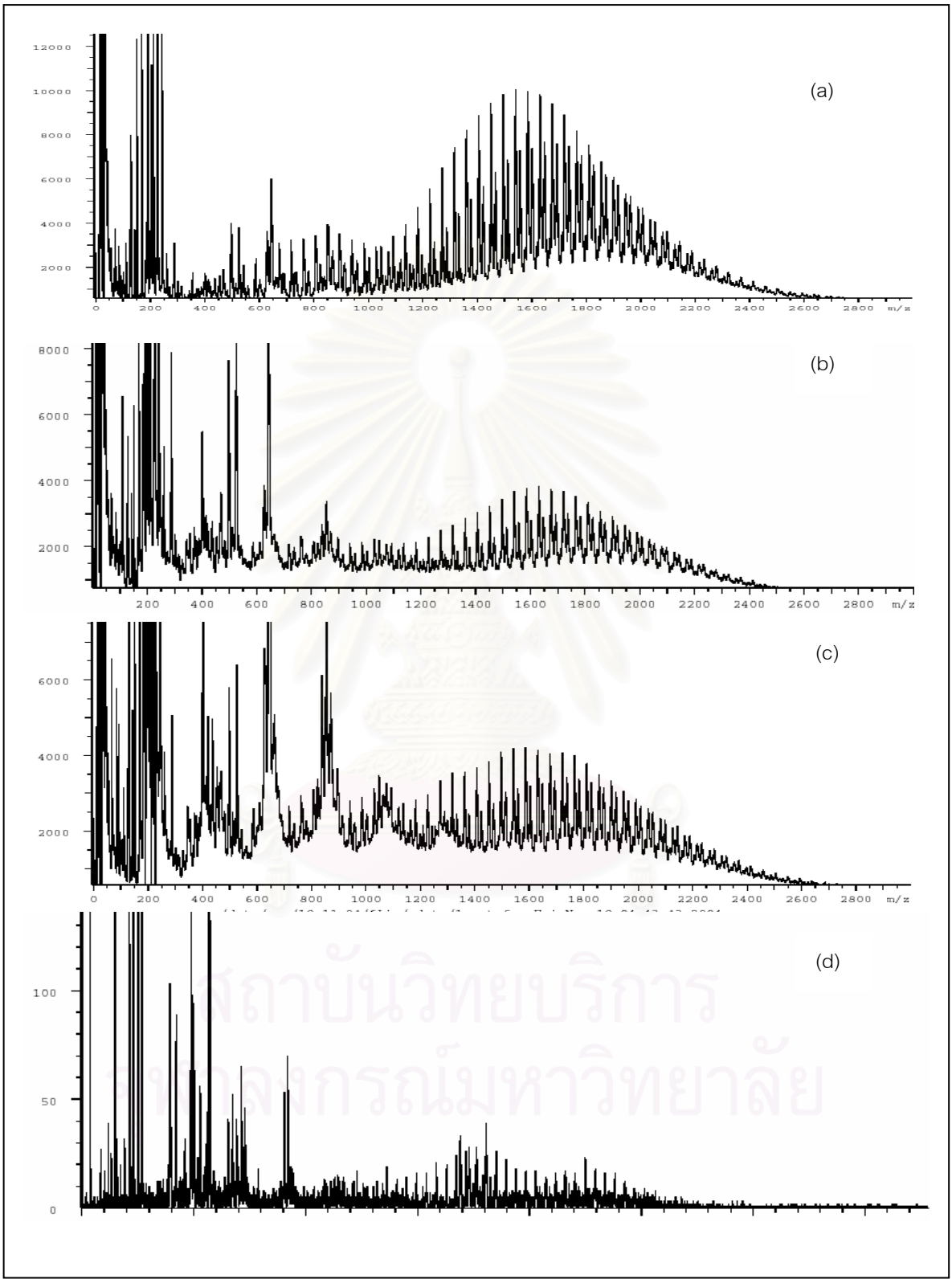


Figure A8 MALDI-MS spectra of various concentration of polysorbate mixture: (a) 41.16, (b) 20.59, (c) 1.37 and (d) 1.03 femtomole.

