ผลของปัจจัยกำหนดต่าง ๆของสูตรตำรับต่อความสามารถในการปลดปล่อยสารอีพิแกลโลแคททิชิน แกลเลท (อีจีซีจี) จากสูตรตำรับอิมัลชันน้ำในน้ำมันเข้มข้น

นางสาว ศรันยา อัครวิทยาภูมิ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE EFFECT OF DIFFERENT FORMULATION PARAMETERS ON THE *IN VITRO* RELEASE OF THE (−)–EPIGALLOCATECHIN GALLATE (EGCG) FROM CONCENTRATED W/O EMULSIONS

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ศรันยา อัครวิทยาภูมิ: ผลของปัจจัยกำหนดต่างๆของสูตรตำรับต่อความสามารถในการปลดปล่อยสารอีพิแกลโลแคททิชิน แกลเลท (อีจีซีจี) จากสูตรตำรับอิมัลชันน้ำในน้ำมันเข้มข้น (THE EFFECT OF DIFFERENT FORMULATION PARAMETERS ON THE IN VITRO RELEASE OF THE (−)–EPIGALLOCATECHIN GALLATE (EGCG) FROM CONCENTRATED W/O EMULSIONS) อ.ที่ปรึกษา: รศ. ปภาวดี คล่องพิทยาพงษ์, 325 หน้า.

สารแคททิชินในชาเขียว (จีทีซี) และ คาเฟอีน เป็นสารประกอบหลักที่สกัดได้จากผงบดละเอียดของใบชาเขียว (*Camellia Sinensis* L.) โดยใช้วิธีการสกัดด้วยน้ำปราศจากไอออนที่อุณหภูมิ 80±1 องศาเซลเซียส ตามด้วยการสกัดด้วยตัวทำละลาย ไดคลอโรมีเทน และไม่ได้ตามด้วยการสกัดด้วยตัวทำละลาย ไดคลอโรมีเทน สารสกัดชาเขียวผงแห้งที่ได้จากการทำแห้งโดยเทคนิค ฟรีซ-ดรายด์ ถูกนำมาวิเคราะห์หาปริมาณสารแคททิชินในชาเขียวได้แก่ อีพิแคททิชิน (อีซี), อีพิแคททิชิน แกลเลท (อีซีจี), อีพิแกลโลแคททิชิน (อีจีซี), อีพิแกลโลแคททิชิน แกลเลท (อีจีซีจี) และ คาเฟอีน โดยใช้วิธีวิเคราะห์ชนิดไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟฟีแบบวัฏภาคกลับ ปริมาณผลผลิตรวมทั้งหมดของสารสกัดผงแห้งที่ได้จากวิธีการสกัดทั้งสองแบบไม่มีความแตกต่างกันอย่างมีนัยสำคัญที่ระดับค่าความเชื่อมั่นน้อยกว่า 0.05 ปริมาณสารแคททิชินทั้งหมดในชาเขียวที่สกัดโดยใช้ตัวทำละลาย ไดคลอโรมีเทนมากกว่าที่พบในสารสกัดที่สกัดด้วยน้ำอย่างเดียวอย่างมีนัยสำคัญที่ระดับค่าความเชื่อมั่นน้อยกว่า 0.05 นอกจากนี้ ปริมาณสารคาเฟอีนที่พบในสารสกัดที่สกัดโดยใช้ตัวทำละลาย ไดคลอโรมีเทนน้อยกว่าที่พบในสารสกัดที่สกัดด้วยน้ำอย่างเดียวอย่างมีนัยสำคัญที่ระดับค่าความเชื่อมั่นน้อยกว่า 0.05 ดังนั้น ตัวทำละลาย ไดคลอโรมีเทน จึงสามารถนำมาใช้ในการสกัดแยกสารคาเฟอีนจากสารสกัดชาเขียวด้วยน้ำร้อนได้อย่างมีประสิทธิภาพ อีกทั้งยังไม่มีผลกระทบต่อปริมาณผลผลิตรวมทั้งหมดของสารสกัดที่ได้อย่างมีนัยสำคัญด้วย

สูตรตำรับอิมัลชันน้ำในน้ำมันเข้มข้นที่ประกอบไปด้วยสารสกัดชาเขียวผงแห้งปริมาณร้อยละ 2.25 และ ปริมาณความเข้มข้นของวัฏภาคน้ำที่ร้อยละ 84, 86 และ 88 ของสูตรตำรับ และ สารทำอิมัลชันที่ความเข้มข้นร้อยละ 3 ของสูตรตำรับ ต่างชนิดกัน 4 ชนิด (ซอบิแทน, ซิลิโคน, ผสม และ กลูโคเอสเทอร์) ถูกเตรียมขึ้นเพื่อใช้ในการศึกษาผลของปัจจัยกำหนดของสูตรตำรับต่อความสามารถในการปลดปล่อยสารอีจีซีจีผ่านเมมเบรนสังเคราะห์ชนิดโพลีซัลโฟน

ปริมาณความเข้มข้นของสารทำอิมัลชันไม่มีผลต่อการปลดปล่อยสารอีจีซีจีอย่างมีนัยสำคัญ เมื่อเปรียบเทียบระหว่างชนิดของสารทำอิมัลชันทั้ง 4 ชนิด พบว่าภายหลังจาก 48 ชั่วโมง อิมัลชันชนิดผสมปลดปล่อยสารอีจีซีจีจากสูตรตำรับได้มากกว่าสูตรตำรับชนิดอื่นๆ นอกจากนั้น พบว่าสูตรตำรับอิมัลชันชนิดผสมที่เตรียมขึ้นที่ทุกความเข้มข้นของวัฏภาคน้ำนั้นไม่คงตัวและโครงสร้างของหยดอนุภาคน้ำภายในสูตรตำรับไม่เป็นรูปทรงหลายด้าน ซึ่งผลจากการปลดปล่อยตัวยาอย่างรวดเร็วนี้น่าจะเกิดขึ้นเนื่องจากแผ่นฟิลม์น้ำมันที่ได้จากขี้ผึ้งที่อยู่ในสารทำอิมัลชันชนิดผสมนั้นมีความแข็งเกร็งและขาดความยืดหยุ่นจึงทำให้แผ่นฟิลม์ที่ได้เกิดการแตกแยกได้เร็วขึ้น ทำให้สารอีจีซีจีถูกปลดปล่อยออกมาได้อย่างรวดเร็ว ผลการศึกษาดังกล่าวแสดงให้เห็นว่าลักษณะจำเพาะในการปลดปล่อยตัวยาจากสูตรตำรับอิมัลชันน้ำในน้ำมันเข้มข้นที่ไม่คงตัว (อิมัลชันชนิดผสม) นั้นขึ้นอยู่กับความคงตัวของสูตรตำรับซึ่งเกี่ยวเนื่องกับความแข็งเกร็งของแผ่นฟิลม์ที่หุ้มระหว่างหยดอนุภาคน้ำภายในสูตรตำรับ

ขนาดเส้นผ่านศูนย์กลางของหยดอนุภาคน้ำลดลงเมื่อปริมาณวัฏภาคน้ำในสูตรตำรับเพิ่มขึ้น ในขณะที่ความหนืดของสูตรตำรับเพิ่มขึ้นเมื่อปริมาณวัฏภาคน้ำในสูตรตำรับเพิ่มขึ้น ปัจจัยรูปทรงของหยดอนุภาคน้ำภายในสูตรตำรับนั้นน่าจะมีบทบาทหลักต่อความสามารถในการปลดปล่อยสารอีจีซีจี จากสูตรตำรับอิมัลชันน้ำในน้ำมันเข้มข้นที่คงตัว พบว่าในสูตรตำรับอิมัลชันน้ำในน้ำมันเข้มข้นที่คงตัวซึ่งเตรียมขึ้นที่ความเข้มข้นของวัฏภาคน้ำร้อยละ 88 ของสูตรตำรับ ยกเว้นอิมัลชันชนิดผสม นั้นมีโครงสร้างของหยดอนุภาคน้ำภายในสูตรตำรับเป็นรูปทรงหลายด้าน นอกจากนั้น สูตรตำรับดังกล่าวยังมีความคงตัวและความข้นหนืดสูงมาก ซึ่งอัตราเร็วในการปลดปล่อยตัวยาน่าจะมีความสัมพันธ์กับโครงสร้างของหยดอนุภาคน้ำภายในสูตรตำรับ โดยที่โครงสร้างดังกล่าวที่พบในสูตรตำรับที่มีปริมาณวัฏภาคน้ำภายในสูงมีผลทำให้การปลดปล่อยตัวยาของสูตรตำรับช้าลง เนื่องจากการแพร่ผ่านได้เองของอนุภาคน้ำระหว่างหยดอนุภาคที่อยู่ติดกัน ดังนั้นสูตรตำรับอิมัลชันน้ำในน้ำมันเข้มข้นที่มีโครงสร้างหลายด้านจึงมีผลช่วยทำให้การปลดปล่อยตัวยาของสูตรตำรับยาวนานขึ้น

สาขาวิชา เทคโนโลยีเภสัชกรรม.....ลายมือชื่อนิสิต.....ศรันยา อัครวิทยาภูมิ.....

ปีการศึกษา.....2549.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

4676853233: MAJOR PHARMACEUTICAL TECHNOLOGY (INTERNATIONAL) PROGRAM
KEYWORD: CONCENTRATED W/O EMULSIONS / *IN VITRO* RELEASE / FORMULATION EFFECTS / EPIGALLOCATECHIN GALLATE (EGCG)

SARANYA UK-KARAWITTAYAPUMI: THE EFFECT OF DIFFERENT FORMULATION PARAMETERS ON THE *IN VITRO* RELEASE OF THE (−)–EPIGALLOCATECHIN GALLATE (EGCG) FROM CONCENTRATED W/O EMULSIONS. THESIS ADVISOR: ASSOC. PROF. PAPAVADEE KLONGPITYAPONG, 325 pp.

Green tea catechins (GTCs) and caffeine, major constituents of green tea were extracted from ground powder of green tea leaves (*Camellia Sinensis* L.) by using 80±1 °C de-ionized water with and without dichloromethane solvent extraction. Freeze-dried green tea extracts (FD-GTE) obtained from freeze-drying technique were determined their GTCs contents, i.e. Epigallocatechin (EGC), Epigallocatechin gallate (EGCG), Epicatechin (EC), Epicatechin gallate (ECG) and caffeine by reversed phase-high performance liquid chromatography analysis. Total yields of FD-GTE residues obtained from both extraction procedures are not significantly different ($p < 0.05$). Content of total GTCs obtained from the extracts with dichloromethane is significantly higher than the one without dichloromethane extraction ($p < 0.05$). As well as, caffeine content of extracts with dichloromethane shows significantly lower extent when compared with the one without dichloromethane extraction ($p < 0.05$). Without significant effect on the total yields of FD-GTE, the dichloromethane, therefore, could be effective solvent for removing caffeine from the hot water extraction of green tea leaves.

Formulations of concentrated water in oil (w/o) emulsions (CEs), containing 2.25% (w/w) of FD-GTE with 84, 86 and 88% dispersed phase (% DP) and 3% of four different emulsifiers (sorbitan, silicone, mixed and glucoester), were prepared to study the effect of formulation parameters on the *in vitro* release of EGCG on synthetic polysulfone membrane.

The concentrations of the emulsifiers do not have a considerable effect on the release of EGCG. Among the four emulsifiers, the mixed emulsions are the only one that gave a considerable faster release of EGCG over 48 h. Furthermore, they allowed the unstable products and could not perform the polyhedral droplets of all ranges of % DP. This faster release could be attributed to the presence of waxes, which give a more rigid oil film. This rigidity could be responsible for an earlier breakage of the film upon application thereby causing a more rapid release of EGCG. Results suggested that the release characteristics of the unstable CEs (the mixed emulsions) depend mainly on their stability due to the rigidity of interfacial films of water droplets.

The droplet diameter decreases and the apparent viscosity increases with the % DP increasing. The shape factor may play an important role on the release of EGCG from stable CEs. With 88% DP, the droplet shape of all stable CEs, except for the mixed type provided the polyhedral structure of water droplets. Moreover, they allowed very stable products with the extreme viscosities. The flux of these systems could be correlated with the structure of water droplet. The polyhedral droplets could retain the release of drug from CEs, containing large amount of water dispersed phase, by self diffusion of water between adjacent droplets. Therefore, the CEs, which possess the polyhedral structures, would prolong the release of drug from the system.

Field of study Pharmaceutical Technology.....Student's signature.....

Academic year.....2006.....Advisor's signature.....

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LIST OF ABBREVIATIONS

Abbreviations of units

cm	=	centimeter (s)
cm^2	=	square centimeter (s)
cP	=	centiPoise (s)
°C	=	degree Celsius (s)
g	=	gram (s)
h	=	hour (s)
K	=	Kalvin
kg	=	kilogram (s)
l	=	liter (s)
m	=	meter (s)
M	=	molar (s)
m^2	=	square meter (s)
m^3	=	cubic meter (s)
µg	=	microgram (s)
µg/ml	=	microgram (s) per milliliter
µl	=	microliter (s)
µm	=	micrometer or micron (s)
µM	=	micromolar (s)
$mAU \cdot s^{-1}$	=	milli-absorbance unit (s) per second
mg	=	milligram (s)
mg/ml	=	milligram (s) per milliliter
min	=	minute (s)
ml	=	milliliter (s)
ml/min	=	milliliter (s) per minute
mm	=	millimeter (s)
mM	=	millimolar (s)
MΩ	=	Mega ome (s)
mPa·s	=	milliPascal-second (1 cP)

mV	=	millivoltage (s)
nm	=	nanometer (s)
Ω	=	ome (s)
%	=	percentage (s)
% DP	=	percentage (s) of dispersed phase
% emulsifier	=	percentage (s) of emulsifier
%, w/v	=	percentage (s) of weight by volume
%, w/w	=	percentage (s) of weight by weight
rpm	=	round (s) per minute

Abbreviations of symbols

α-	=	Alpha
β-	=	Beta
di-	=	two
tetra-	=	four
tri-	=	three
Φ	=	volume fraction

Other abbreviations

Aglycones	=	non-glycosylated forms
ANOVA	=	Analysis of variance
ASTM	=	American Society for Testing and Materials
BHT	=	Butylated hydroxytoluene Butylhydroxytoluene
CEs	=	concentrated emulsions concentrated w/o emulsions
CMC	=	critical micelle concentration
conc	=	concentrate
conc.	=	concentration
CPB	=	citrate–phosphate buffers

Cryo-SEM	=	cryoscanning electron microscopy
CV	=	coefficient of variation
DAD	=	Diode array detector, Diode array detection
DCM	=	Dichloromethane
di-Na.EDTA	=	*di*-Sodium salt ethylenediamine tetra acetate
DI	=	de-ionized
DNA	=	deoxyribonucleic acid
EC	=	(−)–Epicatechin
ECG	=	(−)–Epicatechin gallate
EDTA	=	Ethylenediamine tetraacetic acid
EGC	=	(−)–Epigallocatechin
EGCG	=	(−)–Epigallocatechin gallate
EC_{50}	=	Effective concentration at 50 % inhibition
e.g.	=	for example (example gratia)
EM	=	electronic microscopy
et al.	=	and others (et alii)
etc.	=	and so on (et cetera)
FDA	=	Food and Drug Administration
FD-GTE	=	freeze-dried green tea extract
G	=	gas (state of matter)
GTCs	=	green tea catechins
GTE	=	green tea extract
GTPs	=	green tea polyphenols
HIPRE	=	high internal phase ratio emulsions
HLB	=	hydrophilic-lipophilic balance
HPLC	=	high performance liquid chromatography
i.d.	=	internal diameter
i.e.	=	that is (from Latin ‘id est’)

ICH	=	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
K_p	=	permeability coefficient
L	=	liquid (state of matter)
L1	=	swollen micellar solution of nonionic surfactants
L2	=	swollen micellar solution of ionic surfactants
Max	=	maximum
Med	=	medium
Min	=	minimum
MMD	=	mass median diameter
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole
MW	=	molecular weight
NaCl	=	sodium chloride
NARA	=	United States National Archives and Records Administration
No.	=	number
o/w	=	oil in water
o/w/o	=	oil in water in oil
Paraben Conc.	=	Paraben Concentrate
PDA	=	photodiode array
PG	=	propylene glycol
R^2	=	coefficient of determination
r^2	=	the least squares regression
R.H.	=	relative humidity
RP-HPLC	=	reverse-phase high performance liquid chromatography
R.S.	=	relative span

RSD	=	relative standard deviation
Rx	=	formulations
S	=	solid (state of matter)
S.D.	=	standard deviation
SEM	=	scanning electron microscopy
TFA	=	Trifluoroacetic acid
TFs	=	Theaflavins
TRs	=	Thearubigins
USP	=	United States Pharmacopeia
UV	=	ultraviolet
UV A	=	ultraviolet A
UV B	=	ultraviolet B
UV-vis	=	ultraviolet-visible
UV/VIS	=	ultraviolet/visible
viz.	=	namely (videlicet)
VMD	=	volume mean diameter
vs	=	versus
w/o	=	water in oil
w/o/w	=	water in oil in water
Wt	=	weight
λ_{max}	=	wavelength of maximum absorption

CHAPTER I

INTRODUCTION

Emulsions are mixtures of two immiscible fluids consisting of droplets of one phase dispersed into the other. They belong to the class of non-equilibrium systems, known as metastable systems and tend to come back to their equilibrium state of biphasic mixture (Schramm, 1992; Larson, 1999). They can be of either oil-in-water (o/w) type or water-in-oil (w/o) type. This research was concerned with the latter one. The w/o emulsions are easy to prepare and have a very attractive potential as prolonged release systems for hydrophilic drugs with the oil phase and surfactant layer acting as a release barrier (Davis, Hadgraft, and Palin, 1985). With regard to topical administration for hydrophilic drugs, the w/o emulsions have the potential to increase the therapeutic efficacy of drugs. Therefore, the frequency of administration can be reduced with the w/o emulsions. In addition, the oil phase protects drugs in the internal water phase against degradation in a harsh environment. Furthermore, they offer a wide range of opportunities for modifying and/or controlling release properties by varying formulations or process parameters. The physicochemical parameters influencing the release rate of drugs from the w/o emulsions have been evaluated in many studies. These beneficially supports make the w/o emulsions as a promising topical formulation in which the release of hydrophilic drugs can be controlled.

Additionally, the subtype of w/o emulsions, concentrated w/o emulsions (CEs), which are known as high internal phase ratio emulsions (HIPRE), gel-emulsions, biliquid foams, etc., have been shown to act as a controlled release vehicle for the release of some hydrophilic drug compound (Clément, Pinchon, and Redziniak, 1998). They are a peculiar type of emulsion, containing more than 74% internal phase by volume (volume fraction, Φ up to 0.99) and very low surfactant concentration (as low as 0.5%, w/w) (Kuneida *et al*., 1987; Solans *et al*., 1993). From a cosmetic point of view, these systems have great potential since they allow very high concentrations of internal phase components while retaining the feel of the external phase. In addition, they can be used in many diverse applications like formulation of pharmaceutical, culinary and cosmetic products (Lissant, 1975;

Aronson and Petko, 1986). These aspects make them attractive for economical, environmental and toxicological reasons.

The structure of CEs can be complex with an internal/water phase surrounded by a continuous phase composed of a w/o microemulsion (Kuneida *et al*., 1987; Solans, Azemar, and Parra, 1988a; Solans *et al*., 1988b; Ravey and Stébé, 1990; Pons *et al*., 1993; Solans *et al*. 1993). A w/o microemulsion continuous phase consists of a swollen micellar (L1 or L2) solution of nonionic or ionic surfactants. The functional macroscopic properties of CEs are dependent on the structural parameters of the microemulsion continuous phase as well as of the interfacial properties (interfacial tension, bending modules, spontaneous curvature) of surfactant monolayers. The droplets of these emulsions cannot be spherical and assume some transitional form between spheres and polyhedra (Lissant, 1966).

These emulsions have the structure of biliquid foams and behave as gels since they present viscoelastic and plastic properties (Princen and Kiss, 1986; Princen, 1989; Pons, Solans and Tadros, 1995; Jager-Lezer *et al*. 1998). They are obtained with very little mechanical energy, or simply by heating the system, which contains all components in one-step (Pons *et al*., 1994). The existence of transparent w/o gels has also been reported for a few hydrophobic hydrogenated ethoxylated alcohols with the highest water concentrations (98–99%) and with mixtures of fluorinated nonionic surfactants and fluorocarbons (Kunieda *et al*., 1987; Ravey and Stébé, 1990; Pons *et al*., 1993; Rocca *et al*., 1998).

The stability of these systems can be influenced by many factors. The effect of electrolyte and surfactant concentration, temperature, as well as other physicochemical parameters on the flocculation threshold, stability, and yielding properties of highly CEs is explained by the effect of these parameters on the critical micelle concentration (CMC) and the aggregation number of surfactants, and, consequently, on the depletion interaction. However, it has been observed that electrolytes dissolved in the aqueous phase of CEs dramatically increase emulsion stability (Kunieda *et al*., 1987; Solans *et al*., 1988a; Pons *et al*., 1992; Aronson and Petko, 1993; Aronson *et al*., 1994; Caldero *et al*., 1997). The electrolytes appear to enhance the stability of these emulsions by increasing the resistance of the water droplets to coalescence (Aronson and Petko, 1993) and by the prevention of ice

crystallization at low temperature (Aronson *et al*., 1994). Furthermore, Caldero *et al*. (1997) reported that the stability of CEs could be improved by addition of salting out electrolytes to the aqueous phase ranging from 5% to 15%. Consequently, sodium chloride (NaCl) is preferred to add into the aqueous phase varying from the low concentration (5%) to the high concentration (15%) to candidate the approximate level that might not destabilize the CEs.

The factors affecting the transport properties of these systems have been recently studied with various membranes (Solans *et al*., 1988a, 1993; Caldero *et al*., 1997, 1998; Clément *et al*., 1998; Clément, Laugel, and Marty, 2000) and without membrane (Pons *et al*., 1996; Caldero *et al*., 1997, 1998; Rocca *et al*., 1998; Rocca, Muller and Stébé, 1999). Results from previous studies on hydrogenated and fluorinated CEs indicate that the release rate is strongly system dependent. The release of a hydrophilic probe was found to be higher in hydrogenated systems than in fluorinated ones (Caldero *et al*., 1998). Whereas, when the probe molecule is lipophilic, the contrary have been observed (Rocca *et al*., 1998). These latest studies also showed the possibility of modulating the release of a probe by mixing hydrogenated and fluorinated surfactants in various proportions (Caldero *et al*., 1998; Rocca *et al*., 1998).

It has been well known that formulation parameters can play a role in the release of an active from an emulsion (Kundu *et al*., 1993). Moreover, various physicochemical factors, which may affect drug release from topical formulation, would be evaluated including droplet size, viscosity, physical stability and structure. Consequently, the emulsions prepared have to be characterized in terms of their droplet diameter, apparent viscosity, their physical stability at various temperatures (accelerated aging) and their structure observed by electronic microscopy (EM). Since, normal microscopic examination lacks sufficient resolution and depth of focus to reveal the details of the microstructure of the most CEs. Therefore, the EM could be the practical technique using to observe the structure of these emulsions in depth.

Percutaneous absorption experiments give interesting and valuable information but skin availability and the risks related with its use is a motivation to make preliminary studies with artificial membranes. Diffusion cells without a rate limiting membrane are generally designed to investigate the characteristics of the drug

release from topical formulations (Smith and Haigh, 1989).The *in vitro* tests have limited applicability for estimating the complex process of percutaneous absorption but are useful as screening tools for drug release. Recently, a study of the release of caffeine from CEs on different synthetic membranes shows the importance of the choice of the membrane, which will allow a good differentiation of different formulations of CEs, to use it later in screening experiments (Clément *et al*., 2000). The hydrophilic polysulfone membrane is the only one that allows statistical differentiation of the different formulations. As many beneficial evidences above seem possible to utilize this synthetic membrane as a diffusional membrane to make screening on different CEs. In particular, the *in vitro* diffusion study is mentioned only the release of active molecule from inner formulation to outer environment that is the skin surface. The receptor fluid, therefore, is supposed to be analogous to the skin condition. In generally, the epidermis is the outermost layer of the skin. It has two layers: the outer epidermis and the germinativum. The outer epidermis protects the skin from several harsh environments and manages its own acidity and pH balance, pH 4.5-5.5. Maintaining the skin pH factor helps maintain a proper balance of the acid covering, which aids in protecting the body from bacteria and helps prevent moisture loss. Consequently, pH of the receptor fluid is considered to closely relate with the skin pH, around 4.5-5.5 range is recommended.

Tea is probably one of the most common beverages all over the world. Its popularity is attributed to its sensory properties, relatively low retail price, stimulating effects and potential health benefits. Tea plant is made from the tender leaves of two varieties of the plant *Camellia sinensis*: *assamica* and *sinensis* L., originally from southeast China, gradually expanded to India, Sri Lanka and further into many tropical and subtropical countries. The three main categories of tea, resulted from different processing procedures, include of green, black and oolong. Green tea is manufactured from fresh, unfermented tea leaves.

In recent years, green tea has attracted significant attention because of reported health benefits, in particularly as an antioxidant, as an anticarcinogenic and antiarteriosclerotic agent. One of the most exciting health developments of the nineties has been the discovery of the extraordinary anti-aging properties of green tea. It is generally believed that flavonoids are mainly responsible for these actions.

Green tea contains polyphenolic compounds also known as catechins. Research aimed at finding the active compounds in green tea revealed that its protective effects are due chiefly to catechins.

Powerful polyphenolic antioxidants, catechins are astringent, water-soluble compounds that can be easily oxidized. They are a subgroup of flavonoids, weak phytoestrogenic compounds. The oxidation of catechins is minimal, and hence they are able to serve as antioxidants. The antioxidant potential of green tea, as measured by the phenol antioxidant index, was found to be significantly high.

There has been a growing interest in identifying the pharmacological and physiological effects of green tea, which include antioxidative (Yen and Chen 1995; Kumamoto and Sonda, 1998), antimutagenic (Fujiki, 1999), anticarcinogenic (Fujiki *et al*., 1992, 1998; Pashka *et al*., 1998; Yang *et al*., 1998), hypocholesterolemic (Yang *et al*., 1998) and other clinically relevant activities.

Most research continues to confirm that green tea polyphenols (GTPs) have powerful anticarcinogenic, cardioprotective, neuroprotective and antimicrobial actions. Numerous recent studies have been done on green tea and the activity of its various catechin components. The major and most chemopreventive constituent in green tea responsible for these biochemical or pharmacological effects is (−)-epigallocatechin-3-gallate (EGCG). Recent studies, by computer modeling, indicated that urokinase, one of the proteolytic enzymes needed by human cancer to invade cells, could be inhibited by EGCG (Jankun *et al*., 1997).

Following the successful investigation of physiologically actives found in green tea (Shimamura, 1991; Jankun *et al*., 1997), the incorporation of green tea extracts will provide additional profits for pharmaceutical and cosmetics industries with additional marketing benefits.

Moreover, many laboratories have shown that topical treatment of green tea polyphenols inhibits chemical carcinogen- or ultraviolet radiation-induced skin tumorigenesis in different animal models. Studies have shown that green tea extract (GTE) also possesses anti-inflammatory activity. These anti-inflammatory and anti-carcinogenic properties of green tea are due to their polyphenolic constituents present therein. Topical treatment with EGCG on mouse skin also results in prevention of UV B-induced immunosuppression, and oxidative stress.

From previous study on the effect of polyphenols in Thai GTE regarding cytotoxicity study on melanoma cell line by MTT assay (Aung, 2002), it is indicated that Thai green tea extract (GTE) shows effective concentration at 50 % inhibition level (EC_{50}) on melanoma cell line at approximately 206 micrograms per milliliter (µg/ml). As a result, approximately 400 µg/ml of GTE is referred as an effective dose of GTE (0.04%, w/w).

Based on documented extensive beneficial effects of green tea on mouse skin models and very little in human skin (Katiyar *et al*., 1999), many pharmaceutical and cosmetic companies are supplementing their skin care products with GTE. Consequently, GTE, practically, has a substantial opportunity for further development as an attractive formulation for topical.

As the susceptibility of EGCG to promote self-oxidation itself, topically administration of pure EGCG should be mainly considered to its stability. Furthermore, interactions of EGCG with other pharmaceutical additive components remain unclear, and thus need to be carefully assessed in order that the full potential benefits from topical application of EGCG, in whatever form, can be achieved.

The solubility of GTE in water and ethanol are good. According to the instability of green tea catechins (GTCs) in aqueous solution, GTCs is stable in the aqueous phase at acidic pH (considered at low temperature, i.e. 4 and 25 °C) (Nwuha, 2000).To facilitate protection of oxidation that forces an active form to convert, some antioxidants might be incorporated in the formulations. From the results of the study utilizing glycerin as a solvent (Proniuk, Liederer, and Blanchard, 2002), the solution stability of EGCG is improved by using the organic solvents glycerin. As well, it indicated that the proper selection of an antioxidant could have a significant impact on the stability of EGCG in aqueous solution. It is found that the addition of Butylated hydroxytoluene (BHT) and Ethylenediamine tetraacetic acid (EDTA) in to glycerin could provide the greatest increase in stability compared with the others. These findings suggest that non-aqueous solvents such as glycerin should be utilized in the development of a topical formulation. Particularly, the addition of 0.1% BHT and 0.025% EDTA to glycerin is recommended to maximize the shelf life of the formulation. Subsequently, glycerin combined with BHT (0.1%) and EDTA (0.025%)

is preferred as solvent in an attempt to enhance the stability of EGCG including the humectant (moisturizer) effect.

PURPOSES OF STUDY

The main purpose of this study is to investigate a possibility of the CEs as a topical controlled release system for hydrophilic drug substances with poor stability in aqueous system. For this investigation, EGCG, a major component in GTE could be attractive to employ as a model drug, due to their several physiological activities combined with antitumorgenic, anticancergenic and antimutagenic activity (Shimamura, 1991; Jankun *et al*., 1997). Accordingly, the effects of formulation parameters of cosmetic CEs on the release profile of EGCG would be investigated. The effect of water content, emulsifier type and its concentration would be discussed. Various physico-chemical factors, which may affect drug release from topical cream, would be evaluated including droplet size, viscosity, stability and structure.

The specific purposes of this study are as following:

1. To prepare sufficient stable CEs with different formulation parameters for further investigations in this study.
2. To investigate the effects of formulation parameters on the stability of CEs by evaluating their characteristic properties altered with storage time, e.g., physical stability in various storage temperatures, viscosity, morphology, droplet size and polydispersity.
3. To estimate the effect of formulation parameters of CEs on the *in vitro* release of EGCG.

The results of this thesis research would reveal some descriptive information involved in the effect of formulation parameters of CEs on the *in vitro* release of EGCG, a poor stability hydrophilic substance. This observation could be used as supporting tool for preliminary formulation development based on their release characteristics. Finally, it would provide a novel controlled release system for further studies of other interested hydrophilic substances in Thai herbal extracts as a beneficially cosmetic skin care product.

CHAPTER II

LITERATURE REVIEW

1. Association colloids

Association colloids, which are thermodynamically stable, are solutions of highly surface-active materials (Duncan, 1992: 3).

1.1 Surfactants

Molecules and surfaces are often qualitatively categorized as either *hydrophilic* (water loving) or *hydrophobic* (water fearing). Surface-active agents or surfactants have both characteristics. One end of a simple surfactant is hydrophilic, the polar or ionic head group. The rest of the molecule is the hydrophobic tail, such as an oily hydrocarbon. Having very dissimilar parts in solution attracts the molecule to surfaces, as in detergency (Aston, 2004).

1.2 Adsorption

Surfactants concentrate at interfaces by adsorption to remove *lyophobic* (solvent-fearing) parts from the solvent. This behavior lowers the liquid's surface tension; that is to say, it lessens the imbalance of intermolecular forces between the solvent and its surroundings. The surface tension of pure water causes it to bead up on hydrophobic surfaces. Liquids with lower surface tensions, such as oils and alcohols, bead up to lesser extents. Water can be made to wet hydrophobic material by adding surfactant; this is sometimes called "*breaking*" the surface tension (Aston, 2004).

1.3 Molecular association or aggregation

In diluted solution, the surfactant acts as a normal solute (and in the case of ionic surfactants, normal electrolyte behavior is observed). At fairly well defined concentrations, the critical micelle concentration (CMC), the surfactant ions, in which the lipophilic hydrocarbon chains, are oriented towards the interior of their organized aggregates, termed as *micelles*, leaving the hydrophilic groups in contact with the aqueous medium (Duncan, 1992: 3).

2. Macroemulsions

2.1 Definition

Emulsions are mixtures of two immiscible fluids consisting of droplets of one phase dispersed into the other. They are metastable systems and tend to come back to their equilibrium state of biphasic mixture. They belong to the class of non-equilibrium systems and are subject to aging and slow destruction (Larson, 1999).

2.2 Type of emulsions

Emulsions are formed when one liquid is dispersed in a continuous liquid phase of a different composition. Two types of simple emulsions have been identified depending on which kind of liquid forms the continuous phase (Schramm, 1992).

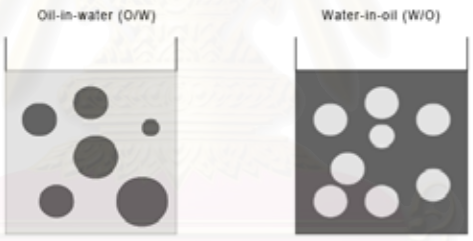


Figure 1 Types of simple emulsions (Schramm, 1992).

Pharmaceutical emulsions usually consist of a mixture of an aqueous phase with various oils and/or waxes. If the oil droplets are dispersed throughout the aqueous phase, the emulsion is termed o/w. A system, in which the water is dispersed throughout the oil, is w/o emulsion. It is also possible to form multiple emulsions. For example, many small water droplets can be enclosed within larger oil droplets, which then dispersed themselves in water. This gives a water-in-oil-in-water (w/o/w) emulsion. The alternative oil-in-water-in-oil (o/w/o) emulsion is also possible.

If the dispersed globules are of colloidal dimensions (1 nm to 1 μm diameter) the preparation, which is quite often transparent or translucent, is called a microemulsion. This type of emulsion has similar properties to a micellar system,

therefore will exhibit the properties of hydrophobic colloids. As the size of the dispersed droplets increases, more of the characteristics of coarse dispersions will be exhibited (Schramm, 1992).

2.3 Thermodynamics of processing emulsions

The formation of emulsions is studied as a two-stage process including of; formation of new droplets (stage 1) and stabilization of the droplets (stage 2) (Sherman, 1968).

In both stages, the mechanism required to produce the emulsion is the thermodynamically unflavored process. The first process involves disrupting the stable bulk liquid to form an unstable dispersed phase. In the second process, the tendency of the droplets to coalesce and reform the stable bulk liquid must be overcome.

2.3.1 Emulsion formation

Emulsifying agents facilitate both stages of emulsification to make thermodynamically unstable emulsions (*metastable*).

Initially emulsifiers lower the interfacial tension making it easier to create the fine dispersion of droplets. A decrease in the interfacial tension reduces the energy required to form a new surface and makes the processes more thermodynamically favorable.

The creation of new droplets is governed by surface forces as (Schramm, 1992):

$$\Delta G_{form} = \Delta A \gamma_{12} - T\Delta S_{conf} \qquad \text{Eq.1}$$

Where,

ΔG_{form} is a free energy of formation of droplets from a bulk liquid.

ΔA is an increase in interfacial area.

γ_{12} is an interfacial tension.

$T\Delta S_{conf}$ is a configurationally entropy contribution.

2.3.2 Emulsion stabilization

The kinetic energy of shaking breaks up the oil into small droplets. However, the oil quickly separates again, aggregating into a large, hydrophobic phase. This is called *coalescence* for droplets since they not only attach but also merge into a single, larger drop. The interfacial tension between oil and water is very high; in other words, they do not mix easily. A surfactant is needed to reduce the oil-water interfacial tension enough for stable emulsion formation (Aston, n.d., cited in Ian and Sydney, 2002).

Emulsifying agents also assist in the stabilization of the droplets by forming a protective coating around the dispersed droplets that prevents coalescence. These agents have a polar head and long non-polar chain. The chains of the agents orientate themselves such that the polar head surrounds the non-polar molecule, which are known as micelles. As a result, the non-polar molecule appears to be a polar molecule to the dispersing liquid and the two phases mix.

2.4 Emulsion destabilization

Emulsions are *kinetically* or *operationally (although not thermodynamically) stable* (Larson, 1999). In general, when a liquid phase disperses in an immiscible liquid to form drops, there is a tendency for the phases to separate again to reduce the augmented surface free energy. With pure phases, this proceeds by rapid coalescence of approaching dispersed entities, as there is no barrier against rupture of the intervening liquid film. Stability (or, more correctly, *metastability*), can be conferred by adsorption of surfactants, polymers, or finely divided solid particles at the interface. By this expedient, *coalescence* can often be suppressed completely, which renders their use attractive in many industrial applications such as cosmetics, road building, or food processing. However, this will not prevent ultimate phase separation, as there is another mechanism for reducing the surface area, namely, *Ostwald ripening*. By this mechanism, large drops grow at the expense of small ones by dissolution and diffusion of the dispersed phase in response to the higher Laplace pressure in the latter ones. Because of slightly solubility and diffusivity of dispersed

liquid in a given continuous liquid, this process is generally quite time-consuming in emulsions. Indeed, drop size distribution of emulsions does not change perceptibly for months or years (Larson, 1999).

A relatively stable emulsion due mostly to high viscosity (more precisely, viscoelasticity), though surfactants are also present. The oil and water in a relatively stable emulsion cannot separate into phases because the emulsion droplets do not have enough energy for much movement. In less viscous emulsions, surfactants are responsible for stability. They reduce interfacial tension for the formation of small particles that either repel or very weakly attract each other. *Brownian motion* must be able to counter the effects of interparticle attraction, *sedimentation*, or *creaming*, which is floatation. Micellar suspensions could also be considered microemulsions, although this is debatable (Aston, n.d., cited in Ian and Sydney, 2002).

2.5 Application

The emulsions can be of either the o/w type or the w/o type. Especially for system containing hydrophilic drugs, there are much concerned with the latter type, w/o emulsions. With the oil phase and surfactant layer acting as a release barrier, they have a very attractive potential as prolonged release systems for hydrophilic drugs. Davis *et al.* (1985) has previously reviewed the use of emulsions as drug delivery systems. The applications mostly include topical and parenteral administration. With respect to topical administration, w/o emulsions have the potential to increase half-life of hydrophilic drugs, especially anti-oxidative substances with poor stability due to oxidation process in a harsh environment. The oil film shelters incorporated drugs in the internal water phase from air exposure. Furthermore, sheltering effect can prolong release of incorporated drugs, so their therapeutic efficacy might be improved. The frequency of administration can be reduced with this effect. These all benefits make the w/o emulsions as a promising topical drug delivery system, in which the release of hydrophilic drugs, can be controlled.

3. (Highly-) concentrated water-in-oil emulsions

(Highly-) CEs have been the topic of several investigations. A rationale for the interest in these systems can be found in the fact that they are used in a multitude of technical applications ranging from pharmaceutical, cosmetic, and food technologies to emulsion explosive. Accordingly, it is of importance to understand the basic properties of such systems.

3.1 Definition

CEs are a peculiar type of emulsions. The characterization of this system is defined by a fraction of dispersed phase in *ternary water/nonionic- surfactant/oil* systems. They are systems containing a large fraction of water-dispersed phase; theoretically, the volume fraction of the dispersed phase is more than 74%. In fact, they can be made to contain in excess of 99% dispersed phase (% DP). Because of its exceedingly high volume fraction (in this case, the percentage of the amount of water is higher than 90%, w/w), this type of emulsion can have the aspect and consistency of gels and has been characterized as *highly concentrated w/o emulsions* (Lissant, 1966).

Highly CEs, which form at the water-rich region of *ternary water/nonionic surfactant/oil* systems, have been termed *gel-emulsions* (Kunieda *et al.*, 1987; Solans, 1988a).

3.2 Physicochemical properties

The formation, structure, stability and rheology of CEs have been subjects of interest in recent years (Kizling and Stenius, 1987). They exhibit peculiar structural, rheological, and optical properties.

3.2.1 Structural property

The structure of some CEs can be complex with an internal/water phase surrounded by a continuous phase composed of a w/o

microemulsion (Kunieda *et al*., 1987; Solans, 1988a, 1988b; Ravey and Stébé, 1990; Pons *et al.*, 1993; Solans *et al.*, 1993).

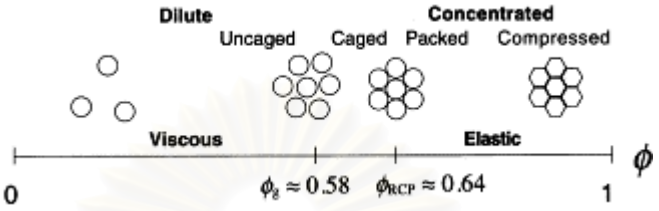


Figure 2 Illustrated image of volume fraction vs droplet structure of emulsions.

Earlier investigations showed that at equilibrium state, they consist of two isotropic liquid phases: i) the dispersed phase is a submicellar surfactant solution in water, and ii) the continuous phase is a swollen reverse micellar solution, i.e. a w/o microemulsion (Solans *et al.*, 1993; Pons *et al.*, 1993, 1994). The droplets of these emulsions cannot be spherical and assume some transitional form between spheres and polyhedra (figure 2) (Lissant, 1966).

These CEs have a foam-like structure where the internal phase consists of polyhedral compartments, filling up the continuous phase making it stiff and gel-like. Hence, these emulsions are called biliquid foams, as proposed by Sebba (1987).

Structurally, they are foam-like materials, they possess a gel appearance, and their visual aspect can vary from transparent to translucent or white depending on composition variables and temperature (Solans *et al.*, 1993; Pons *et al.*, 1994). The existence of transparent w/o gels has also been reported for a few hydrophobic hydrogenated ethoxylated alcohols with the highest water concentrations (98–99%) and with mixtures of fluorinated nonionic surfactants and fluorocarbons (Kunieda *et al.*, 1987; Ravey and Stébé, 1990; Pons *et al.*, 1993; Rocca *et al.*, 1998). The resulting gel-emulsion is transparent regardless of the amount of water. Gel-emulsions may be quite transparent because of a refractive index effect of hydrophobic hydrocarbon or oil phase and nonionic surfactant (Ravey and Stébé, 1990). Indeed, the refractive index of water is intermediate between that of fluorocarbons (the lowest index known) and that of ethoxylated surfactants.

Consequently, a well-chosen mixture of fluorocarbon and surfactant matches the refractive index of water.

3.2.2 Rheological behavior

Numerous rheological studies have been conducted on CEs (Princen and Kiss, 1986; Princen, 1989; Pons *et al.*, 1995; Jager-Lezer *et al.*, 1998). The structure of CEs results in solid-like responses such as elastic behavior at low strains and yield stresses.

The rheological behavior of these systems depends on the liquid fraction of the dispersed phase. At low volume fraction, the droplets are *Brownian* and the emulsion is *Newtonian* with a viscosity close to that of the continuous phase. With such high water content, they behave like viscoelastic gels, due to their foam like structure (Pons *et al.*, 1993), which prompted the *Gel-emulsion* term to designate these systems.

When the volume fraction of the dispersed phase is increased, droplets come into contact and their interfaces are deformed (Pons *et al.*, 1993).

The emulsion undergoes an abrupt transition between a jammed state and a fluid phase. After transient inhomogeneous states that can last for more than one hour, the flow is always homogeneous once equilibrium is reached.

The transition between these two regimes occurs at a critical stress σ_0, called the *yield stress*. When subjected to small strain rates (below the *yield stress*, $\sigma < \sigma_0$), they can support an additional interface deformation and thus store energy, which results in an elastic response. The emulsion is completely jammed and the shear rate is zero. Therefore, the emulsion does not flow and its rheological response is purely elastic. At higher strain rates (above the *yield stress*, $\sigma > \sigma_0$), droplets rearrange irreversibly and the emulsions start to flow and then finally flow homogeneously (Pons *et al.*, 1993).

3.2.3 Formation

These gel-emulsions can be obtained by several methods of preparation (Pons *et al.*, 1994). They are obtained with very little mechanical energy, or simply by heating the system, which contains all components in one-step (Pons *et al.*, 1994). Their formation is closely related to the hydrophilic-lipophilic balance (HLB)-temperature of the corresponding system (Solans, 1988a, 1988b). Gel-emulsions only form at temperatures above the HLB-temperature and present maximum stability at about 20-30 °C, above this temperature.

3.2.4 Stability

The stability of these systems can be influenced by many factors. As described for normal w/o emulsions, the nature of the surfactant also influences gel-emulsion stability; the longer the hydrocarbon chain of the oxyethylenated nonionic surfactant, the higher the stability is.

Moreover, it has been observed that the electrolytes appeared to enhance the stability of these w/o emulsions by increasing the resistance of the water droplets to coalescence (Aronson and Petko, 1993) and by the prevention of ice crystallization at low temperature (Aronson *et al.*, 1994). As mentioned above, the electrolytes dissolved in the aqueous phase of CEs dramatically increased emulsion stability, which was attributed to the dehydration of the polar heads of the surfactant molecules (Pons *et al.*, 1992).

3.2.5 Long-term stability

One of the remarkable properties of CEs is their long-term stability despite very low emulsifier content.

As for ordinary emulsions, the emulsion stability rests on: i) a good adsorption of the emulsifier to the liquid/liquid interface, and ii) a stability against formation of hole in the continuous phase upon the collision of two emulsion droplets. The latter stabilization mechanism is closely related to the *Bancroft* rule stating that the emulsifier should be soluble in the continuous phase, as discussed by

Kabalnov and Wennerström (1996). In CEs, the stability can be greatly enhanced by lowering the *van der Waals* attraction between the emulsion droplets.

The attractive force, *A*, between two droplets of emulsions in a continuous medium can be expressed as,

$$A = a\left(\frac{\varepsilon_1 - \varepsilon_2}{\varepsilon_1 + \varepsilon_2}\right)^2 + b\frac{\left(n_1^2 - n_2^2\right)^2}{\left(n_1^2 + n_2^2\right)^{3/2}} \qquad \text{Eq.2}$$

Where,

n_i is a refractive index.

ε_i is a dielectric constant of phase ε_i.

a and *b* are constants.

Consequently, the attractive force is at a minimum when the refractive index of the two phases is the same. The refractive index of organic phases is in the order of 1.4 while aqueous solutions have a refractive index of approximately 1.3. Hence, the attractive forces between two aqueous emulsion droplets can be lowered by the addition of salts that increase the refractive index of the aqueous solution. In fact, increasing the refractive index of the aqueous phase to be the same as for the organic phase renders transparent emulsions that have a very good stability.

3.2.6 Diffusional characteristic

In the following, it will be useful to discriminate between free and hindered diffusion. In either case the motional process is diffusive (the inertial effects are completely negligible on the time scales considered here).

In the former case, the self-diffusion has its normal Gaussian propagator and the diffusion coefficient is equal to its bulk value. With hindered motion we will imply two cases: i) the diffusional motion is still described by a Gaussian propagator (referred to as a Gaussian diffusion), but with a diffusion coefficient that is reduced from its short time (bulk) value, or ii) the diffusional process is not described by a Gaussian propagator (referred to as non-Gaussian

diffusion). In both cases, the motion is hindered by barriers that are present in the system, and the classification of the motion is of course dependent on the time of the observation and the length scale studied.

3.2.7 Diversity of applications

CEs can be formulated with a very high amount of water (up to ϕ = 0.99 or higher than 99%, w/w), and very low surfactant concentration (as low as 0.5%, w/w). The high water content and low amount of surfactant make them appealing from the economical, environmental and toxicological point of view (Kuneida *et al.*, 1987; Solans *et al.*, 1993).

CEs can be used in many diverse applications like formulation of pharmaceutical, culinary and cosmetic products (Lissant, 1975; Aronson and Petko, 1986). From a cosmetic point of view, these systems have great potential since they allow for very high concentrations of internal phase components while retaining the feel of the external phase.

Furthermore, CEs display a compartment-like structure, with the micellar phase separating the big water droplets of the dispersed phase (Ravey and Stébé, 1990). This separated structure allows them to be new drug delivery systems as they retard and control the release of molecules entrapped in the water droplets of the emulsion. These water droplets would act as a reservoir of active principles in pharmaceutical preparations and in cosmetics. In addition, other researchers have proven the fact that CEs revealed to act as a controlled release vehicle for the release of water-soluble substance (Clément *et al.*, 1998).

4. Green tea extracts as a model drug compounds

Tea has become the most popular beverage after water throughout the world. In recent years, tea has attracted significant attention because of reported health benefits, in particularly as an antioxidant, but also as an anticarcinogenic and antiarteriosclerotic agent. It is generally believed that flavonoids are mainly

responsible for these actions (Balentine, 1997; Dreostic, Wargovich, and Yang, 1997; Jankun *et al.*, 1997; Wiseman, Balentine, and Frei, 1997; Yang, 1997).