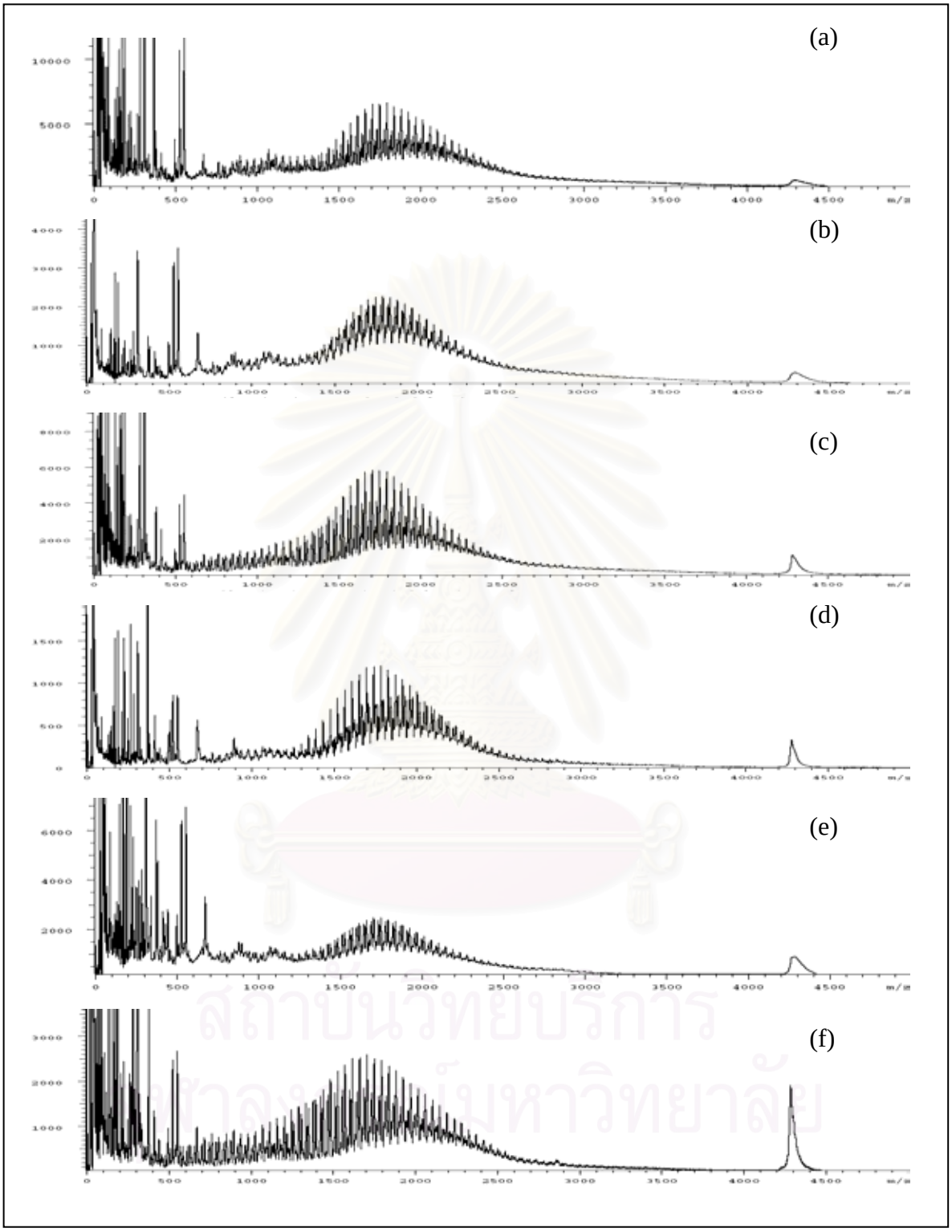


Figure A9 MALDI-MS mass spectra of various concentration of polysorbate mixtures: (a) 50.00, (b) 25.00, (c) 20.00, (d) 15.00, (e) 10.00 and (f) 5.00 mg/ml to quantitative analysis commercial drug A.

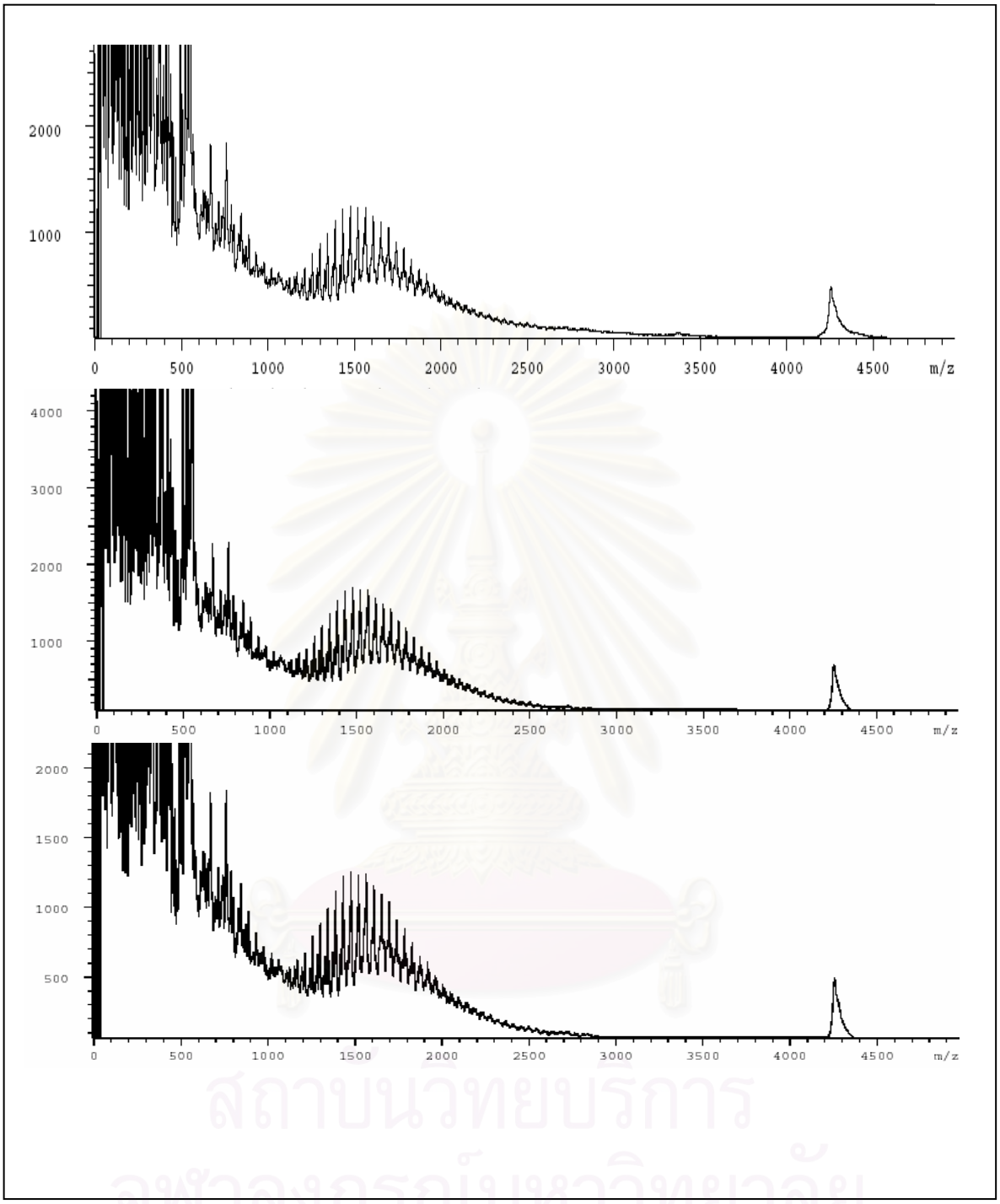


Figure A10 MALDI-MS spectra of polysorbate in commercial drug A.