EGCG is regarded as the most important of the tea catechins because of its high content in tea and the fact that its activity is mirrored by green tea extracts. In addition, it has been suggested that a major part of the beneficial physiological action associated with green tea consumption is attributable to EGCG (Huang, Ho, and Lee, 1992).

4.1 Tea

4.1.1 Botany

The tea shrub is a perennial evergreen plant (Bokuchava and Skobeleva, 1969). It is classified in the *Theaceae* family and the *Camellia* species (*Camellia sinensis*, (L.) O. Kuntze) (Hara *et al.*, 1995; Johns, 1998). *Camellia sinensis* consists mainly of two varieties, *Camellia sinensis* variety *sinensis* and *Camellia sinensis* variety *assamica* (Hara *et al.*; Johns). In nature, tea trees can attain a height of 20-30 m. The plant is kept as an evergreen shrub by pruning. Only the apical bud and the first few leaves are plucked for tea processing. In tropical countries, tea leaves are harvested all year around. In temperate countries, harvesting is seasonal. There are many different kinds of products of different quality arising from different cultivation practices, growing conditions and processing methods (Bhatia and Ullah, 1962; Millin, 1987; Hara *et al.*).

4.1.1.1 Types of tea

There are currently six main types of tea produced including black, green, white, yellow, oolong, and reprocessed teas (Hara *et al.*, 1995). These types are converted into large range of tea products. There are over 300 kinds of reprocessed teas alone, of which some are the well-known scented teas, such as jasmine, and brick teas (Hara *et al.*). White and yellow teas have been regarded as two subclasses of green tea by Harbowy and Balentine (1997). These two types of tea

are different from green tea due to differences in variety, processing, geographical and traditional distributions (Lu, 1987).

4.2 Flavonoids

4.2.1 Occurrence and chemistry of flavonoids

Flavonoids are a group of polyphenolic compounds, diverse in chemical structure and characteristics. They occur in virtually all plant parts, particularly the photosynthesizing plant cells, and are an integral part of both human and animal diets (Cook and Samman, 1996; Bravo, 1998). As plant phytochemicals, flavonoids cannot be synthesized by humans and animals (Harborne, 1967, 1988; Harborne, Mabry, T. J., and Mabry, H., 1975). Flavonoids found in animals are considered to originate from plants upon which the animals feed rather than being biosynthesized in situ (Harborne, 1967). About 4000 individual compounds belonging to this class are already known (Harborne, 1982), with the actual number of flavonoids possibly being closer to 5000 (Cadenas and Packer, 1996). The distribution of flavonoids in plants suggests there is a strong tendency for taxonomically related plants to produce similar types of flavonoids (Markham, 1982).

Naturally, occurring flavonoids are generally classified into six classes according to their chemical structures (Peterson and Dwyer, 1998), including flavanones, flavones, isoflavonoids, flavans (flavanols), anthocyanins and flavonols. These flavonoids vary in their structural characteristics around the heterocyclic oxygen ring, forming a unique carbon skeleton C6-C3-C6 (figure 3).

This structure can have various numbers of hydroxyl substitutions and other functional groups attached to form many different types of flavonoids (Harborne, 1967, 1988). Flavonoids occur as both aglycones and glycosides. In general, all flavonoids are derivatives of the 2-phenylchromone parent compound composed of three phenolic rings referred to as the A, B and C rings, all of which contain varying levels of hydroxylation and methoxylation (Clifford and Cuppett, 1997). The biochemical activities of flavonoids and their metabolites depend

on their chemical structures and the relative orientation of various moieties on the molecule.

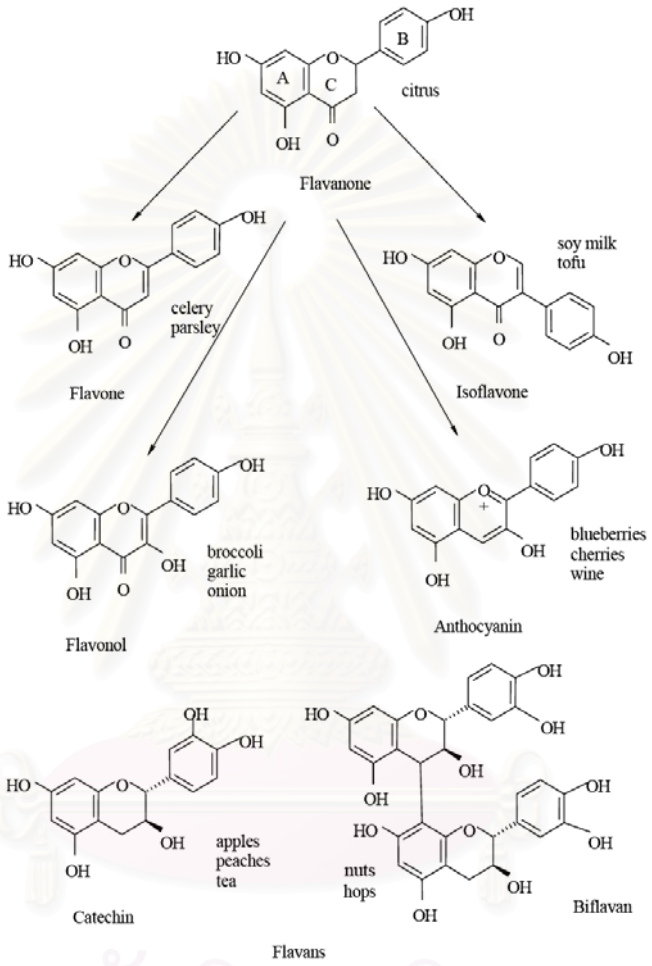


Figure 3 Structure and dietary occurrence of the main classes of flavonoids (arrows indicate biosynthetic path) Source: adapted from Peterson and Dwyer, 1998.

The structure of flavonoids is the basis of many hypotheses about their physiological actions. Flavonoids are easily oxidized at the B ring, which leads to opening of this ring at the oxygen atom (Havsteen, 1983). The high chemical reactivity of flavonoids is expressed in the binding affinity to biological polymers and heavy metal ions, and the ability to catalyze electron transport and to scavenge free radicals (Havsteen). Thus, flavonoids are a group of phenolic compounds that share

some common structural features and physicochemical properties, which create interest in their biological effects.

Flavonoids in plants, such as anthocyanins, impart color as pigments, and ensure pollination, fertilization and seed dispersal by animals (Harborne *et al*., 1975; Harborne, 1988; Mazza, 1997). These compounds act as a light screen against damaging UV radiation in young leaves, provide resistance to pathogens, and act as antioxidants, enzyme inhibitors and precursors of toxic substances. In addition, flavonoids may function as photosensitizing and energy-transferring compounds, and take part in the control of plant growth and development in concert with plant hormones (Harborne *et al*., 1975, Harborne, 1988). Flavonoids appear to be universal chemical tools that plants use to interact with their environment. Furthermore, these compounds have been implicated in defense against other plants, fungi, insects and bacteria; as regulators of interactions between beneficial fungi, herbivores, and insects; as plant hormones; and as important constituents of animal diets, both nutritionally and medically (Berhow, 1998).

Consequently, scientists are showing increased interest in food flavonoids, due to their possible beneficial roles in human health as antioxidants, in the prevention of cancer and cardiovascular diseases and in the treatment and prevention of many other pathological disorders, such as gastric and duodenal ulcers, allergies, vascular fragility, and viral and bacterial infections (Bravo, 1998).

4.2.2 Biochemical systematic of flavonoids

Flavonoids have been regarded as important taxonomic markers in systematic studies since 1962 (Harborne, 1967). The reasons why flavonoids are preferred to most other low molecular weight constituents are: i) they are universally distributed in vascular plants and show considerable structural diversity, ii) they are so chemically stable that they can be detected in herbarium tissue, and iii) they are easily and rapidly identified (Harborne *et al*., 1975; Harborne, 1982; Harborne and Mabry, 1982; Harborne and Turner, 1984; Harborne, 1988).

Distribution and biochemical evolution of flavonoids in the plant kingdom may indicate the evolution of plants. This evolving flavonoid pattern in higher plants has very useful properties (Harborne, 1982).

4.2.3 Dietary occurrence of flavonoids

4.2.3.1 Generic distribution in foods

Flavonoids are not present in animals but occur in all plant foods. Flavonoids occurring in foods are generally responsible for color, taste, the protection of fats against oxidation, the destruction of vitamins, and the inactivation of enzymes (Swain, 1962). Flavanones occur predominantly in citrus while isoflavonoids occur in legumes (Huang *et al.*, 1994). Flavones occur mainly in herbs (Ho *et al.*, 1994); while anthocyanins and catechins are found in teas, fruits and vegetables (Ho *et al.*; Huang *et al.*); and flavonols occur in all fruits and vegetables (Peterson and Dwyer, 1998). Any foods containing natural flavors and colorings, or made from plants, may contain flavonoids. The dietary occurrence of flavonoids in foods is summarized in figure 3.

Rich amounts of natural phenolic compounds are found in teas, fruits, and vegetables, while some amounts of polyphenols exist in red wine and coffee (Ho, Lee, and Huang, 1992).

4.2.3.2 Level of flavonoids in foods

The levels of individual and total flavonoids are influenced by genetic factors such as species, environmental conditions such as light, ripeness, and post-harvest treatments such as processing and storage (Bravo, 1998; Duthie, G. G., Duthie, S. J., and Kyle, 2000). Although most fruits, chocolate, and some legumes contain catechins, the levels vary largely from 4.5 mg/kg in kiwifruit to 610 mg/kg in black chocolate (Arts, Van De Putte, and Hollman, 2000). Here, catechin and epicatechin (EC) are the predominant catechins, whereas gallocatechin (GC), epigallocatechin (EGC) and epicatechin gallate (ECG) are detected only in certain foods.

Tea is the only beverage that contains GC, EGC, ECG, and epigallocatechin gallate (EGCG), in addition to catechin and EC (Arts *et al.*, 2000). Green tea has the highest level of phenolic compounds amongst foods; up to 35% of the dry matter (Bravo, 1998).

4.2.4 Flavonoids and health benefits

4.2.4.1 General benefits of consumption of flavonoid rich food

Food phenolic compounds, particularly flavonoids, are thought to play important roles in human health (Ho *et al.*, 1992, 1994; Huang *et al.*, 1992, 1994). *In vitro* and animal studies have demonstrated that flavonoids have antioxidant and anti-mutagenic activities (Peterson and Dwyer, 1998) and may thus reduce the risk of cardiovascular disease and stroke (Duthie *et al.*, 2000). Isoflavonoids, such as phytoestrogens, have a wide range of hormonal and non-hormonal activities in animals or *in vitro* (Cassidy, Hanley, and Lamuela-Raventos, 2000), suggesting potential human health benefits of diets rich in these compounds.

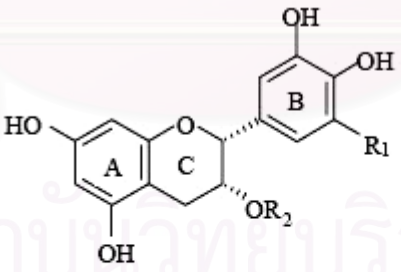
Flavonoids may act as antioxidants to inhibit free-radical mediated cytotoxicity and lipid peroxidation; as anti-proliferative agents to inhibit tumor growth; or as weak estrogen agonists or antagonists to modulate endogenous hormone activity (Lyons-Wall and Samman, 1997). In these ways, flavonoids may confer protection against chronic diseases such as atherosclerosis and cancer, and assist in the management of menopausal symptoms. Thus, flavonoids have been referred to as semi-essential food components (Kühnau, 1976).

Early studies have uncovered some properties of tea polyphenols related to human health (Chow and Kramer, 1990), including a capillary-strengthening property, an antioxidative property responsible for the radioprotective effect, and the antimicrobial property. Hara (1992) showed that the habit of tea drinking could prevent cardiovascular diseases, by increasing plasma antioxidant capacity in humans (Nakagawa *et al.*, 1999; Duthie *et al.*, 2000). Interestingly, tea

polyphenols are rapidly absorbed after drinking with milk, and milk does not impair the bioavailability of polyphenols (Van het Hof *et al.*, 1998).

4.2.4.2 Flavonoid structures and antioxidant activities

Antioxidants can be defined as compounds that inhibit or delay but do not completely prevent oxidation (Clifford and Cuppett, 1997). They can be compounds capable of inhibiting oxygenmediated oxidation of diverse substrates and/or inhibiting free radical chain-propagation reactions (Ho *et al.*, 1994). There are generally two groups of antioxidants, synthetic and natural. The antioxidant mechanisms of the synthetic antioxidants are well established, but only the mechanisms of certain classes of natural ones such as tocopherols and carotenes have been determined. Clifford and Cuppett (1997) classified the antioxidant mechanisms of flavonoids into free radical chain-breaking, metalchelating and singlet oxygen quenching, with the inhibition of enzymatic activity possibly being included. Further, Bors *et al.* (1996) suggested that the mechanisms might include the synergistic effects.



Epicatechin (EC) $R_1 = R_2 = H$
Epicatechin gallate (ECG) $R_1 = H$, R_2 = gallate
Epigallocatechin (EGC) $R_1 = OH$, $R_2 = H$
Epigallocatechin gallate (EGCG) $R_1 = OH$, R_2 = gallate

Figure 4 Structure of EC, ECG, EGC and EGCG (Franke *et al.*, 1998).

Flavonoid compounds with similar chemical structures exhibit comparable trends in antioxidant activity (Fukumoto and Mazza, 2000). This activity usually increases with an increase in the number of hydroxyl groups and a

decrease in glycosylation. Flavonoids such as EC and ECG with a vicinal diphenol structure in the B ring and a saturated C ring exhibit the strongest effects (Figure 4) (Franke *et al.*, 1998).

EGCG with two triphenol components in its structure, one from the B ring and one from the gallate attachment (figure 4), has been found to strongly and dose-dependently inhibit histamine release from rat basophilic leukemia cells (Matsuo *et al.*, 1997). EGC (in the B ring) and ECG (in the gallate attachment) with only one triphenol in the structure, moderately inhibit such release (Matsuo *et al.*). The catechins, such as EC, with only a diphenolic structure in the B ring, do not produce such an effect. Similar findings were reported recently by Toschi *et al.* (2000). These results suggest that the triphenol structure plays an important role in the activities of tea polyphenols (Pannala *et al.*, 2001).

4.2.4.3 Variation in scavenging and antioxidant properties

Flavonoids have been labeled as 'high level' antioxidants based on their abilities to scavenge free radicals and active oxygen species. However, research has shown that antioxidant activity varies among flavonoids (Aruoma, 1997).

Salah *et al.* (1995) showed that the total antioxidative activity and the order of effectiveness of green tea polyphenols as radical scavengers is: ECG > EGCG > EGC > gallic acid > EC = catechin. The oxidation of low-density lipoproteins is inhibited by catechin, EC, ECG and EGCG to a similar degree, but not as much as in the presence of EGC or gallic acid. In addition, Amarowicz and Shahidi (1995) found that the efficacy of antioxidant activity of a reconstituted green tea polyphenol mixture was lower than that of the extracted crude mixture itself. This may indicate that non-catechin components in the mixture possessed their own antioxidant activity or acted synergistically with the catechins.

Recent comparison of different teas showed a wide difference in *in vitro* antioxidant power that was strongly correlated with total

phenolic content (Benzie and Szeto, 1999). Green tea showed the strongest activity, oolong tea the next, while black tea showed the least activity (Chen *et al.*, 1996; Serafini, Ghiselli, and Ferro-Luzzi, 1996; Gardner, McPhail, and Duthie, 1998). Tea flavonoids are more effective scavengers of aqueous and lipophilic stable radicals than many other flavonoids and the antioxidant vitamins (Zhao *et al.*, 1989; Wiseman *et al.*, 1997). It has been reported that tea flavonoids scavenge radicals and protect low-density lipoproteins from oxidation more effectively than established antioxidants (Zhu *et al.*, 1999). In addition, tea flavonoids demonstrate *in vivo* protection in smokers and in rodents exposed to oxidative stress induced by radiation, chemicals or diet (Unilever, 1996).

4.2.4.4 Chelating capacity and redox potential

The chelating properties of flavonoids may be attributed to their antioxidant activities (Aruoma and Cuppett, 1997). Most flavonoids chelate iron (Fe^{2+}), but there is large differences in the chelating capacity (Van Acker *et al.*, 1996). For good scavenging activity, a catechol moiety on ring B is required. Chelation can raise the scavenging activity to the level of the most active scavengers, possibly by site-specific scavenging. Thus, antioxidative capacity of flavonoids increases as their Fe^{2+}-chelating activities increase. However, some of the chelating activity of flavonoids may adversely affect human health (Santos-Buelga and Scalbert, 2000).

Another study showed that green tea extract markedly delayed lipid peroxidation in low-density lipoproteins, with a dose-dependent pattern (Yokozawa and Dong, 1997). Copper chelation was recognized as one of the possible mechanisms of low-density lipoprotein antiperoxidation. An enhanced absorption in the visible region is observed in the case of the iron-digallate complex, but not with copper-digallate complex. For flavonoids, ortho 3′,4′-hydroxyl substitution in the B ring has been shown to be important for Cu^{2+}-chelating formation, thus influencing the antioxidant activity (Brown *et al.*, 1998).

The antioxidative capacity of flavonoids is also associated with their redox potentials (Bors *et al.*, 1996). In many instances, the

reduction potentials of flavonoids, such as catechins of green tea (Jovanovic *et al.*, 1995) are lower than that of vitamin E.

Low reduction potentials and high rates of scavenging of biological oxidant-superoxide radical in neutral media (Unno, Sugimoto, and Kakuda, 2000) are indicative of high antioxidant potentials of tea catechins (Jovanovic *et al.*, 1995, 1996, 1997, 1998).

4.2.4.5 Metabolism and clinical effects

Flavonoids are absorbed by the gastrointestinal tracts of humans and animals, and are excreted either unchanged or as their metabolites in the urine and feces (Cook and Samman, 1996). Colonic bacteria split the heterocyclic ring and degrade flavonoids to phenyl acids, which may be absorbed, conjugated, and excreted or metabolized further by the bacteria (Peterson and Dwyer, 1998). Some flavonoid glycosides are rapidly deglycosylated by enzymes in human tissues whereas others may remain unchanged. The rate and extent of deglycosylation depends on the structure of the flavonoid and the position/nature of the sugar substitutions (Day *et al.*, 1998). Measurement of plasma and urine antioxidant power after ingestion of green tea has shown that absorption of green tea antioxidants is rapid (Benzie *et al.*, 1999). The antioxidants enter the systemic circulation soon after ingestion and cause a significant increase in plasma antioxidant status. Benzie *et al.* suggested that this increase might lower oxidative damage to the deoxyribonucleic acid (DNA) and thus decrease risk of cancer.

Flavonoids have profound effects on the function of immune and inflammatory cells (Middleton and Kandaswami, 1992). In animal studies, two EGCG methyl esters extracted from oolong tea significantly inhibited mice allergic reactions (Sano *et al.*, 1999).

4.2.4.6 Protective activities against heart disease

Possible protective effects of flavonoids against heart disease may be due to their ability to prevent the oxidation of low-density lipoproteins

to an atherogenic form, although anti-platelet aggregation activity and vasodilatory properties are also reported (Muldoon and Kritchevsky, 1996; Chen *et al.*, 2000; Duthie, G. G., Duthie, S. J., and Kyle, 2000; Santos-Buelga and Scalbert, 2000). Flavonoid intake may reduce the risk of death from coronary heart disease in women (Knekt *et al.*, 1996; Duthie *et al.*), postmenopausal women (Yochum *et al.*, 2000a; Yochum, Folsom, and Kushi, 2000b) or elderly men (Hertog *et al.*, 1993, 1995). Differences in flavonoid intake in different countries may partly contribute to differences in coronary heart disease mortality across populations (Hertog *et al.*, 1995).

The habitual intake of flavonoids from food sources such as tea may also protect against stroke (Keli *et al.*, 1996), or lead to a lower risk of atherosclerosis and coronary heart disease (Weisburger, 1996; Tijburg *et al.*, 1997; Duthie *et al.*, 2000). This is because tea pigments can reduce blood coagulability, increase fibrinolysis, prevent platelet adhesion and aggregation, and decrease the cholesterol content in aortic walls *in vivo* (Lou *et al.*, 1992). Green tea is able to protect against nitric oxide toxicity, which may relate the beneficial effects of flavonoid intake to the prevention of coronary heart disease (Paquay *et al.*, 2000).

4.2.4.7 Cancer prevention

Animal studies and epidemiological data indicate that dietary factors play an important role in animal and human health, and in the development of certain diseases, including cancer. Fresh fruits and vegetables are rich in vitamins A, C, and E, *β*-carotene, flavonoids and other constituents that have been studied as cancer chemopreventive agents (Ho *et al.*, 1994). Numerous cell culture and animal models indicate potent anti-carcinogenic activity by certain polyphenols mediated through a range of mechanisms (Duthie *et al.*, 2000). Whether an antioxidant is an anti-carcinogenic agent may depend on its efficacy as an oxygen radical inactivator and inhibitor (Kuo, 1997). Location, concentration in situ, reaction kinetics (rate constants), energetics (redox potentials) and products (intermediate and final) contribute to the efficacy of an antioxidant.

Diets rich in radical scavengers would reduce the cancer-promoting action of some radicals (Sawa *et al.*, 1999). Some flavonoids can modify enzymes and bind carcinogens to DNA, thus exerting an anticarcinogenic effect (Dragsted, Strube, and Leth, 1997). Studies by Dragsted *et al.* showed that a small dose of flavonoids, while ineffective alone, provided an effect when used in combination at the equivalent concentration. Tea is a significant source of flavonoid antioxidants, with a suggested role in prevention of cancer (Oguni *et al.*, 1992; Osawa *et al.*, 1992; Balentine, 1997). Polyphenols present in green tea show cancer chemopreventive effects against tumor initiation (Gensler *et al.*, 1996) and against promotion stages of multistage carcinogenesis in many animal tumor models (Conney *et al.*, 1992; Katiyar, Agarwal, and Mukhtar, 1992a; Katiyar *et al.*, 1992b; Khan *et al.*, 1992; Wang *et al.*, 1992; Yang and Wang, 1993; Mukhtar, Katiyar, and Agarwal, 1994; Dreosti *et al.*, 1997; Kivits, Van der Sman, and Tijburg, 1997; Landau and Yang, 1997). Green tea may protect against cancer by causing cell cycle arrest and inducing apoptosis (Ahmad *et al.*, 1997), while black tea can produce an inhibitory effect on tumor promotion (Nakamura *et al.*, 1992).

The inhibiting effects of tea components may meaningfully reduce the risk for several important types of cancer in the world (Cheng and Ho, 1988; Weisburger, 1992, 1996; Xu *et al.*, 1992a, 1992b). Histopathological examination revealed that green tea was able to inhibit tumor cell proliferation in animal models (Chen, 1992; Chen *et al.*, 1999). EGCG, EGC, and ECG inhibited soybean lipoxygenase, a carcinogen and tumor promoter, most effectively at lower doses (Ho *et al.*, 1992). However, more epidemiological data on the bioavailability, metabolism and intracellular location of polyphenols are required before recommending increasing polyphenol intake for the prevention or treatment of human cancer (Duthie *et al.*, 2000).

4.2.5 Polyphenols in tea

4.2.5.1 Polyphenols in green leaf and green tea

The production of polyphenolic constituents in the tea plant is assumed to be a means of chemical defense against insects, birds, and animals, which would consume the plant as food (Beart, Lilley, and Haslam, 1985).

Green tea is made without enzymatic oxidation of polyphenols, as polyphenol oxidase is inactivated by heat during the early stages of green tea processing (Hara *et al.*, 1995). Thus, the polyphenols present in green tea should be the same as those found in fresh tea leaves. In a broad sense, green tea polyphenols consist of simple and complex compounds, the large majority of which are the flavonoid monomers catechins, catechin gallates and flavonols.

Catechins and their gallates are members of a more general class of flavonoid, the flavan-3-ols or flavanols. The epi-isomers of the catechins and catechin gallates are the principal components found in tea (figure 5). The tea catechins, a term commonly used to refer to both catechins and catechin gallates, make up as much as 30%, w/w of the dry mass of tea (Baruah *et al.*, 1986; Harbowy and Balentine, 1997).

In a more specific sense, catechins include EC, C, EGC, and GC, while catechin gallates include ECG, CG, EGCG, and GCG (Forrest, and Bendall, 1969; Hilton, Palmer-Jones, and Ellis, 1973; Hara *et al.*, 1995). Two minor catechin digallates, epicatechin digallate (ECDG) and epigallocatechin digallate (EGCDG) (Coxon *et al.*, 1972; Nonaka, Kawahara, and Nishioka, 1983; Hashimoto, Nonaka, and Nishioka, 1987) have also been considered as catechin gallates (Opie, 1992). The four most common catechins and catechin gallates are EGCG, EGC, ECG, and EC. Other catechins such as C and GC are present in smaller quantities in tea, whereas the gallates GCG and CG found in tea may be products of racemization and not “native” to the tea plant (Roberts, 1962). Figure 5 shows the structures of the catechins and catechin gallates observed in tea. The methyl esters of ECG and EGCG were also recently identified in tea by Zeeb *et al.* (2000).

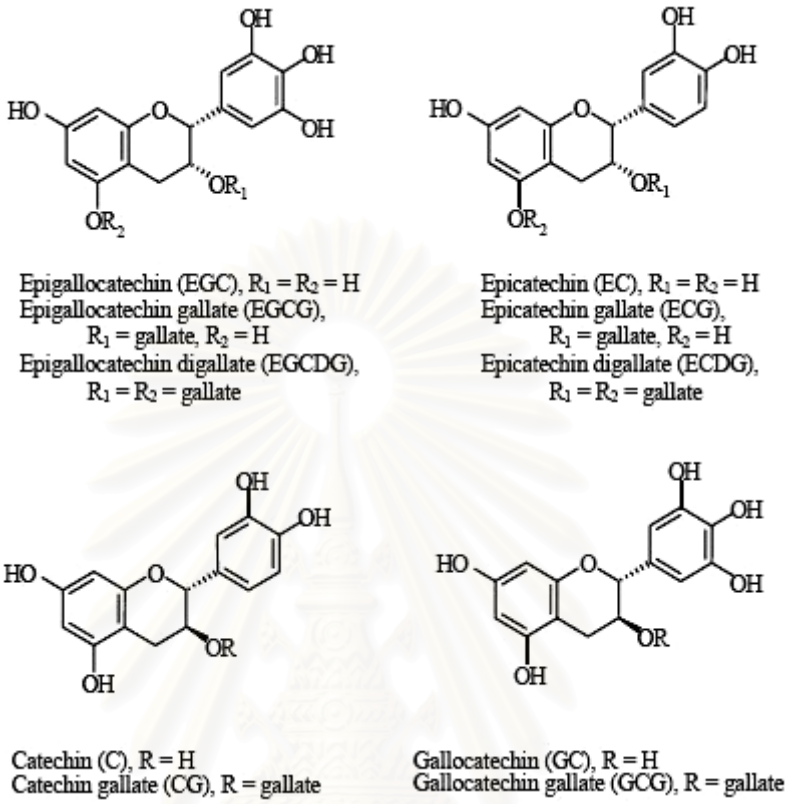


Figure 5 Structures of tea catechins (Adapted from Harbowy and Balentine, 1997).

In an early study, the chemical constituents of polyphenols in tea shoot were shown to be (% of total polyphenols): C 0.4%; EC 1.3%; GC 2.0%; EGC 12.0%; ECG 18.1%; EGCG 58.1%; while the other polyphenols were 6.67% (Bokuchava and Skobeleva, 1969). EGCG is the major constituent in all parts of the tea shoot. Graham (1992) showed that the principal catechins and catechin gallates in fresh leaf were in agreement with Hilton (1973) (% of dry tea): C 1-2%, EC 1-3%, ECG 3-6%, EGC 3-6% and EGCG 7-13%. In another study, the six catechins and catechin gallates, C, GC, EC, ECG, EGC, and EGCG, were found to represent about 80% of the total polyphenols in green tea and in fresh tea leaves (Opie, Robertson, and Davies, 1988).

Seasonal variations in the content of polyphenols in some jats (cultivars) and clones grown in North Eastern India have been observed (Bhatia and Ullah, 1968; Singh, H. P., Ravindranath, and Singh, C., 1999). Towards

the end of a plucking season, the total content of polyphenols and the oxidase activity of Assam tea leaf tends to fall (Wood *et al.*, 1964a, 1964b). Changes in the qualitative and quantitative composition of tea leaf catechins and catechin gallates resulting from the changes in the plucking seasons may result from the biochemical activity during the growth of tea plant. An increase in the content of catechins and catechin gallates in raw tea leaf in summer months is due mostly to an active synthesis of EGCG and ECG (Bokuchava and Skobeleva, 1969). This finding reveals that the accumulation of catechin gallates during summer months may result from the irradiation effects of sunlight.

The precursors of catechins and the gallates, along with theogallin, are presumably present in tea seeds (Bhatia and Ullah, 1962). However, Forrest and Bendall (1969) showed that the only catechins and their gallates that are detectable in the embryo are C and EC. Bhatia and Ullah suggested that the ratio of EGCG/ECG could be used to monitor the development of new organs of the seedling, increasing from 1.7 for epicotyl, to 2.3 for cataphylls and to 6.3-11.7 for the foliage leaf. The relative constancy of this ratio under diverse cultural conditions, and its sole dependence on the nature and origin of the leaf suggested that this ratio could be genetically controlled (table 1).

Studies on the properties of the catechins and their gallates of green tea revealed that these compounds show a strong UV light absorption (Bradfield, Penny, and Wright, 1947; Bradfield and Penny, 1948). The range of the absorption is 266-280 nm (ethanol). The absorption maxima for catechins, catechin gallates and simple phenolic acids in alcohol are: catechol 278 nm; pyrogallol 266 nm; gallic acid 272 nm; EC 280 nm; GC 271 nm; ECG 280 nm; GCG 275 nm and 279.5 nm. Coxon *et al.* (1972) showed similar results with EC 280 nm, ECG 279 nm, ECDG 282 nm, EGC 271 nm, EGCG 275 nm and EGCDG 283 nm.

Table 1 Contents of characteristic constituents of various *Camellia* species leaves (Adapted from Chu, 1997) (g/100g dry basis).

Species	Catechins					Theanine	Caffeine
	C	EC	EGC	ECG	EGCG		
Var. *sinensis*[1]	0.07	1.13	2.38	1.35	8.59	1.21	2.78
Var. *assamica*[1]	0.02	1.44	0.35	3.35	12.10	1.43	2.44
C. taliensis	trace	0.58	0.80	1.90	6.84	0.27	2.54
C. irrawadiensis	0.03	0.72	0.12	0.67	0.21	0.21	0.00

[1] Variety belongs to *Camellia sinensis*

4.2.6 Other chemical components in tea

4.2.6.1 Alkaloids

The study of tea chemistry may be said to have begun with the isolation of the alkaloid caffeine (figure 6) from tea in 1827 (Bradfield, 1946). Tea has been valued historically for its caffeine content, which is between 2 and 5% (w/w) depending on variety. High caffeine content in fresh leaf may be one of the important factors ensuring good quality of the resulting black tea (Bhatia, 1964). Caffeine is regarded as an important constituent of tea, bestowing mood and cognitive-enhancing properties (Bokuchava and Skobeleva, 1969; Chow and Kramer, 1990). In Kenya, caffeine is used as an important quality parameter for the evaluation of plain black tea quality (Owuor *et al.*, 1986). The quantity of caffeine infused into a tea brew is determined by the infusion time and by the leaf style (Harbowy and Balentine, 1997). In green tea, the infusion is slightly affected by tea clones, but significantly affected by the temperature and infusion time (Yao *et al.*, 1992). The temperature and time also affects caffeine infusion in black tea, with water volume being significant (Yao *et al.*, 1993). Thus, the actual content of caffeine in a brew depends on many factors, mainly the method of brewing. No significant differences in caffeine levels have been found when brewing green and black teas under similar conditions (Hicks, Hsieh, and Bell, 1996), discrediting the theory that withering and fermentation have a significant impact on caffeine content (Sanderson, 1972).

Owuor (1987) suggested that seasonal, genetic, agronomic and cultural factors, as well as processing practices might influence the

caffeine content of made teas to some extent. In Argentina, caffeine content in tea leaf decreased gradually during most of the season after an early rapid increase (Malec and Vigo, 1988). In central Africa, the highest level of caffeine was found during the peak harvesting season when shoot growth rate was most rapid (Cloughley, 1982). In addition, studies on Assam tea by Wood *et al.* (1964a) showed that caffeine content decreased progressively through the season. Shoot maturity, variety, season, fertilizers, pruning, processing, grading and location have effects on the caffeine content of tea (Dev Choudhury, Rahman, and Barbora, 1991). Caffeine levels vary from 5.30 % for 1 bud 1 leaf; to 4.20 % for 1 bud 2 leaves; to 3.80 % for 1 bud 3 leaves; and to 3.20 % for 1 bud 4 leaves (Dev Choudhury *et al.*, 1991). Thus, the caffeine content decreases as the leaf ages or matures. Accordingly, teas made from pruned shoots have higher caffeine contents because of more young tender shoots. During withering and the entire processing of black tea, caffeine content increases marginally. Caffeine of an infused brew is responsible for the briskness of the tea liquor; this is due to its association with theaflavins.

Caffeine is one of the most comprehensively studied ingredients in food. Caffeine acts as a diuretic, cardiac muscle stimulant, central nervous system stimulant, smooth muscle relaxant, gastric acid secretion stimulant, elevates plasma free fatty acids and glucose (Harbowy and Balentine, 1997). The caffeine content of a typical tea beverage ranges 20-70 mg/170 ml of infusion, with the infusion being prepared from 2-2.5 g of tea leaves, while a coffee brews typically contain 40-155 mg caffeine/170 ml beverage (Harbowy and Balentine). Caffeine is absorbed rapidly and reaches peak levels in the body about 1 h after ingestion (Graham, 1978).

Theobromine (figure 6) and theophylline are another two alkaloids that occur in tea, but are present in much lower quantities than caffeine. In Assam black tea, alkaloids were estimated on a dry basis (w/w) as 1-5 % caffeine, 0.05 % theobromine, 0.0002-0.0004 % theophylline (Stagg and Millin, 1975). This xanthine content of teas is an area that may require further and more careful research.

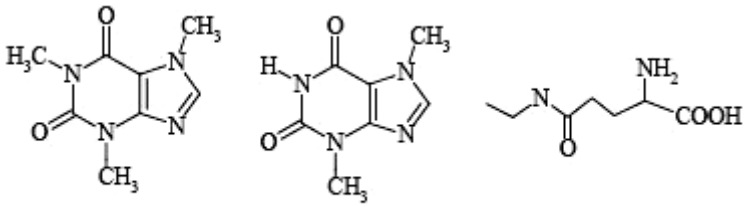


Figure 6 Structures of some nitrogenous tea phytochemicals (Adapted from Harbowy and Balentine, 1997).

5. Freeze drying

Freeze drying (also known as lyophilization) is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport. Freeze drying works by freezing the material and then reducing the surrounding pressure and adding enough heat to allow the frozen water in the material to sublime directly from the solid phase to gas (Harris and Angal, 1989).

5.1 Freeze-drying process

There are three stages in the complete freeze-drying process: freezing, primary drying, and secondary drying (Harris and Angal, 1989).

5.1.1 Freezing

The freezing process consists of freezing the material. In a lab, this is often done by placing the material in a freeze-drying flask and rotating the flask in a bath, called a shell freezer, which is cooled by mechanical refrigeration, dry ice and methanol, or liquid nitrogen. On a larger-scale, freezing is usually done using a freeze-drying machine. In this step, it is important to freeze the material at a temperature below the eutectic point of the material. Since the eutectic point occurs at the lowest temperature where the solid and liquid phase of the material can coexist, freezing the material at a temperature below this point ensures that sublimation rather than melting will occur in the following steps. Larger crystals are easier to freeze dry. To produce larger crystals the product should be frozen slowly or can be cycled up

and down in temperature. This cycling process is called annealing (Harris and Angal, 1989).

5.1.2 Primary drying

During the primary drying phase the pressure is lowered and enough heat is supplied to the material for the water to sublimate. The amount of heat necessary can be calculated using the sublimating molecules' latent heat of sublimation. In this initial drying phase, about 98% of the water in the material is sublimated. This phase may be slow, because if too much heat is added the material's structure could be altered (Harris and Angal, 1989).

In this phase, pressure is controlled through the application of partial vacuum. The vacuum speeds sublimation making it useful as a deliberate drying process. Furthermore, a cold condenser chamber and/or condenser plates provide a surface(s), on which the water vapor can re-solidify. This condenser does not play a role in keeping the material frozen; rather, it prevents water vapor from reaching the vacuum pump, which could degrade the pump's performance. Condenser temperatures are typically below −50 °C (Harris and Angal).

5.1.3 Secondary Drying

The secondary drying phase aims to sublimate the water molecules that are adsorbed during the freezing process, since the mobile water molecules were sublimated in the primary drying phase. This part of the freeze-drying process is governed by the material's adsorption isotherms. In this phase, the temperature is raised even higher than in the primary drying phase to break any physico-chemical interactions that have formed between the water molecules and the frozen material. Usually the pressure is also lowered in this stage to encourage sublimation. However, there are products that benefit from increased pressure as well (Harris and Angal, 1989).

After the freeze-drying process is complete, the vacuum is usually broken with an inert gas, such as nitrogen, before the material is sealed (Harris and Angal).

5.2 Properties of Freeze-dried Products

If a freeze-dried substance is sealed to prevent the reabsorption of moisture, the substance may be stored at room temperature without refrigeration, and be protected against spoilage for many years. Preservation is possible because the greatly reduced water content that results inhibits the action of microorganisms and enzymes that would normally spoil or degrade the substance (Kennedy and Joaquim, 1993).

Freeze-drying also causes less damage to the substance than other dehydration methods using higher temperatures. Freeze-drying does not usually cause shrinkage or toughening of the material being dried. In addition, flavors and smells generally remain unchanged making the process popular for preserving food. Unfortunately, water is not the only chemical capable of sublimation and the loss of other volatile compounds such as acetic acid (vinegar) and alcohols can yield undesirable results (Kennedy and Joaquim).

Freeze-dried products can be rehydrated (reconstituted) much more quickly and easily because it leaves microscopic pores. The pores are created by the ice crystals that sublimate, leaving gaps or pores in its place. This is especially important when it comes to pharmaceutical uses. Lyophilization can also be used to increase the shelf life of some pharmaceuticals for many years (Kennedy and Joaquim).

5.3 Freeze-drying Equipment

There are essentially three categories of freeze dryers: rotary evaporators, manifold freeze dryers, and tray freeze dryers (Harris and Angal, 1989).

Rotary freeze dryers are usually used with liquid products, such as pharmaceutical solutions and tissue extracts (Harris and Angal).

Manifold freeze dryers are usually used when drying a large amount of small containers and the product will be used in a short period. A manifold dryer will dry the product to less than 5% moisture content. Without heat, only primary drying (removal of the unbound water) can be achieved. A heater needs to be added for secondary drying, which will remove the bound water and will produce lower moisture content (Harris and Angal).

Tray freeze dryers are more sophisticated and are used to dry a variety of materials. A tray freeze dryer is used to produce the driest product for long-term storage. A tray freeze dryer allows the product to be frozen in place and performs both primary (unbound water removal) and secondary (bound water removal) freeze drying, thus producing the driest possible product. Tray freeze dryers can dry product in bulk or in vials. When drying in vials, the freeze dryer is supplied with a stoppering mechanism that allows a stopper to be pressed into place sealing the vial before it is exposed to the atmosphere. This is used for long-term storage, such as vaccines (Harris and Angal).

6. Mechanisms of percutaneous absorption

The skin is a highly organized, heterogeneous, and multilayered organ. The sum total of the various layers forming the epidermis and dermis, together with its appendages and underlying microvasculature, constitute a living envelope surrounding the body. Until recently, skin absorption studies focused to a great extent on physico-chemical and biophysical factors. The permeability coefficient (K_p) is a key parameter in estimating percutaneous absorption.

6.1 Structure and function of skin

The general anatomy and morphology of the skin have been well characterized. Several detailed reviews of the physical nature of the skin are available (e.g., Marks, Barton, and Edwards, eds., 1988). However, percutaneous absorption is highly influenced by the microstructure and biochemical composition of the skin.

The skin is composed of two layers: the epidermis, a nonvascular layer about 100 μm thick, and the dermis, a highly vascularized layer about 500 to 3,000 μm thick. The outermost layer of the epidermis, the stratum corneum is about 10-40 μm thick. This layer is thought to provide the major barrier to the absorption into the circulation of most substance deposited on the skin surface. It is composed of dead, partially desiccated, and keratinized epidermal cells. Below this layer lies the viable epidermis, a region about 50-100 μm thick, containing at its base the germinative or basal cell layer whose cells move outward to replace the outer epidermis as it wears away. This layer generates about one new cell layer per day, which results in the stratum corneum beaming totally replaced once every two to three weeks. The viable epidermis contains enzymes that metabolize certain penetrant substances. Enzymes may also be active in the stratum corneum (Marzulli, 1962; Marzulli and Maibach, 1984), if cofactors are not required.

Below the epidermis lies the dermis, a collagenous, hydrous tissue. The hair follicles and sweat ducts (skin appendages) originate deep within the dermis and terminate at the external surface of the epidermis. These occupy only about 1% of the total skin surface, and therefore their role as transport channels for the passage of substances from the external environment to the capillary bed is thought to be negligible for most chemicals (Scheuplein and Blank, 1971). The structure of the skin is shown diagrammatically in figure 7.

As mentioned above, the stratum corneum is generally considered the rate-limiting diffusion barrier for most compounds. Because of the importance of this layer in determining the rate and extent of percutaneous absorption, the following discussion will focus on its structure and function.

Michaels, Chandrasekaran, and Shaw (1975) described the stratum corneum as a heterogeneous structure containing about 40% protein (primarily keratin), 15% to 20% lipids, and 40% water. Lipids in the stratum corneum exist principally in the form of triglycerides, fatty acids, cholesterol, and phospholipids. Michaels *et al.* (1975) conceptualized the stratum corneum as being composed of parallel arrays of proteinaceous cells separated by thin layers of lipoidal material in a “bricks and mortar” arrangement (figure 8).

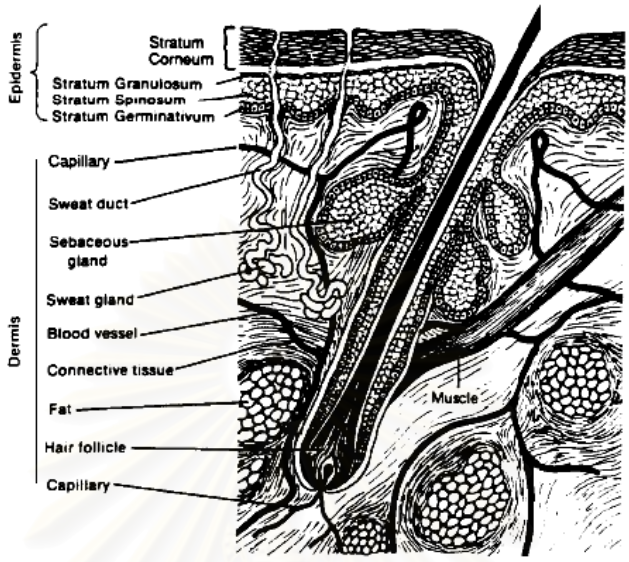


Figure 7 Structure of the skin (Casarett and Doull, 1986).

Raykar, Fung, and Anderson (1988) reported that the lipid content of dry stratum corneum in 35 human skin samples ranged from 3 % to 46% depending on skin site and the individual. Using a factor of four to convert dry to hydrated stratum corneum, the range is 1 % to 11%. This wide range is important with regard to the role of lipophilicity in storage, membrane functions, and skin site.

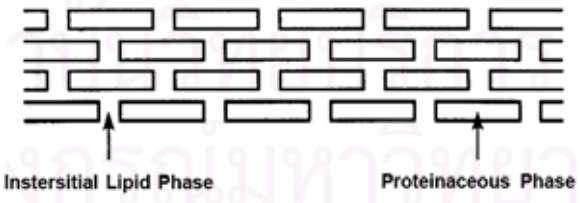


Figure 8 Two-phase model of the stratum corneum (Michaels *et al.*, 1975).

7. Factors that influence percutaneous absorption

Our understanding of skin absorption is largely derived from in vivo and in vitro experiments. The rate and amount of percutaneous absorption of a compound

depend highly on both the physiologic characteristics of the skin and the physico-chemical nature of the compound that is exposed to the skin. This section reviews how skin-specific factors (e.g., skin thickness, hydration, and temperature) and compound-specific factors (e.g., lipophilicity, polarity, volatility, and volubility) are involved in determining the rate and amount of absorption by the cutaneous route.

7.1 Skin-specific factors

As discussed below, a number of factors (e.g., species, gender, age, site of application, and the condition of the skin, i.e., degree of hydration and temperature) can have a marked effect on the extent and rate of percutaneous absorption.

7.1.1 Site of application or exposure

However, as reported by Feldmann and Maibach (1967), the extent of absorption of a compound such as hydrocortisone in humans is dependent on the anatomical site to which the compound is applied. Feldmann and Maibach later extended their investigation to include pesticides (Maibach *et al*., 1971). A marked variation exists in the dose of parathion and malathion absorbed at different anatomical sites in humans.

As expected, the percutaneous absorption of compounds also demonstrates regional variation in experimental animal species. Franklin, Somers, and Chu (1989) have shown that the absorption of a series of pesticides applied to the foreheads of *rhesus* monkeys was approximately twice that observed when the compound was applied to the forearms of these animals.

Although gender-related permeability differences have not been measured directly in humans, animal data that demonstrate gender differences are frequently noted in toxicity studies, and these differences are taken into account when extrapolating animal toxicity to humans. Any regional permeability differences that are observed may be due to the gender- and site-related differences in the thickness of the stratum corneum and/or whole skin. However, a competent stratum corneum is

expected to provide better barrier capacity than a thick, disorganized stratum corneum. Thus, thickness is not the only regional variation factor in skin permeability.

7.1.2 Age of skin

Infants and children represent a population at high risk for the toxic effects of environmental pollutants because of, among other reasons, their immature detoxification pathways and rapidly developing nervous systems. Infants and children are also at increased risk for dermal exposure to toxic compounds, because of their greater surface-to-volume ratio. Reports of toxic effects occurring in infants after the topical application of various drugs or pharmaceutical agents are not common in the literature. These toxic effects, however, are most likely the result of the increased surface-to volume ratio in infants resulting in greater total absorption of the compound, rather than to the increased permeability of the skin of infants relative to adults Full-term infants have been shown to have a completely functional stratum corneum with excellent barrier properties (Atherton and Rook, 1986).

7.1.3 Skin condition

For most compounds, the rate of percutaneous absorption is limited by diffusion through the stratum corneum. However, the epidermal barrier may not be intact in diseased or damaged skin. Persons with diseased or damaged skin may be at special risk for the toxic effects of environmental pollutants because of increased percutaneous absorption. Damage to the skin may occur from mechanical injury (cuts, wounds, abrasions) or other insults such as sunburn. Any skin condition that compromises the capability of the stratum corneum to serve as a permeability barrier, including psoriasis, eczema, rashes, or dermatitis, may also result in increased percutaneous absorption in affected individuals (Brown, Bishop, and Rowan, 1984).

7.1.3.1 Hydration

As discussed previously, the thickness of the stratum corneum is a major determinant of the dermal permeation. The permeability is

inversely proportional to the thickness of the stratum corneum. However, thickness of the stratum corneum *in vivo* and *in vitro* is positively correlated with the relative environmental humidity and degree of hydration of this layer.

Therefore, one would expect well-hydrated skin to be less permeable than relatively dry skin because of its increased thickness. This, however, is not generally the case. As a rule, hydration increases the permeability of skin for most compounds. Therefore, there is an increased potential for percutaneous absorption of environmental pollutants in scenarios such as bathing, swimming, or showering where the skin is well hydrated.

7.1.3.2 Circulation to the skin

Prolonged skin exposure to organic solvents is known to result in vasodilatation in areas that are exposed to these compounds. If the rate of chemical accumulation in the epidermis (via diffusion across the stratum corneum) is equal to or greater than the circulatory perfusion rate, then the rate-limiting step for skin permeation could become that of capillary transfer. The relationship between the rates of capillary transfer and diffusion can be described by the following equation (Scheuplein and Blank, 1971):

$$-D\frac{dc}{dx} = \frac{\varnothing(C_t - C_c)}{L} \qquad \text{Eq.3}$$

Where,

D is an average membrane diffusion coefficient (cm^2/min).

dc/dx is a change in chemical concentration over the change in unit distance through the layers (mg/cm^2).

$\varnothing$ is a peripheral blood flow (ml/min), showering with warm water.

C_t is concentration of the diffusing compound in tissue adjacent to the capillary walls.

C_c is concentration (ng/ml) of the diffusing compound in capillary blood.

L is thickness (cm) of the capillaries below the stratum corneum.

The ratio of *Ø/L* represents the transfer coefficient (for the epidermal-dermal junction) into capillary circulation and, in practical terms, is inversely proportional to the resistance of capillary wall permeability. If this resistance were small relative to resistance to diffusion across the stratum corneum, then the latter would be the rate-limiting step. For all situations except those involving gases and small, highly lipophilic compounds, the diffusion resistance (across the stratum corneum) is likely to be substantially greater than capillary resistance. Thus, circulatory flow should not be rate limiting in most cases.

7.1.3.3 Skin temperature

Humans, who are exposed to ambient or drinking water supplies during activities such as bathing, showering, or swimming may differ markedly in skin surface temperature. Therefore, it is important to consider the potential impact that water temperature may have on the rate or extent of percutaneous absorption of the compound of interest. To mimic the physiological state, the receptor solution is maintained at 37 °C.

Frequently, in the absence of skin damage, a lo-fold increase in temperature results in a doubling of skin permeability. Depending on the magnitude of activation energy for diffusion, an increase in temperature may result in a different extent of increase in skin permeability.

7.1.3.4 Miscellaneous factors

In addition to the variables discussed in this section, there are several other factors that may affect the rate and degree of skin penetration, including the release rate of the compound from the vehicle, in which it is formulated and multiple vs single-dose application (Wester and Maibach, 1983).

7.2 Compound-specific factors

In addition to the skin-specific factors discussed above, the physico-chemical nature of the penetrant compound also plays a role in the rate and extent of absorption of that compound. These factors are reviewed below.

7.2.1 Partition coefficients

The best penetrants are those that are soluble in both lipids and water, whereas compounds that are largely soluble only in either lipids or water, but not both, are not as good penetrants. The relative volubility of a compound in an organic or water phase can be represented by a partition coefficient. Several investigators have attempted to demonstrate a correlation between percutaneous absorption and partitioning behavior. K_p values tend to increase with increasing lipophilicity. This relationship also exists for compounds such as steroids (Scheuplein *et al*., 1969).

7.2.2 Polarity

The capacity of a substance to penetrate the skin is at least partially dependent on the polarity of a compound; that is, the extent to which the substance, at a molecular level, is associated with a nonsymmetrical distribution of electron density. Polar compounds are generally poorly absorbed through the skin, whereas nonpolar compounds are more readily absorbed. The extent of the polarity of a molecule can be expressed quantitatively through its dipole moment, which is a function of the magnitude of the partial charges on the molecule and the distance between the charges. The degree of polarity associated with a molecule is a function of spacing and proportion of electronegative atoms (e.g., nitrogen, oxygen, and fluorine), particularly if they are ionizable, vs the occurrence of non-electronegative atoms (e.g., hydrogen, carbon). Thus, placing an electronegative functional group on a nonpolar compound will increase its polarity, but in many cases, the molecular size and structure will also determine a compound's polarity. The greater the polarity of a

compound, the lower is the lipophilicity; lipophilicity can most readily be measured based on partition coefficients (see above).

The polar compounds (i.e., those least able to penetrate the skin) are those that spontaneously dissociate to form ions in an aqueous environment; such compounds are referred to as electrolytes. Electrolytes can be inorganic salts, which are readily dissociated, or weak organic acids and bases, whose state and extent of ionization depend on the pH of the environment. Weak organic acids or bases in their non-ionized form are much more soluble in lipids and are absorbed more readily through the skin than when in their ionized forms. Generally, the smaller the pKa for an acid and the larger the pKa for a base, the more extensive will be the dissociation in aqueous environments at normal pH values, and the greater will be the electrolytic nature of the compound. Thus, the potential for absorption through the skin can be at least qualitatively determined from the ratio of the ionized to unionized compounds as defined by the Henderson-Hasselbach equation:

$$pH = pKa + \log \frac{[ionized]}{[unionized]} \qquad \text{Eq.4}$$

Several investigators have shown that electrolytes in dilute solution (and therefore in the ionized form) penetrate the skin poorly. It is interesting to note that small ions such as sodium, potassium, bromine, and aluminum penetrate the skin with permeability constants of about 10^{-3} cm/h, similar to the rate reported by Scheuplein (1965) for water. Wahlberg (1968) and Skog and Wahlberg (1964) reported similar results for the chloride salts of cobalt, zinc, cadmium, and mercury; sodium chromate; and silver nitrate applied to guinea pig skin *in vivo* or *in vitro*. In addition, if the pH value of the applied solution results in very acidic or alkaline conditions on the skin, there is a potential to increase the rate of absorption of a compound because of destruction of the barrier layer (Zatz, 1983).

7.2.3 Chemical structure

Changes in chemical structure across a series of homologous compounds have the potential to alter the permeability characteristics of these

compounds. For example, Blank, Scheuplein, and MacFarlane (1967) demonstrated the effect of increasing chain length on the permeability coefficient of aqueous solutions of normal alcohols. This change in K_p is most likely a result of the increase in lipophilicity.

Schaefer *et al*. (1987) also have shown how minor modifications in chemical structure can markedly alter the percutaneous absorption of a series of closely related androgens. For example, the addition of two hydrogen molecules to a double bond in the A ring of testosterone to yield dihydrotestosterone, results in a 30-fold decrease in the relative absorption of the latter compound over the former.

For compounds of molecular weight of 400 or more, the molecular size and weight of a compound appear to have less of an effect on the rate or extent of percutaneous absorption than lipophilicity. Large macromolecules penetrate skin slowly because of a combination of molecular size and poor lipid volubility. Summarizing the work of several researchers, Grasso and Lansdown (1972) noted that macromolecules such as colloidal sulfur, albumin, dextran, and polypeptides penetrate the skin poorly if applied in an aqueous solvent. However, these macromolecules will permeate the skin more readily if applied in a solvent with high lipid volubility.

7.2.4 Volatility

Volatilization of the compound will alter the amount on the skin surface available for absorption, estimates of percutaneous uptake should account for this loss process.

Volatilization can be prevented in experimental studies by the application of occlusive wraps or devices over the site of compound exposure. However, occlusion generally results in enhanced absorption of the test compound.

7.2.5 Compound concentration

A major determinant of the amount of a compound absorbed across the skin is the concentration or the amount of the compound at the skin surface.

Wester and Maibach (1976) demonstrated that the total amount of a compound absorbed increases as a function of the applied amount per unit area. Taylor (1961) estimated that at least 1 mg/cm2 liquid must be applied to fill the holes in surface of skin. Above this amount, an applied liquid forms a pool on the skin surface.

Furthermore, when Fick's first law of diffusion is applicable, skin penetration at steady state is proportional to the concentration (driving force) of the penetrant (Tregear, 1966). Fick's first law does not apply when the penetrant damages the skin.

Liron and Cohen (1984) reported that the penetration of propionic acid from *n* –hexane solution through porcine skin in vitro was relatively high at higher concentrations. The authors postulate that this effect may be the result of a breakdown of the skin barrier by exposure to the acid used in the study.

The skin barrier can also be damaged by the delipidizing effect of organic solvents. Numerous investigators (Scheuplein and Blank, 1973; Roberts *et al*., 1977; Baranowska-Dutkiewicz, 1982; Behl *et al*., 1983; Huq *et al*., 1986) demonstrated increased flux rates for various compounds, which dissolved in organic solvents across both human and animal skin relative to the permeability of more diluted aqueous solutions of the same compounds. Each of these researchers attributed this increased permeability to the delipidization and subsequent damage of the stratum corneum.

8. *In vitro* diffusion study

In recent years, it has been shown that the skin is a useful route for drug delivery to the systemic circulation. Advances in the sciences of drug design, drug delivery technology, penetration enhancement and topical vehicle formulation have allowed the stratum corneum, classically considered a total barrier to the ingress of chemical substances, to be used as a portal for the delivery of a selected group of drugs.

The stratum corneum is the principal barrier for cutaneous penetration and allows only slow absorption for the majority of drugs. In any case, the use of

appropriate vehicles allows drug absorption to be increased by changing either the permeability of the stratum corneum (Roberts and Anderson, 1975) or the thermodynamic activity of the drug (Schaefer *et al.*, 1978). In this respect, the best vehicle for topical controlled release would be the one, which contributes to a reversible decrease in the stratum corneum resistance and allows the controlled diffusion of molecules into the vehicle itself.

The increased interest in topical drug delivery systems has necessitated the development of new experimental procedures for the assessment of these products. Optimally, the testing of topical delivery systems should be performed *in vivo*. This is often impossible, especially in the developmental stages when the toxicity or irritancy of new drugs, excipients or devices may not be documented.

Bronaugh and Maibach (1983) reported advantages of using *in vitro* methods for obtaining percutaneous absorption rates. For example, these techniques permit an investigation of percutaneous absorption separate from other pharmacokinetic factors that affect cutaneous uptake; larger numbers of assays; sampling directly under the skin; and measurements of the permeability of highly toxic compounds using human tissues. Furthermore, *in vitro* techniques are rapid, inexpensive, and easy to perform.

The main objective of the *in vitro* experimentation is to simulate diffusion conditions in human, thus obviating the requirement for *in vivo* research using humans or animals. Where human or animal skin is difficult to obtain, or where a large number of experiments are to be carried out, particularly with regard to pre-formulation screening experiments, synthetic membranes have been employed widely.

The advantage of utilizing a diffusion cell is that the experimental conditions (i.e. normalization of baseline and temperature) are controlled, and thus yield results that are more precise. Furthermore, direct measurement of an analyte may be obtained in this kind of study. Most often, the advantage of the *in vitro* methodology is that *in vivo* measurements cannot easily be made. However, in the *in vitro* study, the skin persistence measurement can easily to be conducted *in vivo*, but does not correspond directly to the percutaneous absorption. Both biological and synthetic membranes have been investigated for potential usefulness in the *in vitro* test systems. These membranes range in barrier function from negligible resistivities to permeabilities

approximating that of human skin. Generally, membranes from animal sources tend to have higher resistivities to drug diffusion compared with synthetic membranes, probably because of the more complex biochemical composition of the former. Accordingly, laboratory test systems require a membrane to mimic the barrier function of the stratum corneum. Although, percutaneous absorption experiments give interesting and valuable information but skin availability and the risks related with its use is a motivation to make preliminary studies with artificial membranes. Therefore, *in vitro* experimental procedures have become increasingly important in this field because of the multitude of problems associated with *in vivo* protocols.

8.1 *In vitro* apparatus: Diffusion cells

In vitro percutaneous absorption studies are most often carried out using diffusion cells (e.g., glass, teflon, stainless steel). The various types of diffusion cells have recently been reviewed (Franz, 1990). A wide variety of diffusion cell apparatus has been employed to measure drug release and percutaneous penetration *in vitro*.

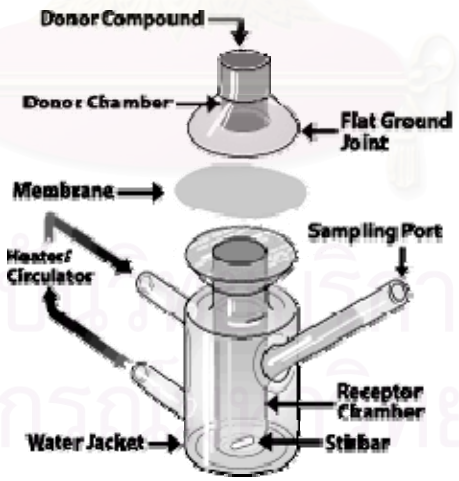


Figure 9 A side view of a modified Franz diffusion cell (adapted from Franz, 1990).

Despite variations in design, most cells have several common features. They generally employ two chambers, one containing the active formulation, and the other, stirred, chamber containing the receptor solution. The two chambers are separated by the rate-limiting barrier, usually excised skin or a synthetic membrane.

Cells are commonly arranged side-by-side or vertically. In side-by-side chambers, both cells are usually stirred independently.

The vertical Franz diffusion cell (Franz, 1975) is a commonly used apparatus for evaluating transdermal delivery. It is comprised of two major compartments, a donor chamber and a receptor chamber (figure 9 and figure 10).

Figure 10 A side view of a joint adapter-jacketed modified Franz diffusion cell (adapted from Franz, 1975).

Stirring of the receptor cell is essential to remove or minimize the unstirred static diffusion boundary layers and to avoid high local concentrations of drug. It often carried out by using magnetic bar coupled with magnetic stirrer (figure 11). Maintenance of sink conditions is also paramount for accurate *in vitro* permeation studies.

Temperature control is essential for accurate *in vitro* studies, and is usually maintained by the use a controlling temperature water-bath flow-through the surrounding water-jackets of diffusion chambers or immersion in the water bath.

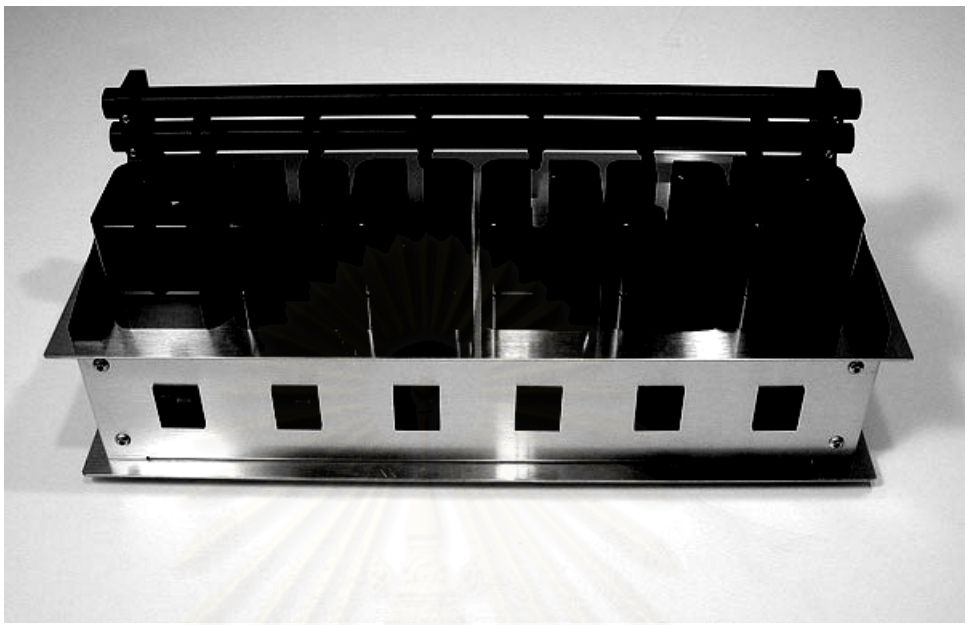


Figure 11 A top-side view of a 6-stationed stand stirrers.

8.2 Design experiment

Generally, this type of study assumes an infinite sink condition in the receptor fluid, negligible donor phase depletion and a homogeneous membrane (Brain, Walters, and Watkinson, 1999). In order to achieve these assumptions, a well-design experiment should be constructed.

Diffusion cells without a rate limiting membrane are generally designed to investigate the characteristics of the drug release from topical formulations (Smith and Haigh, 1989).The *in vitro* tests have limited applicability for estimating the complex process of percutaneous absorption but are useful as screening tools for drug release.

8.2.1 Diffusion membrane

The release rate of drugs from topical preparations depends directly on the physical and chemical properties of the vehicle and drug employed. The physiological availability of topically applied drugs is dependent on both the release rate from the vehicle and the permeability through the skin. The type of membrane utilized for the *in vitro* evaluation of drug release is of primary

significance since permeability depends on the nature of membrane materials. Synthetic membranes are commonly employed for the *in vitro* diffusion studies due to their accessibility and reproducibility, although many investigators use excised skin to measure permeability. The variety of compounds examined illustrates the scope of the *in vitro* diffusion studies through synthetic membranes.

The most commonly used artificial membranes are polydimethylsiloxane (PDMS) and cellulose acetate (porous dialysis tubing) (Kurosaki *et al*., 1991; Megrab, Williams, and Barry, 1995a, 1995b; Stott, Williams, and Barry, 1996; Van Hal *et al.*, 1996; Esposito *et al*., 1998; Woolfson, McCafferty, and Moss, 1998; Minghetti *et al*., 1999). Cellulose acetate membranes have similarly found use in such experiments and in the characterization of iontophoretic delivery (Cichon and Janicki, 1991; Yuk *et al*., 1991; Foley, Corish, and Corrigan, 1992; Bayon, Corish, and Corrigan, 1993; Ramis *et al*., 1997). However, these membranes have often been shown to overestimate significantly the flux across skin and their use is significantly limited. Recently, a study of the release of caffeine from concentrated emulsion on different synthetic membranes showed the importance of the choice of the membrane, which will allow a good differentiation of different formulations of CEs, to use it later in screening experiments (Clément *et al*., 2000). The hydrophilic polysulfone membrane was the only one that allows statistical differentiation of the different formulations. As many beneficial evidences above seem possible to utilize this synthetic membrane as the diffusion membrane to make screening on different CEs.

8.2.2 Receptor fluid

The method of analysis used to detect the penetrant should cause few issues of variability. Such a method should be able to allow full extraction of the penetrant from the receptor phase. Thus, issues of receptor solvent choice are important. In fact, the nature of the receptor phase should mimic as closely as is realistic, within experimental constraints, the ultimate receptor sink in the body. A number of other factors should also be represented including the systemic circulation, and important processes within the skin that can affect the ability of an exogenous

chemical to reach the circulation, such as active transport or metabolism within the skin. Somewhat idealistically, the most representative receptor fluid may be either human or animal blood.

In practical, the use of additives to the receptor phase in order to enhance flux *in vitro* is potentially an unrealistic element of experimental design, which may affect flux in the laboratory, and could make correlations to *in vivo* experiments more difficult to estimate.

To obtain an infinite sink capacity, it is crucial to select the proper receptor fluid. For a hydrophilic analyte, phosphate buffered saline, pH 7.4, is commonly used in the receptor phase. In contrast, when the analyte is lipophilic, modifying of the receptor fluid is recommended in order to achieve an adequate solubility. A non-ionic surfactant such as polyethylene glycol oleyl-ether (Oleth-20), solvents such as methanol, ethanol, or polyethylene glycol and a physiological compound such as serum or albumin could be added to improve solubility (Bronaugh and Swart, 1985).

The composition of the receptor phase appears to be very important in influencing percutaneous penetration, not just by its volume but also its content. For example, Kasting, Smith, and Cooper (1987) employed sodium azide (0.02%, w/w), as an antimicrobial agent, and ethanol (50%, v/v), to ensure solubility of their penetrant in the receptor phase. Dal Pozzo *et al*. (1991) suggested that incorporation of BSA into the receptor phase would ensure a more efficient collection of the penetrant in the receptor phase. This method was also employed by Sartorelli *et al*. (1998) who incorporated BSA (4%, w/w) and gentamicin sulphate into their receptor phase.

Further, Lin, Hsu, and Chen (1996) employed a sodium acetate receptor phase buffered at pH 6, ostensive to ensure adequate solubilization of their penetrant.

Since the *in vitro* diffusion study is mentioned only the release of active molecule from inner formulation to outer environment that is the skin surface. The receptor fluid, therefore, is supposed to be analogous to the skin condition. In generally, the epidermis is the outermost layer of the skin. It has two

layers: the outer epidermis and the germinativum. The outer epidermis protects the skin from several harsh environments and manages its own acidity and pH balance, pH 4.5-5.5. Maintaining the skin pH factor helps maintain a proper balance of the acid covering, which aids in protecting the body from bacteria and helps prevent moisture loss. Consequently, pH of the receptor fluid considers to be closely related with the skin pH, ranging between pH 4.5-5.5 is recommended.

8.2.3 Receptor temperature

The effect of skin temperature of percutaneous absorption is well-understood (Barry, 1983; Woolfson and McCafferty, 1993). Normally, but not universally, in skin delivery experiments the receptor cell temperature is maintained at the body temperature, 37±1 °C and the skin temperature, 32±1 °C. Inefficient or incomplete heating may create temperature gradients, affecting flux (Friend, 1992). To simulate *in vivo* conditions, diffusion membrane is mounted between the two chambers while the receptor temperature is controlled at 32 ºC, resulting in a skin surface temperature between 30-32 ºC (Gettings, Howes, and Walters, 1999).

8.2.4 Measurement of the penetrant

Penetrant concentration is determined *in vitro* by measurement of the concentration in the receptor or donor chambers at given time intervals, yielding information concerning the steady state flux and lag times of penetrants. The penetrants may be quantified by instrumental analysis, including chromatography, UV/visible and fluorescence detection, and by scintillation counting and specific assays (Nugent andWood, 1980).

8.2.5 Finite dose vs infinite dose

The finite dose experiment is generally employed for the measurement of percutaneous absorption. However, several authors have used infinite or side-by-side diffusion cells, for skin delivery experiments (Feldman and Maibach, 1969; Michaels *et al*., 1975). The differentiation between finite and infinite dose

experiments *in vitro* has been investigated by several researchers, and may be an important aspect of the accuracy and reliability of such data. Walters *et al*. (1997) investigated the percutaneous penetration of octyl salicylate *in vitro* in a range of representative cosmetic formulations. They demonstrated significant differences between K_p values in finite (6.6×10^{-7} cm/h) and infinite dose (4.7×10^{-6} cm/h) experiments when delivered from a hydroalcoholic lotion. Further, these values are significantly different from the predicted K_p value (1.35×10^{-7} cm/h). They also demonstrated the effect of formulation on K_p, by demonstrating that the apparent permeability coefficients of octyl salicylate from an oil-in-water emulsion was 1.7×10^{-5} cm/h and 6.6×10^{-7} cm/h for infinite and finite dose experiments, respectively.

8.2.6 Static vs flow-through cells

Several other researchers have examined the effect of the experimental method on the flux of model penetrants. Bronaugh and Maibach (1985) reported no significant difference in flux from static and flow-through cells. Similar findings have been reported by Hughes *et al*. (1993). A large variety of experimental methods has been employed in the percutaneous absorption literature. Hughes *et al*. compared flux from static (exposed surface area 0.32 cm^2) and flow-through cells (exposed surface area 0.64 cm^2) with bovine serum albumin (BSA) and gentamicin in the receptor phase. The authors determined that there were no differences in these systems but commented that data can vary significantly, particularly if the penetrant is absorbed well.

8.2.7 Perfusion of receptor phase: Sink condition

Nevertheless, such findings are perhaps unexpected, in that perfusion has been shown previously to increase the flux of a penetrant (Crutcher and Maibach, 1969). The authors observed that perfusion prevented the build-up of a significant concentration of the penetrant in the receptor phase, obviating most concerns pertaining to sink conditions (Martin, Swarbrick, and Cammarata, 1983).

The continual removal of the penetrant prevents a rise in its concentration in the receptor phase and prevents ‘‘sink conditions’’ occurring. This phenomenon can reduce the rate of penetration significantly, by reducing the concentration gradient across the diffusing membrane. Sink conditions exist when, in diffusion experiments, the receptor phase of the diffusion cell is constantly removed and replenished. This is done in order to keep the concentration of the penetrant at as low a concentration as possible in the receptor compartment. Without removal of the penetrant from the receptor compartment, its concentration will decrease in the donor compartment and increase in the receptor compartment until equilibrium is achieved, altering the rate of diffusion across a membrane.

In order to maintain the concentration gradient across the skin, flux may be increased by perfusion, regular sampling or replacement of the receptor solution, or by employing a larger receptor phase in the experimental design. For example, Martin *et al*. (1983) compared their receptor to the uptake of a penetrant by the dermal blood supply, which maintains a substantial concentration gradient across the skin.

8.2.8 Release models

There are several models, which can be used for description of the release profiles from controlled release systems. The choice of a specific model for the data set from a particular controlled release formulation depends on shape of the graphics and the underlying controlling mechanism.

8.2.8.1 Baker and Lonsdale equation (Higuchi's model for spherical matrices)

The Baker and Lonsdale equation (Baker and Lonsdale, 1974) which was derived from Higuchi's model (Higuchi, 1963) describes the drug release from sustained release microspheres:

$$\tfrac{3}{2}\left[1-\left(1-F\right)^{2/3}\right]-F=kt \qquad \text{Eq.5}$$

Where,

F is the fraction of drug released at any time t.

k is the constant that equal to $3DC_s/r_o^2C_o$.

D is the diffusion coefficient.

C_s is the drug solubility in the polymer.

r_o is the radius of the device.

C_o is the initial concentration of the drug in the polymer matrix.

The equation has been fitted to release data from various microspheres formulations (Jun and Lai, 1983; Leelarasamee *et al*., 1986; Chang, Price, and Whitworth, 1986; Shukla and Price, 1989, 1991; Duberuet *et al*., 1990). Linear fitting was usually used after calculating the quantity corresponding to the expression on the left side of the equation at each time point.

8.2.8.2 Peppas equation

The Peppas equation (Peppas, 1985) represents a general data fitting approach for drug release:

$$F = kt^n \qquad \text{Eq.6}$$

Where,

F is the fraction released at time t.

k is a constant incorporating structural and geometric characteristics of the controlled release device.

n is the release exponent, that may be used to indicate the mechanism of drug release.

This general semi-empirical equation is not based on a certain model, certain geometry or a single mechanism. It is usually used to analyze release data from polymeric devices, when the mechanism of release is not well known or when more than one type of release may be involved (Orienti and Zecchi, 1993; Franz *et al*., 1987).

8.2.8.3 Hixon and Crowell equation

Hixon and Crowell originally derived the following equation to describe the dissolution of solid particles (Hixon and Crowell, 1931).

$$1-\left(1-F\right)^{1/3}=kt \qquad \text{Eq.7}$$

Where,

F is the fraction dissolved at time t.

k is a constant.

This equation has been used by some researchers to describe the release of drugs from spherical matrices that have been compressed into tablets and satisfactory results were obtained (Touitou and Donbrow, 1982; Franz *et al*., 1987). The use of this model is based on the assumption that the rate of release is limited by the rate of dissolution of the drug particles and not by diffusion through the polymer matrix.

8.2.8.4 Higuchi equation of square root of time

The following equation was derived by Higuchi (1961) to describe the release of drugs diffusing through a planar system and it has occasionally been used to fit release data from some microspheres formulations.

$$F=k\sqrt{t} \qquad \text{Eq.8}$$

Where,

F is the fraction dissolved at time t.

k is the constant that equal to $(2ADC_s)^{½}/M_o$.

A is the surface area of the device.

D is the diffusion coefficient.

C_s is the solubility of the drug in the polymer.

M_o is the amount of the drug per unit area present initially in the system.

Although the use of this equation for spherical matrices does not reflect any single release mechanism, some data showed good fitting (Mortada *et al*., 1988). This may indicate the possibility of superposition of two mechanisms or more. In this case, it may correspond to an exponent n = 0.5 in the Peppas equation. A square root model may also describe the release from a monolithic solution in a spherical device, where the early time approximation results in a square root of time equation (Baker and Lonsdale, 1974).

8.2.8.5 First order equation

Occasionally, drug release data are fitted to a first-order decline model (Shah, De Gennaro, and Suryakasuma, 1987; Mortada *et al*., 1988). Since most microspheres consist of drug particles embedded in a polymer matrix, a first order release does not conform with a known mechanism for drug release from spherical matrices. One situation that can be described by first-order release kinetics is the nonconstant activity reservoir spherical device, where the drug solution is enclosed within a porous membrane through which diffusion occurs (Baker and Lonsdale, 1987).

For the fitting purpose, the first order release equation is

$$F = 1 - e^{-kt} \qquad \text{Eq.9}$$

Where,

F is the fraction dissolved at time t.

k is a constant.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. **ASTM Type II water (Ultra-pure Water), 18.2 MΩ or triple-distilled water or sterile water for injection (HPLC grade):** The water used in HPLC and sampling was prepared with an ultra-purifier water system with a resistivity over 18.2 MΩ (Maxima UF, ELGAStat) (ELGA Ltd., England) or purchased from local hospital
2. **Butylated hydroxytoluene (BHT)** (Analytical grade) (Sigma-Aldrich, USA)
3. **Citric acid** ($HOC(COOH)(CH_2COOH)_2.H_2O$) (Analytical grade) (MW = 210.14) (Ajax Finechem, Australia)
4. **De-ionized water (DI water) (Reagent grade):** The water used in common purpose was prepared by pre-filtration using in-house water purifier and further purified with a water purifier (ELGAStat Option 3B) (ELGA Ltd., England)
5. **Dichloromethane (DCM)** (CH_2Cl_2) (Analytical grade) (FW = 84.93) (Lab-Scan Asia Co., Ltd., Thailand) (Under license & Q.A. by Lab-Scan Ltd., Ireland)
6. **Ethylenedinitrilo tetraacetic acid, *di*-sodium salt (*di*-Na.EDTA)** (Pharmaceutical grade) (Sigma-Aldrich, Italy)
7. ***di*-Sodium hydrogen orthophosphate anhydrous** (Na_2HPO_4) (Analytical grade) (MW = 141.96) (Ajax Finechem, Australia)
8. **Dried green tea leaves:** (Namchai Tea, Doi Mae Salong, Thailand) were purchased from a local teashop in Thailand
9. **Emulsifying agent** (Pharmaceutical grade)

    9.1 **Cetyl dimethicone copolyol (Abil EM 90™)** was a gift from Goldschmidt, Germany

    9.2 **Sorbitan sesquioleate (Arlacel 83™)** (CAS 8007-43-0; Fp 113 °C (235 °F); d 0.989) (Sigma-Aldrich, USA)

    9.3 **Methyl glucose dioleate (Isolan DO™)** was a gift from Goldschmidt AG, Germany

9.4 **A mixture of sorbitan oleate, beeswax, hydrogenated castor oil, stearic acid (Montane 481 VG™)** was a gift from Adinop Co., Ltd., Thailand (Seppic, France)

9.5 **PEG-7 hydrogenated castor oil (Simulsol 989™)** was a gift from Adinop Co., Ltd., Thailand (Seppic, France)

10. **Freeze-dried green tea extract (FD-GTE)** was obtained from the infusion of grind-dried green tea leaves, provided by the present manufacturer as reported herein
11. **Glycerin (glycerol, *1,2,3*-propanetriol)** ($C_3H_8O_3$) (Pharmaceutical grade) (FW = 92.10) (Sigma-Aldrich, Germany)
12. **Isohexadecane (Arlamol HD™)** (Pharmaceutical grade) (Sigma-Aldrich, USA)
13. **Methanol** (CH_3OH) (Analytical grade) (FW = 32.04) (Lab-Scan Asia Co., Ltd., Thailand) (Under license & Q.A. by Lab-Scan Ltd., Ireland)
14. **Methanol** (CH_3OH) (HPLC grade) (FW = 32.04; Minimum Transmission Levels compared against HPLC Water at 210 nm, 60% Transmission) (Lab-Scan Asia Co., Ltd., Thailand) (Under license & Q.A. by Lab-Scan Ltd., Ireland)
15. **Methyl paraben (methyl-*p*-hydroxybenzoate) and propyl paraben (propyl-*p*-hydroxybenzoate)** for performing ready-to use preservatives (Carlo Erba, Italy): 10% methyl paraben–1% propyl paraben in **propylene glycol** (Pharmaceutical grade)
16. ***ortho*-Phosphoric acid**, 85% (H_3PO_4) (Analytical grade) (CAS 7664-38-2; FW = 98.00) (Mallinckrodt AR® (ACS), USA)
17. ***Poly (ethylene glycol) bis (carboxymethyl) ether** (Pharmaceutical grade) (CAS 39927-08-7; Fp 113 °C (235 °F); d 1.185) (Sigma-Aldrich, Germany)
18. **Reference standard green tea catechins;** (−)-epigallocatechin [(−)-EGC], (−)-epigallocatechin gallate [(−)-EGCG], (−)-epicatechin [(−)-EC], (−)-epicatechin gallate [(−)-ECG], were purchased from Sigma Chemical Co (Dorset, UK)
19. **Reference standard caffeine** was purchased from Sigma Chemical Co (Dorset, UK)

20. **Sodium Chloride volumetric standard, 0.1014 N Solution in Water** (Analytical grade) (CINa: FW 58.44; mp 801 °C; d 1) (Sigma-Aldrich, USA)
21. **1.09945 Titrisol® Sodium Chloride solution** (Analytical grade) ($_C$(NaCl) = 0.1 N (mol/l); 5.844 g NaCl for 1000 ml) (Merck, Germany)

EQUIPMENTS

1. **Analytical balance** (Sartorius Basic, BA 210S, S/N 21203485) (Scientific Promotion Co., Ltd., Thailand)
2. ***6-positions* Analytical balance** (Mettler) (Mettler Toledo Ltd., Thailand) (Mettler Toledo AG, Switzerland)
3. ***7-positions* Analytical balance** (Max. 2.1 g, d = 0.1 μg) (Mettler MT/UMT) (Mettler Toledo Ltd., Thailand) (Mettler Toledo AG, Switzerland)
4. **Bench shakers, horizontal motion** (GFL®, 3006) (GFL®, Germany)
5. **Freeze-dryer** (LyoLab w/PC, Lyophilization Systems Inc., USA)
6. **Glass pasteur pipettes**
7. **5 ml-Glass test tubes**
8. **Heating magnetic stirrer** (ARE, S/N 22665) (VELP® Scientifica, Italy)
9. **Heating water bath** (Memmert, Germany)
10. **High-Performance Liquid Chromatography (HPLC)** (Shimadzu, Japan) (Bara Scientific Co., Ltd., Thailand)

    10.1 **Liquid Chromatography:** LC-10AD*VP* (binary high pressure gradient mixing); with FCV-10AL*VP* gradient mixer

    10.2 **System controller:** SCL-10A*VP* **and chromatography workstation interface:** CLASS-*VP*™ (V. 6.14 SP1)

    10.3 **Photodiode-array UV-vis detector:** SPD-M10A*VP*

    10.4 **Sample auto injector:** SIL-10AD*VP*

    10.5 **Column oven:** CTO-10AS*VP*

    10.6 **Solvent degasser:** DGU-14A

11. **High-speed ultracentrifuge** (Max. 13400 min^{-1}) (Eppendorf® AG 22331, minispin, Model 5452, S/N 17156) (Mondotech Co., Ltd., Thailand) (Eppendorf™, Germany)
12. **Hot air oven** (Memmert, BE 200, S/N e201.0619) (Memmert, Germany)
13. **HPLC column compartment**

    13.1 **Column:**

    13.1.1 **Analytical column: Nova-Pak® C18**, Dimethyl-octadecylsilyl bonded amorphous silica bed, **60 Å, 4 µm, 3.9 mm x 150 mm** Steel column (Waters Corp., Ireland)

    13.2 **Guard column including guard column holder:**

    13.2.1 **Guard column: Waters Sentry™ guard column Nova-Pak® C18**, Dimethyl-octadecylsilyl bonded amorphous silica bed, **60 Å, 4 µm, 3.9 mm x 20 mm** (Waters Corp., Ireland)

    13.2.2 **Universal guard column holder:** (Waters Corp., Ireland)
14. **Injection vials and caps** (Waters Corp., Ireland)
15. **Laser light scattering spectroscopy** (Mastersizer S long bed version 2.11, Malvern Instruments Ltd., UK)
16. **Magnetic stirrer** (ARE, S/N 47819) (VELP® Scientifica, Europe)
17. **Micropipette: BP1000 (100-1000 µl), BP200 (20-200 µl) and BP20 (2-20 µl)** (BioPette A™) (Labnet International, Inc.), Gilson pipettes and tips
18. **Modified-Franz diffusion cells** (Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)
19. **Nylon membrane filters: 0.45 µm, 47 mm** (Whatman International Ltd., England)
20. **Overhead stirrer** (RW 10R, 0-2000 min^{-1}) (Janke & Kunkel, Kika® Labortechnik)

21. **pH meter, multi-parameter analyzer** (Consort®, C832T, S/N 73655) (Consort®, Belgium)
22. **Polysulfone membrane**, hydrophilic-synthetic membrane (Tuffryn® membrane filter; 47 mm, 0.45 µm, HT-450) (Gelman Laboratory, Pall Corp., USA)
23. **Refrigerated centrifuge** (Centurion, K3) (Centurion Scientific Ltd., Germany)
24. ***75 liter*-Reservoir water purifier** (ELGAStat Option 3B) (ELGA Ltd., England)
25. **Rotary evaporator** (Büchi Rotavapor, R-200) **including heating bath** (Büchi Heating bath, B-490) **and vacuum pump** (Büchi Vac, V-500) **with condenser** (Büchi , Switzerland)
26. **1.5 ml-Safe lock micro test tubes** (Eppendorf™, Germany)
27. **Scanning electron microscope** (SEM) (JSM-5410LV, JEOL Co., Ltd., Japan)
28. **Ultra-purifier water system** (Maxima UF, ELGAStat) (ELGA Ltd., England)
29. **Ultrasonic bath** (TranssonicDigital, D-78224) (Elma®, Germany)
30. **Viscometer** (Brookfield™ viscometer: Spindle ASTM7)
31. **Vortex mixing** (Vortex-Genie™, K-550-GE) (Scientific Industries, Inc., USA)

METHODS

1. Extraction of major components in dried green tea leaves

In general, green tea usually contains relatively simple polyphenols, mainly catechins, their catechin gallates and some residues of caffeine, so it is also relatively simple to measure the content of these components. Therefore, major components, provided by an extraction of dried green tea leaves, were mainly considered to green tea catechins (GTCs) together with caffeine.

The extraction of GTCs together with caffeine was performed by using hot water. After that, the next process was divided into two procedures: with and without solvent extraction using dichloromethane (DCM) (procedure A and B, respectively). The final process was a drying step using a freeze-drying technique. The extractions, with and without DCM solvent extraction, were all carried out in triplicates.

Since the moisture of crude drugs often varies from batch to batch, they, therefore, were dehydrated before utilizing to ensure a uniformity of moisture content. By using a hot air oven, green tea leaves were dried out at 110±1 °C for 1 h and then kept in a cool, dry and dark place until further processing. Figure 12 shows a schematic diagram of green tea extraction procedure.

Extraction with hot water

In order to improve efficiency of extraction, an extraction temperature was preferred at 80 °C, as recommended by most researchers (Roberts, Cartwright, and Wood, 1956; Wood, and Roberts, 1964). Accordingly, both of extraction procedures were performed by using de-ionized water (DI water) with maintaining temperature constantly at 80 °C.

Dried green tea leaves were ground to a fine powder with a mortar and pestle. Fifty grams of ground green tea leaves were immediately extracted with 1,000 ml of 80±1 °C DI water.

Approximately 250 ml of 80±1 °C DI water were poured over ground green tea leaves in a 2,000-ml flat-bottom Erlenmeyer flask. Maintaining temperature constantly at 80±1 °C, the whole mixture was stirred at 1,000 rpm for 10 min by using

a heat-controlled magnetic stirrer. The hot DI water extraction process was repeated four times to reach the final volume of 1,000 ml. The extraction times were 10+10+10 and 20 min, respectively. An extraction solution was kept stirring for 2 h. After that, the extracted solution was allowed to cool to room temperature and kept incubating over night in the dark place. The incubated mixture was filtered through cotton wool and the residues were washed with DI water (3×10 ml). Subsequently, the collected filtrate was centrifuged at 2,000 rpm at −10±1 °C for 30 min and then taken the supernatant. The supernatant was filtered under vacuum through 0.45 µm PTFE (Teflon™) filter membrane.

Solvent extraction with dichloromethane (Procedure B)

For the procedure A, this step was skipped to the next process. While, procedure B was followed by DCM solvent extraction process. The filtrate from the former step was introduced into a separating funnel and re-extracted with equal volume portion of DCM to remove caffeine and some residual components, if any, such as some pigments, aromatic compounds, etc. The beaker formerly contained some residual liquid were rinsed with 10 ml of DCM (3×10 ml). The rinsing was then added to the separating funnel. The whole mixture of solvent extraction in the funnel was then swirled gently (5×5 min) and the vapor produced was released after a few shakes. Some emulsions were broken up by agitation with a glass rod. The mixture in the funnel was allowed to separate for 10 min. Then, the DCM layer at lower part together with the emulsions, if any, was discarded. The aqueous layer left in the separating funnel was re-extracted twice with two equal volume portions of DCM. The three portions of aqueous fraction were collected and allowed to stand over night for completely separation in the dark place. After that, all aqueous fractions were collected into amber-glass bottle and then kept in dark at −20 °C of freezer temperature until further processing.

Solvent residual evaporating step

Approximately 250 ml of final aqueous fraction was transferred into round-bottom flask equipped with vacuum rotary evaporator. The aqueous solvent was evaporated under vacuum (with pressure of 10 mbar) at 30±1 °C of water bath in

dark room. The final dried residues were kept in dark at −20 °C of freezer temperature until further processing.

Freeze-drying step

Freeze drying process was performed using a laboratory freeze-dryer (LyoLab w/PC, Lyophilization Systems Inc., USA). The dried residue of extract was reconstituted with 20 ml of DI water and then dried with freeze-drying technique. The frozen samples were dehydrated to moisture content below 0.5% (w/w) using a freeze dryer. The dry extract was stored in dark at −20 °C of freezer for further analysis.

The storage of tea extracts at −20 °C in a dry form was considered, to produce less decomposition of extracted GTCs than storing in solution.

1.1 Determination of total yield of freeze-dried green tea extract

Total yield (%, w/w) of freeze-dried extracted solids obtained from both procedures was determined by calculating in terms of the percent recovery of ground tea leaves (%, w/w), based on dry basis.

Calculation of total yield of freeze-dried green tea extract

The percentage of total yield (%, w/w) was carried out according to the following equation:

$$Total\ yield\ (\%,\ w/w) = \frac{W_{final}}{W_{initial}} \times 100 \qquad \text{Eq.10}$$

Where,

W_{final} is the total weight of solid matters (g), obtained at the end of processing.

$W_{initial}$ is the total weight of ground tea leaves (g), added in the initial extracting processing.

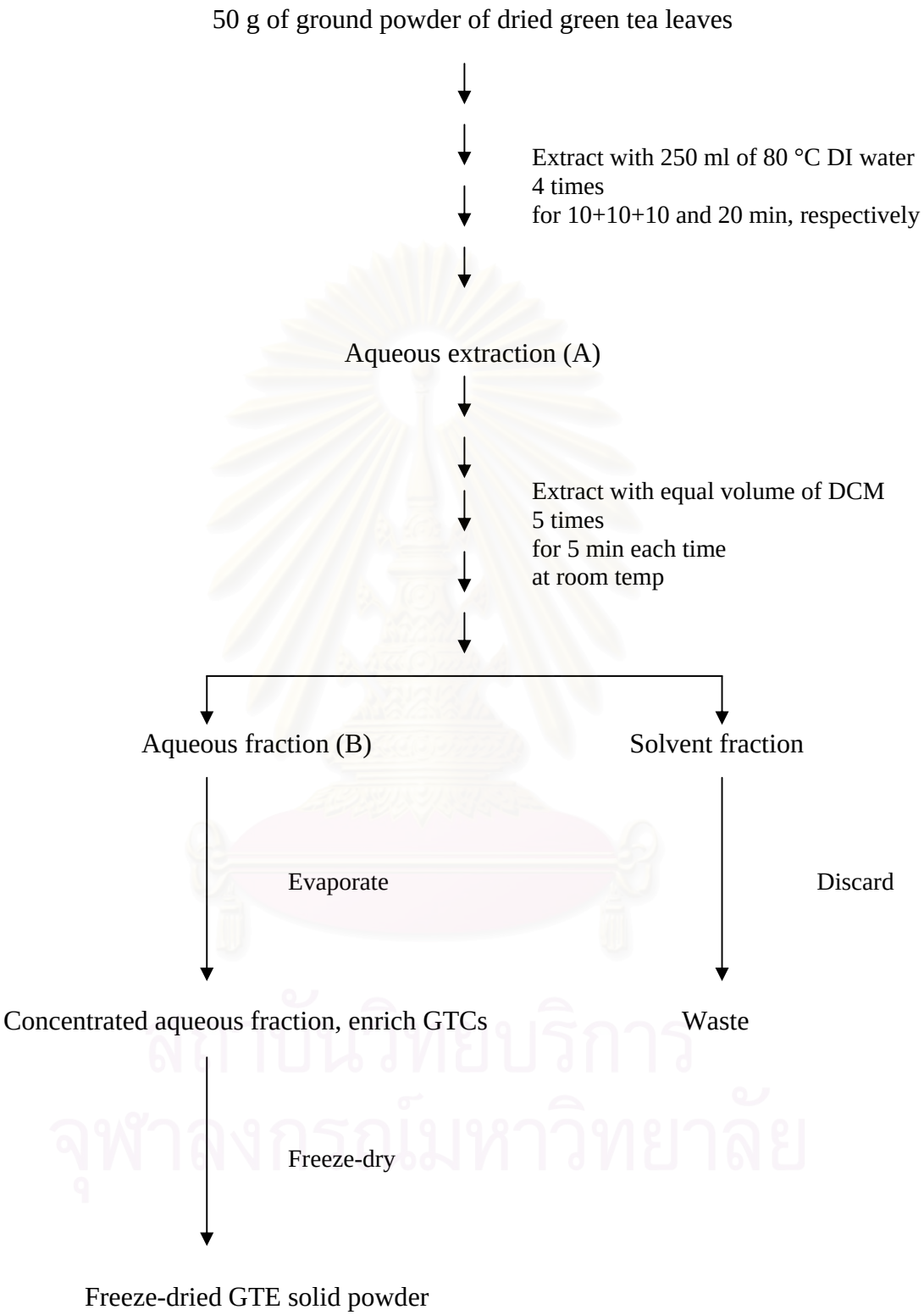


Figure 12 Schematic diagram of green tea extraction procedure.

1.2 Statistical analysis

For statistical analysis, program SPSS, version 14.0 (Statsoft Inc., USA) was used. The independent t test analysis (when only two groups had to be compared) was used to test significant difference between the means (n = 3) of total yield of freeze-dried GTE (FD-GTE) obtained from both procedures. The tests were all carried out at a level $P < 0.05$.

1.3 Determination of green tea catechins and caffeine contents in freeze-dried green tea extract samples

The analysis of GTCs in FD-GTE included two components of catechins, EGC and EC; and two components of catechin gallates, EGCG and ECG. The content of individual catechins and their gallates were determined. In addition, the total GTCs content was also determined by summing the contents of two catechins and two catechin gallates. In generally, caffeine content is usually found in green tea samples with considerably high content level. Consequently, the caffeine content should be also included in the analysis of green tea.

The analysis of GTCs together with caffeine in all samples was performed by Reversed Phase High Performance Liquid Chromatography (RP-HPLC). Diode-array detection (DAD) was employed in all analysis.

Identification of these compounds were done by comparing the UV absorption and retention times to those of reference standards as stated below in table 2.

Quantification of these compounds was done by comparing the peak area due to each GTC and caffeine in the sample to all those of standard curves. The analyses were all carried out in duplicate samples. Each sample was injected through the HPLC in triplicates.

Preparation of sample solutions

For sample preparation, duplicate samples were performed. The FD-GTE solids were accurately weighed followed by the dilution of all samples solutions. The

diluting solvent, composed of 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) with 10% methanol, was used throughout the HPLC analysis.

The FD-GTE solids equivalent to 20 mg was accurately weighed into 50-ml volumetric flask. Approximately 30 ml of diluting solvent was added into the FD-GTE solids to re-dissolve the GTCs. Then, the volume was adjusted to 50 ml by the diluting solvent to obtain stock dilution of sample at approximately 0.4000 mg/ml. The stock sample solutions were prepared in duplicates. Then the stock sample solutions were subsequently diluted with the diluting solvent to obtain final dilution of sample solution at approximately 0.0400 mg/ml.

The final dilution of sample solutions were all centrifuged at 12,000 rpm at −10±1 °C for 20 min and then taken the supernatant. The content of each GTC and caffeine in FD-GTE samples was immediately determined by injection through the HPLC. The analyses were done in triplicate injections for each sample.

Calculation

The analysis of total GTCs included the four compounds, i.e. EGC, EGCG, EC and ECG.

The observed amount of each constituent found in samples (mg) was quantified according to the following equation:

$$Amount\ found\ (mg) = \frac{(PA_{un} - c)}{m} \times \frac{V_3}{V_2} \times V_1 \qquad \text{Eq.11}$$

Where,

PA_{un} is peak area due to each constituent found in the unknown sample ($mAU \cdot s^{-1}$).

c and m are a constant ($mAU \cdot s^{-1}$) and slope ($mAU \cdot s^{-1} \cdot mg^{-1} \cdot ml$) obtained from the standard curve of each GTC, respectively.

V_3 is a 50 ml of second dilution volume of sample solution (ml).

V_2 is a 5 ml of pipetted volume of sample solution (ml).

V_1 is a 50 ml of first dilution volume of sample solution (ml).

The actual amount of analyzed samples applied (mg) was quantified according to the following equation:

$$Amount\ added\ (mg) = \frac{W_{un} \times X}{100} \quad \text{Eq.12}$$

Where,

W_{un} is a weight of unknown sample added (mg).

X is a purity content (%, w/w on dry basis) of each analyte found in the unknown samples.