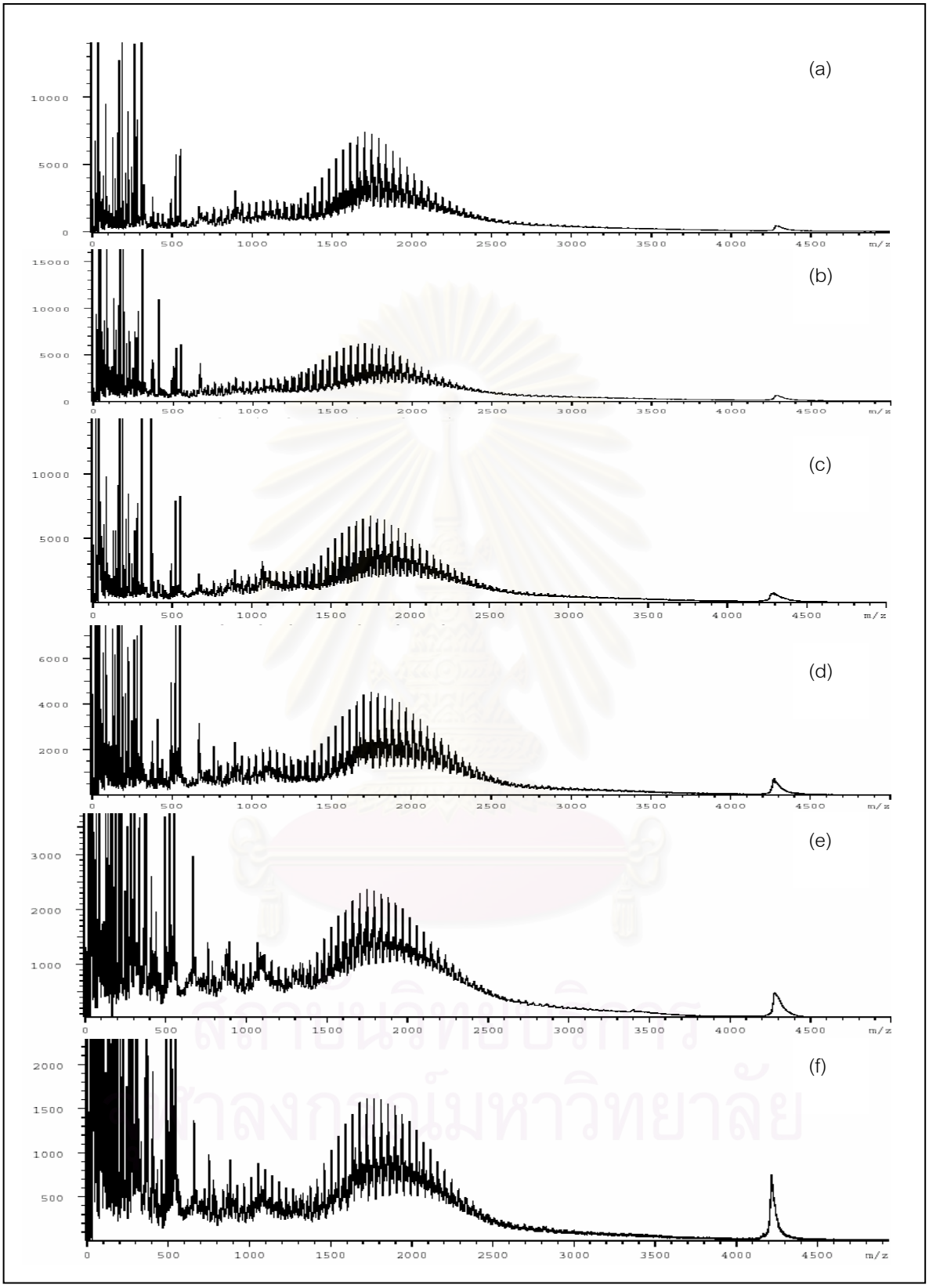


Figure A11 MALDI MS spectra of various concentration of polysorbate mixture: (a) 50.00, (b) 25.00, (c) 20.00, (d) 15.00, (e) 10.00 and (f) 5.00 mg/ml to quantitative analysis of commercial drug B.