The quantification of each GTC content in the samples (%, w/w), on dry basis, was carried out according to the following equation:

$$Content\ (\%,\ w/w) = \frac{[Amount\ found]}{[Amount\ added]} \times 100 = \frac{(PA_{un} - c)}{m} \times \frac{V_3}{V_2} \times \frac{V_1}{W_{un}} \times 100 \quad \text{Eq.13}$$

Where,

PA_{un} is peak area due to each constituent found in the unknown sample ($mAU \cdot s^{-1}$).

c and m are a constant ($mAU \cdot s^{-1}$) and slope ($mAU \cdot s^{-1} \cdot mg^{-1} \cdot ml$) obtained from the standard curve of each GTC, respectively.

V_3 is a 50 ml of second dilution volume of sample solution (ml).

V_2 is a 5 ml of pipetted volume of sample solution (ml).

V_1 is a 50 ml of first dilution volume of sample solution (ml).

W_{un} is a unknown sample weight added (mg).

By summing the contents of two catechins and two catechin gallates, the content of total GTCs was carried out according to the following equation:

$$Content\ of\ total\ GTCs\ (\%,\ w/w) = Content\ of\ EGC + Content\ of\ EGCG + Content\ of\ EC + Content\ of\ ECG \quad \text{Eq.14}$$

Notably, the content of each GTC and caffeine was based on the dry weight of FD-GTE solids applied.

In addition, the content of each GTC and caffeine was also calculated based on the dry weight of dried tealeaves applied. It was transformed to the actual content found in dried tea leaves. It was multiplied by the total yield (% (w/w), based on dry basis of tea leaves) obtained from each extraction procedure as shown in the following equation:

$$Content\ (\%, w/w\ of\ dried\ tea\ leaves) = \frac{Content\ in\ FD\text{-}GTE}{100} \times Total\ yield \quad \text{Eq.15}$$

Where,

Content in FD-GTE is a purity content (% (w/w), based on dry basis of extract) of each analyte found in the unknown samples.

Total yield is a total yield (%, w/w) of FD-GTE samples.

1.4 Statistical analysis

For statistical analysis, program SPSS, version 14.0 (Statsoft Inc., USA) was used. The independent t test analysis (when only two groups had to be compared) was used to test significant difference between the means (n = 2) of percent content of major components in FD-GTE from two different procedures, including EGC, EGCG, EC, ECG, total GTCs and caffeine. The tests were all carried out at a level $P < 0.05$.

2. Development of analytical procedures for qualitative and quantitative determinations of green tea catechins and caffeine constituents in freeze-dried green tea extract or other test samples by HPLC analysis

The major constituents in FD-GTE, mainly considered to GTCs and caffeine in all samples were analyzed. A principle of analytical procedure was based on RP-HPLC analysis. Separation was binary gradient elution and detection was performed by UV-vis (Ultraviolet-visible) detector.

2.1 HPLC systems

RP-HPLC analysis was employed to develop the analytical method. All experiments were performed using a *Shimadzu VP* series liquid chromatograph (LC) equipped with a SCL-10A*VP* system controller and CLASS-*VP*™ (V. 6.14 SP1) chromatography workstation interface. A liquid chromatograph system comprising a DGU-14A vacuum degasser, LC-10AD*VP* high pressure liquid chromatography binary pump combined with a FCV-10AL*VP* gradient mixer, a SIL-10AD*VP* auto-sampler (20 µl sampling loop), a thermostatted column compartment using CTO-10AS*VP* air bath column oven, and a SPD-M10A*VP* UV-vis photo diode array detector (photo-DAD) were used.

The column used was a C18 RP-column, Nova-Pak®, 4 µm (3.9 mm, i.d. x 150 mm) with a Nova-Pak® 4 µm C18 (3.9 mm, i.d. x 20 mm) Waters Sentry™ Guard column (Waters Corp., Ireland).

2.2 Chromatographic conditions development (study I)

The HPLC chromatographic conditions were developed according to the following effects:

2.2.1 Effect of mobile phase on chromatographic separation and elution of green tea catechins together with caffeine constituents

2.2.1.1 Acidic aqueous buffer solution

It was reported that the presence of acetic acid in the mobile phase is essential for the complete separation and elution of GTCs together with caffeine constituents (Dalluge *et al*., 1998). In addition, it was confirmed by Wang, Helliwell, and You (2000); if *o*-phosphoric acid was added to mobile phases, the chromatographic separations could be considerably improved. There was some different from the result given by Dalluge *et al*. Although, acetic acid has been found to have the same effect on the separation, but it is not as effective as *o*-phosphoric acid. The effective amount of *o*-phosphoric acid in the mobile phase is ranging from 0.04 to 0.10% (v/v). Accordingly, buffer systems consisting of *o*-phosphoric acid with different concentration, varied from 0.04 to 0.10% (v/v) were evaluated.

2.2.1.2 Organic modifier

The compositions of mobile phase used, composed of different ratios of the acidic aqueous buffer solution to an organic modifier, were evaluated.

Mobile phases consisted of 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) with 10% methanol (eluent A) and 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) with 20% methanol (eluent B). By using the binary gradient elution system, both eluent were programmed to vary the ratio of total methanol eluted in binary gradient system at any elution time. The solvent gradient elution program was described in study II.

2.2.2 Effect of diluting solvent compositions on chromatographic separation and elution of green tea catechins together with caffeine constituents

All FD-GTE sample solutions were prepared in acidified DI water (with *o*-phosphoric acid). This was also required because catechins are more stable in acidic media as reported by Zhu *et al*. (1997).

In addition, methanol was also added into the diluting solvent. The methanol addition in the diluting solvent is not to obtain good efficiency of sample extraction, but minimize dissimilarity from mobile phase when passing through column system. Wang *et al*. (2000) suggested that it is important to make the concentration of methanol in the sample solution less than 15% when HPLC analysis of catechins was carried out. With higher concentration of organic solvent used, the chromatogram could not perform a satisfactorily good separation of earlier peaks that eluted close to the void volume. This might due to the dissimilarity effect of organic solvent used in the mobile phase when passing through column system.

The diluting solutions composed of 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution with 10 and 20% (v/v) of methanol (Me 10 and Me 20) were selected to re-dissolve FD-GTE samples to compare dissimilarity effect of organic solvent concentration on the efficiency of elution and separation between all peaks detected in chromatogram of samples.

2.2.3 Effect of column temperature on chromatographic separation and elution of green tea catechins together with caffeine constituents

By using the air bath column oven (CTO-10AS*VP*), the column compartment was controlled its temperature, ranged from 33±1.0 °C to 40±1.0 °C. With different column temperature, all the peaks eluted from a complex of compounds in chromatogram were compared the efficiency of elution and separation.

2.2.4 Effect of wavelength of detector on peak absorbance

By using photo-DAD, all the peaks eluted from a complex of compounds in chromatogram at any wavelength of interest between 210-370 nm range were compared the maxima absorbance obtained.

2.3 Chromatographic conditions (study II)

The modified chromatographic conditions, which had already been evaluated in study I, were employed for all the HPLC analysis throughout this study.

For binary gradient elution, the mobile phases composed of eluent A and B, which were programmed to vary the ratio of total methanol eluted in binary gradient system at any elution time. The eluent A was consisted of 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) and 10% (v/v) methanol. The eluent B was approximately 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) with 20% (v/v) methanol.

For all mobile phase used in HPLC analysis, the acidic aqueous buffer solution, containing approximately 0.10% (v/v) of *o*-phosphoric acid (pH 2.50±0.01) was prepared by combining 2.50 ml of *o*-phosphoric acid (~85% (v/v)) with ASTM Type II water to a total volume of 2,000 ml. This solution was then degassed by sonication under ultrasonic bath. This solution was subsequently used to prepare the mixture of mobile phase. Approximately 0.10% (v/v) of *o*-phosphoric acid (pH 2.50±0.01) was mixed with the methanol (organic modifier). Final mixtures of mobile phase solutions were prepared as followed: Approximately 900 ml of degassed acidic aqueous buffer solution was combined with 100 ml of methanol to perform an eluent A (10% (v/v) methanol). For eluent B (20% (v/v) methanol), approximately 800 ml of degassed acidic aqueous buffer solution was combined with 200 ml of methanol. After that, both solutions were thoroughly mixed, and then adjusted to pH 2.50±0.01. As needed, both solutions were degassed for at least 30 min prior to use.

The diluting solvent used in all test sample and reference standard preparations was the same as composition of eluent A (0.10% (v/v) *o*-phosphoric acidic aqueous buffer solution (pH 2.50±0.01) with 10% (v/v) methanol).

Elution was performed at a constantly solvent flow rate of 0.8 ml/min. The column compartment was maintained at 33±1.0 °C. The sample injection volume was 20 µl. Detection was accomplished with photo-DAD. The spectroscopic data were detected from 190 nm to 370 nm, and chromatograms were monitored at 210 and 270 nm with 1 milli-absorbance unit per second ($mAU \cdot s^{-1}$) full scale; bandwidth was 8 nm, sampling period and time constant was 0.64 sec, suitable for minimum peak width at base of 0.21 min.

Modified chromatographic conditions of the analysis (Wang *et al*., 2000).

Column : Novapak C18, 4 µm, 4.6 x 150 mm.

Column temperature : 33±1.0 °C.

Solvent elution : Binary concentration gradient elution.

Mobile phase : Eluent A: 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) with 10% methanol.
Eluent B: 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) with 20% methanol.

Flow Rate : 0.8 ml/min.

Injection Volume : 20 µl.

Detection Wavelength : 210 nm, UV spectra in the 190–370 nm range.

Post running Time : 20 min.

Total running Time : 55 min.

Binary gradient elution was programmed by CLASS-*VP*™ (V. 6.14 SP1) chromatography workstation interface. The gradient elution was performed by programming percentages of the eluent B's concentration, as shown in table 2. The time programs of gradient elution were as following: 0.01–21 min, 25%B; 21.01 min, concentration gradient of B was immediately increased from 25 to 80%B; 21.01-36 min, concentration gradient of B was maintained simultaneously at 25%B; 36.01–55 min, 25%B. Post-run time was 20 min at 25%B (re-equilibrate column with 10-15 times of column volume (~1.7 ml) is general recommended for gradient elution).

Table 2 Modified chromatographic conditions of the analysis (Wang *et al*., 2000): Gradient solvent system program.

Retention time (min)	Total methanol (%, v/v)	Eluent A (%, v/v)	Eluent B (%, v/v)
0.01	12.50	75.00	25.00
21.00	12.50	75.00	25.00
21.01	18.00	20.00	80.00
36.00	18.00	20.00	80.00
36.01	12.50	17.00	25.00
55.00	12.50	75.00	25.00

The reference standards of green tea components used in this study, with their purity contents, % (w/w) based on dry basis are all listed in table 3. All of them were received from Sigma Chemical Co., ltd.

Table 3 Listing of reference standards using for HPLC analysis of green tea components.

Product name	**Purity content, % (w/w) on dry basis**	**Standard ID**
Epigallocatechin (EGC)	98.00% on dry basis	Std EGC
Caffeine (Caffeine)	95.00% on dry basis	Std Caff
Epigallocatechin gallate (EGCG)	91.70% on dry basis	Std EGCG
Epicatechin (EC)	95.00% on dry basis	Std EC
Epicatechin gallate (ECG)	98.60% on dry basis	Std ECG

3. Qualitative determination of freeze-dried green tea extract samples (Study III)

3.1 Identification of green tea catechins and caffeine in freeze-dried green tea extract samples

Multi-wavelength detection (Photo-DAD) was used for the identification of compounds. The PDA detector was used in the role of a coupled chromatographic-spectroscopic technique to obtain information about the complex tea liquor analyzed during this study.

UV spectra obtained from all peaks of interest, after subtraction of the corresponding UV base spectrum, were computer normalized and the plots were superimposed. Chromatographic peaks in the samples were identified by comparing their retention times and UV spectra in the 190–370 nm range with those of the reference standards.

FD-GTE sample solutions equivalent to 0.04 mg/ml were prepared in triplicates. In addition, the mixed solution of EGC, EGCG, EC, ECG and caffeine reference standards was constituted up to approximately 0.00604, 0.02488, 0.02060, 0.00348, and 0.00399 mg/ml, respectively. As well as, standard solutions of EGC, EGCG, EC, ECG and caffeine reference standards were also prepared separately at approximately 0.02 mg/ml. After that, triplicate sample preparations of FD-GTE samples, mixed standards and individual standards were all injected in triplicate injections (3×3) through the HPLC system.

The chromatograms obtained were computerized by using CLASS-*VP*™ (V. 6.14 SP1) program. The retention times and UV spectra in 190-370 nm range obtained from the chromatogram of FD-GTE samples were compared with those of reference standards to identify each peak observed.

4. Quantitative determination of freeze-dried green tea extract samples (Study IV)

4.1 Determination of green tea catechins and caffeine contents in freeze-dried green tea extract samples

Quantification was carried out by comparing between the integrated peak areas of EGC, EGCG, EC, ECG and caffeine, obtained from the FD-GTE samples and their corresponding standard curves.

Standard curve of green tea catechins and caffeine

Standard curves of EGC, EGCG, EC, ECG and caffeine were constructed by plotting peak area response ($mAU \cdot s^{-1}$) vs concentration (mg/ml).

To quantify each GTC and caffeine in FD-GTE samples using the developed HPLC condition, triplicates of FD-GTE samples and six dilution concentrations of mixed standards solution were all injected in triplicate injections (3×3) through the HPLC system.

4.1.1 Preparation of serial dilutions of mixed standards solution

Reference standards of green tea components used in this study, with their purity contents, % (w/w) based on dry basis are all previously listed in table 3. All reference standards were combined together to obtain the mixture of all standards similar to the real constituents in the FD-GTE sample solution. All of them were diluted to the expected final concentrations to cover the range of each analyte (the interval between the upper and lower concentration (amounts) of analyte in the sample).

Firstly, individual standard stock solutions of EGC, EGCG, EC, ECG and caffeine were prepared by dissolving each reference standard in diluting solvent and then adjusted to expected concentration. Then each standard stock solution was combined together to obtain mixed standards solution. For the calibration curves, the mixed standards solution was subsequently diluted with

diluting solvent to obtain six dilution concentrations (1.5, 15, 25, 50, 75 and 100%, v/v of mixed standard solution).

Individual standard stock solutions

Reference standards of EGC, EGCG, EC, ECG and caffeine were separately dissolved in the diluting solvent and then adjusted to expected concentration. Using *7-positions* analytical balance (Max. 2.1 g, d = 0.1 μg) (Mettler MT/UMT), all reference standards were accurately weighed by addition technique. All dilutions were performed by using volumetric micropipette (100-1,000 μl) (BioPette A™).

Approximately 2 mg of EGC, EGCG and caffeine reference standards were accurately weighed into a 1.5-ml safe lock micro test tube (Eppendorf™, Germany). Dilution was performed by accurately pipetting 1,000 μl of diluting solvent into the micro test tube and then sonicated for 10 min in dark. Dilution concentration of first stock solution was approximately 2 mg/ml.

An approximately 2 mg of EC reference standard was accurately weighed into a 1.5-ml safe lock micro test tube (Eppendorf™, Germany). Dilution was performed by accurately pipetting 1,500 μl of diluting solvent into the micro test tube and then sonicated for 10 min in dark. Dilution concentration of first stock solution was approximately 1.33 mg/ml.

An approximately 1 mg of ECG reference standard was accurately weighed into a 1.5-ml safe lock micro test tube (Eppendorf™, Germany). Dilution was performed by accurately pipetting 1,500 μl of diluting solvent into the micro test tube and then sonicated for 10 min in dark. Dilution concentration of first stock solution was approximately 0.67 mg/ml.

Calculation

The actual concentration of each constituent added in each standard stock solution (mg) was quantified according to the following equation:

$$Actual\ conc.\ of^{1st} stock\ (mg \cdot ml^{-1}) = \frac{W_{std}}{V_1} \times \frac{X}{100} \quad \text{Eq.16}$$

Where,

W_{std} is a standard weight added (mg).

V_1 is a first dilution volume of standard stock solution (ml).

X is a purity content (%, w/w on dry basis) of each analyte found in the standard samples.

The actual concentrations of each constituent in the first stock solution were 2.01272, 1.91378, 1.37364, 0.69651 and 1.99481 mg/ml for EGC, EGCG, EC, ECG and caffeine, respectively.

Mixed standards solution

Mixed standards solution composed of GTCs and caffeine was performed by combining each standard stock solution of EGC, EGCG, EC, ECG and caffeine.

Dilution series of mixed standards solution were prepared following to table 4. The mixed standards solution were prepared by combining 15, 65, 75, 25 and 10 µl of EGC, EGCG, EC, ECG and caffeine standard stock solutions, respectively. These dilutions of stock solutions were accurately transferred into a 5-ml amber-glassed vial, and then brought to final volume (5 ml) with diluting solvent. To obtain consistent mixture, the final mixture was subsequently sonicated for 10 min in dark.

Table 4 Dilution series of mixed standards stock solution.

Reference standard stock solution			Diluting solvent (µl)	Total vol. (ml)	Second dilution conc. (mg/ml)
ID. Label (% Purity)	First stock conc. (mg/ml)	Added vol. (µl)			
Std EGC (98.00%)	2.01272	15	4,810	5	0.00604
Std EGCG (91.70%)	1.91378	65			0.02488
Std EC (95.00%)	1.37364	75			0.02060
Std ECG (98.60%)	0.69651	25			0.00348
Std Caff (95.00%)	1.99481	10			0.00399

Calculation

The actual concentration of each constituent added in mixed standards stock solution (mg) was quantified according to the following equation:

$$Actual\ conc.\ of^{2nd} stock\ (mg \cdot ml^{-1}) = \left(\frac{W_{std}}{V_1} \times \frac{X}{100} \right) \times \frac{V_2}{V_3} \quad \text{Eq.17}$$

Where,

W_{std} is a standard weight added (mg).

V_1 is a first dilution volume of standard stock solution (ml).

X is a purity content (%, w/w on dry basis) of each analyte found in the standard samples.

V_2 is a pipetted volume of standard stock solution (ml).

V_3 is a 5 ml of the second dilution volume of mixed standards solution (ml).

The actual concentrations of each constituent in the mixed standards solution were 0.00604, 0.02488, 0.02060, 0.00348 and 0.00399 mg/ml for EGC, EGCG, EC, ECG and caffeine, respectively.

Serial dilutions of mixed standards solution

The mixed standards solution, which composed of EGC, EGCG, EC, ECG and caffeine at approximately 0.00604, 0.02488, 0.02060, 0.00348, and 0.00399 mg/ml, respectively, was subsequently used to prepare the serial dilutions of mixed standards solution.

It was diluted with diluting solvent to obtain six dilution concentrations (1.5, 15, 25, 50, 75 and 100%, v/v of mixed standards solution) for the calibration curve. The mixed standards solution was performed in triplicates for each dilution concentration. Dilution series were all performed as shown in table 5. All standard samples were centrifuged at 12,000 rpm at −10±1 for 20 min and then taken the supernatant prior to the HPLC analysis.

Triplicates of mixed standards solution with dilution concentrations at 1.5, 15, 25, 50, 75 and 100%, v/v were all injected in triplicates through the HPLC system. The mean of peak area responses obtained from each dilution concentration were plotted, as a function of concentration.

Table 5 Serial dilutions of mixed standards solution.

Mixed reference standard solution ID. Label (% Dilution concentration)	Dilution vol. (µl)	Added diluting solvent (µl)	Total volume (ml)
Std 1 (1.5%)	15	985	1
Std 2 (15%)	150	850	1
Std 3 (25%)	250	750	1
Std 4 (50%)	500	500	1
Std 5 (75%)	750	250	1
Std 6 (100%)	1,000	0	1

4.1.2 Preparation of sample solutions

Quantification of these compounds was done by comparing the peak area due to each GTC and caffeine in the sample to all those of standard curves. The analyses were all carried out in duplicate samples. Each sample was injected through HPLC in triplicates.

For the analysis of FD-GTE sample, the freeze dried extracted solids were re-dissolved using the same diluting solvent as used throughout the HPLC analysis.

For sample preparation, duplicate samples were performed. The FD-GTE solids were accurately weighed followed by the dilution of all samples solutions.

The FD-GTE solids equivalent to 20 mg was accurately weighed into 50-ml volumetric flask. Approximately 30 ml of diluting solvent was added into the FD-GTE solids to re-dissolve the GTCs. Then, the volume was adjusted to 50 ml by the diluting solvent to obtain stock dilution of sample solution at approximately 0.40 mg/ml. The stock sample solutions were prepared in duplicates. Then the stock sample solutions were subsequently diluted with the diluting solvent to obtain final dilution of sample solution at approximately 0.04 mg/ml.

The final dilution of sample solutions were all centrifuged at 12,000 rpm at −10±1 °C for 20 min and then taken the supernatant. The content of each GTC and caffeine in FD-GTE samples was immediately determined by injection through the HPLC. The analyses were done in triplicate injections for each sample.

Calculation

All calculations are previously shown in equation 11-15.

5. Validation of analytical method for qualitative and quantitative determination of green tea catechins and caffeine in freeze-dried green tea extract samples by HPLC analysis (Study V)

Developed analytical method for an assay of EGC, EGCG, EC, ECG and caffeine in FD-GTE were validated, according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidance Q2A (ICH, 1995) and Q2B (ICH, 1996). Types of analytical procedures to be validated were intended for qualitative and quantitative tests of active moieties in samples for drug substance and drug product.

5.1 Validation of protocols

The ICH guidance Q2A text on validation of analytical procedures (ICH, 1995) and Q2B validation of analytical procedures & methodology (ICH, 1996) provide recommendations on validation of analytical procedures. The validation characteristics that should be addressed during validation of analytical procedures were depending on the type of test. The validation information should support the intended purpose of the test.

In this study, the analytical procedure was performed by using HPLC analysis in order to identify the identity and quantitate the content of active substances in the unknown samples.

The recommended validation characteristics under identification and assay tests, i.e. accuracy, precision, selectivity, linearity and range would apply.

In this study, the FD-GTE sample solutions and the diluting solvent as used throughout the HPLC analysis were used to validate the developed analytical method. For the calibration curves, the preparations of serial dilutions of mixed standards solution at 1.5, 15, 25, 50, 75 and 100%, v/v were all previously stated in study IV.

Accuracy

Accuracy of an analytical method is the degree of closeness between the true value of analytes in the sample and the value determined by the method. Accuracy

can be measured by analyzing samples with known concentrations and comparing the measured values with the true values.

The accuracy should be determined by a minimum of five determinations for at least three concentrations (low, medium, high) in the range of test concentrations. The mean values should be within 2% from the true value.

Accuracy represented by the percentages of absolute recoveries was performed with five concentrations of duplicates sample solutions without a serial dilution.

The recovery was obtained from the mean of observed concentrations calculated from the calibration curve divided by the mean of actual concentrations and multiplied by 100. The results were calculated in percentage terms. According to guidance, the recovery should be coverage in the range of 98-102%.

Precision

Precision was obtained in terms of repeatability (intra-day precision) when the analysis was performed in one laboratory by one analyst using the same equipment at the same day. Repeatability was measured by the analysis through six determinations without a serial dilution at 100%, v/v of the test concentration.

The precision of an analytical method was calculated as coefficient of variation (C.V.), i.e. relative standard deviation (RSD) as percentage terms (the percentage of RSD (% RSD)). Precision should be better than 2% of RSD value.

The recovery (%) was obtained by dividing the mean of observed concentrations by the mean of actual concentrations and multiplying by 100. The recovery should be coverage in the range of 98-102% recovery (100% recovery ± 2% RSD).

Linearity and Range

The linearity of an analytical method was evaluated in the concentration span 80-120% of the test concentration without a serial dilution. The range of an analytical method is the concentration interval that has been validated according to accuracy, precision and linearity as previous described. For the assay of a drug substance, the

specified range was derived from the linearity studies, which covered the range between 80-120% of the test concentration without a serial dilution.

The linearity, the R^2 value, was calculated by plotting the observed concentration against the actual concentration. The linear correlation coefficient (R^2) should be greater than 0.9995 accordingly to guidance.

Selectivity

Selectivity refers to a method that gives responses for a number of substances and can distinguish the analyte(s) response from all other responses. Selectivity of the method should be evaluated by processing blank samples with and without the addition of analytes and inject them to test for interferences. Selectivity of the chosen system was investigated by injection each reference standard solution and comparing chromatograms of pure matrix solution with matrix solution to which analytes had been added. Resolution between peaks of GTCs together with caffeine should not less than 1.5. As well as, asymmetry should not more than 1.5.

Table 6 Summarized criteria of analytical method validation.

Test	Parameter	Criteria
Accuracy	% Recovery	98–102%
Precision	% RSD	NMT 2%
Linearity and Range	Slope	0.9–1.1
	Intercept	−0.001 to 0.001
	R^2	NLT 0.9995
	RSS	−0.01 to 0.01
	80-120% of test concentration	Method must have linearity covering this range
Selectivity	Resolution	NLT 1.5
	Asymmetry	NMT 1.5

The analytical method for qualitative and quantitative determination of GTCs and caffeine in FD-GTE using HPLC analysis was validated according to ICH guidance (Q2A and Q2B) (ICH, 1995, 1996). The summarized criteria are shown in table 6.

Preparation of sample solutions

For accuracy and linearity, duplicates sample solutions were prepared independently into five points (80, 90, 100, 110, and 120% of the test concentration) without a serial dilution. With duplications, the freeze-dried extracted solids equivalent to 16, 18, 20, 22 and 24 mg were accurately weighed into each 50-ml volumetric flask separately; except for the flask with approximately 20 mg of the extract powder were six replicated for the precision study. Each flask was added with approximately 30 ml of diluting solvent and then sonicated for 10 min until completely dissolved. The sample solutions were adjusted to volume with diluting solvent to obtain stock sample solutions.

For precision, the sample solutions at 100% of the test concentration were all prepared in six replicates without a serial dilution. With six replications, the freeze-dried extracted solids equivalent to 20 mg was accurately weighed into each 50-ml volumetric flask separately. Each flask was added with approximately 30 ml of diluting solvent and then sonicated for 10 min until completely dissolved. The sample solutions were adjusted to volume with diluting solvent to obtain stock sample solutions. To perform final dilution of sample solutions, approximately 5.0 ml of stock sample solutions were accurately transferred into each 50-ml volumetric flask, and then adjusted to final volume with diluting solvent (Final dilution concentrations ~ 0.032, 0.036, 0.040, 0.044, 0.048 mg/ml for 80, 90, 100, 110, and 120% of the test concentration).

For selectivity, the diluting solution used throughout this analysis (as blank samples) and the sample solutions at 100% of the test concentration (as unknown samples) were all prepared in triplicates. To define each peak detected in the chromatogram of sample, triplicates of the mixed standards solution at 100% of the dilution concentration (Std 6) (as standard samples) were all prepared.

The final dilution of all sample solutions performed were centrifuged at 12,000 rpm at −10±1 °C for 20 min and then taken the supernatant prior to inject through the HPLC system. A 20 µl of each sample was directly injected in triplicates. All injections were performed using the same chromatographic condition as previous

described. The concentrations (amounts) of each GTC and caffeine in FD-GTE samples were immediately determined by injection through the HPLC system.

Calculation

The observed concentration of each constituent found in samples (mg/ml) was carried out according to the following equation:

$$Observed\ conc.\ (mg \cdot ml^{-1}) = \frac{(PA_{un} - c)}{m} \times \frac{V_3}{V_2} \qquad \text{Eq.18}$$

Where,

PA_{un} is peak area due to each constituent found in the unknown sample ($mAU \cdot s^{-1}$).

c and m are a constant ($mAU \cdot s^{-1}$) and slope ($mAU \cdot s^{-1} \cdot mg^{-1} \cdot ml$) obtained from the standard curve of each GTC, respectively.

V_3 is a 50 ml of second dilution volume of sample solution (ml).

V_2 is a 5 ml of pipetted volume of sample solution (ml).

The actual concentration of each constituent found in samples (mg/ml) was carried out according to the following equation:

$$Actual\ conc.\ (mg \cdot ml^{-1}) = \frac{W_{un}}{V_1} \times \frac{X}{100} \qquad \text{Eq.19}$$

Where,

W_{un} is a weight of unknown sample added (mg).

X is a purity content (%, w/w on dry basis) of each analyte found in the unknown samples.

V_1 is a 50 ml of first dilution volume of sample solution (ml).

The recovery of each constituent found in samples (%, w/w), on dry basis, was carried out according to the following equation:

$$\text{Recovery (\%, } w/w) = \frac{[Amount\ found]}{[Amount\ added]} \times 100 = \frac{(PA_{un} - c)}{m} \times \frac{V_3}{V_2} \times \frac{V_1}{W_{un}} \times 100 \quad \text{Eq.20}$$

Where,

PA_{un} is peak area due to each constituent found in the unknown sample ($mAU \cdot s^{-1}$).

c and m are a constant ($mAU \cdot s^{-1}$) and slope ($mAU \cdot s^{-1} \cdot mg^{-1} \cdot ml$) obtained from the standard curve of each GTC, respectively.

V_3 is a 50 ml of second dilution volume of sample solution (ml).

V_2 is a 5 ml of pipetted volume of sample solution (ml).

V_1 is a 50 ml of first dilution volume of sample solution (ml).

W_{un} is a unknown sample weight added (mg).

The observed amount of each constituent found in samples (mg) was quantified according to the previous equation as shown in the equation 11.

The actual amount of analyzed samples applied (mg) was quantified according to the previous equation as shown in the equation 12.

Standard deviation (SD) was carried out according to the following equation:

$$SD = \sqrt{\frac{\sum_{i=1}^{N} (x_i - \bar{x})^2}{N-1}} \quad \text{Eq.21}$$

Where,

x_i is % recovery in each replicate.

$\bar{x}$ is an overall mean of % recoveries of all replicates.

N is a number of replicate, e.g. 3 and 6 replicates for accuracy and precision studies, respectively.

The % RSD was carried out according to the following equation:

$$\%RSD = \frac{SD \times 100}{\bar{x}} \qquad \text{Eq.22}$$

Where,

SD is a standard deviation of % recoveries of all replicates.

$\bar{x}$ is an overall mean of % recoveries of all replicates.

6. Pre-characterization of concentrated w/o emulsions on achievability in processing, apparent viscosity and short-term stability

To perform fifteen representative formulations for further study, formulations with various formulation compositions were preliminary prepared and then characterized their general properties due to short-term stability of final products.

6.1 Effect of different formulation parameters

This research was considered to study the effect of different formulation parameters on the *in vitro* diffusion of EGCG, hydrophilic model drug, from CEs containing FD-GTE. Possible determinants that may affect release of the systems, i.e. water content, emulsifier type and its concentration were evaluated.

6.1.1 Effect of water content

The range of % DP was preferred to be as large as possible for these emulsions. Therefore, CEs with 80-90% (w/w) DP were prepared in triplicates (fixed with other formulation components). The emulsions with three ranges (minimum (min), medium (med) and maximum (max)) of % DP were chosen by the following criterions. The lowest concentration of water phase selected was the one that allowed the production of stable products (for at least 1 week). The highest concentration was the one that could be achieved with most emulsifiers and was therefore chosen as the max % DP.

6.1.2 Effect of emulsifier type

For studying the influence of emulsifier type, four different emulsifiers were selected as following:

i) Sorbitan, namely, a sorbitan ester type (sorbitan sesquioleate, Arlacel 83™).

ii) Silicone, namely, a silicone polymer type (cetyl dimethicone copolyol, Abil EM 90™).

iii) Glucoester, namely, a glucoester type (methyl glucose dioleate, Isolan DO™).

iv) Mixed, namely, two emulsifiers coupled type (a mixture of sorbitan oleate, beeswax, hydrogenated castor oil, stearic acid, Montane 481™ and PEG-7 hydrogenated castor oil, Simulsol 989™).

The choice of selection made to cover the major groups of emulsifier used in the cosmetic industry.

6.1.3 Effect of emulsifier concentration

A study of the effect of emulsifier concentration was also conducted with one emulsifier system (glucoester in the range of 1%, 2%, 3% and 4% of emulsifier), fixed at max % DP. All samples were performed in triplicates.

6.1.4 Effect of other additives

Other formulation compositions that could affect the stability of CEs, esp., an electrolyte (Caldero *et al*., 1997) were also evaluated. Therefore, CEs with 5%, 10% and 15% of 0.1 M NaCl electrolyte solution were performed in triplicates (fixed with other formulation components).

Moreover, the moisturizer used (glycerin) that may affect general aspects of CEs, i.e. their stiffness due to viscosity was also evaluated. Therefore, CEs with 5%, 10% and 15% of glycerin were performed in triplicates (fixed with other formulation components).

6.2 Preparation of concentrated w/o emulsions

CEs were manufactured by varying formulation compositions as previous detailed. Briefly, they composed of two immiscible phases.

The aqueous phase consisted of the following components. An approximately 5%, 10% and 15% (w/w) of glycerin were used as the moisturizer. As well as, a 0.1 M NaCl electrolyte solution was varied between 5%, 10% and 15% (w/w). An antioxidant was 0.025%, w/w of *di*-sodium EDTA (*di*-Na.EDTA).

The oil phase was composed of an organic hydrocarbon oil (isohexadecane, Arlamol HD™) with four different emulsifiers (Arlacel 83™, Abil EM 90™, Montane 481™,coupled with Simulsol 989™ and Isolan DO™), combined with 0.1% (w/w) of BHT, as a chelating agent. An approximately 1% (w/w) of paraben concentrate (paraben conc.) in propylene glycol (PG) was used as preservatives. The CEs compositions with all in concentrations under study are shown in table 7.

Table 7 Concentrated w/o emulsions compositions.

Continuous oil phase (External phase)					
Composition	**Chemical name**	**Trade name**	**% Amount in Rx (w/w)**		
Emulsifier	Sorbitan sesquioleate	Arlacel 83™	3%		
	Cetyl dimethicone copolyol	Abil EM 90™	3%		
	A mixture of sorbitan oleate, beeswax, hydrogenated castor oil, stearic acid	Montane 481™	2.2%		
	PEG-7 hydrogenated castor oil	Simulsol 989™	0.8%		
	Methyl glucose dioleate	Isolan DO™	3%		
Organic hydrocarbon	Isohexadecane	Arlamol HD™	10-20%		
Antioxidant (oil soluble)	1%, w/v of BHT stock solution in Arlamol HD™ (10 ml of stock solution ~ 0.1 g BHT)		0.1%*		
Aqueous dispersed phase (Internal phase)					
Moisturizer	Glycerin		5%	10%	15%
Electrolyte	0.1 M NaCl solution		5%	10%	15%
DI water	DI water		80-90%		
Antioxidant (water soluble)	0.25%, w/v of *di*-Na.EDTA stock solution in DI water (10 ml of stock solution ~ 0.025 g *di*-Na.EDTA)		0.025%*		
Preservative	Paraben conc. in propylene glycol		1%*		

*, Not considered the effect of those formulation parameters.

In general, formulation can be simply prepared by weighing all compositions and then mixed altogether. However, there are considerable disadvantages of this manner. It is not only providing inconsistent addition, but promoting the salting out effect occurred during mixing. Consequently, other solid additives were all prepared as stock solutions prior to use. To reach a certain concentration by volume solution, all solutions were prepared by addition a part of solvent used in formulations to a known weight of those solids.

The additives in the continuous oil phase composed of BHT, which considerably miscible in organic hydrocarbons. Therefore, BHT was diluted to 1%, w/v of stock solution with part of the Arlamol HD™ oil in the formulations.

The additives in the aqueous phase composed of NaCl and *di*-Na.EDTA. To reach concentration at 0.1 M of electrolyte solution, NaCl was dissolved in part of the DI water in the formulations. As well as, *di*-Na.EDTA was also diluted with DI water to reach concentration at approximately 0.25%, w/v of stock solution.

The preservative used in this study was paraben conc., which composed of 1%, w/v of methyl paraben and 10%, w/v of propyl paraben in PG. To slow down light-decomposition, all of them were kept in amber glass bottles, tightly sealed with screw caps and stored in refrigerator as possible. However, they should be freshly prepared and did not use in case of more than 1 week storage.

The samples were prepared in glass vials (volume of 20 ml and length of 5 cm) tightly sealed with screw caps. The emulsifier was added into the vials with known amount of emulsifier as shown in table 7. After that, an organic hydrocarbon was transferred into the vial using glass dropper. A few amount of glass beads (diameter of 5 mm, about 5 beads) were added into these sample vials. Using a heavy-duty vortex shaker, the sample vials were vigorously shaken until homogeneous. Occasionally, some of emulsifiers that could not be miscible in the continuous oil phase at room temperature were liquefied at approximately 70±5 °C using water bath to promote thorough mixing. The homogeneous mixture of emulsifier and continuous oil phase was named as an oil part. As such a high melting point of some emulsifiers, they always come back to their solid form at room temperature, so the whole mixture appears to separate again. Consequently, they were kept at about 40±1 °C in the hot air oven before using.

An aqueous phase mixture was prepared in 15-ml conical flask to reduce water loss during manufactured. To keep hold water, glycerin was firstly added into the conical flask. After that, NaCl electrolyte and *di*-Na.EDTA stock solutions were added by using measuring pipette. After each addition, the whole mixture was constantly agitated on the magnetic stirrer set at low speed around 40-50 rpm. The final mixture was named as an aqueous part.

The oil part of formulations was allowed to come back to room temperature. Occasionally, some of them were melted in the hot water bath to promote thorough mixing. To avoid thermal decomposition of BHT, BHT stock solution in Arlamol HD™ oil was added when the mixture was cooled down. The entire mixture was suddenly agitated by a heavy-duty vortex mixer until homogeneous.

The order of mixing that could be suitable for these formulations was performed. All the samples were prepared following the same procedure. In order to obtain the most stable emulsions, the water-swollen micellar solutions were obtained by the addition of a small amount of water (4–5%, w/w of Rx) into a mixture of continuous oil phase. Then, the aqueous part was added gradually into the micellar solution under a gently mechanical stirring to obtain reverse emulsions with the water content, ranging from 80 to 90% DP (w/w).

6.3 Characteristic assessment of concentrated w/o emulsions

In general, the internal phase is slowly added to the external phase under continuous mixing. The critical stage occurs during the processing at an internal phase volume up to 75% DP. Not only do the emulsions have a tendency to invert, but also one can easily form metastable double emulsions, in which the water phase becomes continuous. These duplex emulsions are difficult to break and one generally has to start over. Accordingly, the production achievement of emulsions has to be observed during the manufacturing process.

In case of, all emulsions that success to be achieved during the processing were subsequently transferred to amber glass vials tightly sealed with screw caps and then stored in dark place at ambient. The obtained emulsions were assessed their property due to the stability, i.e. a degree of phase separation in short-term physical stability (for at least 1 week).

Furthermore, the changing of physical property that related to their quality and appearance of final products, i.e. a degree of apparent viscosity changing compared with blank formulation (without electrolyte) was investigated after emulsions manufacture. All the experimental work was carried out at ambient.

6.3.1 Production achievement of concentrated w/o emulsions

Under continuous mixing of two phases, the production achievement of the emulsions was observed during the critical stage of manufacturing process, which generally occurs after an internal phase volume reached to 75% DP.

6.3.2 Short-term physical stability of concentrated w/o emulsions

A short-term physical stability of CEs was observed at ambient. At definite time interval (1, 2, 3, 4, 5, 6, 7 days), the physical stability was assessed by visual observation. Typically, creaming, aggregation and phase separation have traditionally been used to monitor the physical stability of emulsions. The degree of phase separation of water under storage condition was used as one measure of emulsions stability and was determined as following, i.e. 2-phase and 3-phase separations.

6.3.3 Apparent viscosity of concentrated w/o emulsions

After emulsions manufacture, an apparent viscosity was investigated at room temperature with a Brookfield viscometer (Brookfield, USA). An approximately 150 g of samples were measured in triplicate. Spindle ASTM 7 was used for all samples. A shear rate of instrument status was set at 100 s^{-1} and kept spindle rotating for about 60 s or until constantly mean viscosity was obtained. The mean viscosity (mPa·s) was recorded.

Degree of changing in apparent viscosity was carried out according to the following equation:

$$D = \frac{D_{Blank}}{D_{Estimated}} \qquad \text{Eq.23}$$

Where,

D is degree of changing on apparent viscosity.

D_{Blank} is an apparent viscosity of blank formulation (mPa·s).

$D_{Estimated}$ is an apparent viscosity of estimated formulation (mPa·s).

If,

D = 1, an apparent viscosity doesn't change compared with blank formulation (without electrolyte or moisturizer).

D > 1, an apparent viscosity decreases compared with blank formulation.

D < 1, an apparent viscosity increases compared with blank formulation.

7. Preparation of concentrated w/o emulsions containing freeze-dried green tea extract for *in vitro* diffusion and characterization studies

From a previous study, the suitable compositions of CEs that obtained superior short-term stability of all formulations were preferred to use for *in vitro* diffusion and characterization studies. With some considered modifications, all in concentrations were as followed in table 8.

Table 8 Compositions of developed concentrated w/o emulsions, containing 2.25% (w/w) of freeze-dried green tea extract for *in vitro* diffusion and characterization studies.

Rx ID no.	Estimated formulation parameters		
	Emulsifier type	% Emulsifier	% Dispersed phase
1*	Sorbitan (Arlacel 83™)	3%	84%
2*		3%	86%
3*		3%	88%
4*	Silicone (Abil EM 90™)	3%	84%
5*		3%	86%
6*		3%	88%
7*	Glucoester (Isolan DO™)	3%	84%
8*		3%	86%
9*		3%	88%
10*	Mixed (Montane 481™-Simulsol 989™)	3%	84%
11*		3%	86%
12*		3%	88%
13*	Glucoester (Isolan DO™)	1%	88%
14*		2%	88%
15*		4%	88%

*; 5% (w/w) Glycerin, 10% (w/w) NaCl electrolyte, 0.025% (w/w) *di*-Na.EDTA, 0.1% (w/w) BHT and 1% (w/w) paraben conc. in PG.

FD-GTE, which composed of GTCs together with caffeine, obtained from the extraction study was incorporated into all formulations and only EGCG was investigated as a model drug substance in this study.

The amount of FD-GTE incorporated into CEs was referred to the definite dose (based on the solubility of EGCG in receptor fluid used in the *in vitro* diffusion study). An approximately 2.25% w/w of FD-GTE was incorporated into each formulations. All samples were performed in triplicates.

The compositions of each formulation are reported in table 8. All the samples were prepared followed the similar procedure as stated above.

Indeed, CEs were prepared by two alternative methods depending on the quantities required. The conditions of preparation were kept as similar as possible. Approximately 10 g and 600 g of CEs containing 2.25% FD-GTE were prepared for *in vitro* diffusion and characterization studies of CEs, respectively.

In the small scale, the CEs were prepared in amber glass vials (volume of 20 ml and length of 5 cm) tightly sealed with screw caps. In order to enhance stability of GTCs in aqueous solution, the FD-GTE solids was re-dissolved with glycerin in the formulations in the presence of 0.025% of *di*-Na.EDTA and then stirred until homogeneous. This step was supposed to avoid a direct contact of GTCs with a pure aqueous solution that possibly promotes hydrolysis degradation process during mixing. Afterward solution from this step was added into the rest of water part. The electrolyte solution from previous step was added slowly into the whole mixture. To avoid the salting out effect, esp., at the pouring area, the electrolyte solution was dropped slowly and suddenly mixed well. The vial was vigorously shaken, using a heavy-duty vortex shaker, until the content became homogeneous. Finally, CEs were prepared by slow addition of the internal phase (the aqueous phase) to the external phase (the oil phase) under continuous mechanical stirring. After that, the aqueous phase was added gradually into the continuous oil phase, i.e. a mixture of the emulsifier and hydrocarbon in the presence of 0.1% of BHT. After each addition, the emulsions were thoroughly mixed. When the emulsions became consistent, the preservative (1% of paraben conc. in PG) was added slowly and gently stirred until homogeneous. Thereafter, the samples were centrifuged in a table centrifuge (approximately 1,200 rpm) in order to remove air bubbles.

When greater quantities of emulsions were desired, the above procedure was scaled up and a tall form beaker fitted with a constant speed overhead stirrer was employed for mixing.

About 18 g of an emulsifier solution (typically 3%, w/w of Rx) was charged to a tared beaker fitted with a constant speed overhead stirrer. The aqueous phase (typically 504, 516 and 528 g) was then added dropwise from a separatory funnel over a period of about 1 h under constant mixing. After the water was added, the emulsions were mixed for another 30 min and stored in PTFE jars. A “V” blade was used for mixing and the overhead stirrer was set at a speed that produced about 120 rpm.

The characteristics of these emulsions were reproduced from one batch to another as verified by their apparent viscosity. The production achievement of the emulsions was observed during the manufacturing process. In case of, emulsions that succeed to be achieved during the processing were subsequently transferred to amber glass vials tightly sealed with screw caps and then stored in dark place at ambient. Moreover, the obtained emulsions were assessed their property due to the stability, i.e. a degree of phase separation in short-term physical stability (for at least 1 week) and degree of phase separation of emulsions, which was determined by optical observation after 1 week. All the experimental work was carried out in triplicates at room temperature.

8. Characterization of concentrated w/o emulsions

All formulations were characterized by the following methods (Clément *et al.*, 2000). These characterizations would allow us to make correlation between the formulation parameters of CEs and the diffusion profiles of model drug.

All emulsions obtained were transferred to amber glass vials tightly sealed with screw caps and then stored in dark place at ambient and two accelerated aging, −20±1 °C (freezing temperature) and 40±1 °C (high temperature). At definite time, intervals as described below, all samples storage at various temperatures were allowed to come to room temperature for physical stability observations. For other characterization studies, the emulsions storage at ambient were ready for measurements. Triplicate samples of each formulation were measured in duplicates.

8.1 Long-term physical stability of concentrated w/o emulsions

The long-term physical stability of the CEs were observed at three points of temperature; ambient and two accelerated aging, -20±1 °C (freezing temperature) and 40±1 °C (high temperature).

At definite time intervals (1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months), the emulsions were allowed to come back to room temperature and the physical stability were assessed by visual observation. Typically, creaming, aggregation and phase separation have traditionally been used to monitor the physical stability of emulsions. The degree of phase separation was investigated.

8.2 Apparent viscosity of concentrated w/o emulsions

At the same definite time intervals (1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months), an apparent viscosity was investigated at room temperature with a Brookfield viscometer (Brookfield, USA). An approximately 150 g of samples were measured in triplicate. Spindle ASTM 7 was used for all samples. A shear rate of instrument status was set at 100 s^{-1} and kept spindle rotating for about 60 s or until constantly mean viscosity was obtained. The mean viscosity (mPa·s) was recorded. The obtained data were all shown in Appendix II.

8.3 Determination of morphology: Droplet diameter and shape

A high-resolution cryoscanning electron microscope (cryo-SEM), JSM-5410LV, was employed to obtain images of the CEs for studying a change of droplet diameter and shape during time intervals (1, 8, 15 days, 3 and 6 months). A small amount of samples were filled in the mini-tubing cell, and then frozen in sub-cooled nitrogen, immediately fractured at 110 K under vacuum condition. After that, all samples were immediately observed in viewing chamber of cryo-SEM. The obtained data were all shown in Appendix III.

8.4 Determination of mean droplet diameter and droplet size distribution: Laser diffraction analysis (wet dispersion)

For droplet size analysis, all samples were determined in triplicate. Mean droplet diameter and droplet size distribution were determined by laser diffractometry using a Malvern Mastersizer S long bed version 2.11. (Malvern Instruments Ltd., UK). All the samples of CEs were prepared and analyzed at the same definite time intervals (1, 8, 15 days, 3 and 6 months) after preparation.

For wet dispersion of the CEs samples, the dispersion of CEs samples were performed by dispersing with light mineral oil, which similar to the oil continuous phase of formulation as a dispersant. A dispersing medium used was only supposed to pre-disperse the samples, but not dissolve them. A sample amount of approximately 200 mg was pre-dispersed in an appropriate volume of the dispersant. Then the pre-dispersion was introduced in the wet dispersion module until an adequate obscuration level was obtained. Fresh dispersions were made for all measurements. This dilution of dispersion did not affect the size of the droplet as confirmed by the scanning electronic micrographs prior to the measurement.

8.4.1 Average mean droplet diameter in μm (D [v, 0.5])

The mean diameter in termed of the volume median diameter (VMD) and the droplet size distribution in termed of polydispersity were measured by mean of laser light scattering spectroscopy. The obtained data were reported in termed of the equivalent volume diameters at the 10, 50 and 90% of cumulative droplets

volume counted undersize; D[v,0.1], D[v,0.5] and D[v,0.9] (μm), as shown in Appendix IV.

8.4.2 Polydispersity (Relative span)

The polydispersity, i.e. the width of the droplet size distribution, of the CEs was accordingly measured by the relative span. The obtained data were all shown in Appendix IV.

The D [v, 0.1] and D [v, 0.9] values enable the calculation of the span, or, more correctly, the relative span is a dimensionless measure of the spread of droplet sizes in the emulsions. The larger the value of the relative span, the wider the range of droplet sizes present in the emulsions. In contrast, the lower the value of the relative span, the narrower the range of droplets produced. It was calculated using the formula as following:

$$\text{Relative span} = \frac{D[v,0.9] - D[v,0.1]}{D[v,0.5]} \qquad \text{Eq.24}$$

Where,

D [v, 0.1] = the equivalent volume diameters at 10% cumulative volume.

D [v, 0.5] = the equivalent volume diameters at 50% cumulative volume.

D [v, 0.9] = the equivalent volume diameters at 90% cumulative volume.

9. *In vitro* diffusion study

In vitro diffusion study was used as a tool for determining the effect of formulation on the release of EGCG from different formulations of CEs with different emulsifier types, percentages of dispersed phase and percentages of emulsifier used. The study was performed by using modified Franz diffusion cells.

Procedure of *in vitro* diffusion study

9.1 Membrane diffusion experiments

9.1.1 Preparation of samples

CEs containing FD-GTE powder, approximately to 2.25%, w/w of total formulations, were used as samples in this study (The exactly amount of drug content added in the formulations had not to be considered in this case, just mentioned the source of FD-GTE powder, which was be produced from the same batch). There were fifteen formulations, manufactured by varying the formulation parameters, which divided into four main categories as shown in table 8.

9.1.2 Diffusion membrane & membrane pre-treatment

Diffusion membrane

A commercially available synthetic hydrophilic polysulfone membrane was utilized as diffusion membrane (Tuffryn® Membrane filter, HT-450, pore size 0.45 µm; 165 µm thick (47 mm), Lot No. 127301) (Gelman Laboratory, Pall Corp., USA).

Membrane pre-treatment

Process carried out on a membrane after the completion of its preparation and prior to its use in a separation application, by means of pre-equilibration in actual feed solutions or a solution similar to the feed stream it will contact. Subsequently, membranes were allowed to equilibrate with the receptor phase for 6 h before charging each donor compartment with the formulations.

9.1.3 Diffusion cells

Vertical modified-Franz diffusion cells used as an apparatus to perform the diffusion test through the synthetic membrane with a receiver chamber of 13.50±0.50 ml (a diameter of 17 mm, contact surface area 2.27 cm^2).

9.1.4 Receptor phase

0.05 M, pH 3.0 citrate-phosphate buffer (CPB) solution was utilized as receptor medium. To ensure the stability of EGCG and its solubility, pH 3.0 was selected as optimum pH of receptor medium as confirmed by a good stability of EGCG in strong acidic aqueous buffer pH (Nwuha, 2000). The medium prepared was degassed before using.

9.1.5 Definite dose

Definite dose was 600 mg of CEs, containing 2.25%, w/w FD-GTE (with EGCG content at approximately 13.26% (w/w), on dry basis), which calculated based on the solubility of EGCG in receptor fluid. The solubility measurement would be performed to ensure that the receptor fluid was not rate limiting in the diffusion process and the experiment was conducted under sink conditions. The solubility of EGCG was approximately 1 mg/ml in the receptor fluid (pH 3.0 CPB) at 32±1 °C.

9.1.6 Procedure

The studies were carried out at 32±1 °C using 6 diffusion cell assemblies. The receiver chamber was filled to overflowing with a degassed receptor phase. The overflow allowed the cells to be bubble-free.

The membrane, which had been occluded with the receptor phase for 6 h, was placed over the orifice of each cell cap (with the upper side in contact with the donor chamber, and the lower side in contact with the receiver medium). According to a leakage of sample, a couple of silicone-typed o-rings were placed between membranes, and then both compartments were tightly held in position by a stainless-steel clamp. The air bubbles generated at underside of membrane

surface had to be removed by flipping cell several time until it move along the sampling port of cell.

The system was maintained at a controlled temperature of 32±1 °C, corresponding to the temperature of the human skin surface. Each cell body surrounded by water jacket were joined together and then connected to a water bath. In order to perform a completely mixing of fluid on downstream side of the membrane, the 6-stationed stand stirrers were set at constantly 600 rpm throughout the experiment. At last, the cells were allowed to equilibrate for 3 h before the donor formulation was added and defined as the starting point of the experiment.

Approximately 600 mg of CEs containing 2.25% FD-GTE (definite dose) was applied on the membrane surface as donor phase. With a glass rod, test sample was applied to the open cap of cell and tamped down on the equilibrated membrane. The tamping was continued until the view of the underside of the membrane showed no channeling. The dose was determined by weighing the rod before and after application.

During the study, the open cap of donor chamber and the sampling port of receiver chamber were covered by Parafilm™ in order to allow the cell to be occluded throughout the experiment.

Predetermined time intervals of 0, 15, 30, 45 min, 1, 2, 3, 4, 5, 6 h, 8, 10, 12 h, 15, 18 h, 24, 36, 48, 60,72 h were chosen as sampling times. The samples were removed simultaneously from the lower region of the receptor chamber in triplicates and replaced with the same withdrawn volume of fresh receptor fluid. An approximately 1.5 ml of sampling aliquot was removed using a syringe and 0.5 mm i.d. 4.5 cm flexible Teflon tubing and transferred into the safety-locked capping micro test tube. The samples were stored at −20 °C until HPLC analysis. The EGCG content was determined by using HPLC assay. All experiments were performed in triplicates.

9.2 Determination of EGCG amount released from concentrated w/o emulsions containing freeze-dried green tea extract (Study VI)

Approximately 1.5 ml of sampling aliquot was centrifuged at 12,000 rpm at −10±1 °C for 20 min and then taken the supernatant prior to inject through the HPLC system. Without any dilution, 20 µl of sample solution was directly injected

through HPLC. The concentrations of EGCG were determined by the HPLC. The analyses were all carried out in triplicate samples. Each sample was injected through HPLC in triplicates.

9.2.1 Analysis

The presence of the major catechins and caffeine in the extract was confirmed by qualitative analysis using RP-HPLC. The identification of samples from the diffusion experiments were performed by comparing individual response signals with reference standard. Quantitative analysis of samples from the diffusion experiments was by RP-HPLC. Calibration curves, prepared in receptor phase, were linear over the observed range, with R^2 values of ≥ 0.9999. Analytes were identified through a combination of comparison of retention times and sample spiking. All analytes were baseline resolved under conditions as previously described.

9.2.2 Data processing

Data obtained by HPLC analysis were collected for release profile. Q_t and Q_t/Q_0 were plotted as a function of time. In addition, the amount released at 48 h was stated as Q_{48} value.

For non-linear transformation using Microsoft® Office Excel 2003 program, release data from all formulations of CEs were fitted to the Peppas equation (Peppas, 1985). Each data point was a mean of three replicates.

Peppas equation:

$$Q_t = kt^n \qquad \text{Eq.25}$$

Where,

Q_t is a cumulative amount of drug released per unit surface area at any time t.

k is a release rate constant incorporating structural and geometric characteristics of the controlled release formulations.

n is a release exponent, that may be used to indicate the mechanism of drug release.

Cumulative amount released (Q_t, $\mu g \cdot cm^{-2}$) of EGCG per contact surface area of membrane was as following:

$$Q_t = \left(\frac{C_t \times V}{A} \right) \quad \text{Eq.26}$$

Where,

Q_t is a cumulative amount of EGCG released per unit surface area at anytime t ($\mu g \cdot cm^{-2}$).

C_t is a concentration of EGCG determined at anytime t ($\mu g \cdot ml$).

V is a volume of individual Franz cell (ml), which was 13.50±0.50 ml in this study.

A is a contact surface area of sample well (cm^2), which was 2.27 cm^2 in this study.

According to variation of initial amount of drug loading, the cumulative amount of EGCG released should be divided by the amount of EGCG presented initially in loading dose to correct the values of all data.

Cumulative amount released (Q, $\%\cdot cm^{-2}$) of EGCG per contact surface area of membrane per % unit initial drug loading dose was as following:

$$Q, \%\cdot cm^{-2} = \frac{Q_t}{Q_0} = \left(\frac{C_t \times V}{A} \right) \times \frac{100}{Q_0} \quad \text{Eq.27}$$

Where,

Q_0 is an amount of EGCG presented initially in the system (μg).

Q_t is a cumulative amount of EGCG released per unit surface area at anytime t ($\mu g \cdot cm^{-2}$).

C_t is a concentration of EGCG determined at anytime t ($\mu g \cdot ml$).

V is a volume of individual Franz cell (ml), which was 13.50±0.50 ml in this study.

A is a contact surface area of sample well (cm^2), which was 2.27 cm^2 in this study.

100 is unit transformation (%).

At the end of the in vitro release study, the cumulative amount released of EGCG was determined at 48 h, stated as Q_{48}. The calculation was as followed below:

$$Q_{48}, \%\cdot cm^{-2} = \frac{Q_{48}}{Q_0} = \left(\frac{C_{48} \times V}{A}\right) \times \frac{100}{Q_0} \quad \text{Eq.28}$$

Where,

Q_0 is an amount of EGCG presented initially in the system (µg).

Q_{48} is a cumulative amount of EGCG released per unit surface area at 48 h (µg·cm^{-2}).

C_{48} is a concentration of EGCG determined at 48 h (µg·ml).

V is a volume of individual Franz cell (ml), which is 13.50±0.50 ml in this study.

A is a contact surface area of sample well (cm^2), which is 2.27 cm^2 in this study.

100 is unit transformation (%).

CHAPTER IV

RESULTS AND DISCUSSION

1. Extraction of major components in dried green tea leaves

Most of researchers usually use boiling water for the extraction of major components from green tea, mainly considered to GTCs (Roberts *et al.*, 1956; Wood, and Roberts, 1964). In an early study, Roberts and Wood (1951) boiled fresh green tea leaves with water to study the constituents of catechins in green tea but found that the boiling of a green tea leaf infusion resulted in epimerization of the simple catechins and their galloyl esters.

However, there is no systematic and detailed method available for the extraction of catechins from green tea. Accordingly, hot water was trialed as a solvent to extract green tea and an extraction temperature was preferred at approximately 80 °C.

Extraction was carried out from 50 g of ground green tea leaves with 1,000 ml of 80 °C DI water. After that, aqueous fractions of ground green tea leaves were followed by an extraction with DCM to remove caffeine and some residues, if any, such as some pigments, aromatic compounds, etc. To assure completely removal of these compounds, the extraction was repeated at least three times or until color and odor turned pale. After the third DCM solvent extraction, color and odor considerably diminished. The entire GTCs rich aqueous fraction was collected. By using a vacuum rotary evaporator, the final extract was subsequently evaporated DCM solvent residual out. The evaporating process is not only removing of DCM solvent residual, but also performs concentrating of the final extract. Dark brown solid matters were obtained, as concentrated extracts. They were kept in freezing temperature at about −20 °C for further processing.

The freezing extract was allowed to come back to room temperature. The dried residue of extract was re-extracted with 20 ml of DI water, followed by completely drying in a laboratory freeze dryer. The light brown solid matters were obtained. The freeze-dried water extracted solids were stored in a freezer (−20 °C) before further analysis.

1.1 Determination of total yield of freeze-dried green tea extract

Total yields of FD-GTE solid matters obtained from both extraction procedures (procedure A and B) were quantified as a weight of FD-GTE obtained per 50 g of dried tea leaves and as a percentage of total yield based on weight of dried tea leaves.

A total yield of 17.0634 g extract per 50 g dried tea leaves with total GTCs at approximately 8.8883% (w/w), based on dried tea leaves could be obtained from procedure A. By comparison, procedure B provided the negligible higher extent of total yield around 17.1534 g extract per 50 g dried tea leaves with total GTCs at approximately 9.9119% (w/w), based on dried tea leaves. The total yields of freeze-dried extracted solids, obtained from procedure A and B were 34.1267 and 34.3067% (w/w) of dried tea leaves, respectively (table 9).

Table 9 Total yield of freeze-dried green tea extract from two different procedures.

Extraction procedure	% Total yield[1], w/w of FD-GTE (on dry basis of dry leaves)		
Procedure A	34.1267	±	0.1845
Procedure B	34.3067	±	0.7447

[1], Means ± SD (n = 3)

Furthermore, it showed that the total yields of FD-GTE from both procedures were above 30% (w/w) of dried tea leaves. This may suggest that some of extracted matters were negligible loss during the DCM solvent extraction. However, the loss yield during the extracting process showed not significantly affecting overall yield of product obtained.

1.2 Statistical analysis

Although, the total yield of FD-GTE from procedure B was slightly higher than the one obtained from procedure A, but it did not show a significant difference between both procedures.

From the independent t test analysis (appendix I), a significance value of 0.7053 (sig. > 0.05) indicated that there was no significant difference between two group means. Accordingly, it could be concluded that the total yield of extracted

solids obtained from FD-GTE with DCM solvent extraction was not significantly different to the one without DCM ($P < 0.05$).

1.3 Determination of green tea catechins and caffeine contents in freeze-dried green tea extract samples

The freeze-dried water extracted solids were determined for the contents of individual GTCs, including EGC, EGCG, EC and ECG. Furthermore, the residual content of caffeine, which was not completely remove from the extraction processing, was also determined to investigate the effect of DCM solvent on decaffeinated processing. The amount of each GTC and caffeine presented in FD-GTE was calculated from the peak area of each substance obtained from the HPLC chromatogram with the substitution in the equation of each standard curve.

The contents of individual GTCs and caffeine in FD-GTE as percent purity, based on weight of dried tea leaves and dried extracts, are all presented in table 10 and table 11, respectively.

The percent content of total GTCs in FD-GTE, obtained from procedure A was between 26.0087% and 26.0810%, with an average of 26.0449% (w/w) of dried extracts. This was slightly lesser than the total GTCs content detected in FD-GTE from procedure B, which ranged from 28.7716% to 29.0129%, with an average of 28.8923% (w/w) of dried extracts.

Whereas, the percent content of total GTCs in dried tea leaves, obtained from procedure A was between 8.8759% and 8.9006%, with an average of 8.8883% (w/w) of dried tea leaves. This was slightly lesser than the total GTCs content detected in FD-GTE from procedure B, which ranged from 9.8705% to 9.9533%, with an average of 9.9119% (w/w) of dried tea leaves.

It also showed that the percent content of caffeine in FD-GTE, obtained from procedure A was 5.9924% (w/w) of dried extracts and 2.0451% (w/w) of dried tea leaves. By comparison, the caffeine content in FD-GTE, obtained from procedure B was considerably high at approximately 0.4796% (w/w) of dried extracts and 0.1646% (w/w) of dried tea leaves.

As a result, it showed that hot DI water (80 °C) extracted considerably higher amounts of all components examined, excepted ECG.

However, significant difference of caffeine content was observed in comparison of FD-GTE with and without DCM solvent extraction. The percent content of caffeine obtained from FD-GTE with DCM solvent extraction was significantly less than the one without DCM. Therefore, it could be concluded that DCM solvent could be effective to extract considerably higher amount of caffeine.

Table 10 Contents of major components in dried green tea leaves from two different procedures, including EGC, EGCG, EC, ECG and caffeine.

Major components in dried green tea leaves	Procedure A			Procedure B		
	Content (%, w/w) (dry basis of tea leaves)[a]			Content (%, w/w) (dry basis of tea leaves)[a]		
EGC[*]	2.3323	±	0.0062	2.4612	±	0.0132
EGCG[*]	3.9732	±	0.0093	4.5484	±	0.0168
EC[*]	0.6769	±	0.0028	0.7362	±	0.0017
ECG	1.0593	±	0.0031	1.2215	±	0.0228
Total GTCs[b*]	8.8883	±	0.0175	9.9119	±	0.0585
Caffeine[*]	2.0451	±	0.0114	0.1646	±	0.0018

[a], Means ± SD (n = 2).
[b], Total GTCs does not include caffeine.
[*], Statistical significantly difference at $P < 0.05$.

Table 11 Contents of major components in freeze-dried green tea extracts from two different procedures, including EGC, EGCG, EC, ECG and caffeine.

Major components in FD-GTE	Procedure A			Procedure B		
	Content (%, w/w) (dry basis of extracts)[a]			Content (%, w/w) (dry basis of extracts)[a]		
EGC[*]	6.8340	±	0.0181	7.1740	±	0.0386
EGCG[*]	11.6425	±	0.0274	13.2580	±	0.0488
EC[*]	1.9834	±	0.0083	2.1460	±	0.0050
ECG	3.1041	±	0.0092	3.5603	±	0.0666
Total GTCs[b*]	26.0449	±	0.0511	28.8923	±	0.1706
Caffeine[*]	5.9924	±	0.0334	0.4796	±	0.0052

[a], Means ± SD (n = 2).
[b], Total GTCs does not include caffeine.
[*], Statistical significantly difference at $P < 0.05$.

By comparing the percentage of each catechin content as shown in the table 10 and table 11, it showed that there were significant differences of the percentage of each catechin between both FD-GTE samples, obtained from extraction with and without DCM solvent extraction.

1.4 Statistical analysis

From the independent t test analysis (appendix I), a significance value of 0.0232, 0.0023, 0.0044 and 0.0021 for EGC, EGCG, EC and caffeine, respectively (sig. < 0.05), indicated that there were significant differences between two group means. Whereas, only significance value of 0.0611 for ECG (sig. > 0.05) indicated that there was no significant difference between two group means.

Accordingly, it could be concluded that the percent content of GTCs (excepted for ECG) and caffeine obtained from FD-GTE with DCM solvent extraction were significantly different to the one without DCM ($P < 0.05$). The percent content of ECG was the only one that showed no significant difference between two extraction procedures.

2. Development of analytical procedures for qualitative and quantitative determinations of green tea catechins and caffeine constituents in freeze-dried green tea extracts or other test samples by HPLC analysis

The major components in FD-GTE, mainly considered to GTCs and caffeine in all samples were analyzed by RP-HPLC system.

Derived from previous study on an isocratic elution system for the determination of catechins together with caffeine (Wang *et al.*, 2000), a binary gradient elution system was developed for the separation of catechins together with caffeine.

2.1 HPLC systems

Separations were performed with a C18 RP-analytical column (Nova-Pak®), 4 µm silica bonded phase, dimethyl-octadecylsilyl (dimethyl-ODS) bonded amorphous silica bed, protected with C18 RP-guard column (Waters Sentry™).

The C18-Nova-Pak® column was not tested by comparing with other types. However, this type of column provided optimal separation of catechins together with caffeine, it, therefore, could be employed in this study.

2.2 Chromatographic conditions development (study I)

The HPLC chromatographic conditions were developed according to the following effects:

2.2.1 Effect of mobile phase on chromatographic separation and elution of green tea catechins together with caffeine constituents

2.2.1.1 Acidic aqueous buffer solution

From the results (not shown herein), the highest amount of *o*-phosphoric acid (0.10%, v/v) provided the best separation between all peaks detected in each chromatogram. Since the structures of the catechins and

caffeine constituents, found in FD-GTE samples are all weak acidic drugs that can ionize themselves in the alkaline system. The very low pH at approximately 2.50±0.01, obtained from an aqueous buffer system containing 0.10% (v/v) *o*-phosphoric acid could protect the ionization of the weak acidic compounds. The two tailing peaks, therefore, could not be obtained by avoiding the interaction between the free hydroxyl groups of the ionized polyphenols and the stationary phase. Therefore, an aqueous phase containing 0.10% (v/v) *o*-phosphoric acid was preferred to use in this study.

2.2.1.2 Organic modifier

By using the solvent gradient elution, full separation (results not shown herein) could be achieved in the different ratios of the methanol/water system with *o*-phosphoric acid addition. The solvent gradient elution program has been already shown in table 12.

According to the results of all effects of the mobile phase compositions, a system consisting of methanol as an organic modifier, water with an approximately 0.10% (v/v) of *o*-phosphoric acid as pH control substance was preferred in this study.

2.2.2 Effect of diluting solvent compositions on chromatographic separation and elution of green tea catechins together with caffeine constituents

The diluting solutions composed of 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution with 20% (v/v) methanol (Me 20) provided higher extent of dissimilarity effect of organic solvent when passing through the HPLC system. The first peak eluted through HPLC system, EGC, could be overlapped with the solvent peak occurred at void volume of injection. By comparison, the chromatogram obtained from the FD-GTE samples diluted with 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution in 10% (v/v) methanol (Me 10), did not show considerably interfering of the solvent peak to the peak of EGC (results not shown herein). There was not a considerable overlap between the peak of EGC and the solvent peak of

methanol. Accordingly, the diluting solvent, composed of 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) with 10% (v/v) methanol, was preferred to use throughout the HPLC analysis.

2.2.3 Effect of column temperature on chromatographic separation and elution of green tea catechins together with caffeine constituents

The faster elution could be performed when increased the column temperature. Although, the faster elution would save the time of analysis, but the separation between adjacent peaks could not still be satisfied. As the results (not shown herein), the chromatogram obtained from the elution with 33±1.0 °C showed a sufficiently good separation between adjacent peaks, e.g. caffeine, EGCG and EC. With column temperature higher than 33±1.0 °C, these peaks tended to overlap each other. Thus, the column compartment should be controlled at approximately 33±1.0 °C.

2.2.4 Effect of wavelength of detector on peak absorbance

All catechins and caffeine were found to have maximum absorbance at 210 nm and in the range of 275-280 nm. However, chromatograms recorded at 210 nm (results not shown herein) showed considerable improvement in signal-to-noise ratio. This was especially useful for the determination of EC and ECG, which were very difficult to detect, either because of their low content found in green tea samples or because of their low response (Wang *et al*., 2000). Accordingly, all chromatograms obtained throughout this study were recorded and computerized under detector wavelength at 210 nm.

2.3 Chromatographic conditions (study II)

Modified chromatographic conditions of the analysis with the gradient solvent system were as followed. In addition, the compositions of mobile phase used, composed of different ratios of the acidic aqueous buffer solution to an organic modifier, were evaluated. Ratios of aqueous buffer to organic solvent that provided

the best separation between all peaks detected in each chromatogram are shown in table 12 as binary gradient solvent system.

Modified chromatographic conditions of the analysis (Wang *et al*., 2000)

Column : Nova-Pak® C18, 4 µm, 4.6 x 150 mm.

Column temperature : 33±1.0 °C.

Solvent elution : Binary concentration gradient elution.

Mobile phase : Eluent A: 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) with 10% methanol.
Eluent B: 0.10% (v/v) *o*-phosphoric acid aqueous buffer solution (pH 2.50±0.01) with 20% methanol.

Flow Rate : 0.8 ml/min.

Injection Volume : 20 µl.

Detection Wavelength : 210 nm, UV spectra in the 190–370 nm range.

Post running Time : 20 min.

Total running Time : 55 min.

Table 12 Modified chromatographic conditions of the analysis (Wang *et al*., 2000): Gradient solvent system program.

Retention time (min)	Total methanol (%, v/v)	Eluent A (%, v/v)	Eluent B (%, v/v)
0.01	12.50	75.00	25.00
21.00	12.50	75.00	25.00
21.01	18.00	20.00	80.00
36.00	18.00	20.00	80.00
36.01	12.50	17.00	25.00
55.00	12.50	75.00	25.00

3. Qualitative determination of freeze-dried green tea extract samples (Study III)

3.1 Identification of green tea catechins and caffeine in freeze-dried green tea extract samples

Identification of GTCs and caffeine was carried out using Photo-DAD. UV spectra were extracted from the principal peaks of the chromatogram of freeze-dried extracts from dried green tea leaves (figure 13).

These PDA UV spectra extracted were used in combination with retention times and spectral data of available reference standards to identify the compounds responsible for the peaks.

Chromatogram of green tea catechins and caffeine in freeze-dried green tea extract samples compared with individual and mixed reference standards

The FD-GTE samples equivalent to 0.04000 mg/ml were identified. To set each standard concentration close to the real concentration of sample, mixed reference standard solutions of EGC, EGCG, EC, ECG and caffeine were constituted up to approximately 0.00604, 0.02488, 0.02060, 0.00348, and 0.00399 mg/ml, respectively. While, each reference standard solution of EGC, EGCG, EC, ECG and caffeine was also prepared individually at approximately 0.02000 mg/ml.

After that, they were then injected through the RP-HPLC. Chromatographic peaks in the samples were identified by comparing their retention times and UV spectra in the 190–370 nm range with those of the reference standards. Peaks were considered to chromatographically pure when there was exact coincidence of their corresponding UV spectra. Discussion of the identification of these peaks is detailed below.

From the analysis of FD-GTE samples in figure 13, the chromatogram showed that there were five peaks (1, 2, 3, 4 and 5) between 8 and 35 min. Retention times were in the range of 6.413 min for peak 1, 11.703 min for peak 2, 13.455 min for peak 3, 15.748 min for peak 4 and 31.356 min for peak 5.

The oxidation products of green tea polyphenols should be the slowest eluting compounds during HPLC analysis based on the work of Bailey, McDowell,

and Nursten (1990). However, there were no obvious peaks in the chromatogram of figure 13 after 35 min of elution, suggesting that the extraction of polyphenols caused little oxidation.

The chromatograms of mixed reference standard with final concentration of EGC, EGCG, EC, ECG and caffeine at approximately 0.00604, 0.02488, 0.02060, 0.00348, and 0.00399 mg/ml, respectively, were shown in figure 14. Retention times were in the range of 6.514 min for EGC, 11.773 min for caffeine, 13.712 min for EGCG, 16.038 min for EC and 31.806 min for ECG.

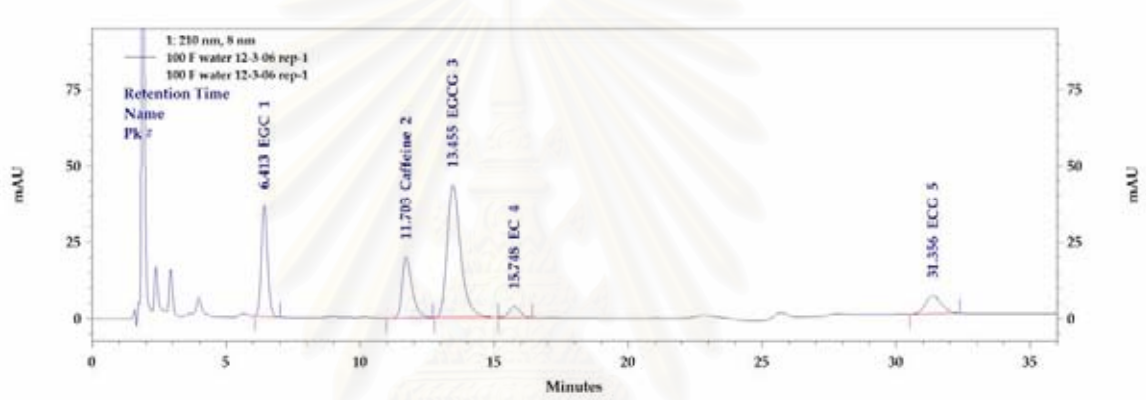


Figure 13 HPLC chromatogram of a freeze-dried extract from dried green tea leaves monitored at 210 nm (eluted in the first 35 min). Mobile phase: 0.10% (v/v) *ortho*-phosphoric acid buffer solution (pH 2.50±0.01) with 10% methanol (eluent A) and 0.10% (v/v) *ortho*-phosphoric acid buffer solution (pH 2.50±0.01) with 20% methanol (eluent B). Binary gradient elution as follows: maintained at 25% B for 21 min; switched to 80% B over 15 min, and then returning to 25% B for 20 min. The dried green tea leaves (*Camellia sinensis*) were obtained from Namchai Tea (Doi Mae Salong, Thailand).

While, the chromatograms of individual reference standard with final concentration at approximately 0.02 mg/ml in dilution solvent were shown in figure 16, 17, 18, 19 and 20. Retention times were in the range of 6.443 min for EGC, 11.414 min for caffeine, 13.108 min for EGCG, 15.402 min for EC and 30.895 min for ECG. Figure 21 showed that it was not found peak of impurities in chromatogram of diluting solvent (blank sample).

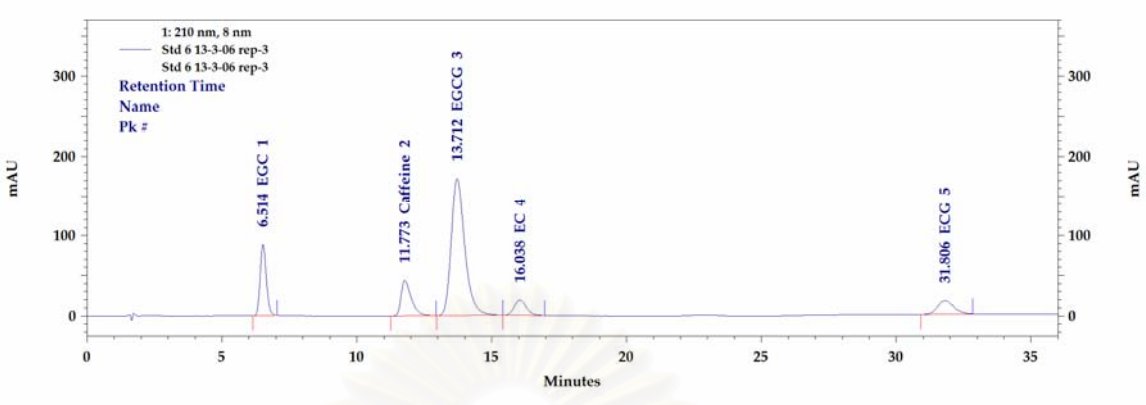


Figure 14 HPLC chromatogram of mixed reference standards: EGC, EGCG, EC, ECG and caffeine at 100% dilution concentration.

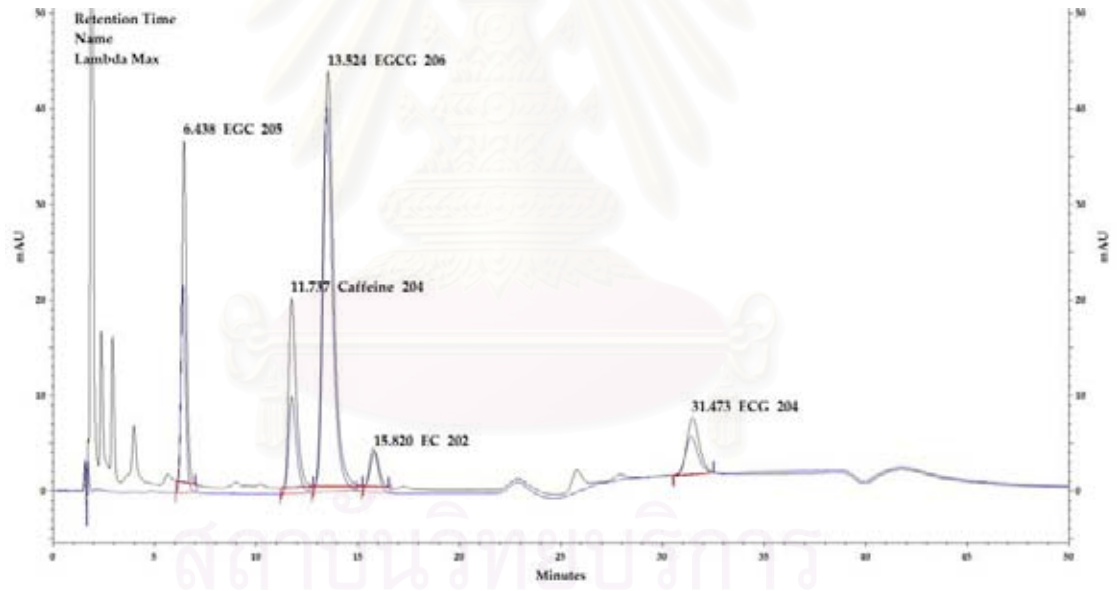


Figure 15 Superimposed chromatograms of mixed reference standards: EGC, EGCG, EC, ECG and caffeine at 25% dilution concentration and freeze dried green tea extract samples equivalent to 0.04 mg/ml.

It should be noted that there was, in some cases, a small shift between the peak retention time and the corresponding peak spectrum time because of the photodiode array software, which divides the time scale of the chromatogram into very small sections. As a result, the spectrum was sometimes extracted from slightly off the top of the peak. This shift or difference, however, was usually very small.

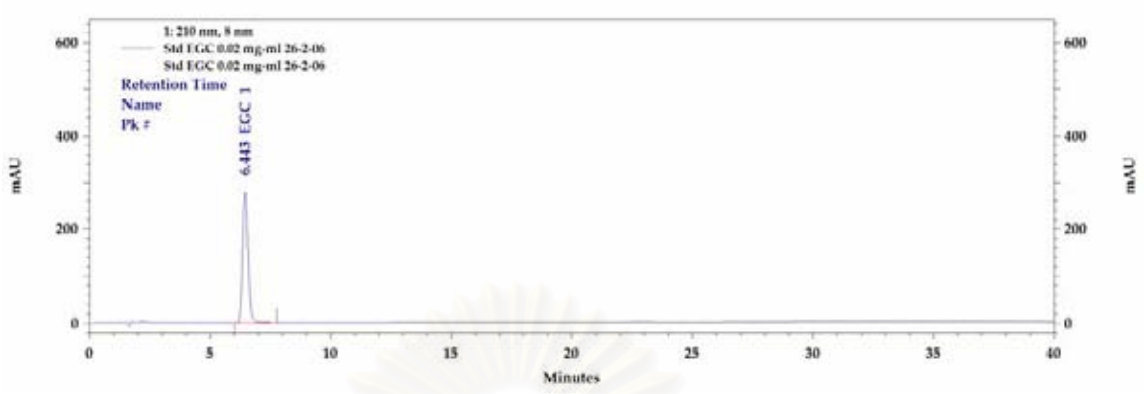


Figure 16 Chromatogram of individual EGC reference standard.

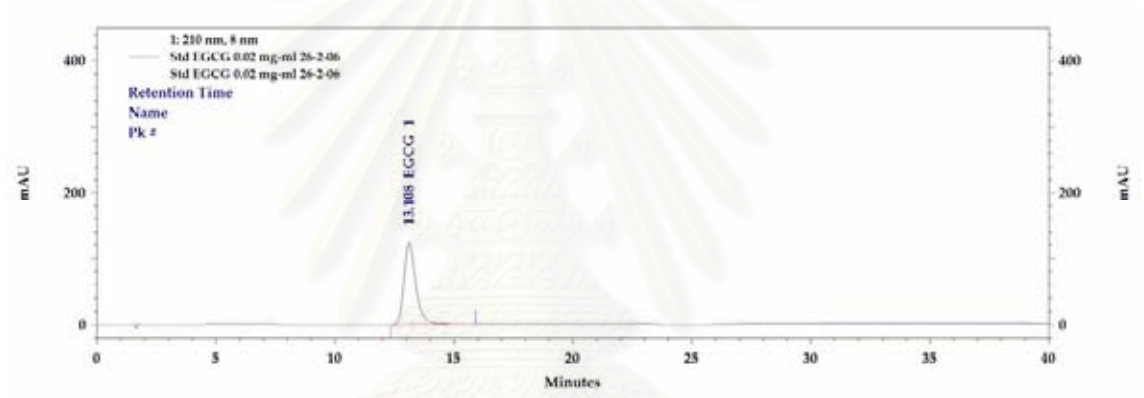


Figure 17 Chromatogram of individual EGCG reference standard.

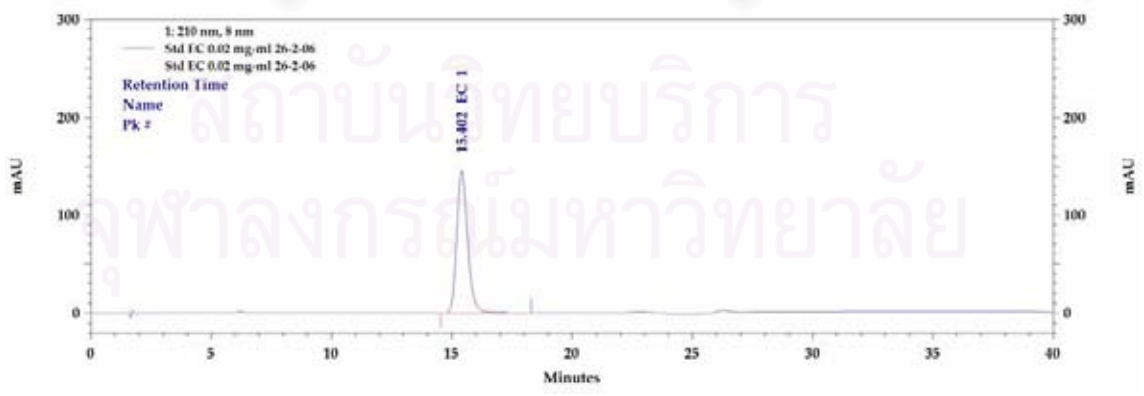


Figure 18 Chromatogram of individual EC reference standard.

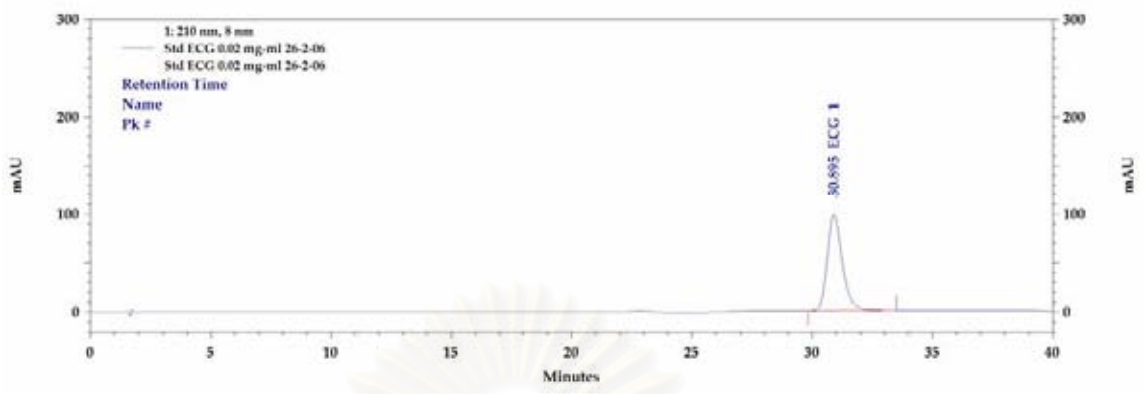


Figure 19 Chromatogram of individual ECG reference standard.

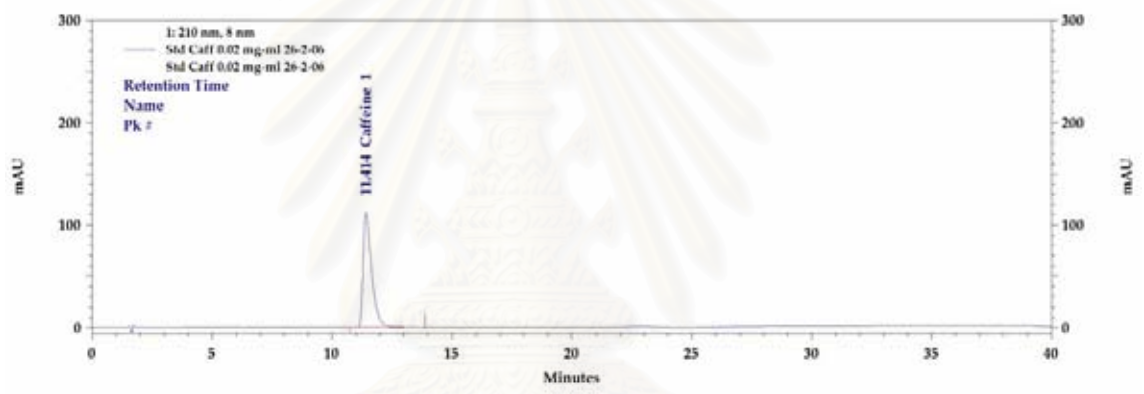


Figure 20 Chromatogram of individual caffeine reference standard.

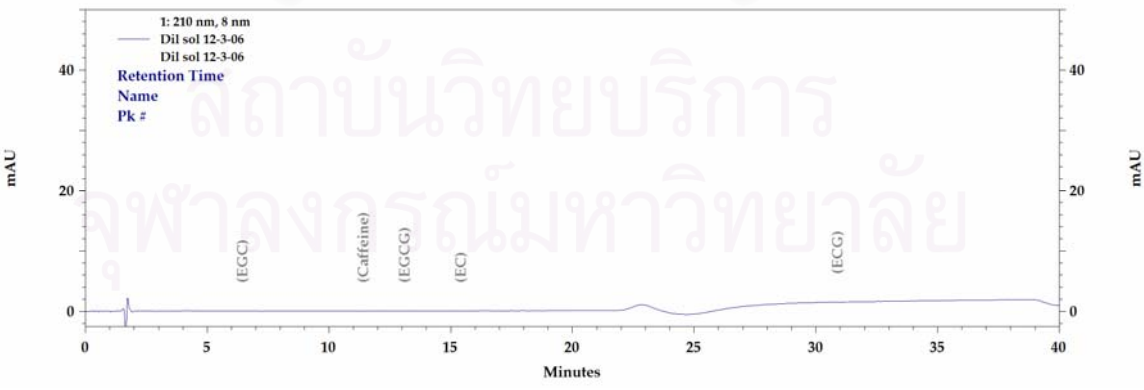


Figure 21 Chromatogram of diluting solvent (blank).

The retention times, spectral data and the identity for the peaks in the chromatogram are shown in table 13 for samples, table 14 for mixed reference standards and table 15 for individual reference standards.

Table 13 UV absorption peak maxima and identity of peaks in the chromatogram of samples.

Peak no.	Retention time (min)	Peak maxima (nm)	Identity chromatogram
1	6.413	205, 230sh, 269	Epigallocatechin (EGC)
2	11.703	205, 230sh, 272	Caffeine
3	13.455	206, 272	Epigallocatechin gallate (EGCG)
4	15.748	202, 227sh, 277	Epicatechin (EC)
5	31.356	204, 220sh, 275.5	Epicatechin gallate (ECG)

sh-shoulder.

Table 14 UV absorption peak maxima and identity of peaks in the chromatogram of mixed reference standards of catechins and caffeine.

Peak no.	Retention time (min)	Peak maxima (nm)	Identity chromatogram
1	6.514	205, 230sh, 269	Epigallocatechin (EGC)
2	11.773	205, 230sh, 272	Caffeine
3	13.712	206, 272	Epigallocatechin gallate (EGCG)
4	16.038	202, 227sh, 277	Epicatechin (EC)
5	31.806	204, 220sh, 275.5	Epicatechin gallate (ECG)

sh-shoulder.

Table 15 UV absorption peak maxima and identity of peaks in the chromatogram of individual reference standard of catechins and caffeine.

Peak no.	Retention time (min)	Peak maxima (nm)	Identity chromatogram
1	6.443	205, 230sh, 269	Epigallocatechin (EGC)
2	11.414	205, 230sh, 272	Caffeine
3	13.108	206, 272	Epigallocatechin gallate (EGCG)
4	15.402	202, 227sh, 277	Epicatechin (EC)
5	30.895	204, 220sh, 275.5	Epicatechin gallate (ECG)

sh-shoulder.

Based on the HPLC elution order, retention times and UV spectra (table 13), the assignment of peaks 1, 2, 3, 4 and 5 to the relevant GTCs and caffeine listed in table 13 was possible.

The PDA spectra of these five peaks were compared with those of reference standards using HPLC.

These PDA UV spectra extracted were used in combination with retention time and spectral data of available reference standards to identify the compounds responsible for the peaks.

The retention time and the spectrum of each peak were proved very strong evidence for the identity of an unknown compound. Thus, criteria for the identification of tea compounds were established based on comparisons of the retention time and spectrum of an unknown compound with the previously generated HPLC data library of standards. The resolution and asymmetry of each peak were determined to ensure correct identification. For closely eluting peaks in the chromatogram, an integration program in the HPLC software was used to split the peaks and produce data for the calculation. For well-resolved or symmetric peaks, the resolutions between closely eluting peaks, such as peaks caffeine, EGCG and EC in the chromatogram were all not less than 1.5.

By utilizing linear PDA technology, the multi-wavelength detector can monitor continuously between 190 and 800 nm, which enables the UV and visible regions to be monitored simultaneously. However, the major constituents in green tea samples were only considered to GTCs and caffeine, which permitted absorbance in the range of UV spectrum. To coverage the range of UV spectrum, the wavelength of detector was monitor ranging between 190 and 370 nm.

The PDA UV spectra of peaks 1, 3, 4 and 5 are characteristic spectra of GTCs and their gallates. All of them have two specific resolved bands (figure 24, 25, 26 and 27). Band I ranges between 200-210 nm and is little affected by the galloyl status of the compounds. Band II ranges 265-280 nm and the shift of absorption depended on the galloyl status of the compounds. The retention times and spectra of a number of reference standard catechins and their gallates compare satisfactorily with the spectra and retention times of these compounds permitting their identification (table 14 and table 15). The four principal compounds present in dried green tea leaves are EC, ECG, EGC, and EGCG (figure 22).

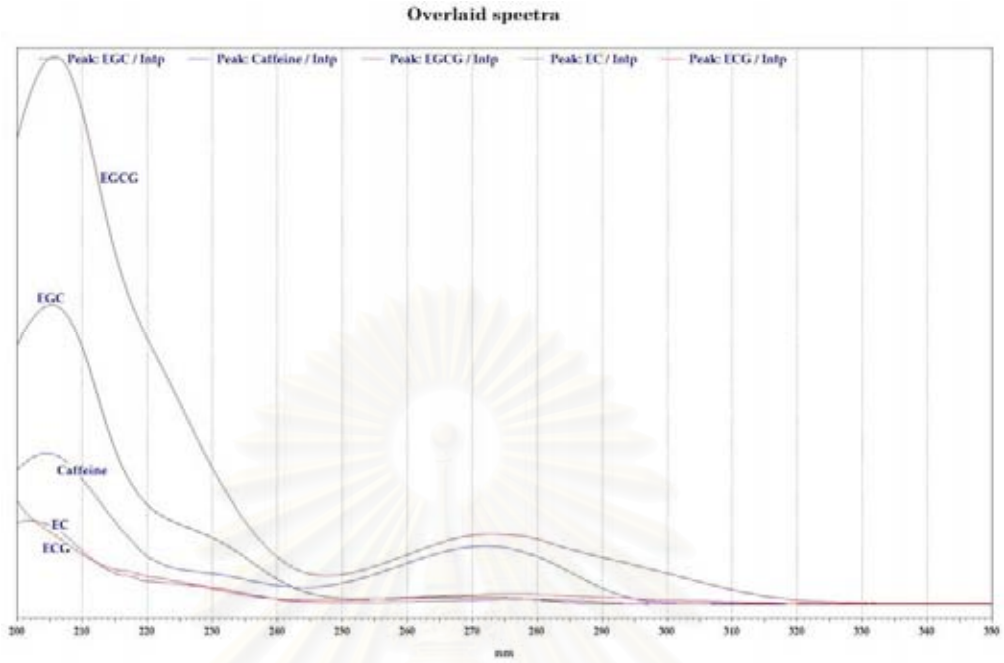


Figure 22 Photodiode array UV spectra of the GTCs and caffeine in freeze-dried green tea extract samples equivalent to 0.04 mg/ml.

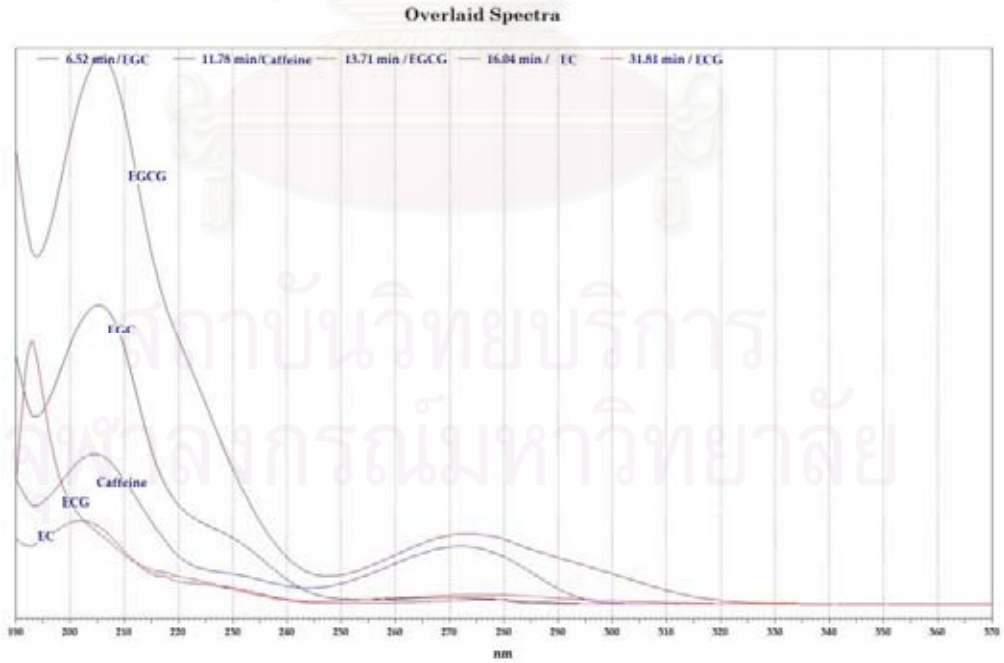


Figure 23 Photodiode array UV spectra of mixed reference standards: EGC, EGCG, EC, ECG and caffeine at 100% dilution concentration.