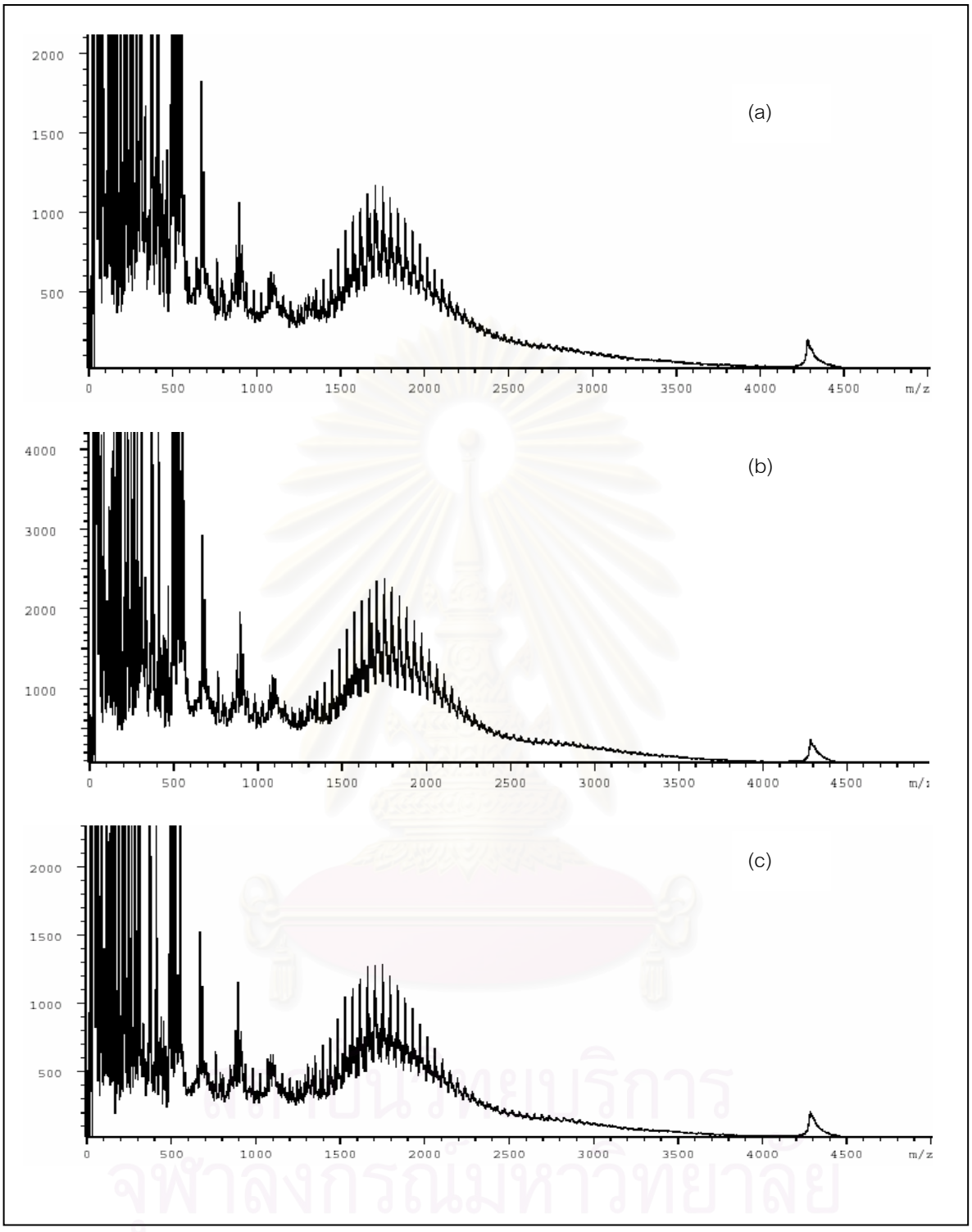


Figure A12 MALDI MS spectra of commercial drug B

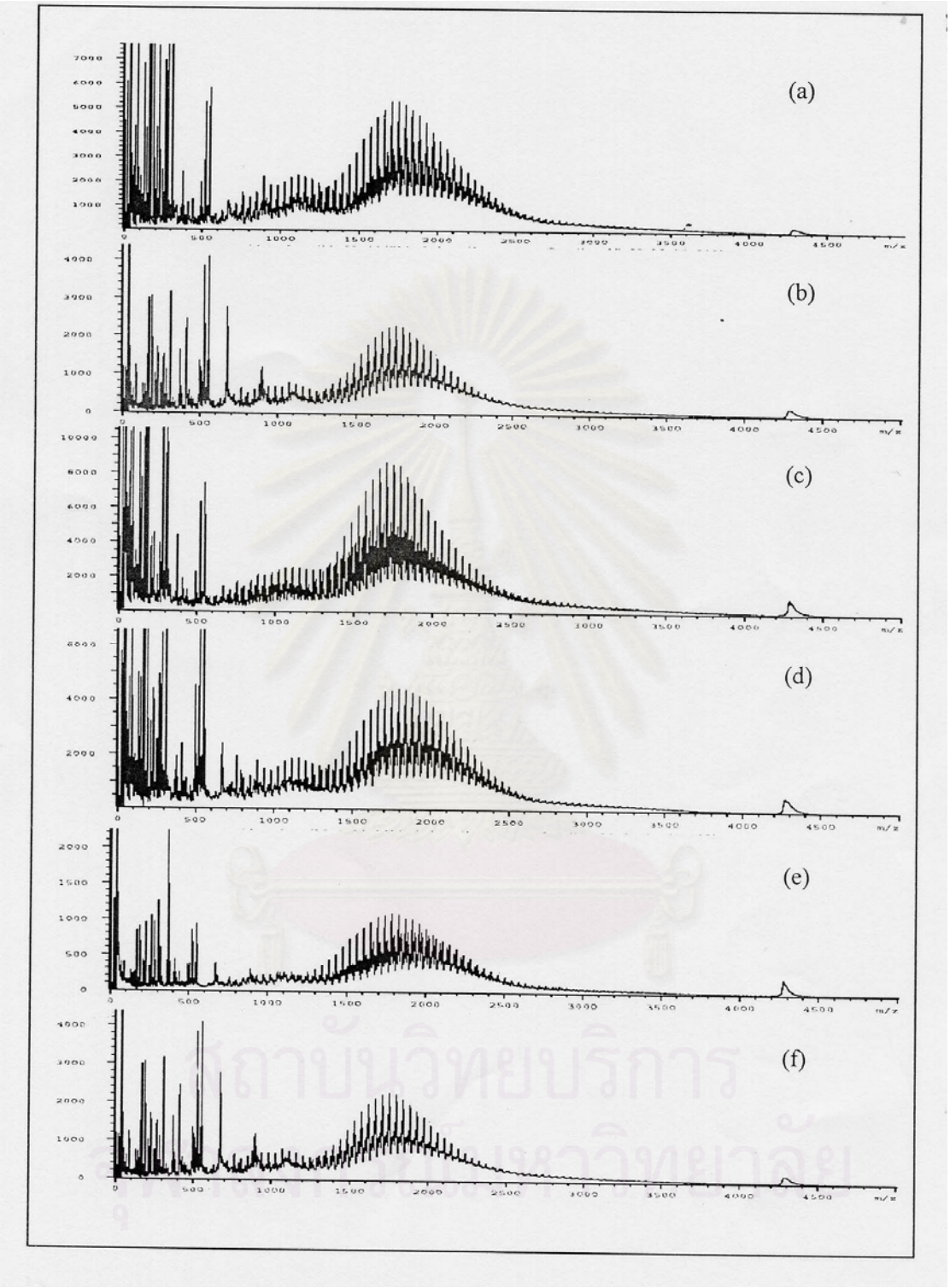


Figure A13 MALDI MS spectra of various concentration of polysorbate mixture: (a) 50.00, (b) 25.00, (c) 20.00, (d) 15.00, (e) 10.00 and (f) 5.00 mg/ml to quantitative analysis of commercial lotion A.