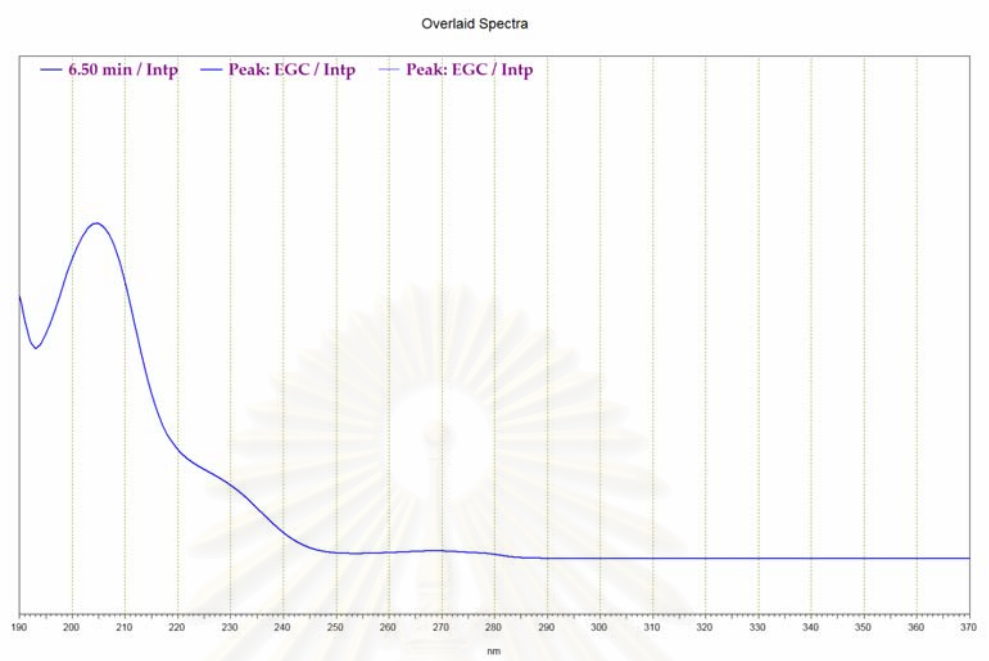


Figure 24 Photodiode array UV spectra of the principal green tea catechins: EGC (peak 1).

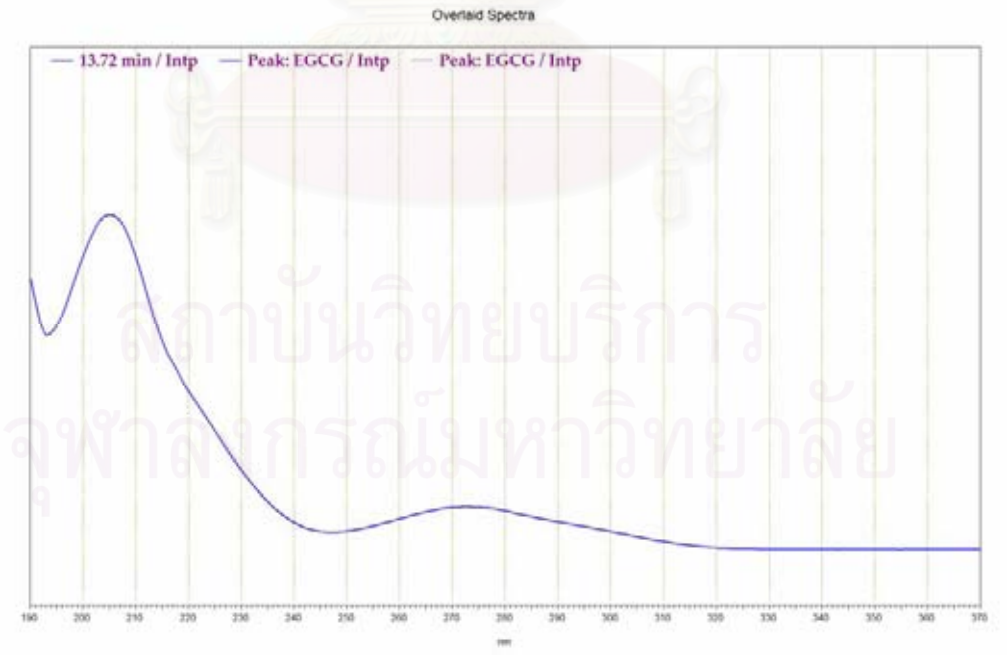


Figure 25 Photodiode array UV spectra of the principal green tea catechins: EGCG (peak 3).

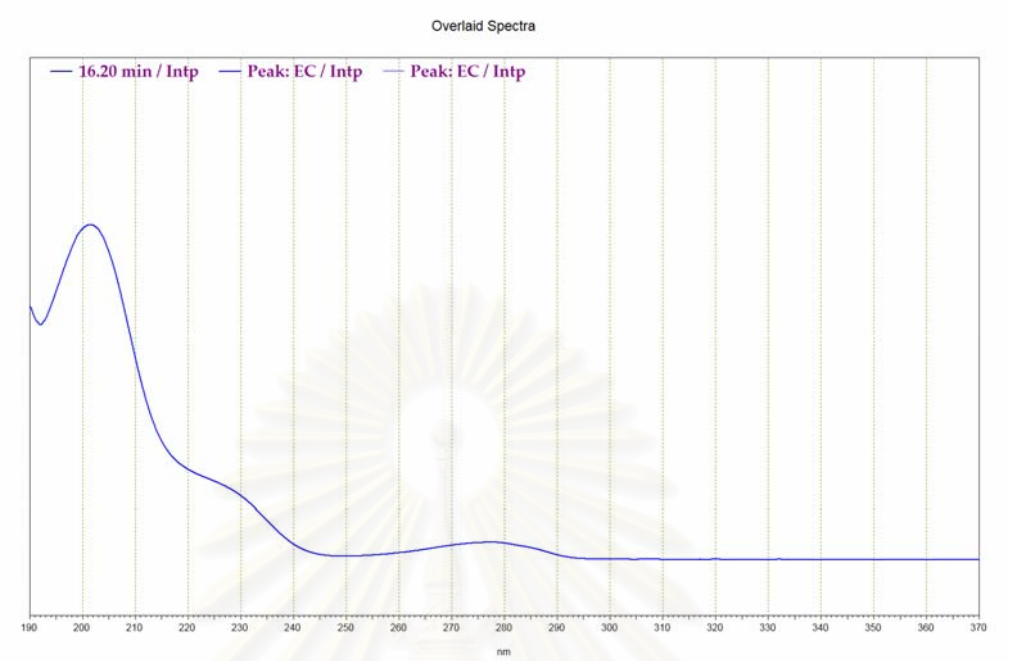


Figure 26 Photodiode array UV spectra of the principal green tea catechins: EC (peak 4).

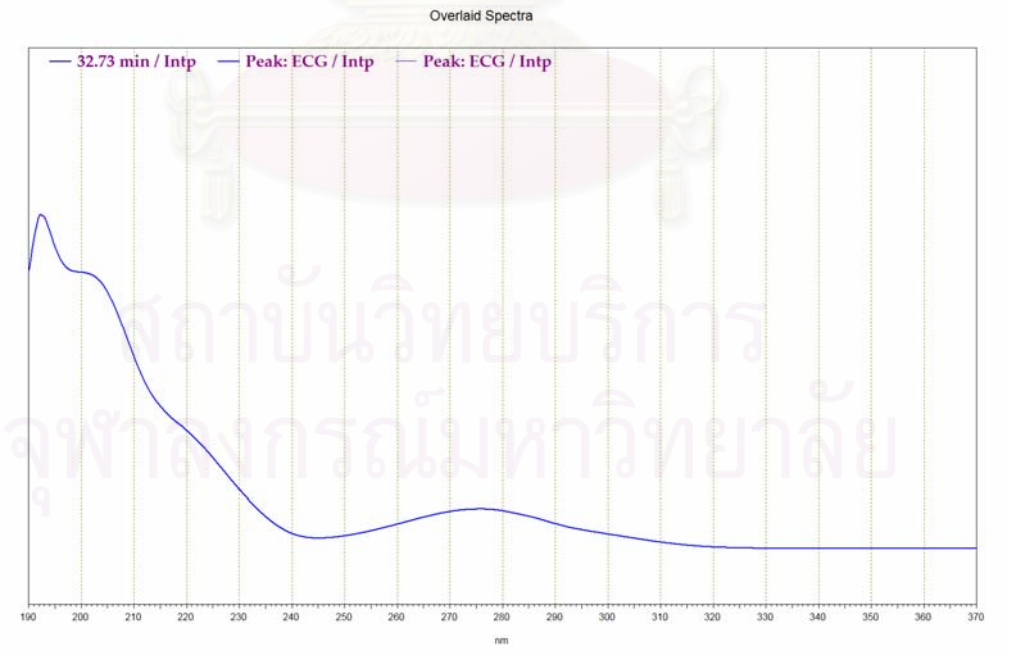


Figure 27 Photodiode array UV spectra of the principal green tea catechins: ECG (peak 5).

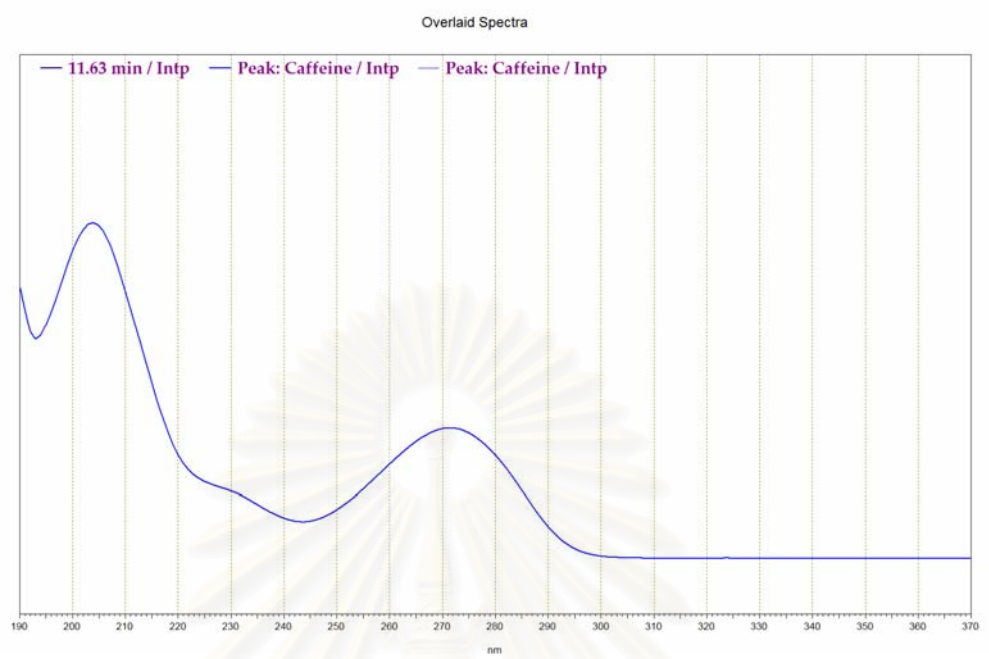


Figure 28 Photodiode array UV spectra of the principal green tea alkaloids: caffeine (peak 2).

It is remarkable that the peak UV absorption maxima of the reference standard catechins and catechin gallates contain only a UV absorption peak maximum between 200-210 nm. The UV absorption maxima of those compounds at 200-240 nm are not listed in the literature. This is because in the past most of the UV absorption maxima were measured photometrically or under a UV light with a certain wavelength. In their systematic identification of GTCs using the UV spectra, Mabry, Markham, and Thomas, (1970) showed that only the maxima for those peaks at wavelengths longer than 240 nm were tabulated. The UV absorption of GTCs is usually extracted with their maximum absorption regions, such 270-280 nm.

4. Quantitative determination of freeze-dried green tea extract samples (Study IV)

4.1 Determination of green tea catechins and caffeine contents in freeze-dried green tea extract samples

The composition of GTCs and caffeine from FD-GTE or other test samples was determined by RP-HPLC. Chromatographic conditions used were as described above in study II. Mixed reference standard solutions were injected in triplicate into the HPLC, and peak area responses were obtained. Quantification was carried out from integrated peak areas of the sample and corresponding standard curve. To quantify each GTC in FD-GTE using the developed HPLC condition, the sample and standard solutions were injected through the system. Standard curve of EGC, EGCG, EC, ECG and caffeine were constructed by plotting peak area of chromatogram versus concentration. Percentage of each catechin was calculated by comparing peak area of sample with standard.

The final sample solutions of FD-GTE were performed in duplicate at concentration equivalent to 0.04 mg/ml. The FD-GTE sample solutions were injected in duplicate into the HPLC, and peak area responses were obtained.

Standard curve of green tea catechins and caffeine

A standard curve for each component was prepared by plotting concentration versus area as shown in table 16. Quantification was carried out from integrated peak areas of the sample and corresponding standard curve.

The standard curves were performed by plotting peak areas versus concentration as shown in figure 29-33.

R^2 (correlation) of EGC, EGCG, EC, ECG and caffeine standard curves were 0.9999, 0.9999, 0.9999, 0.9999 and 0.9999 respectively.

Table 16 Average peak area of green tea catechins and caffeine reference standards at six different concentration dilutions (standard curve).

Mixed Std point	EGC		Caff		EGCG		EC		ECG	
	Conc.	Area	Conc.	Area	Conc.	Area	Conc.	Area	Conc.	Area
Std 1	0.00009	19,749	0.00006	10,210	0.00037	81,795	0.00031	64,648	0.00005	9,208
Std 2	0.00091	197,540	0.00060	80,537	0.00373	840,198	0.00309	649,970	0.00052	98,848
Std 3	0.00151	326,475	0.00100	133,505	0.00622	1,391,335	0.00515	1,076,458	0.00087	168,185
Std 4	0.00302	659,811	0.00199	271,843	0.01244	2,818,436	0.01030	2,180,335	0.00174	339,130
Std 5	0.00453	986,525	0.00299	408,597	0.01866	4,219,658	0.01545	3,262,237	0.00261	512,714
Std 6	0.00604	1,314,638	0.00399	546,062	0.02488	5,625,791	0.02060	4,347,888	0.00348	684,672

Area, average peak area response ($mAU \cdot s^{-1}$); Conc., final concentration at each dilution points (mg/ml).

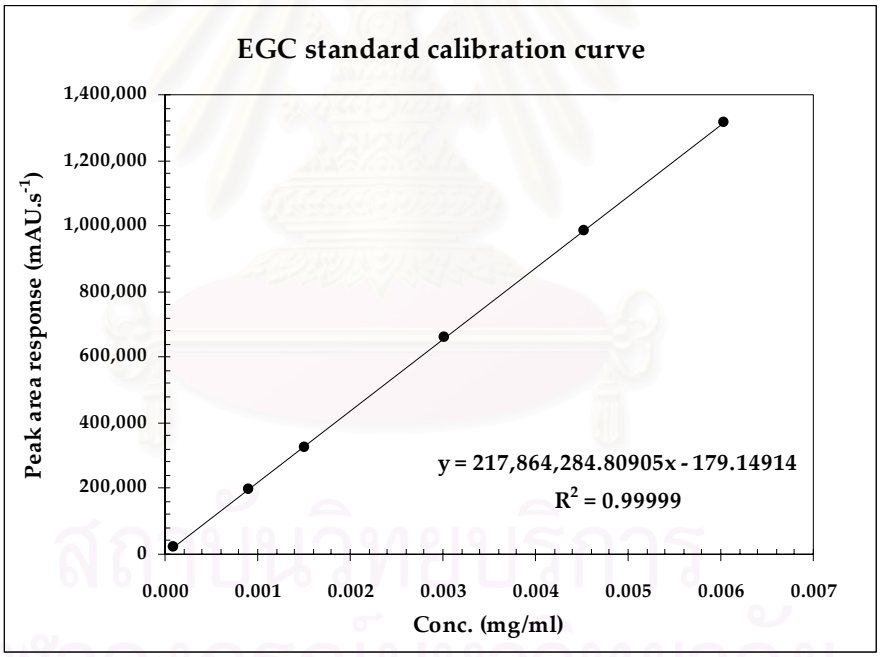


Figure 29 Standard curve of EGC, Epigallocatechin.

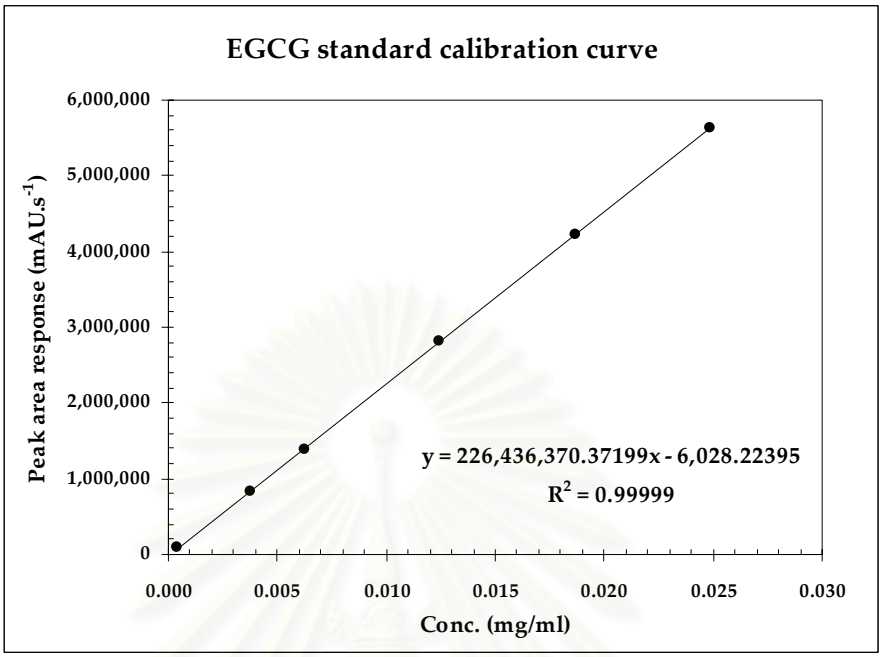


Figure 30 Standard curve of EGCG, Epigallocatechin gallate.

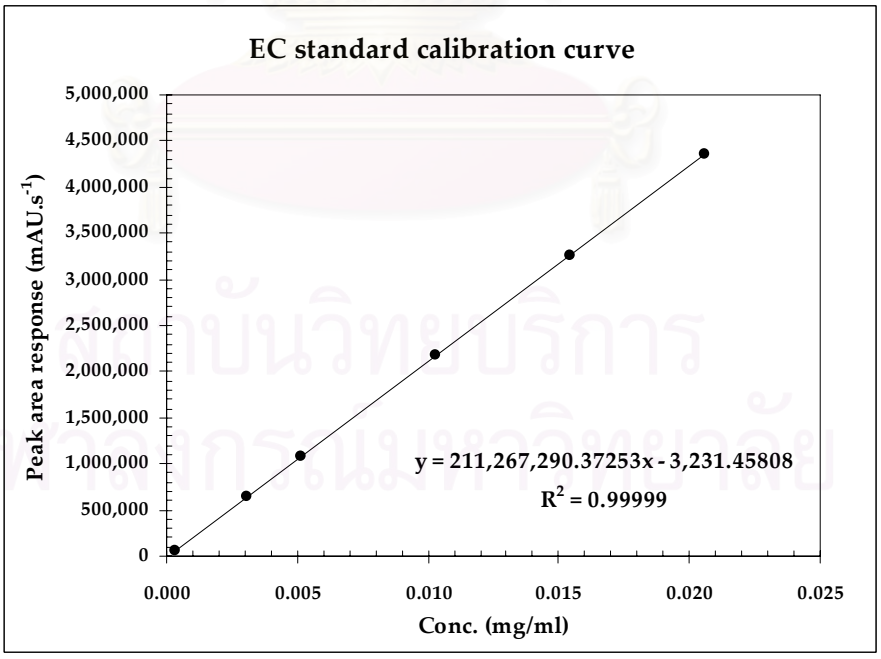


Figure 31 Standard curve of EC, Epicatechin.

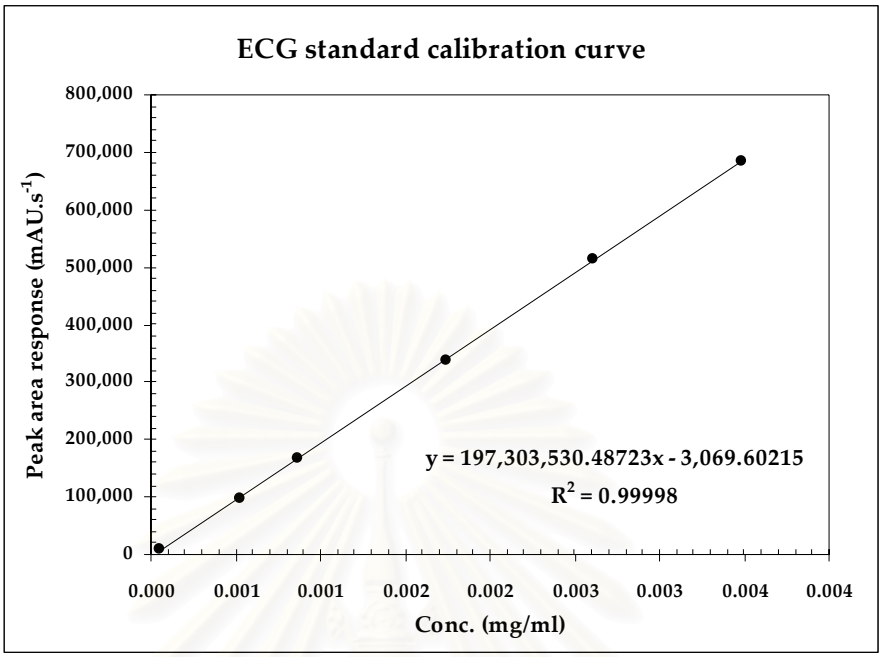


Figure 32 Standard curve of ECG, Epicatechin gallate.

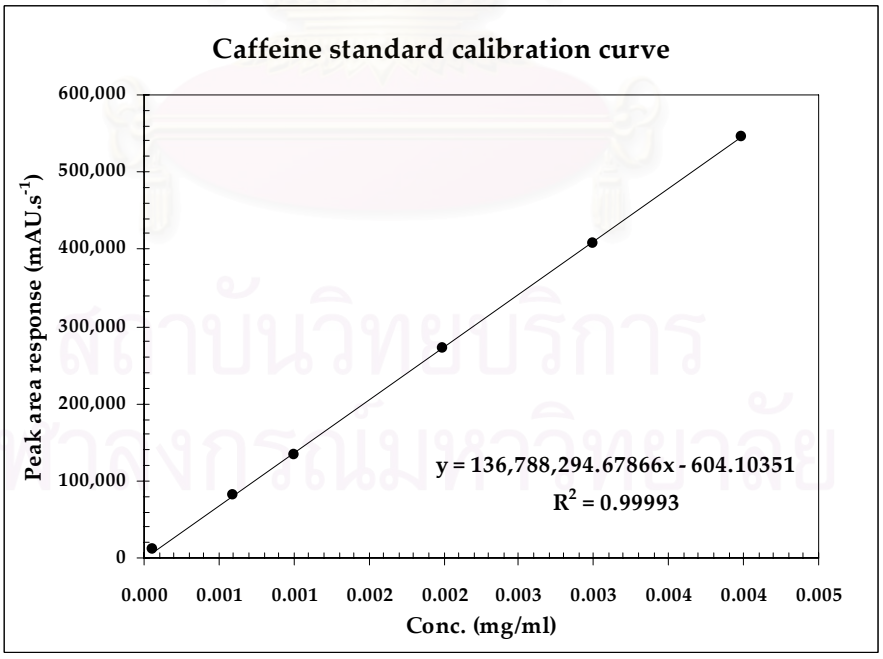


Figure 33 Standard curve of caffeine.

5. Validation of analytical method for qualitative and quantitative determination of green tea catechins and caffeine in freeze dried green tea extract samples by HPLC analysis (Study V)

Developed analytical method for an assay of EGC, EGCG, EC, ECG and caffeine in FD-GTE were validated, according to ICH guidance Q2A (ICH, 1995) and Q2B (ICH, 1996).

5.1 Validation of protocols

HPLC analysis was used for the purpose of identification and assay for drug substance and drug product.

Accuracy

Accuracy of the method was studied at five different concentrations of sample solution without a serial dilution. From the results summarized in table 17-21, it showed that % recovery of each catechin (EGC, EGCG, EC, ECG and caffeine) at five concentrations were ranging between 99.15-100.39% and % RSD were in range of 0.01-1.42%. Accuracy study of analytical method of GTCs and caffeine at five concentrations was carried out. The results were shown in table 17-21.

From the accuracy results of EGC in table 17, the average of % recovery at approximately 100.17%, 100.01%, 99.24%, 100.28% and 99.85% were obtained by the determinations of EGC at 80, 90, 100, 110 and 120% levels of the test concentration, respectively. The % RSD of each concentration level were 0.10%, 0.15%, 0.09%, 0.33% and 0.22% for the determinations of EGC at 80, 90, 100, 110 and 120% levels of the test concentration, respectively.

From the accuracy results of EGCG in table 18, the average of % recovery at approximately 99.47%, 99.66%, 99.98%, 99.56% and 99.35% were obtained by the determinations of EGCG at 80, 90, 100, 110 and 120% levels of the test concentration, respectively. The % RSD of each concentration level were 0.42%, 0.11%, 0.05%, 0.70% and 0.01% for the determinations of EGCG at 80, 90, 100, 110 and 120% levels of the test concentration, respectively.

From the accuracy results of EC in table 19, the average of % recovery at approximately 99.74%, 99.80%, 100.39%, 100.01% and 99.62% were obtained by the determinations of EC at 80, 90, 100, 110 and 120% levels of the test concentration, respectively. The % RSD of each concentration level were 0.23%, 0.64%, 0.31%, 0.97% and 1.31% for the determinations of EC at 80, 90, 100, 110 and 120% levels of the test concentration, respectively.

From the accuracy results of ECG in table 20, the average of % recovery at approximately 99.37%, 100.20%, 100.18%, 100.31% and 99.83% were obtained by the determinations of ECG at 80, 90, 100, 110 and 120% levels of the test concentration, respectively. The % RSD of each concentration level were 0.70%, 0.57%, 1.00%, 0.78% and 1.42% for the determinations of ECG at 80, 90, 100, 110 and 120% levels of the test concentration, respectively.

From the accuracy results of caffeine in table 21, the average of % recovery at approximately 99.20%, 99.57%, 99.84%, 99.15% and 99.22% were obtained by the determinations of caffeine at 80, 90, 100, 110 and 120% levels of the test concentration, respectively. The % RSD of each concentration level were 0.85%, 0.69%, 0.33%, 1.14% and 0.34% for the determinations of caffeine at 80, 90, 100, 110 and 120% levels of the test concentration, respectively.

Table 17 Accuracy of EGC.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
80 A	0.0201	0.0201	100.24	100.17	0.10
80 B	0.0195	0.0195	100.10	(100.10 - 100.24)	
90 A	0.0218	0.0218	99.90	100.01	0.15
90 B	0.0222	0.0222	100.12	(99.90 - 100.12)	
100 A	0.0243	0.0241	99.18	99.24	0.09
100 B	0.0244	0.0242	99.30	(99.18 - 99.30)	
110 A	0.0271	0.0272	100.51	100.28	0.33
110 B	0.0272	0.0273	100.04	(100.04 - 100.51)	
120 A	0.0290	0.0289	99.70	99.85	0.22
120 B	0.0289	0.0289	100.01	(99.70 - 100.01)	

Table 18 Accuracy of EGCG.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
80 A	0.0522	0.0521	99.77	99.47	0.42
80 B	0.0507	0.0503	99.18	(99.18 - 99.77)	
90 A	0.0566	0.0564	99.58	99.66	0.11
90 B	0.0576	0.0575	99.74	(99.58 - 99.74)	
100 A	0.0632	0.0632	99.94	99.98	0.05
100 B	0.0633	0.0634	100.01	(99.94 - 100.01)	
110 A	0.0703	0.0704	100.06	99.56	0.70
110 B	0.0708	0.0701	99.07	(99.07 - 100.06)	
120 A	0.0753	0.0748	99.34	99.35	0.01
120 B	0.0752	0.0747	99.35	(99.34 - 99.35)	

Table 19 Accuracy of EC.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
80 A	0.0044	0.0044	99.58	99.74	0.23
80 B	0.0043	0.0043	99.91	(99.58 - 99.91)	
90 A	0.0048	0.0048	100.25	99.80	0.64
90 B	0.0048	0.0048	99.34	(99.34 - 100.25)	
100 A	0.0053	0.0053	100.17	100.39	0.31
100 B	0.0053	0.0053	100.61	(100.17 - 100.61)	
110 A	0.0059	0.0059	99.33	100.01	0.97
110 B	0.0059	0.0060	100.70	(99.33 - 100.70)	
120 A	0.0063	0.0062	98.70	99.62	1.31
120 B	0.0063	0.0063	100.54	(98.70 - 100.54)	

Table 20 Accuracy of ECG.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
80 A	0.0101	0.0101	99.87	99.37	0.70
80 B	0.0098	0.0097	98.88	(98.88 - 99.87)	
90 A	0.0110	0.0110	100.61	100.20	0.57
90 B	0.0112	0.0111	99.79	(99.79 - 100.61)	
100 A	0.0122	0.0122	99.47	100.18	1.00
100 B	0.0123	0.0124	100.89	(99.47 - 100.89)	
110 A	0.0136	0.0136	99.76	100.31	0.78
110 B	0.0137	0.0138	100.86	(99.76 - 100.86)	
120 A	0.0146	0.0144	98.83	99.83	1.42
120 B	0.0145	0.0147	100.83	(98.83 - 100.83)	

Table 21 Accuracy of caffeine.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
80 A	0.0293	0.0292	99.80	99.20	0.85
80 B	0.0284	0.0280	98.61	(98.61 - 99.80)	
90 A	0.0317	0.0317	100.05	99.57	0.69
90 B	0.0323	0.0320	99.08	(99.08 - 100.05)	
100 A	0.0354	0.0353	99.61	99.84	0.33
100 B	0.0355	0.0355	100.08	(99.61 - 100.08)	
110 A	0.0394	0.0394	99.94	99.15	1.14
110 B	0.0397	0.0390	98.35	(98.35 - 99.94)	
120 A	0.0422	0.0420	99.46	99.22	0.34
120 B	0.0421	0.0417	98.98	(98.98 - 99.46)	

Precision

The determination of precision of the analytical method for the assay of GTCs and caffeine was performed with six replicated sample solutions without a serial dilution at 100%, v/v of the test concentration.

The actual concentrations, observed concentrations, % recoveries and % RSD results are all shown in table 22-26. Precision should be better than 2% of RSD value and the recovery should be coverage in the range of 98-102%.

According to the results of EGC as shown in table 22, the recovery was between 99.18% and 101.20%, with an average of 100.15%. The % RSD was 0.84%.

For the results of EGCG (table 23), the recovery was between 99.94% and 101.45%, with an average of 100.66%. The % RSD was 0.63%.

For the results of EC (table 24), the recovery was between 98.81% and 100.92%, with an average of 100.14%. The % RSD was 0.73%.

For the results of ECG (table 25), the recovery was between 98.91% and 101.33%, with an average of 100.04%. The % RSD was 0.90%.

For the results of caffeine (table 26), the recovery was between 99.61% and 102.05%, with an average of 101.01%. The % RSD was 0.96%.

Table 22 Precision of EGC.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
100 A	0.0243	0.0241	99.18	100.15 (99.18 - 101.20)	0.84
100 B	0.0244	0.0242	99.30		
100 C	0.0246	0.0249	101.20		
100 D	0.0244	0.0246	101.01		
100 E	0.0248	0.0248	100.11		
100 F	0.0249	0.0249	100.10		

Table 23 Precision of EGCG.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
100 A	0.0632	0.0632	99.94	100.66 (99.94 - 101.45)	0.63
100 B	0.0633	0.0634	100.01		
100 C	0.0638	0.0647	101.45		
100 D	0.0633	0.0641	101.31		
100 E	0.0644	0.0647	100.52		
100 F	0.0648	0.0652	100.71		

Table 24 Precision of EC.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
100 A	0.0053	0.0053	100.17	100.14 (98.81 - 100.92)	0.73
100 B	0.0053	0.0053	100.61		
100 C	0.0054	0.0054	100.31		
100 D	0.0053	0.0053	100.05		
100 E	0.0054	0.0055	100.92		
100 F	0.0054	0.0054	98.81		

Table 25 Precision of ECG.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
100 A	0.0122	0.0122	99.47	100.04 (98.91 - 101.33)	0.90
100 B	0.0123	0.0124	100.89		
100 C	0.0123	0.0125	101.33		
100 D	0.0123	0.0122	99.80		
100 E	0.0125	0.0124	99.84		
100 F	0.0125	0.0124	98.91		

Table 26 Precision of caffeine.

Sample ID	Actual conc. (mg/ml)	Observed conc. (mg/ml)	%Recovery	Average (Range)	%RSD
100 A	0.0354	0.0353	99.61	101.01 (99.61 - 102.05)	0.96
100 B	0.0355	0.0355	100.08		
100 C	0.0358	0.0365	102.05		
100 D	0.0355	0.0361	101.78		
100 E	0.0361	0.0366	101.47		
100 F	0.0363	0.0367	101.06		

Linearity and Range

The linearity of the method was studied at five different concentrations of sample solutions (80, 90, 100, 110, and 120% levels of the test concentration) without a serial dilution. The selected concentrations covered the ranges of each analyte in all test samples. The results are shown in table 27-31. The graph of each components (figure 34-38) obtained by plotting the observed concentration vs the actual concentration was straight line with R^2 of EGC, EGCG, EC, ECG and caffeine were 0.9996, 0.9999, 0.9996, 0.9996 and 0.9996, respectively.

With $R^2 \geq 0.9996$, linear regression equation $y = 0.9908x + 0.0002$ and RSS ≤ 0.0004, the analytical method provided the linear relationship, covering the range of EGC concentrations between 0.0198 and 0.0288 mg/ml, as shown in table 27 and figure 34.

With $R^2 \geq 0.9999$, linear regression equation $y = 0.9877x + 0.0006$ and RSS ≤ 0.0001, the analytical method provided the linear relationship, covering the range of EGCG concentrations between 0.0514 and 0.0748 mg/ml, as shown in table 28 and figure 35.

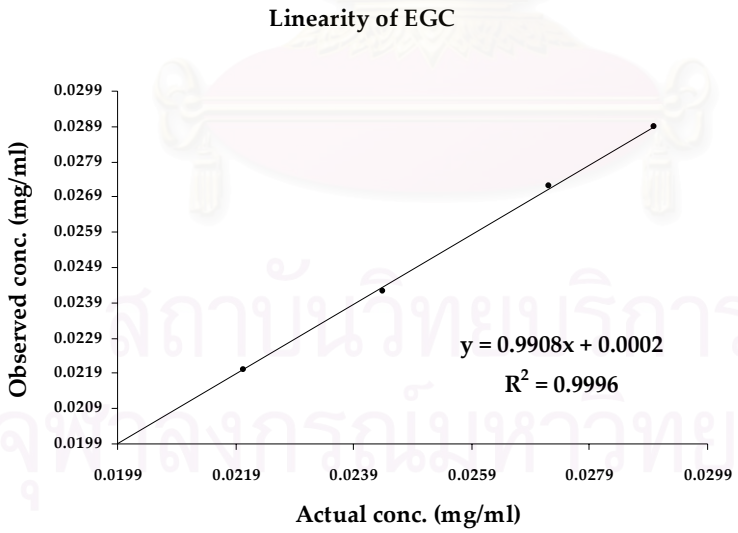
With $R^2 \geq 0.9996$, linear regression equation $y = 0.9975x + 0.0000$ and RSS ≤ 0.0004, the analytical method provided the linear relationship, covering the range of EC concentrations between 0.0043 and 0.0063 mg/ml, as shown in table 29 and figure 36.

With $R^2 \geq 0.9996$, linear regression equation $y = 1.0085x + 0.0001$ and RSS ≤ 0.0004, the analytical method provided the linear relationship, covering the range of ECG concentrations between 0.0099 and 0.0145 mg/ml, as shown in table 30 and figure 37.

With $R^2 \geq 0.9996$, linear regression equation $y = 0.9876x + 0.0002$ and RSS ≤ 0.0004, the analytical method provided the linear relationship, covering the range of caffeine concentrations between 0.0286 and 0.0418 mg/ml, as shown in table 31 and figure 38.

Table 27 Linearity of EGC.

Sample ID	Actual conc. (mg/ml)	Average actual conc. (mg/ml)	Observed Conc. (mg/ml)	Average observed conc. (mg/ml)
80A	0.0201	0.0198	0.0201	0.0198
80B	0.0195		0.0195	
90A	0.0218	0.0220	0.0217	0.0220
90B	0.0222		0.0222	
100A	0.0243	0.0244	0.0241	0.0242
100B	0.0244		0.0242	
110A	0.0271	0.0272	0.0271	0.0271
110B	0.0272		0.0271	
120A	0.0290	0.0290	0.0288	0.0288
120B	0.0289		0.0289	

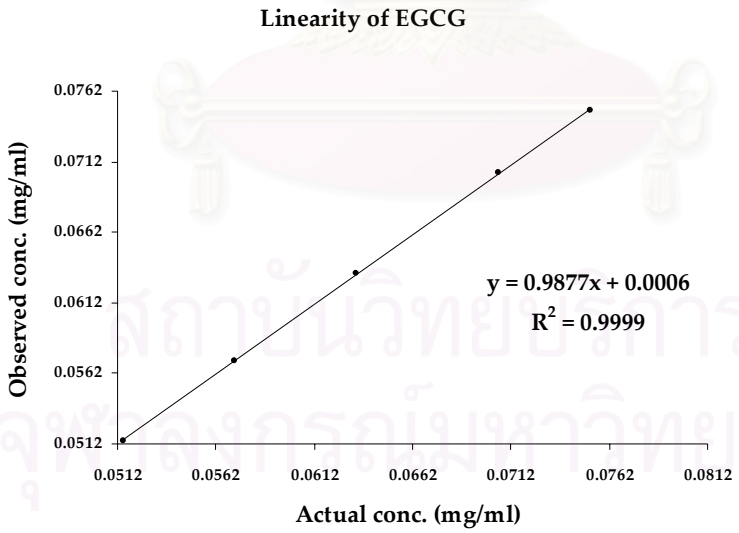


Constant	**0.0002**
R Squared	**0.9996**
Slope	**0.9908**
RSS	**0.0004**

Figure 34 Linearity graph of EGC.

Table 28 Linearity of EGCG.

Sample ID	Actual conc. (mg/ml)	Average actual conc. (mg/ml)	Observed Conc. (mg/ml)	Average observed conc. (mg/ml)
80A	0.0522	0.0515	0.0523	0.0514
80B	0.0507		0.0505	
90A	0.0566	0.0571	0.0566	0.0571
90B	0.0576		0.0575	
100A	0.0632	0.0633	0.0632	0.0633
100B	0.0633		0.0634	
110A	0.0703	0.0705	0.0705	0.0703
110B	0.0708		0.0702	
120A	0.0753	0.0752	0.0749	0.0748
120B	0.0752		0.0748	

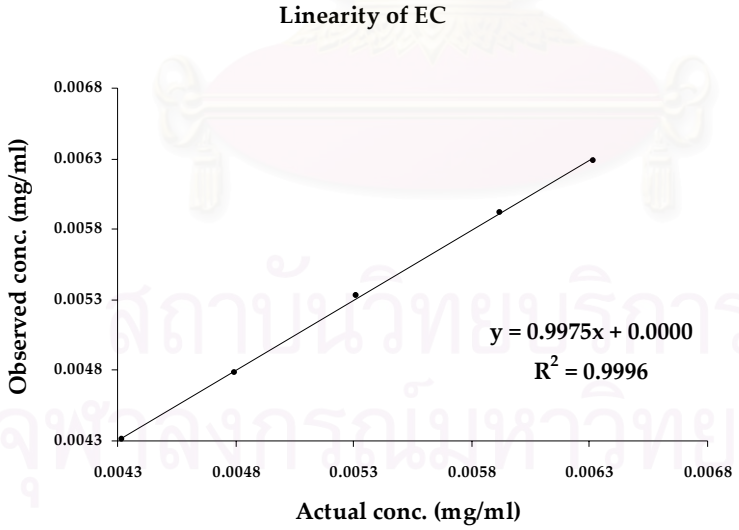


Constant	**0.0006**
R Squared	**0.9999**
Slope	**0.9877**
RSS	**0.0001**

Figure 35 Linearity graph of EGCG.

Table 29 Linearity of EC.

Sample ID	Actual conc. (mg/ml)	Average actual conc. (mg/ml)	Observed Conc. (mg/ml)	Average observed conc. (mg/ml)
80A	0.0044	0.0043	0.0044	0.0043
80B	0.0043		0.0043	
90A	0.0048	0.0048	0.0048	0.0048
90B	0.0048		0.0048	
100A	0.0053	0.0053	0.0053	0.0053
100B	0.0053		0.0053	
110A	0.0059	0.0059	0.0059	0.0059
110B	0.0059		0.0060	
120A	0.0063	0.0063	0.0062	0.0063
120B	0.0063		0.0063	

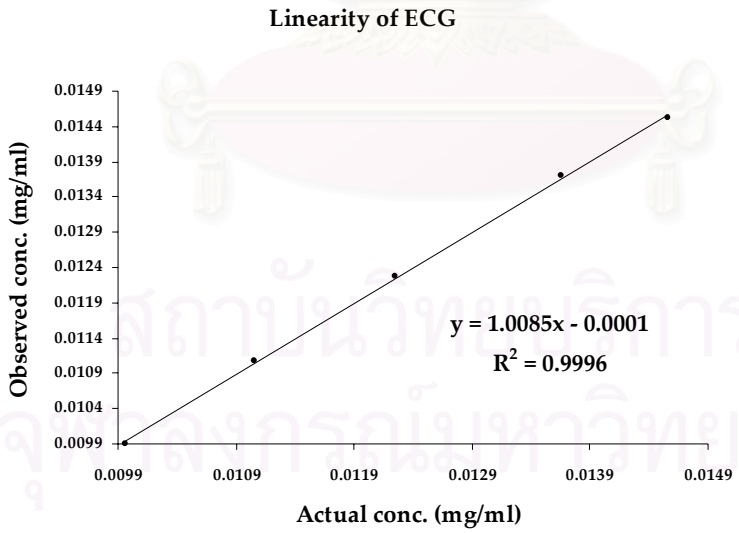


Constant	**0.0000**
R Squared	**0.9996**
Slope	**0.9975**
RSS	**0.0004**

Figure 36 Linearity graph of EC.

Table 30 Linearity of ECG.

Sample ID	Actual conc. (mg/ml)	Average actual conc. (mg/ml)	Observed Conc. (mg/ml)	Average observed conc. (mg/ml)
80A	0.0101	0.0100	0.0101	0.0099
80B	0.0098		0.0097	
90A	0.0110	0.0111	0.0110	0.0111
90B	0.0112		0.0111	
100A	0.0122	0.0122	0.0122	0.0123
100B	0.0123		0.0124	
110A	0.0136	0.0137	0.0136	0.0137
110B	0.0137		0.0138	
120A	0.0146	0.0146	0.0144	0.0145
120B	0.0145		0.0147	

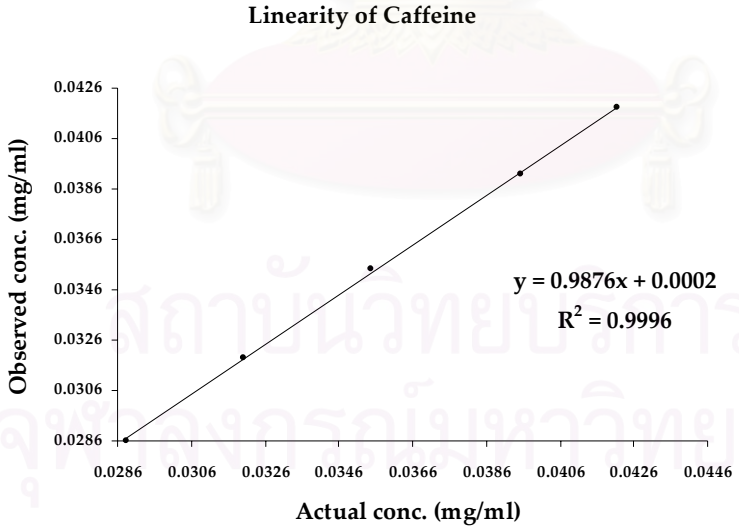


Constant	**-0.0001**
R Squared	**0.9996**
Slope	**1.0085**
RSS	**0.0004**

Figure 37 Linearity graph of ECG.

Table 31 Linearity of caffeine.

Sample ID	Actual conc. (mg/ml)	Average actual conc. (mg/ml)	Observed Conc. (mg/ml)	Average observed conc. (mg/ml)
80A	0.0293	0.0288	0.0292	0.0286
80B	0.0284		0.0280	
90A	0.0317	0.0320	0.0317	0.0319
90B	0.0323		0.0320	
100A	0.0354	0.0355	0.0353	0.0354
100B	0.0355		0.0355	
110A	0.0394	0.0395	0.0394	0.0392
110B	0.0397		0.0390	
120A	0.0422	0.0422	0.0420	0.0418
120B	0.0421		0.0417	



Constant	**0.0002**
R Squared	**0.9996**
Slope	**0.9876**
RSS	**0.0004**

Figure 38 Linearity graph of caffeine.

Selectivity

Selectivity of the analytical method was investigated by injection mixed standards solution of EGC, EGCG, EC, ECG and caffeine reference standards and then comparing chromatograms of pure diluting solution with diluting solution together with the FD-GTE sample.

The chromatogram of the FD-GTE sample (unknown sample) with final concentration equivalent to 0.04 mg/ml in diluting solvent was shown in figure 39. Retention times were in the range of 6.413 min for EGC, 11.703 min for caffeine, 13.455 min for EGCG, 15.748 min for EC and 31.356 min for ECG.

Figure 40 showed that it was not found peak of impurities in chromatogram of diluting solvent (blank sample).

While, the chromatograms of mixed reference standard (standard sample) with final concentration of EGC, EGCG, EC, ECG and caffeine at approximately 0.006, 0.026, 0.021, 0.003 and 0.004 mg/ml, respectively, were shown in figure 41. For mixed reference standards, the retention times of EGC, EGCG, EC, ECG and caffeine were 6.514, 13.712, 16.038, 31.806 and 11.773 min, respectively.

Resolution and asymmetry between peaks of green tea catechins together with caffeine

Resolution and asymmetry were calculated by integrating program. Resolution between peak of EGC and caffeine was 9.69; caffeine and EGCG was 2.24; EGCG and EC was 2.71; EC and ECG was 16.29. As a result, the developed HPLC condition provides resolution between peak of the individual GTCs and caffeine more than 1.5.

Asymmetry of peak EGC, caffeine, EGCG, EC and ECG were 1.14, 1.43, 1.28, 1.13 and 1.13, respectively. Asymmetries, as referred to the tailing factor of peak were all less than 1.5.

From these results, it could be concluded that peak of each catechins and caffeine did not overlap, the developed analytical method was, therefore, selective for analyzing GTCs together with caffeine in this study.

Freeze-dried green tea extract sample, compared with blank

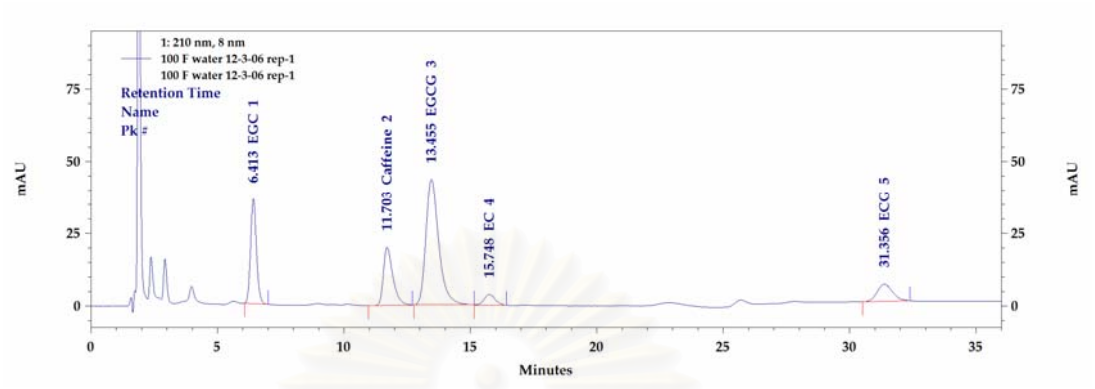


Figure 39 Chromatogram of freeze-dried green tea extract samples equivalent to 0.04 mg/ml.

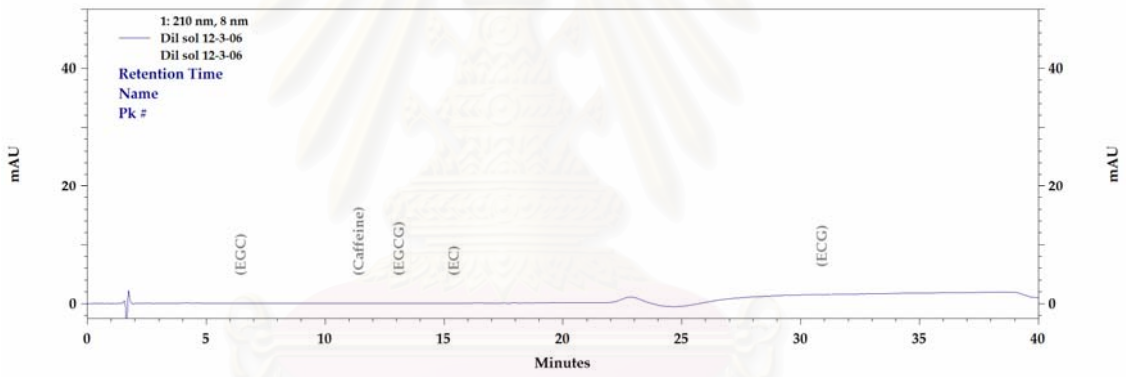


Figure 40 Chromatogram of diluting solvent (blank).

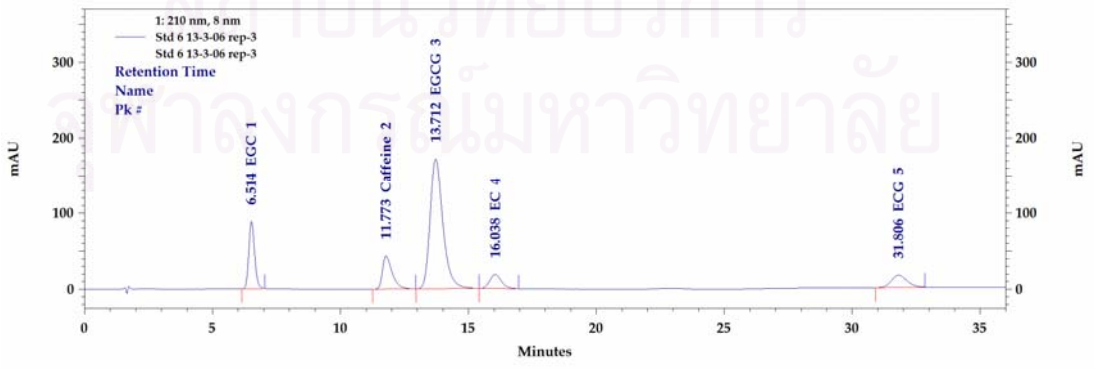


Figure 41 Chromatogram of mixed reference standards at 100% dilution concentration.

In the testing and evaluation of the method chosen as well as in the analysis of GTCs together with caffeine in FD-GTE and other test samples (study I), PDA UV-vis detector was used for the identification and quantification of the compounds. Retention times and UV spectra of the peaks were compared with those of the standards.

The Shimadzu CLASS-*VP*™ (V. 6.14 SP1) software, used for the HPLC system with a PDA detector in this study, could collect information from one chromatographic run as following:

(1) UV spectrum at any retention time, including each selected peak. It also provided a complete UV spectrum for each data point.

(2) A three-dimensional plot (absorbance vs time vs wavelength), which permits monitoring of the absorption profile of a selected peak at any wavelength as the elution progresses or after the elution.

(3) A chromatogram at any wavelength, which permits comparison of the chromatograms of all the peaks eluted from a complex of compounds at any wavelength of interest to justify the chromatogram for analytical purposes.

(4) Mixed view of the above three types of information, which allows loading of a particular wavelength at a particular elution time to obtain the above information for a particular peak.

To ensure the identification to be accurate, the integrated data of the peak in the chromatogram was performed the following manipulations. It obtained the information about the resolution of a peak within a particular elution system. Moreover, it not only permitted the spectra of components within a chromatogram, compared with those of standards, but also the matches of the spectra of the unknown compounds to those of standards for identification. In addition, the superimposition of chromatograms at different wavelengths allowed comparison of the chromatographic behavior of all the compounds at different wavelengths during or after the elution.

The mixed view of each catechin chromatograms compared with blank samples are all shown in figure 42-51.

Individual reference standard of EGC, compared with blank

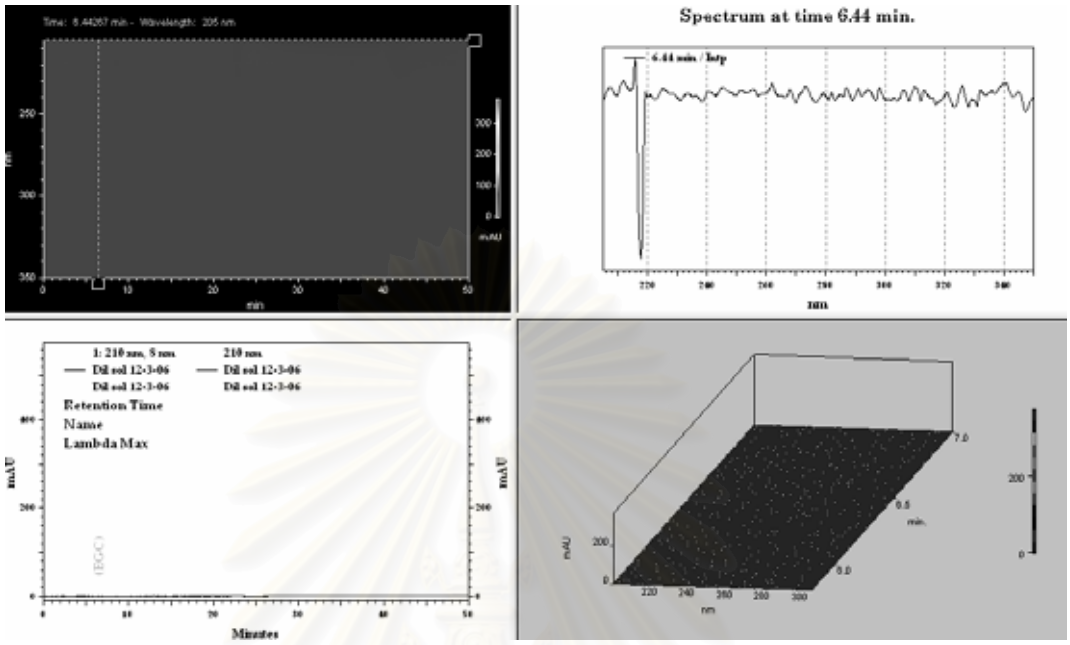


Figure 42 Mixed view of diluting solvent (blank sample) at retention time of EGC chromatogram (R_t = 6.443 min).

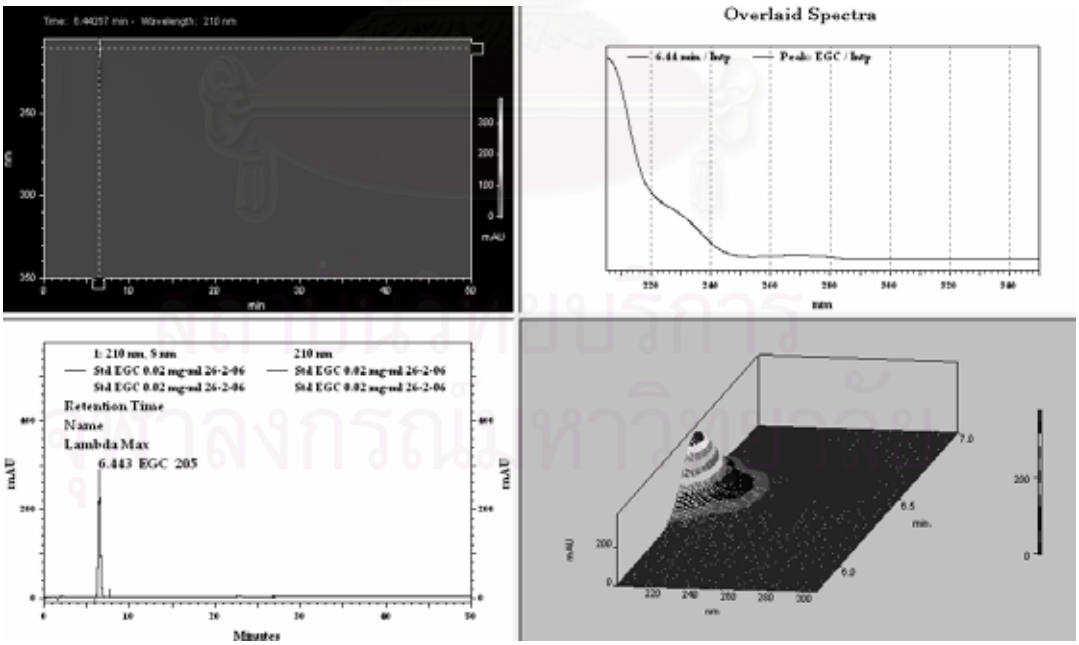


Figure 43 Mixed view of EGC standard at approximately 0.02 mg/ml.

Individual reference standard of EGCG, compared with blank

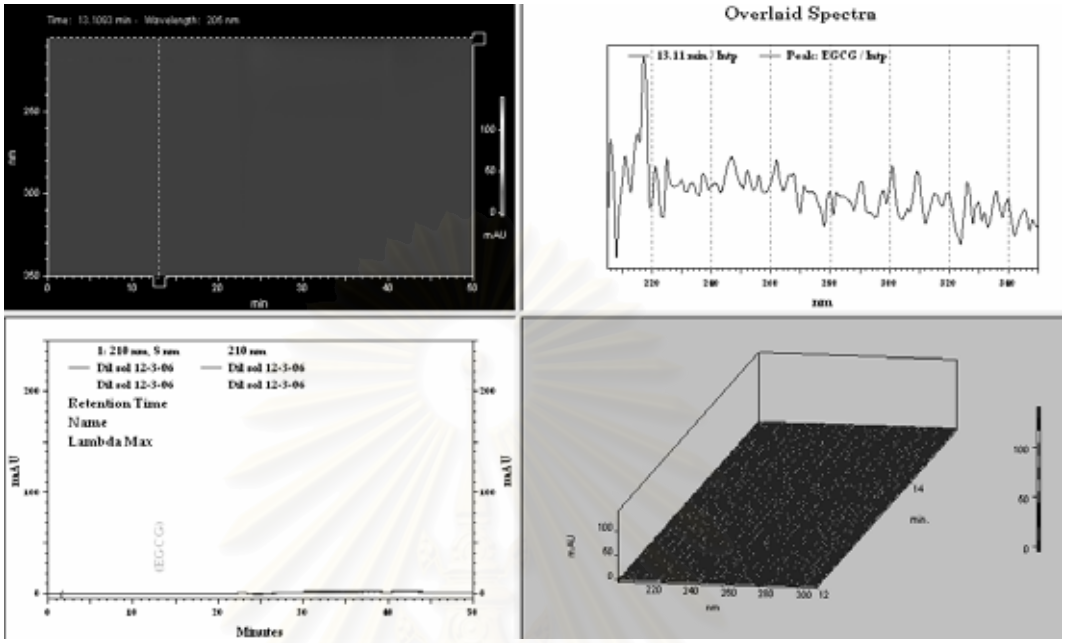


Figure 44 Mixed view of diluting solvent (blank sample) at retention time of EGCG chromatogram (R_t = 13.108 min).

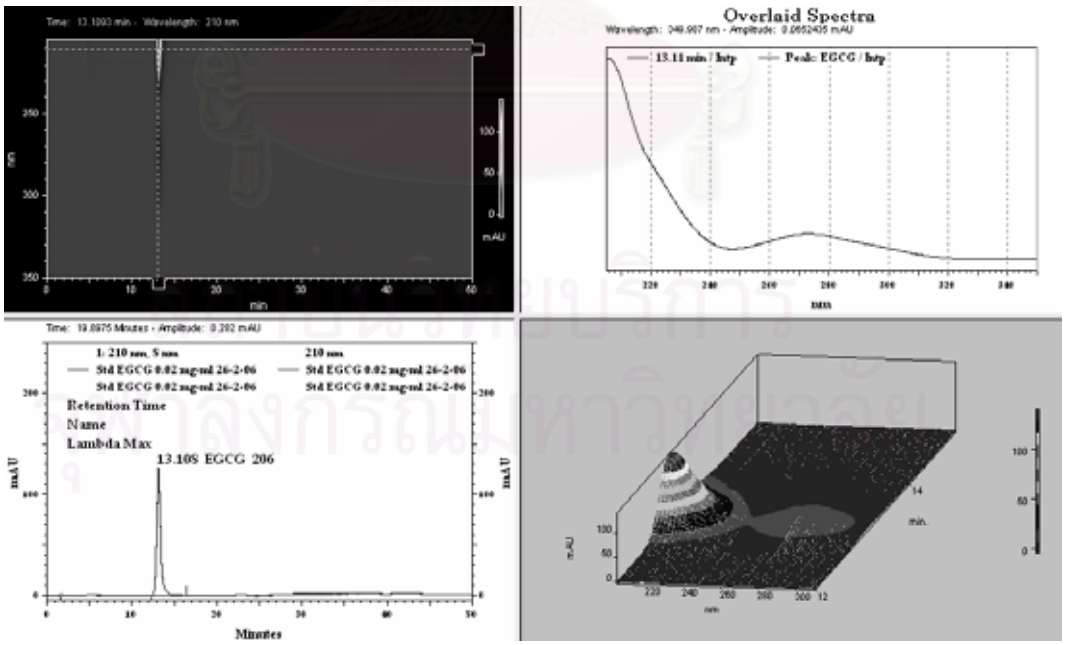


Figure 45 Mixed view of EGCG reference standard at approximately 0.02 mg/ml.

Individual reference standard of EC, compared with blank

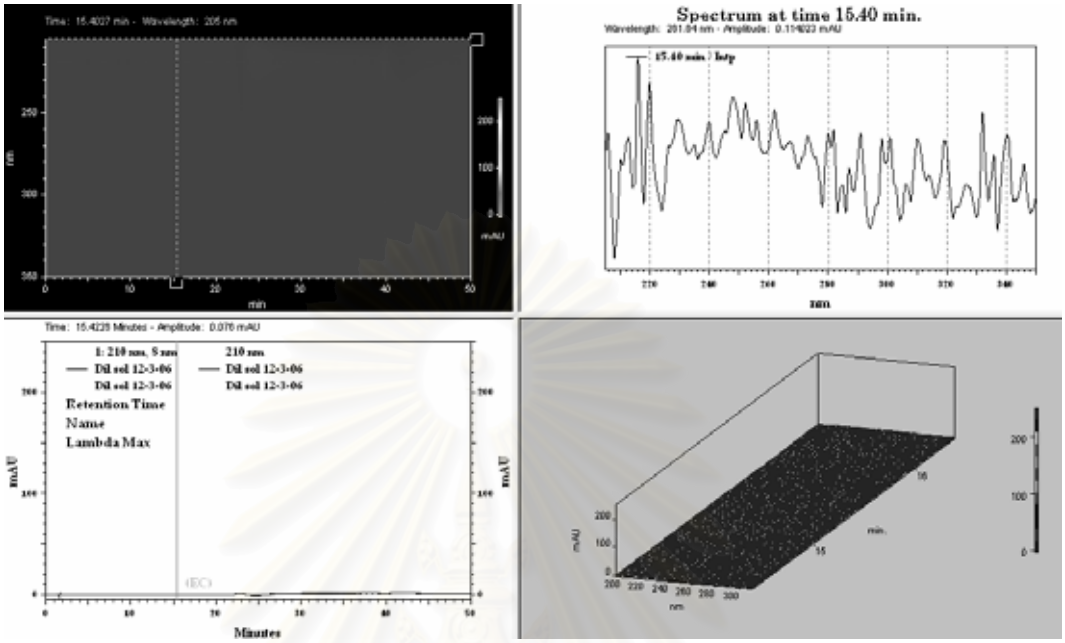


Figure 46 Mixed view of diluting solvent (blank sample) at retention time of EC chromatogram (R_t = 15.402 min).

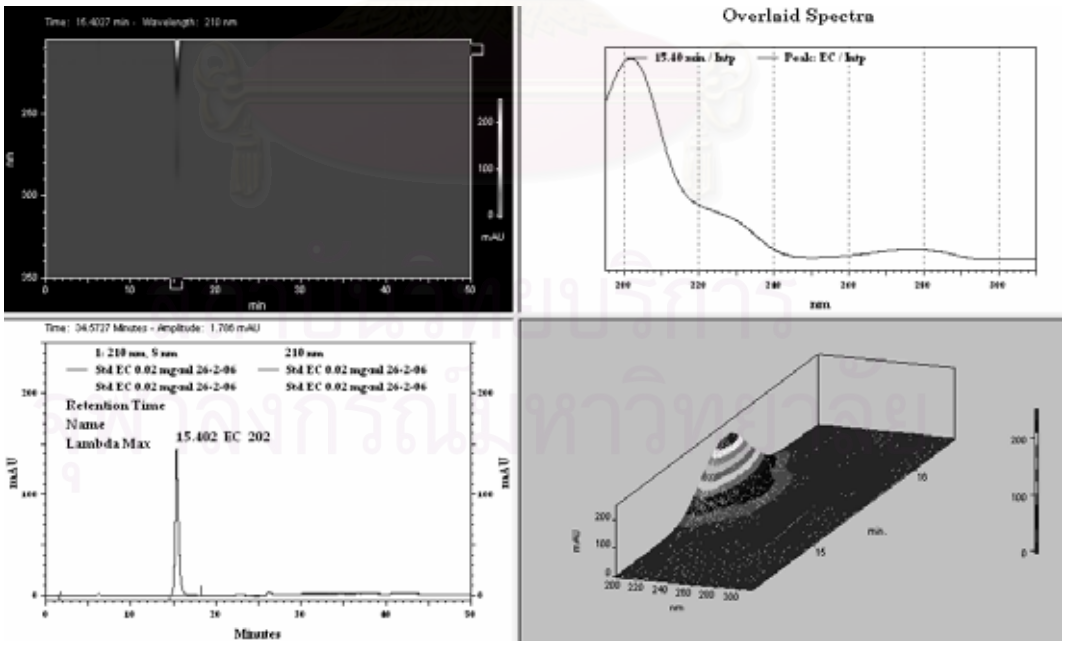


Figure 47 Mixed view of EC reference standard at approximately 0.02 mg/ml.

Individual reference standard of ECG, compared with blank

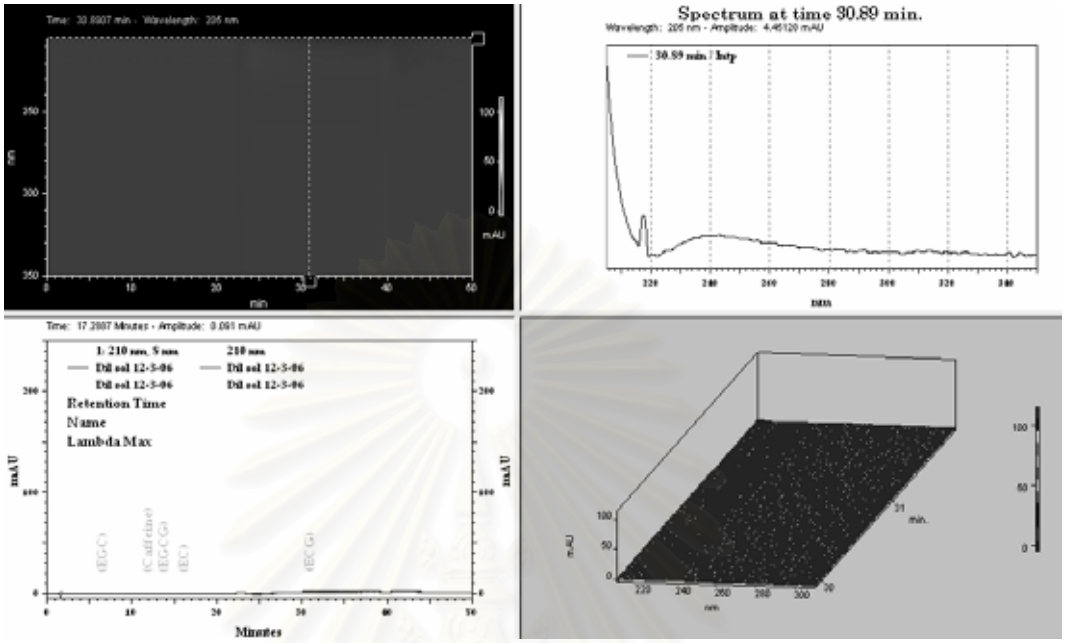


Figure 48 Mixed view of diluting solvent (blank sample) at retention time of ECG chromatogram (R_t = 30.895 min).

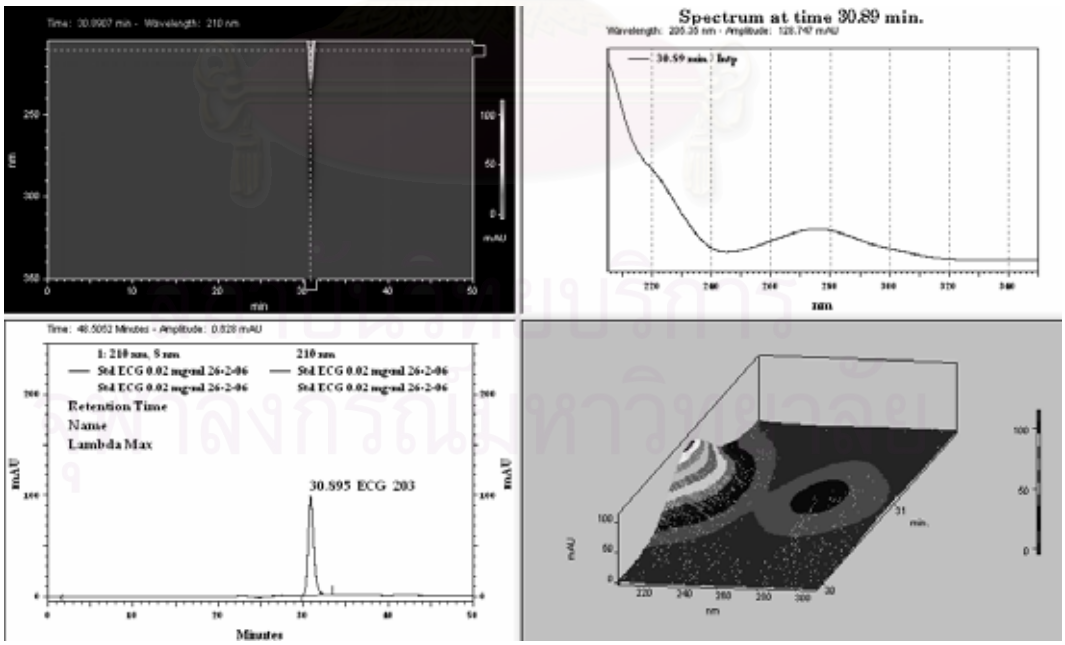


Figure 49 Mixed view of ECG reference standard at approximately 0.02 mg/ml.

Individual reference standard of caffeine, compared with blank

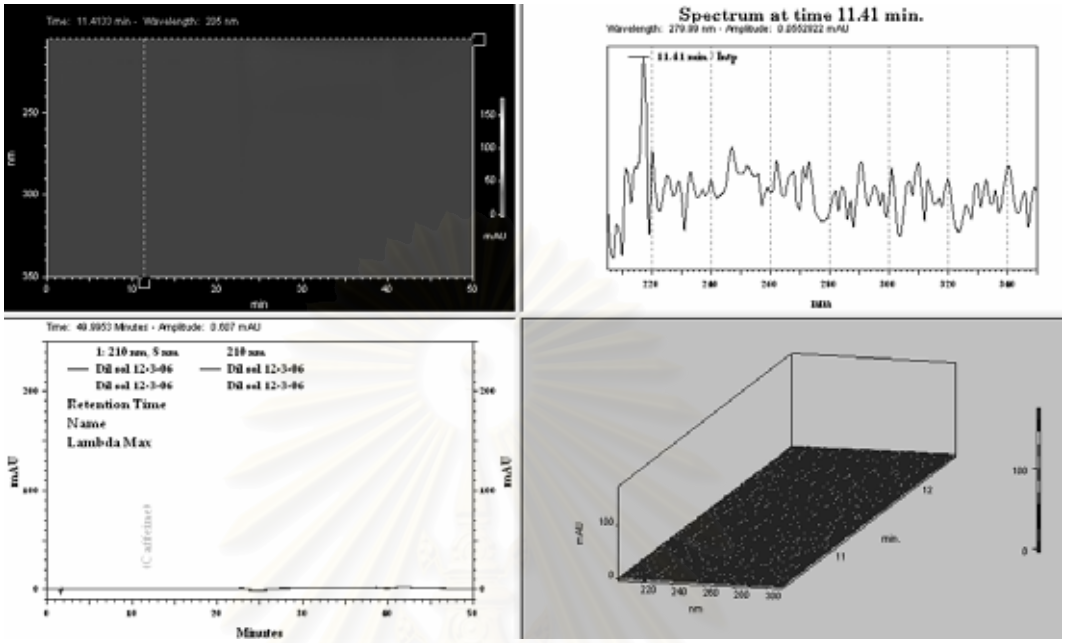


Figure 50 Mixed view of diluting solvent (blank sample) at retention time of caffeine chromatogram (R_t = 11.414 min).

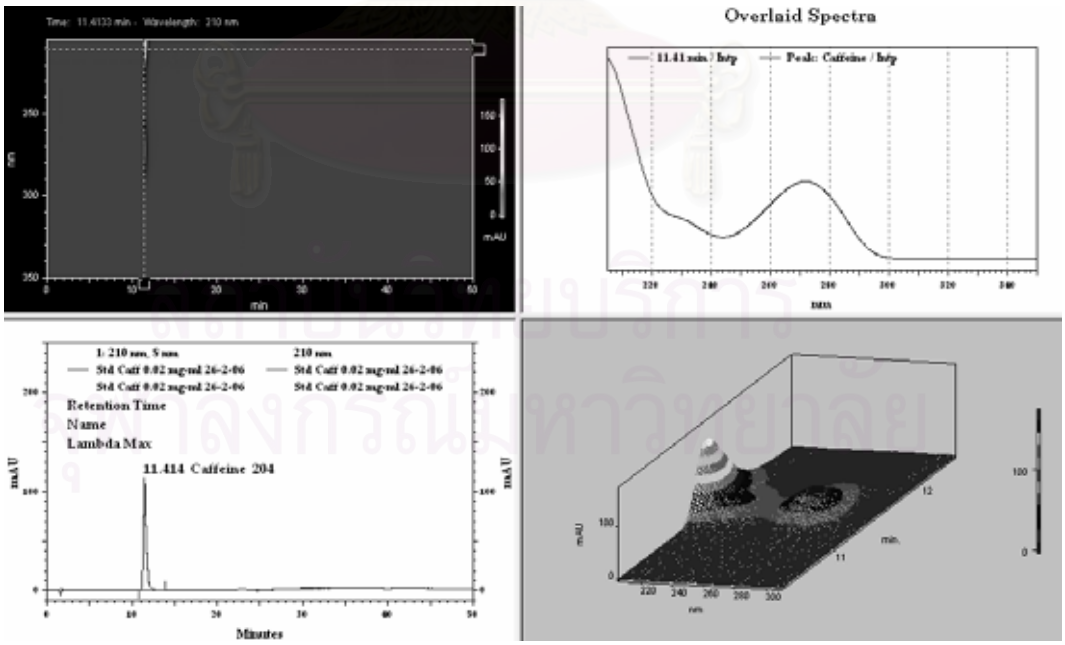


Figure 51 Mixed view of caffeine reference standard at approximately 0.02 mg/ml.

The analytical method for qualitative and quantitative determination of GTCs together with caffeine in FD-GTE using HPLC analysis was validated according to ICH guidance (Q2A and Q2B) (ICH, 1995, 1996). The summarized criteria are shown in table 32-36. The gradient solvent system method developed for the determination of catechins in green tea was ideally suited for most analysis in this research. With this method, good repeatability of the results was established, and four different catechins together with caffeine could be determined at the same time. Furthermore, this method is simple, sensitive and accurate and can be applied to all kinds of tea and tea products.

Table 32 Summarized results of analytical method validation for an assay of EGC.

Test	Parameter	Criteria	Result	Conclusion
Accuracy	% Recovery	98-102%	99.91%	Passed
Precision	% RSD	NMT 2%	0.84%	Passed
Linearity and Range (0.0198 – 0.0290 mg/ml)	Slope	0.9-1.1	0.9908	Passed
	Intercept	−0.001 to 0.001	0.0002	Passed
	R^2	NLT 0.9995	0.9996	Passed
	RSS	−0.01 to 0.01	0.0004	Passed
Selectivity	Resolution	NLT 1.5	9.69	Passed
	Asymmetry	NMT 1.5	1.14	Passed

Table 33 Summarized results of analytical method validation for an assay of EGCG.

Test	Parameter	Criteria	Result	Conclusion
Accuracy	% Recovery	98-102%	99.60%	Passed
Precision	% RSD	NMT 2%	0.63%	Passed
Linearity and Range (0.0515 – 0.0752 mg/ml)	Slope	0.9-1.1	0.9877	Passed
	Intercept	−0.001 to 0.001	0.0006	Passed
	R^2	NLT 0.9995	0.9999	Passed
	RSS	−0.01 to 0.01	0.0001	Passed
Selectivity	Resolution	NLT 1.5	2.71	Passed
	Asymmetry	NMT 1.5	1.28	Passed

Table 34 Summarized results of analytical method validation for an assay of EC.

Test	Parameter	Criteria	Result	Conclusion
Accuracy	% Recovery	98-102%	99.91%	Passed
Precision	% RSD	NMT 2%	0.73%	Passed
Linearity and Range (0.0043 – 0.0063 mg/ml)	Slope	0.9-1.1	0.9975	Passed
	Intercept	−0.001 to 0.001	0.0000	Passed
	R^2	NLT 0.9995	0.9996	Passed
	RSS	−0.01 to 0.01	0.0004	Passed
Selectivity	Resolution	NLT 1.5	16.29	Passed
	Asymmetry	NMT 1.5	1.13	Passed

Table 35 Summarized results of analytical method validation for an assay of ECG.

Test	Parameter	Criteria	Result	Conclusion
Accuracy	% Recovery	98-102%	99.98%	Passed
Precision	% RSD	NMT 2%	0.90%	Passed
Linearity and Range (0.0100 – 0.0146 mg/ml)	Slope	0.9-1.1	1.0085	Passed
	Intercept	−0.001 to 0.001	−0.0001	Passed
	R^2	NLT 0.9995	0.9996	Passed
	RSS	−0.01 to 0.01	0.0004	Passed
Selectivity	Resolution	NLT 1.5	–	Passed
	Asymmetry	NMT 1.5	1.13	Passed

Table 36 Summarized results of analytical method validation for an assay of caffeine.

Test	Parameter	Criteria	Result	Conclusion
Accuracy	% Recovery	98-102%	99.39%	Passed
Precision	% RSD	NMT 2%	0.96%	Passed
Linearity and Range (0.0288 – 0.0422 mg/ml)	Slope	0.9-1.1	0.9876	Passed
	Intercept	−0.001 to 0.001	0.0002	Passed
	R^2	NLT 0.9995	0.9996	Passed
	RSS	−0.01 to 0.01	0.0004	Passed
Selectivity	Resolution	NLT 1.5	2.24	Passed
	Asymmetry	NMT 1.5	1.43	Passed

6. Pre-characterization of concentrated w/o emulsions on achievability in processing, apparent viscosity and short-term stability

To perform fifteen representative formulations for further study, formulations with various formulation compositions were preliminary prepared and then characterized their general properties due to the short-term stability of final products.

6.1 Effect of different formulation parameters

The experiment was carried out by varying the formulation compositions with four emulsifier systems (sorbitan, silicone, mixed and glucoester types) at approximately 3% (w/w) of Rx, combined with 80-90% DP. After that, different percentages of emulsifier (% emulsifier) used were also performed at 1%, 2%, 3% and 4% with one emulsifier system (glucoester), fixed at maximum % DP.

According to the work of Caldero *et al*. (1997), the stability of CEs could be improved by addition of salting out electrolytes to the aqueous phase ranging from 5% to 15%. Consequently, 0.1 M NaCl electrolyte solution was preferred to add into the aqueous phase varying between 5%, 10% and 15% to candidate the approximate level that might not destabilize the CEs.

In addition, glycerin that may affect general aspects of CEs was carried out by varying their percentages into three ranges of 5%, 10% and 15% of Rx.

6.1.1 Effect of water content

The range of % DP (w/w) was selected to be as large as possible for all emulsions. The min % DP selected was the one that allowed the production of stable products. While, the max % DP selected was the one that could be achieved with most emulsifiers.

The pre-characterizations of CEs with different % DP and different emulsifier types (fixed with other formulation components) were performed in triplicates, including the following issues: production achievement throughout manufacturing processes, short-term stability for at least 1 week and, if any, degree of

phase separation of CEs after manufactured for 1 week. Table 37 shows the detailed information of characteristic assessments of those formulations (n = 3).

Table 37 Characteristic assessments of concentrated w/o emulsions with different percentages of dispersed phase, combined with different emulsifier types (n = 3).

Sam no.	Sample Description			Production achievement	Short-term stability (over 1 week)	Degree of phase separation (after 1 week)
	% Emulsifier	Emulsifier type	% DP			
1	3	Sorbitan (Arlacel 83™)	80	O	U	2Φ
2	3		81	O	U	2Φ
3	3		82	O	U	3Φ
4	3		83	O	U	3Φ
5	3		84	O	S	n/c
6	3		85	O	S	n/c
7	3		86	O	S	n/c
8	3		87	O	S	n/c
9	3		88	O	S	n/c
10	3		89	O	S	n/c
11	3		90	O	S	n/c
12	3	Silicone (Abil EM 90™)	80	O	S	n/c
13	3		81	O	S	n/c
14	3		82	O	S	n/c
15	3		83	O	S	n/c
16	3		84	O	S	n/c
17	3		85	O	S	n/c
18	3		86	O	S	n/c
19	3		87	O	S	n/c
20	3		88	O	S	n/c
21	3		89	X	n/a	2Φ*
22	3		90	X	n/a	2Φ*

O = achieved, X = not achieved.
S = stable, U = not stable for at least 1 week.
n/a = not available.
n/c = not significantly changing on estimated parameter, 2Φ = 2-phase separation (completely separation) and 3Φ = 3-phase separation (* phase separation immediately occurred during production process).

Table 37 (Continued)

Sam no.	Sample Description			Production achievement	Short-term stability (over 1 week)	Degree of phase separation (after 1 week)
	% Emulsifier	Emulsifier type	% DP			
23	3	Mixed (Montane 481™ - Simulsol 989™)	80	O	S	n/c
24	3		81	O	S	n/c
25	3		82	O	S	n/c
26	3		83	O	S	n/c
27	3		84	O	S	n/c
28	3		85	O	S	n/c
29	3		86	O	S	n/c
30	3		87	O	S	n/c
31	3		88	O	S	n/c
32	3		89	X	n/a	2Φ*
33	3		90	X	n/a	2Φ*
34	3	Glucoester (Isolan DO™)	80	O	U	3Φ
35	3		81	O	U	3Φ
36	3		82	O	S	n/c
37	3		83	O	S	n/c
38	3		84	O	S	n/c
39	3		85	O	S	n/c
40	3		86	O	S	n/c
41	3		87	O	S	n/c
42	3		88	O	S	n/c
43	3		89	O	S	n/c
44	3		90	O	S	n/c

O = achieved, X = not achieved.
S = stable, U = not stable for at least 1 week.
n/a = not available.
n/c = not significantly changing on estimated parameter, 2Φ = 2-phase separation (completely separation) and 3Φ = 3-phase separation (* phase separation immediately occurred during production process).

From the short-term stability's results, CEs with % DP higher than 84% obtained such a good stability for 1 week. In contrast, CEs with % DP lesser than 84% were not sufficiently stable for at least 1 week. As a result, 84% DP was preferred to be the minimum DP for all formulations studied.

88% DP was the highest percentages of DP that could be achieved with all emulsifier types and not separated throughout the manufacturing processes. With higher than 88% DP, the excess amount of aqueous phase could not incorporated into constituted two phase mixtures, and then squeezed out from the

whole mixture with vigorously agitation. In some cases, the separated aqueous phase on top of cream can be incorporated into the final mixtures by constantly stirring at very low speed. Although, higher % DP could be achieved with patience but it not effectively deliberated in practical and provided such a poor reproducibility between batches. Therefore, 88% DP was preferred to be the maximum DP for all formulations studied.

The three ranges of % DP were selected to cover ranging from 84 to 88% DP. The CEs with 84, 86 and 88% DP (w/w) were prepared with 3% of each emulsifier used.

6.1.2 Effect of emulsifier type

This research selected four different emulsifiers, e.g. sorbitan (Arlacel 83™), silicone (Abil EM90™), glucoester (Isolan DO™) and mixed (Montane 481™ and Simulsol 989™). From the results as shown in table 37, it indicated that all CEs with 84, 86 and 88% DP (w/w), combined with 3% of each emulsifier were stable over 1 week.

6.1.3 Effect of emulsifier concentration

The effect of the emulsifier concentration was performed with glucoester emulsions (fixed at 88% DP). These formulations were varied their percentages of glucoester in the range of 1%, 2%, 3% and 4% of Rx.

The pre-characterizations of CEs with different percentages of emulsifier (fixed with other formulation components) were performed in triplicates, including the following issues: production achievement throughout manufacturing processes, short-term stability for at least 1 week and, if any, degree of phase separation of CEs after manufactured for 1 week. Table 38 shows the detailed information of characteristic assessments of those formulations (n = 3). The results indicated that all ranges of percentages of emulsifier performed good production achievement and short-term stability over 1 week.

Table 38 Characteristic assessments of concentrated w/o emulsions with different percentages of emulsifier (n = 3).

Sam no.	Sample Description			Production achievement	Short-term stability (over 1 week)	Degree of phase separation (after 1 week)
	Emulsifier type	% DP	% Emulsifier (Isolan DO™)			
I 1	Glucoester (Isolan DO™)	84	1	O	S	n/d
I 2			2	O	S	n/d
I 3			3	O	S	n/d
I 4			4	O	S	n/d
I 5		86	1	O	S	n/d
I 6			2	O	S	n/d
I 7			3	O	S	n/d
I 8			4	O	S	n/d
I 9		88	1	O	S	n/d
I 10			2	O	S	n/d
I 11			3	O	S	n/d
I 12			4	O	S	n/d

O = achieved, X = not achieved.
S = stable, U = not stable for at least 1 week.
n/d = not detected.

6.1.4 Effect of other additives

Other components in formulation could also affect the stability of CEs, esp., an electrolyte. In addition, the moisturizer used also changed the physical appearance, the stiffness, the apparent viscosities, etc., of final products. It has already known that the stability of CEs depend mainly on their high viscosity. The moisturizer used in formulations, therefore, was also considered their effect to the stability of CEs as previous discussed.

The pre-characterizations of CEs with different percentages of 0.1 M NaCl electrolyte solution (fixed with other formulation components) were performed in triplicates, including the following issues: production achievement throughout manufacturing processes, degree of apparent viscosity changing compared with blank formulation (without electrolyte) of CEs after manufactured and short-term stability for at least 1 month. Table 39 shows the detailed information of characteristic assessments of these formulations (n = 3).

Table 39 Characteristic assessments of concentrated w/o emulsions with different percentages of 0.1 M NaCl electrolyte solution (n = 3).

Sam no.	Sample Description			Production achievement	Degree of changing in viscosity	Short-term stability (over 1 month)
	% DP	Emulsifier type	% NaCl electrolyte			
E 1	84	3% Sorbitan (Arlacel 83™)	5	O	n/c	S
E 2	86			O	n/c	S
E 3	88			O	n/c	S
E 4	84		10	O	n/c	S
E 5	86			O	n/c	S
E 6	88			O	n/c	S
E 7	84		15	O	D>	S
E 8	86			O	n/c	S
E 9	88			O	n/c	S
E 10	84	3% Silicone (Abil EM 90™)	5	O	n/c	S
E 11	86			O	n/c	S
E 12	88			O	n/c	S
E 13	84		10	O	n/c	S
E 14	86			O	n/c	S
E 15	88			O	n/c	S
E 16	84		15	O	n/c	S
E 17	86			O	D<	S
E 18	88			O	D<	S
E 19	84	3% Mixed (Montane 481™ -Simulsol 989™)	5	O	n/c	U, 3Φ
E 20	86			O	n/c	S
E 21	88			O	n/c	S
E 22	84		10	O	n/c	S
E 23	86			O	n/c	S
E 24	88			O	n/c	S
E 25	84		15	O	D>	U, 3Φ
E 26	86			O	n/c	U, 3Φ
E 27	88			O	D<	S

O = achieved, X = not achieved.
S = stable, U = not stable for at least 1 week: 2Φ = 2-phase separation (completely separation) and 3Φ = 3-phase separation.
n/c = not significantly changing on estimated parameter, D< = an apparent viscosity increased and D> = an apparent viscosity decreased.

The results showed that a suitable amount of NaCl electrolyte that provided a superior stability of all formulations for at least 1 month was 10% (w/w) of Rx.

Table 39 (Continued)

Sam no.	Sample Description			Production achievement	Degree of changing in viscosity	Short-term stability (over 1 month)
	% DP	Emulsifier type	% NaCl electrolyte			
E 28	84	3% Glucoester (Isolan DO™)	5	O	n/c	S
E 29	86			O	n/c	S
E 30	88			O	n/c	S
E 31	84		10	O	D>	U, 3Φ
E 32	86			O	D>	S
E 33	88			O	n/c	S
E 34	84		15	O	D>	U, 3Φ
E 35	86			O	D>	U, 3Φ
E 36	88			O	D>	S

O = achieved, X = not achieved.
S = stable, U = not stable for at least 1 week: 2Φ = 2-phase separation (completely separation) and 3Φ = 3-phase separation.
n/c = not significantly changing on estimated parameter, D< = an apparent viscosity increased and D> = an apparent viscosity decreased.

Table 40 Characteristic assessments of concentrated w/o emulsions with different percentages of glycerin, fixed at 10%, w/w of 0.1 M NaCl electrolyte solution (n = 3).

Sam no.	Sample Description			Production achievement	Degree of changing in viscosity	Short-term stability (over 1 month)
	% DP	Emulsifier type	% Glycerin			
M 1	84	3% Sorbitan (Arlacel 83™)	5	O	n/c	S
M 2	86			O	n/c	S
M 3	88			O	n/c	S
M 4	84		10	O	D>	S
M 5	86			O	D>	S
M 6	88			O	n/c	S
M 7	84		15	O	D>	S
M 8	86			O	D>	S
M 9	88			O	n/c	S

O = achieved, X = not achieved.
S = stable, U = not stable for at least 1 week: 2Φ = 2-phase separation (completely separation) and 3Φ = 3-phase separation.
n/c = not significantly changing on estimated parameter, D< = an apparent viscosity increased and D> = an apparent viscosity decreased.

Table 40 (Continued)

Sam no.	Sample Description			Production achievement	Degree of changing in viscosity	Short-term stability (over 1 month)
	% DP	Emulsifier type	% Glycerin			
M 10	84	3% Silicone (Abil EM 90™)	5	O	n/c	S
M 11	86			O	n/c	S
M 12	88			O	n/c	S
M 13	84		10	O	n/c	S
M 14	86			O	n/c	S
M 15	88			O	n/c	S
M 16	84		15	O	n/c	S
M 17	86			O	n/c	S
M 18	88			O	n/c	S
M 19	84	3% Mixed (Montane 481™ -Simulsol 989™)	5	O	D>	S
M 20	86			O	n/c	S
M 21	88			O	n/c	S
M 22	84		10	O	D>	U, 3Φ
M 23	86			O	D>	U, 3Φ
M 24	88			O	n/c	S
M 25	84		15	O	D>	U, 3Φ
M 26	86			O	D>	U, 3Φ
M 27	88			O	n/c	S
M 28	84	3% Glucoester (Isolan DO™)	5	O	D>	S
M 29	86			O	n/c	S
M 30	88			O	n/c	
M 31	84		10	O	D>	U, 3Φ
M 32	86			O	D>	S
M 33	88			O	n/c	S
M 34	84		15	O	D>	U, 3Φ
M 35	86			O	D>	S
M 36	88			O	n/c	S

O = achieved, X = not achieved.
S = stable, U = not stable for at least 1 week: 2Φ = 2-phase separation (completely separation) and 3Φ = 3-phase separation.
n/c = not significantly changing on estimated parameter, D< = an apparent viscosity increased and D> = an apparent viscosity decreased.

Moreover, CEs with different percentages of glycerin, fixed with other formulation components (fixed at 10%, w/w of 0.1 M NaCl electrolyte solution) were also performed in triplicates and then characterized based on the subjects as previous detailed. Table 40 shows the detailed information of characteristic assessments of these formulations (n = 3).

The results showed that CEs with 5% (w/w) of glycerin did not provide a considerable changing on their emulsions' aspects, esp., physical appearance, stiffness and viscosities (for at least 1 month).

From all the results as previous described, fifteen formulations of CEs that allowed the stable emulsions for at least 1 week was selected to use for further study in characterization and diffusion studies. Table 41 shows all the developed compositions of CEs.

Table 41 Compositions of developed concentrated w/o emulsions in preliminary formulation study.

Rx ID no.	Estimated formulation parameters		
	Emulsifier type	% Emulsifier	% DP
1*	Sorbitan (Arlacel 83™)	3%	84%
2*		3%	86%
3*		3%	88%
4*	Silicone (Abil EM 90™)	3%	84%
5*		3%	86%
6*		3%	88%
7*	Glucoester (Isolan DO™)	3%	84%
8*		3%	86%
9*		3%	88%
10*	Mixed (Montane 481™-Simulsol 989™)	3%	84%
11*		3%	86%
12*		3%	88%
13*	Glucoester (Isolan DO™)	1%	88%
14*		2%	88%
15*		4%	88%

*; % NaCl electrolyte = 10%, w/w and % Glycerin = 5%, w/w

7. Preparation of concentrated w/o emulsions containing freeze-dried green tea extract for *in vitro* diffusion and characterization studies

From a previous study, the suitable compositions of CEs (table 41) that allowed stable products over 1 week were preferred for *in vitro* diffusion and characterization studies.

Consequently, CEs with 84, 86 and 88% DP were prepared with 3% of each emulsifier. Approximately 1, 2, 3 and 4% of emulsifier concentration were also conducted with glucoester, fixed at 88% DP.

FD-GTE composed of GTCs together with caffeine from a previous study was incorporated into all formulations. In this study, only EGCG in the FD-GTE was determined as a model drug substance.

The aqueous phase included of 5% of glycerin, 10% of electrolyte solution (0.1 M NaCl electrolyte solution), 1% of paraben conc. in PG and 0.1% of BHT, combined with 0.025% of *di*-Na EDTA. As well as, the oil phase was composed of isohexadecane (Arlamol HD™) with four type emulsifiers. The characteristics of these emulsions were reproduced from one batch to another batch as verified by their apparent viscosity.

All the samples were prepared following with the same compositions as stated in table 41. Except to the aqueous phase was added FD-GTE. An approximately 2.25%, w/w of FD-GTE was incorporated into all formulations. All samples were performed in triplicates. The amount of FD-GTE incorporated into CEs was referred to the infinite dose as detailed in the solubility and stability of EGCG in receptor fluid used in the *in vitro* diffusion study.

Due to the poor stability of EGCG in aqueous, FD-GTE was reconstituted by DI water including EDTA-glycerin before emulsion preparations were performed. This solvent system was recommended to enhance the stability of EGCG in aqueous solution (Proniuk *et al*., 2002).

Table 42 shows the detailed information of production achievement throughout manufacturing processes, short-term stability for at least 1 week and degree of phase separation of all emulsions studied with different % DP (fixed with

other formulation components). It demonstrated that all emulsions incorporated with approximately 2.25%, w/w of FD-GTE allowed the stable products for at least 1 week. The diffusion study was just only determined over 48 h. This, therefore, ensured the sufficient stability of all formulations during the end of experiment.

Table 42 Characteristic assessments of developed concentrated w/o emulsions, containing 2.25% (w/w) of freeze-dried green tea extract (n = 3).

Sam no.	Sample Description			Production achievement	Short-term stability (over 1 week)	Degree of phase separation (after 1 week)
	Emulsifier type	% Emulsifier	% DP			
G 1	Sorbitan (Arlacel 83™)	3	84	O	S	n/d
G 2		3	86	O	S	n/d
G 3		3	88	O	S	n/d
G 4	Silicone (Abil EM 90™)	3	84	O	S	n/d
G 5		3	86	O	S	n/d
G 6		3	88	O	S	n/d
G 7	Glucoester (Isolan DO™)	3	84	O	S	n/d
G 8		3	86	O	S	n/d
G 9		3	88	O	S	n/d
G 10	Mixed (Montane 481™ - Simulsol 989™)	3	84	O	S	n/d
G 11		3	86	O	S	n/d
G 12		3	88	O	S	n/d
G 13	Glucoester (Isolan DO™)	1	84	O	S	n/d
G 14		2	86	O	S	n/d
G 15		4	88	O	S	n/d

*; % NaCl Electrolyte = 10%, w/w and % Glycerin = 5%, w/w.
O = achieved, X = not achieved.
S = stable, U = not stable for at least 1 week.
n/d = not detected.