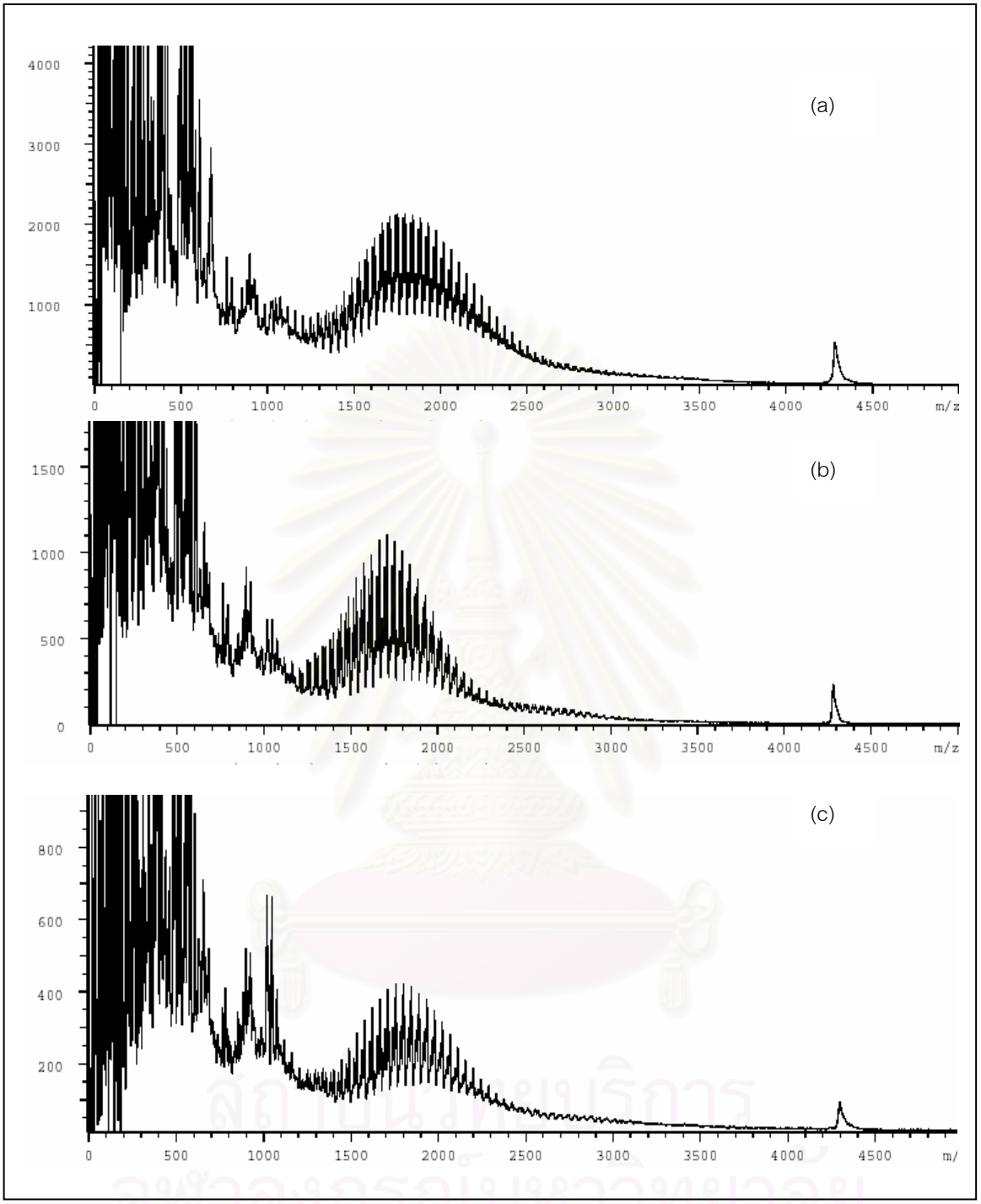


Figure A14 MALDI-MS spectra of polysorbate in commercial lotion A.

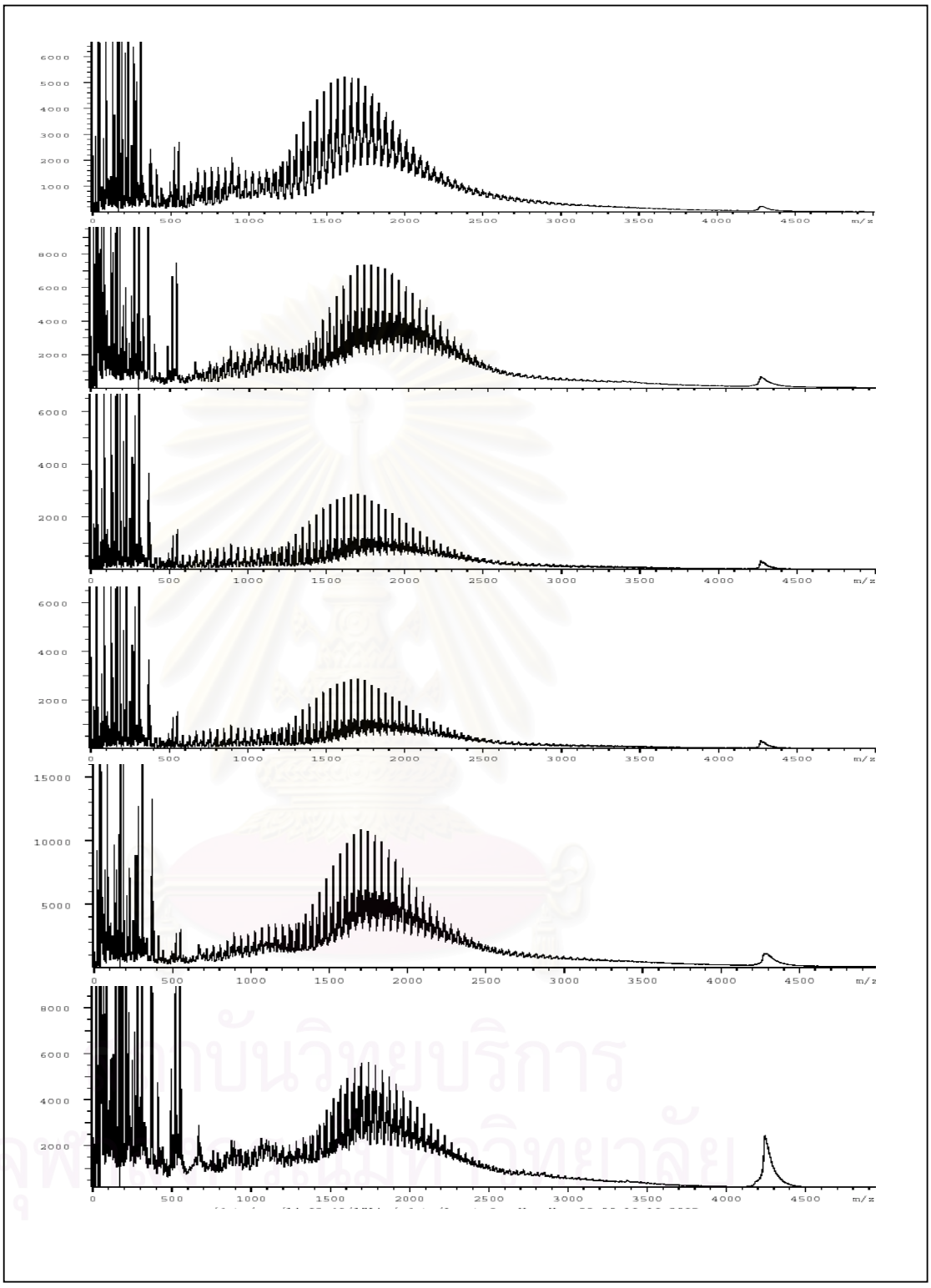


Figure A15 MALDI MS spectra of various concentration of polysorbate mixture: (a) 50.00, (b) 25.00, (c) 20.00, (d) 15.00, (e) 10.00 and (f) 5.00 mg/ml to quantitative analysis of commercial lotion B.

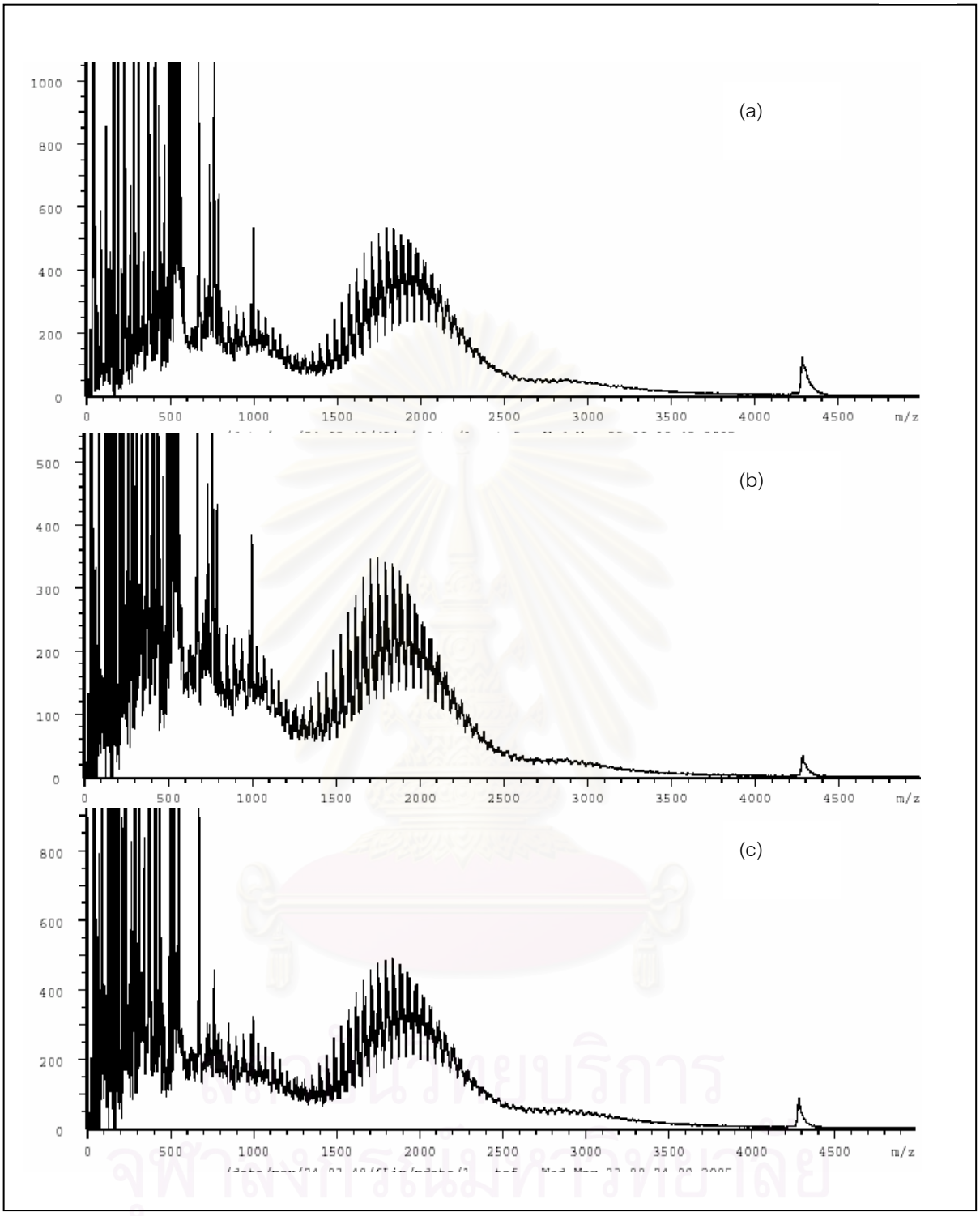


Figure A16 MALDI-MS spectra of polysorbate in commercial lotion B.

VITA

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