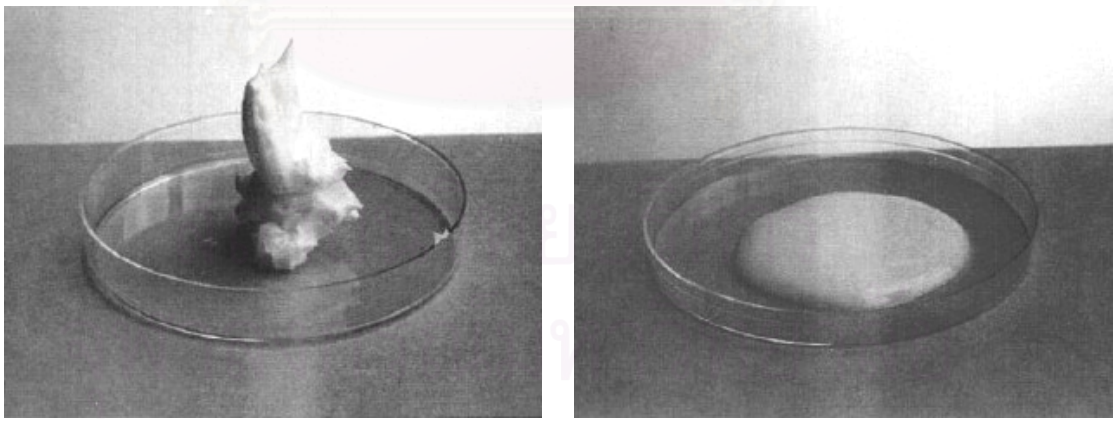
8. Characterization of concentrated w/o emulsions

The obtained emulsions were all characterized in termed of their long-term physical stability during storage at various temperatures (accelerated aging), apparent viscosity, droplet diameter and morphology. The actual results are all presented in appendix II for apparent viscosities, appendix III for images of SEM and appendix IV for mean droplet diameter and particle size distribution. No rheograms will be presented since rheology of CEs is not the subject of this study.

8.1 Long-term physical stability of concentrated w/o emulsions

Long-term physical stability of CEs was observed at three points of temperature; ambient and two accelerated aging, −20±1 °C (freezing temperature) and 40±1 °C (high temperature).

At definite time intervals (1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months), all emulsions were allowed to come back to room temperature and their physical stability were assessed by visual observation. In general, creaming, aggregation and phase separation have been used to monitor the physical stability of emulsions. The degree of phase separation was investigated.



a b

Figure 52 Illustrated images of concentrated w/o emulsions with silicone type emulsifier (a) and sorbitan type emulsifier (b).

The appearance and the spreadability of these emulsions vary: the silicone emulsions were very stiff and potentially broke if the shear applied was too

high whereas the others were much more fluid and did not break upon application. The spreadability of sorbitan emulsions was closely similar to glucoester emulsions, while mixed emulsion provided a high stiffness similar to silicone emulsions. Figure 52 shows the physical appearance and the spreadability of CEs with silicone (a) and sorbitan (b) emulsifier types.

Table 43, 44 and 45 present the results of long-term physical stability for all formulations under study at freezing temperature (−20±1 °C), high temperature (40±1 °C) and ambient, respectively.

Table 43 Long-term stability results at ambient (in days, D, or months, M) and degree of phase separation of concentrated w/o emulsions with different formulation parameters (n = 3).

Sam no.	Sample Description			Long-term stability of CEs	Degree of phase separation
	Emulsifier type	% Emulsifier	% DP		
A 1	Sorbitan (Arlacel 83™)	3	84	5 M	C*, 3Φ
A 2		3	86	5 M	C*, 3Φ
A 3		3	88	5 M	C, 3Φ
A 4	Silicone (Abil EM 90™)	3	84	> 6 M	n/d
A 5		3	86	> 6 M	n/d
A 6		3	88	> 6 M	n/d
A 7	Glucoester (Isolan DO™)	3	84	6 M	C*, 3Φ
A 8		3	86	6 M	C*, 3Φ
A 9		3	88	3 M	C, 3Φ
A 10	Mixed (Montane 481™-Simulsol 989™)	3	84	3 M	C*, A, 3Φ
A 11		3	86	3 M	C*, A, 3Φ
A 12		3	88	3 M	C*, A, 3Φ*
A 13	Glucoester (Isolan DO™)	1	88	2 M	C, 2Φ
A 14		2	88	2 M	C, 3Φ*
A 15		4	88	2 M	C, 3Φ

D = Day, M = Month, > = more than and < = less than.
n/d = not detected.
A = aggregation, C = creaming, 2Φ = 2-phase separation (completely separation) and 3Φ = 3-phase separation (* creaming or phase separation significantly occurred).

At ambient, the sorbitan emulsions were all stable over 5 months in all ranges of % DP. The silicone emulsions provided the best stability over all type emulsifiers under studied; they were all stable over more than 6 months. The

glucoester emulsions with 88%DP (over 3 months) provided the stability lower than 84% and 86% DP (over 6 months). The stability of mixed emulsions showed the lowest lifetime duration (over 3 months). The order of the physical stability at ambient is: silicone > glucoester > sorbitan > mixed emulsions.

For glucoester emulsions (fixed at 88% DP) with different emulsifier concentration, 1%, 2% and 4% emulsifier provided the less stable products only 2 months. While, 3% emulsifier showed a clear superiority of stability over all ranges (over 3 months).

Table 44 Long-term stability results at 40±1 °C (high temperature) (in days, D, or months, M) and degree of phase separation of concentrated w/o emulsions with different formulation parameters (n = 3).

Sam no.	Sample Description			Long-term stability of CEs	Degree of phase separation
	Emulsifier type	% Emulsifier	% DP		
A 1	Sorbitan type (Arlacel 83™)	3	84	2 M	C*, 3Φ
A 2		3	86	2 M	C*, 3Φ
A 3		3	88	2 M	C, 3Φ
A 4	Silicone type (Abil EM 90™)	3	84	6 M	C*, 3Φ
A 5		3	86	2 M	C*, 3Φ
A 6		3	88	2 M	3Φ*
A 7	Glucoester type (Isolan DO™)	3	84	3 M	C*, 3Φ
A 8		3	86	3 M	C*
A 9		3	88	3 M	C*
A 10	Mixed type (Montane 481™-Simulsol 989™)	3	84	1 M	C*, A, 3Φ
A 11		3	86	1 M	C*, A, 3Φ
A 12		3	88	1 M	C*, A, 3Φ*
A 13	Glucoester type (Isolan DO™)	1	88	2 M	C*, 2Φ
A 14		2	88	2 M	C*, 3Φ*
A 15		4	88	2 M	C*, 3Φ

D = Day, M = Month, > = more than and < = less than.
n/d = not detected.
A = aggregation, C = creaming, 2Φ = 2-phase separation (completely separation) and 3Φ = 3-phase separation (* creaming or phase separation significantly occurred).

At high temperature, the sorbitan emulsions were all stable over 2 months in all ranges of % DP. The silicone emulsions with 84% DP (over 6 months) provided the stability greater than 86% and 88% DP (over 2 months). The glucoester

emulsions provided the best stability over all type emulsifiers under studied; they were all stable over 3 months. The stability of mixed emulsions showed the lowest lifetime duration (over 1 month). The order of the physical stability is: glucoester > silicone > sorbitan > mixed emulsions.

For glucoester emulsions (fixed at 88% DP) with different emulsifier concentration, 1%, 2% and 4% emulsifier provided the less stable products only 2 months. While, 3% emulsifier showed a clear superiority of stability over all ranges (over 3 months).

Table 45 Long-term stability results at -20±1 °C (freezing temperature) (in days, D, or months, M) and degree of phase separation of concentrated w/o emulsions with different formulation parameters (n = 3).

Sam no.	Sample Description			Long-term stability of CEs	Degree of phase separation
	Emulsifier type	% Emulsifier	% DP		
A 1	Sorbitan type (Arlacel 83™)	3	84	M 5	C*
A 2		3	86	M 5	C*
A 3		3	88	M 5	C*
A 4	Silicone type (Abil EM 90™)	3	84	M 6	3Φ
A 5		3	86	M 3	3Φ*
A 6		3	88	M 2	3Φ*
A 7	Glucoester type (Isolan DO™)	3	84	M 1	C*, 2Φ
A 8		3	86	M 2	C*, 2Φ
A 9		3	88	M 2	C, 2Φ*
A 10	Mixed type (Montane 481™-Simulsol 989™)	3	84	8 D	C*, 3Φ*
A 11		3	86	8 D	C*, 3Φ*
A 12		3	88	8 D	C*, 3Φ
A 13	Glucoester type (Isolan DO™)	1	88	M 1	C*, 3Φ*
A 14		2	88	M 1	C*, 3Φ*
A 15		4	88	M 1	C*, 3Φ

D = Day, M = Month, > = more than and < = less than.
n/d = not detected.
A = aggregation, C = creaming, 2Φ = 2-phase separation (completely separation) and 3Φ = 3-phase separation (* creaming or phase separation significantly occurred).

At freezing temperature, the sorbitan emulsions were all stable over 5 months in all ranges of % DP. The silicone emulsions with 88%, 86% and 84% DP provided the stability over 2, 3 and 6 months, respectively. The glucoester emulsions

with 84% DP (over 1 month) provided the stability lesser than 86% and 88% DP (over 2 months). The stability of mixed emulsions showed the lowest lifetime duration (only 8 days). The order of the physical stability is: sorbitan > silicone > glucoester >> mixed emulsions.

For glucoester emulsions (fixed at 88% DP) with different emulsifier concentration, 1%, 2% and 4% emulsifier provided the less stable products only 1 month. While, 3% emulsifier showed a clear superiority of stability over all ranges (over 2 months).

8.1.1 Influence of emulsifier type

The chemical names of all emulsifier used in this study were as followed:

Arlacel 83™ is sorbitan sesquioleate.

Abil EM 90™ is cetyl dimethicone copolyol.

Montane 481™ is a mixture of sorbitan oleate, beeswax, hydrogenated castor oil, stearic acid, and coupled with Simulsol 989™ that is PEG-7 hydrogenated castor oil.

Isolan DO™ is methyl glucose dioleate.

Emulsifiers that are soluble in the external oil phase and form highly elastic films work best, and there is a critical level of emulsifier required to prevent inversion and to achieve the desired phase ratio.

A difference in stability is observed with the different emulsifiers used. The stability observed with different storage temperature showed some differences. It indicated that each emulsifier type behaved in different ways when exposed with different temperature.

From the results, the sorbitan and glucoester emulsifiers are small molecules, which allow the production of elastic interfacial films. Although the physical appearances of these products are better than the silicone and mixed emulsifiers, but due to a lack of rigidity of their interfaces, these systems are less stable. The sorbitan emulsifier was very sensitive to high temperature. While, the

glucoester emulsifier was very sensitive to both high and low temperatures. However, both of them work best in the case of ambient.

The mixed system allows the production of less stable products, probably caused by the stearic acid and beeswax presenting in the commercial mixture could not perform sufficiently strong interfacial film for the high internal volume system. Especially for low temperature, the stability of all silicone emulsions was only stable over 8 days. Although, the emulsions obtained with this type showed the rapidly decreased stability at all temperatures studied, but they were very efficient for the production of CEs when compared with the silicone emulsifier. However, the mixed emulsifier still allowed the production of stable products, at least for the duration of the diffusion study (48 hours).

Although, the silicone emulsifier was less efficient for the production of CEs, but the data obtained showed a clear superiority of the stability. The silicone emulsifier gave the more stable products. This emulsifier is a silicone polymer, which produces an emulsion with a very strong interfacial film due to steric crowding. However, this emulsifier type has some problem with the accelerated aging, esp., when employed with high amount of water-dispersed phase (86% and 88% DP).

8.1.2 Influence of percentage dispersed phase

From the results, it showed that the % DP have considerably affected to the stability of the silicone emulsions, esp., at high and low temperatures. The greater amount of water internal phase was performed, the lesser stability of emulsions was obtained.

8.1.3 Influence of emulsifier concentration

As the results, it indicated that the suitable amount of emulsifier for CEs with 88% DP was 3% of formulation. With higher or lesser than 3% of emulsifier, the stability of emulsions obtained decreased rapidly even at any temperature studied.

8.2 Apparent viscosity of concentrated w/o emulsions

After 1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months, the apparent viscosities of all formulations were measured at room temperature with a Brookfield viscometer (Brookfield, USA). A shear rate of system was set at approximately 100 s^{-1}. The results from triplicate samples of CEs were all recorded in termed of mean viscosity (mPa·s).

The average of apparent viscosities of each formulation was plotted as a function of the estimated time (days).

To compare the changing degree of all results, the graphs obtained from all formulations were transformed by linear regression method using Microsoft® Office Excel 2003 program. The good correlation coefficients for all formulations were obtained with $R^2 \geq 0.9$ as shown in table 46 for Rx 1-15. The slope of each graph, defined as rate of changing was used to see how much changing over time of each formulation. The plots of the average apparent viscosities (mPa·s) for all formulations (Avg. $\bar{\eta}$ ± S.D., n = 3), changing after 1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months, as a function of the estimated time, are shown in figure 53, 54 and 55.

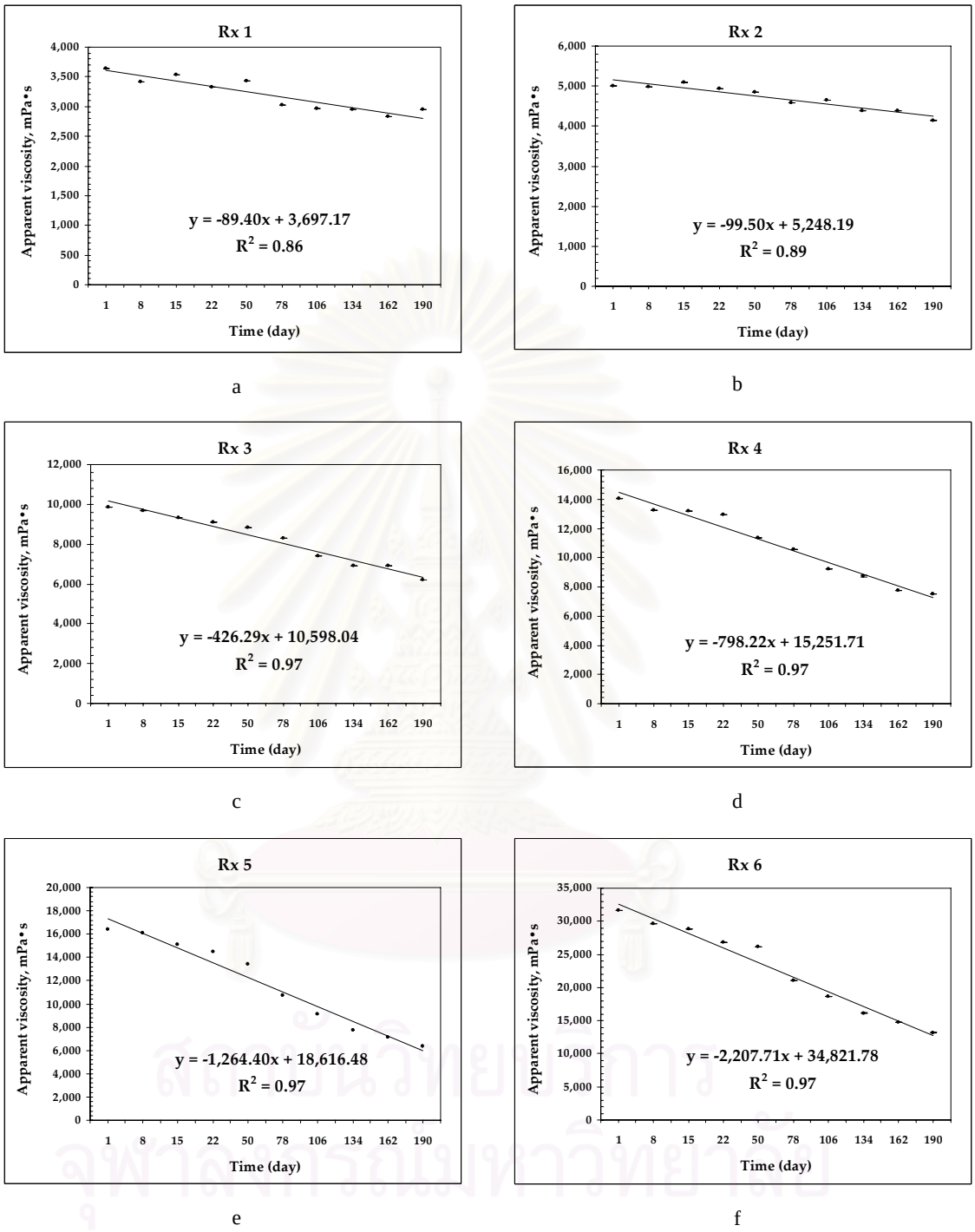


Figure 53 Average apparent viscosity (mPa·s) (Avg. $\dot{\eta}$ ± S.D., n = 3), changing after 1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months for Rx 1 (a), Rx 2 (b), Rx 3 (c), Rx 4 (d), Rx 5 (e) and Rx 6 (f).

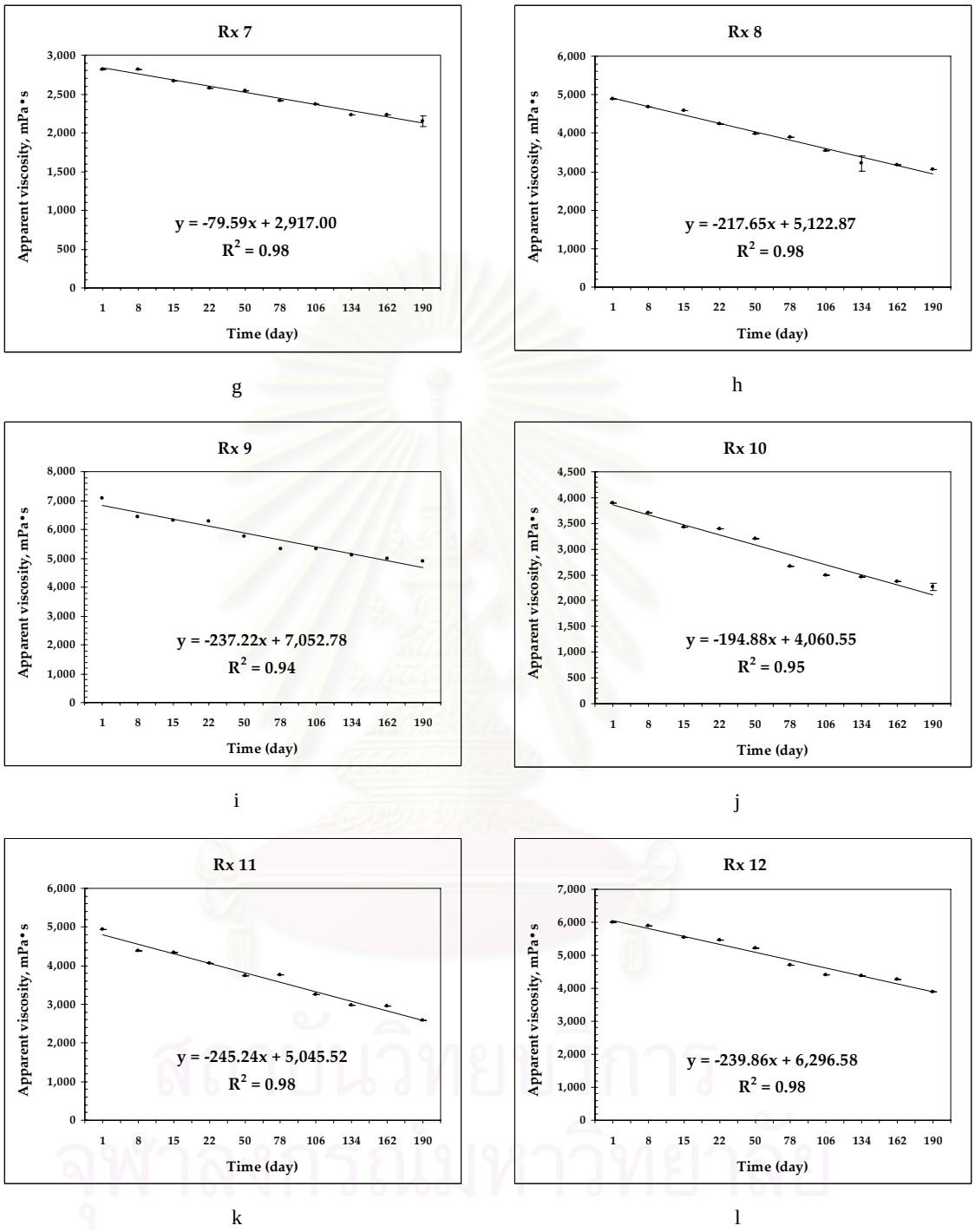
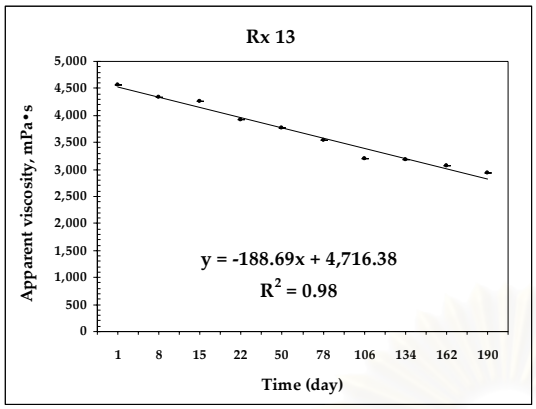
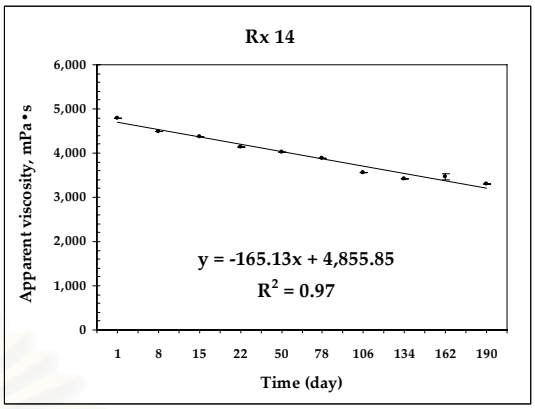


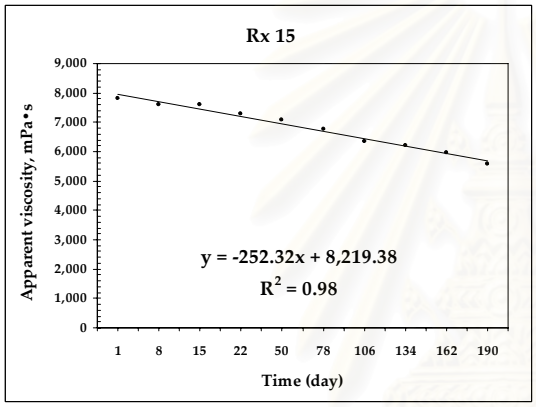
Figure 54 Average apparent viscosity (mPa·s) (Avg. $\bar{\eta}$ ± S.D., n = 3), changing after 1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months for Rx 7 (g), Rx 8 (h), Rx 9 (i), Rx 10 (j), Rx 11 (k) and Rx 12 (l).



m



n



o

Figure 55 Average apparent viscosity (mPa·s) (Avg. **ή** ± S.D., n = 3), changing after 1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months for Rx 13 (m), Rx 14 (n) and Rx 15 (o).

Table 46 Estimated parameters of the linear transformation, obtained from the plots of the average apparent viscosities (mPa·s) (Avg. **ή** ± S.D., n = 3), as a function of time (days) for all formulations under studied.

Type of emulsifier	% of Dispersed phase	Rx. No.	Trendline		
			Slope	Intercept	R^2
3% Arlacel 83™	84% DP	Rx 1	-89.40	3697.17	0.86
	86% DP	Rx 2	-99.50	5248.19	0.89
	88% DP	Rx 3	-426.29	10598.04	0.97
3% Abil EM 90™	84% DP	Rx 4	-798.22	15251.71	0.97
	86% DP	Rx 5	-1264.40	18616.48	0.97
	88% DP	Rx 6	-2207.71	34821.78	0.97
3% Isolan DO™	84% DP	Rx 7	-79.59	2917.00	0.98
	86% DP	Rx 8	-217.65	5122.87	0.98
	88% DP	Rx 9	-237.22	7052.78	0.94
2.2% Mon:0.8% Si	84% DP	Rx 10	-194.88	4060.55	0.95
	86% DP	Rx 11	-245.24	5045.52	0.98
	88% DP	Rx 12	-239.86	6296.58	0.98
1% Isolan DO™	88% DP	Rx 13	-188.69	4716.38	0.98
2% Isolan DO™		Rx 14	-165.13	4855.85	0.97
4% Isolan DO™		Rx 15	-252.32	8219.38	0.98

8.2.1 Influence of the emulsifier type

From table 46, the slopes of the glucoester, mixed, sorbitan and silicone emulsions with 88% DP were −237.22, −239.86, −426.29 and −2207.71, respectively. This indicated that the changing of the apparent viscosity after 6 months of the glucoester and mixed emulsions were similar, while the sorbitan emulsions were twofold of the formers. It was interesting to note that the apparent viscosity of the silicone emulsions changed dramatically after 6 months.

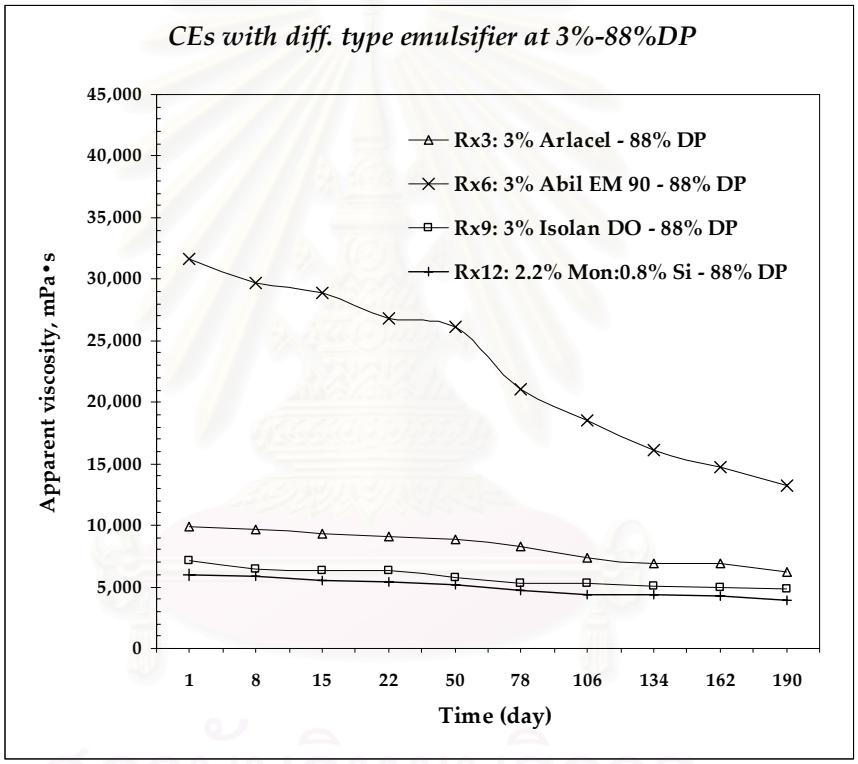


Figure 56 Average apparent viscosity (mPa·s) (Avg. **ή** ± S.D., n = 3), changing after 1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months in CEs with 3% of sorbitan (Rx 3), silicone (Rx 6), glucoester (Rx 9) and mixed (Rx 12) emulsifiers.

The apparent viscosities of silicone emulsions are extremely high as shown in figure 56. Although their apparent viscosity drastically decreased, their viscosities after 6 months of the fabrication are higher than the other types at first day of the production. The superiority of apparent viscosity, therefore, confirmed the good stability observed with the accelerated aging experiments as previously

described. The only problem with the emulsions produced with this system is their instability at very high and very low temperature (−20°C).

8.2.2 Influence of the percentage dispersed phase

Figure 57 shows the effect of % DP on the changing of the apparent viscosities with time for all type emulsifiers used under study.

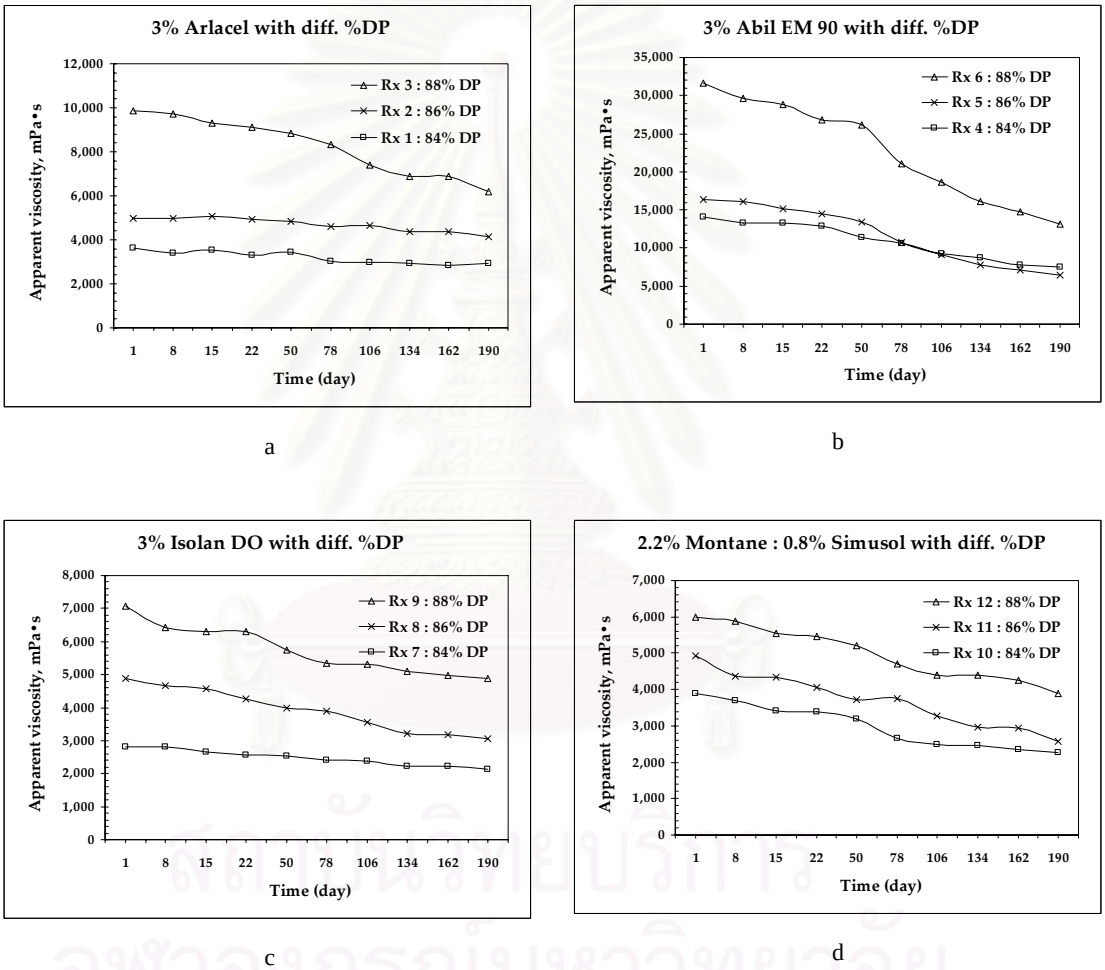


Figure 57 Average apparent viscosity (mPa·s) (Avg. **ή** ± S.D., n = 3), changing after 1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months in CEs with 3% of sorbitan (a), silicone (b), glucoester (c) and mixed (d) emulsifiers at 84, 86 and 88% of dispersed phase.

All of them showed the same tendency of the effect that the high amount of water phase gave the high apparent viscosities. The order of the effect of % DP is: 88% > 86% > 84% DP.

8.2.3 Influence of the percentage of emulsifier

Figure 58 showed that the higher percentages of emulsifier employed, the greater apparent viscosities obtained.

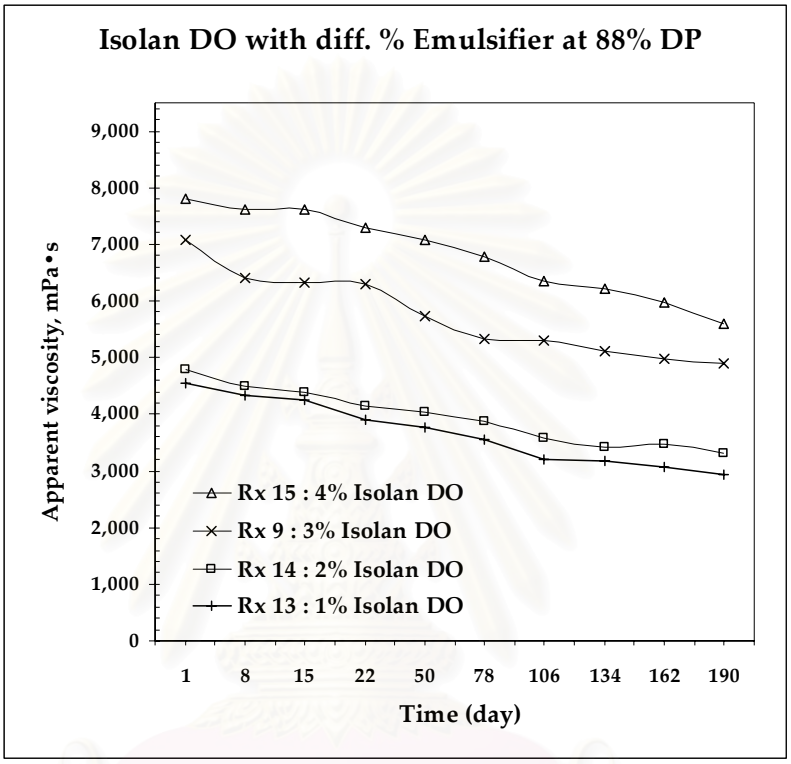


Figure 58 Average apparent viscosity (mPa·s) (Avg. **ή** ± S.D., n = 3), changing after 1, 8, 15, 22 days, 1, 2, 3, 4, 5 and 6 months in glucoester emulsions with 1, 2, 3 and 4% emulsifier.

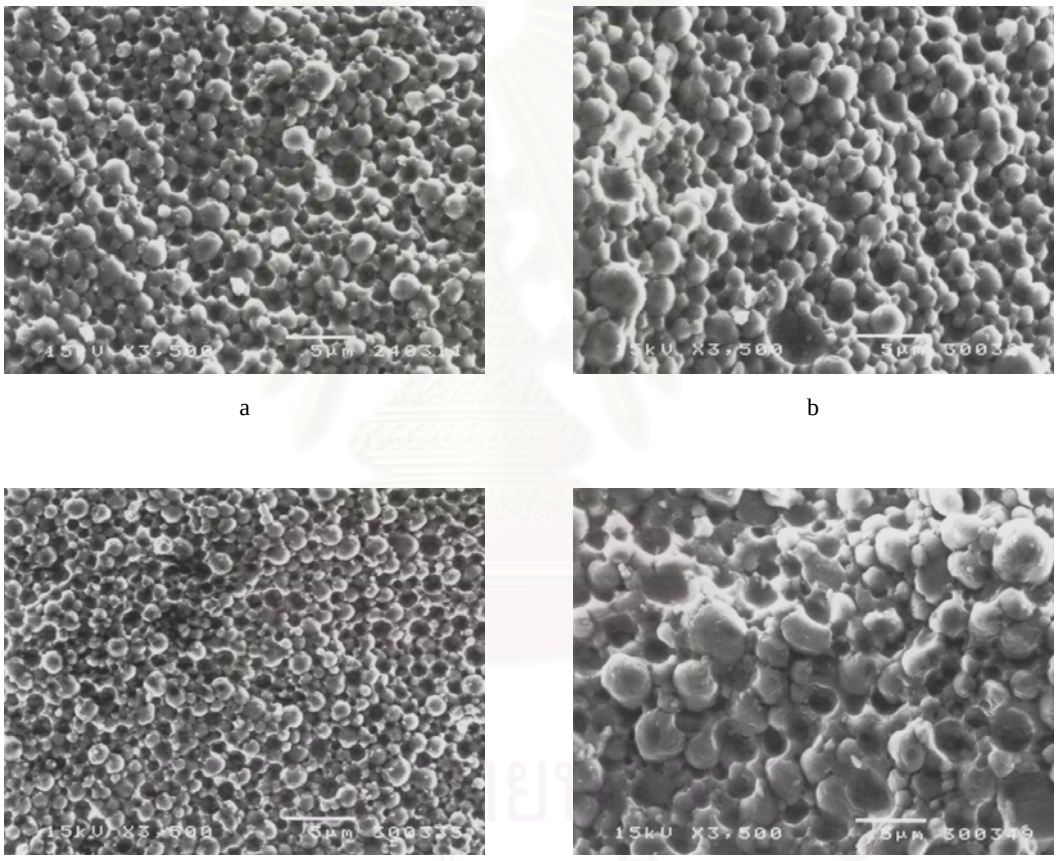
The slopes of the glucoester with 1%, 2%, 3% and 4% emulsifier (−188.69, −165.13, −237.22 and −252.32, respectively) in table 46 indicated that the changing of the apparent viscosity after 6 months increased with the percentages of emulsifier concentration. Although, 4% emulsifier allowed the less stable emulsions as discussed in the long-term stability at three temperatures, but the changing of the apparent viscosity was better than 3%. From this observation, it has to note that the changing of the apparent viscosity of CEs was not the only one parameter to indicate the stability of final products. It also depended on the apparent viscosity obtained after fabrication.

8.3 Determination of morphology: Droplet diameter and shape

SEM images of all formulation, as displayed in appendix III, showed the changing of droplet diameter and shape after 1, 8, 15 days, 3 and 6 months.

8.3.1 Influence of the emulsifier type

From figure 59, the sorbitan (a) and glucoester (c) showed the homogeneity of the water droplets, surrounding with emulsifier and oil film.



a b

c d

Figure 59 SEM images of CEs with 3% of sorbitan (a), silicone (b), glucoester (c) and mixed (d) emulsifiers, fixed at 88% of dispersed phase (day 1).

The silicone (b) and mixed (d) emulsions showed the heterogeneity of the water droplets, surrounding with emulsifier and oil film.

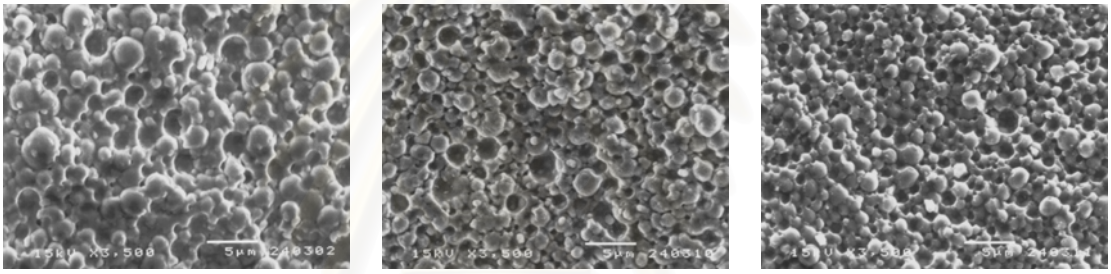
Each droplet was merged to each other with neighborhoods. The shape of water droplets in mixed emulsions was spherical, while the others were all hexagonal. As the results, it could confirm the stability of mixed emulsions, which

are quite low due to the spherical shape. In other word, the hexagonal shape could be the characteristic of very high internal phase emulsions, namely CEs in this study.

8.3.2 Influence of the percentage dispersed phase

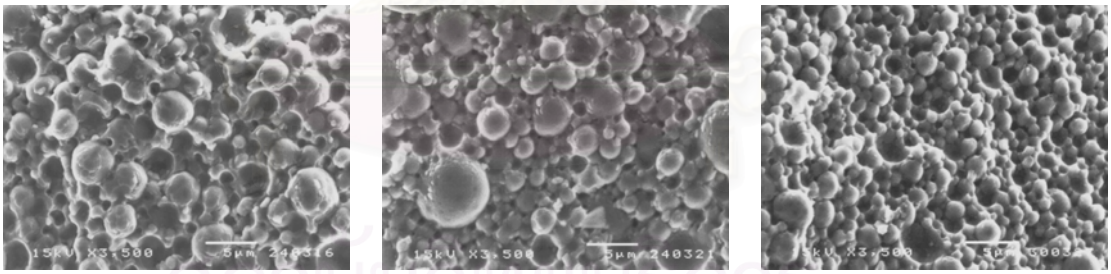
From figure 60 and figure 61, it indicated that the heterogeneity of water droplets obtained decreased with the % DP. In addition, the shape of water droplets tended to transform to hexagon when the % DP increased, except for the mixed emulsions (d). The droplets of the mixed emulsions are still sphere for all ranges of % DP.

a



84% DP 86% DP 88%DP

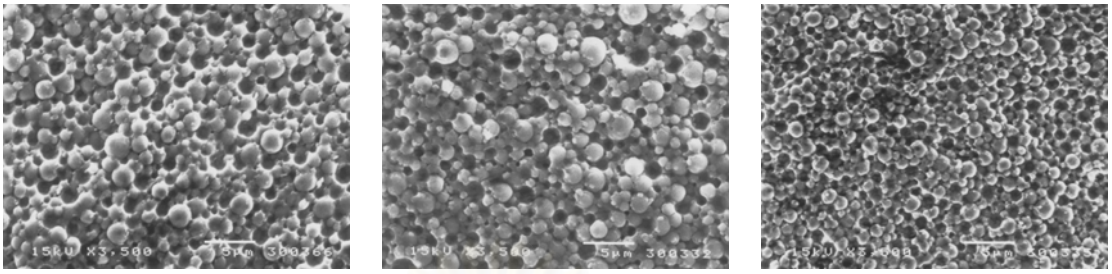
b



84% DP 86% DP 88% DP

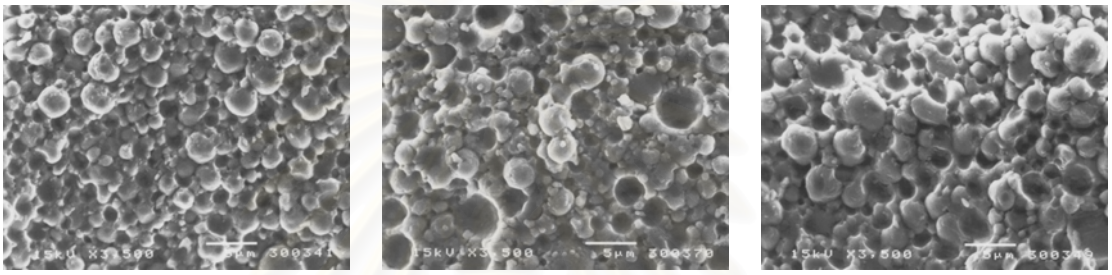
Figure 60 SEM images of CEs with 3% of sorbitan (a) and silicone (b) emulsifiers at 84, 86 and 88% of dispersed phase (day 1).

c



84% DP 86% DP 88% DP

d

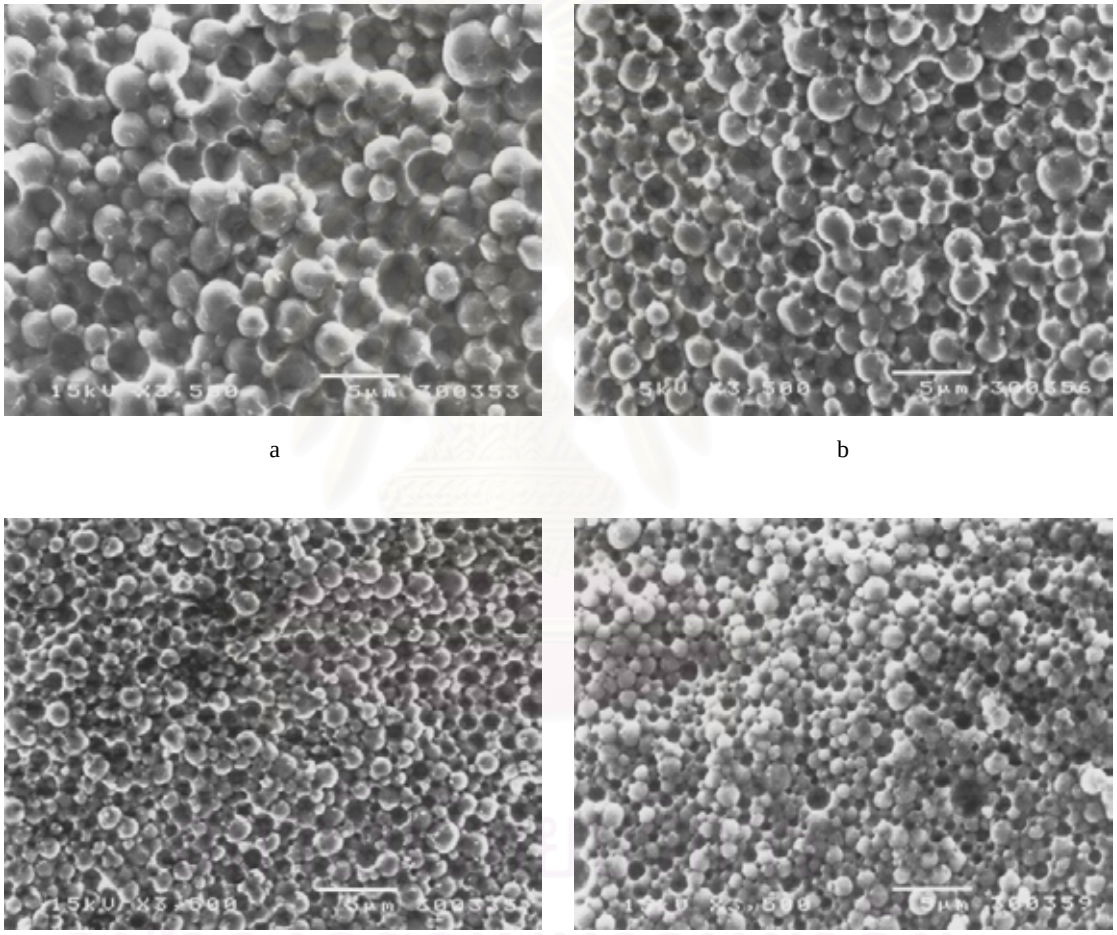


84% DP 86% DP 88% DP

Figure 61 SEM images of CEs with 3% of glucoester (c) and mixed (d) emulsifiers at 84, 86 and 88% of dispersed phase (day 1).

8.3.3 Influence of the percentage of emulsifier

Figure 62 showed that the droplet diameter of the glucoester emulsions (at 88% DP) decreased with the percentages of emulsifier concentration used. It indicated that the higher concentration of emulsifier used could affect to the curvature of the interfacial film. Therefore, very small droplet diameter could be obtained with the suitable agitation during fabrication.



a b

c d

Figure 62 SEM images of the glucoester emulsions with 1% (a), 2% (b), 3% (c) and 4% (d) emulsifier, fixed at 88% of dispersed phase (day 1).

8.4 Determination of mean droplet diameter and droplet size distribution: Laser diffraction analysis (wet dispersion)

The present study investigated the role of the formulation parameters on the size and polydispersity of CEs containing FD-GTE. The mean diameter and droplet size distribution of the CEs were measured in wet dispersions using a laser light scattering spectrometer, Mastersizer S long bed version 2.11. (Malvern, UK). Light mineral oil was used as a dispersion medium.

In droplet size statistics (Bateman, 1993), the most widely used statistic is possibly labeled “D [v, 0.5]”. This is the way that the VMD (volume median diameter) is expressed in scientific terms. This simply means that 50% of the droplet volume is composed of larger droplet sizes and 50% of smaller sizes. D [v, 0.9] refers to a value such that 90% of the droplet volume is made of smaller sizes (with the remaining 10% in larger). Conversely, the D [v, 0.1] value gives the droplet size such that only 10% of the droplet volume is in smaller, while the remaining 90% is composed of larger sizes. It should be noted that these are median rather than average (mean) values. The droplet size of CEs was described by D [v, 0.5], which is related to the mass median diameter (MMD) by the density of the particles (assuming a size independent density for the particles).

8.4.1 Average mean droplet diameter in µm (D [v, 0.5])

The average of mean droplet diameters of each formulation was plotted as a function of the estimated time (days).

To compare the changing degree of all results, the graphs obtained from all formulations were transformed by linear regression method using Microsoft® Office Excel 2003 program. The good correlation coefficients for all formulations were obtained with $R^2 \geq 0.9$ as shown in table 47 for Rx 1-15. The slope of each graph, defined as rate of changing was used to see how much changing over time of each formulation. The plots of the average mean droplet diameters in µm, stated as D [v, 0.5] (Avg. D [v, 0.5] ± S.D., n = 3), changing after 1, 8, 15 days, 3 and 6 months, as a function of the estimated time, are shown in figure 63, 64 and 65.

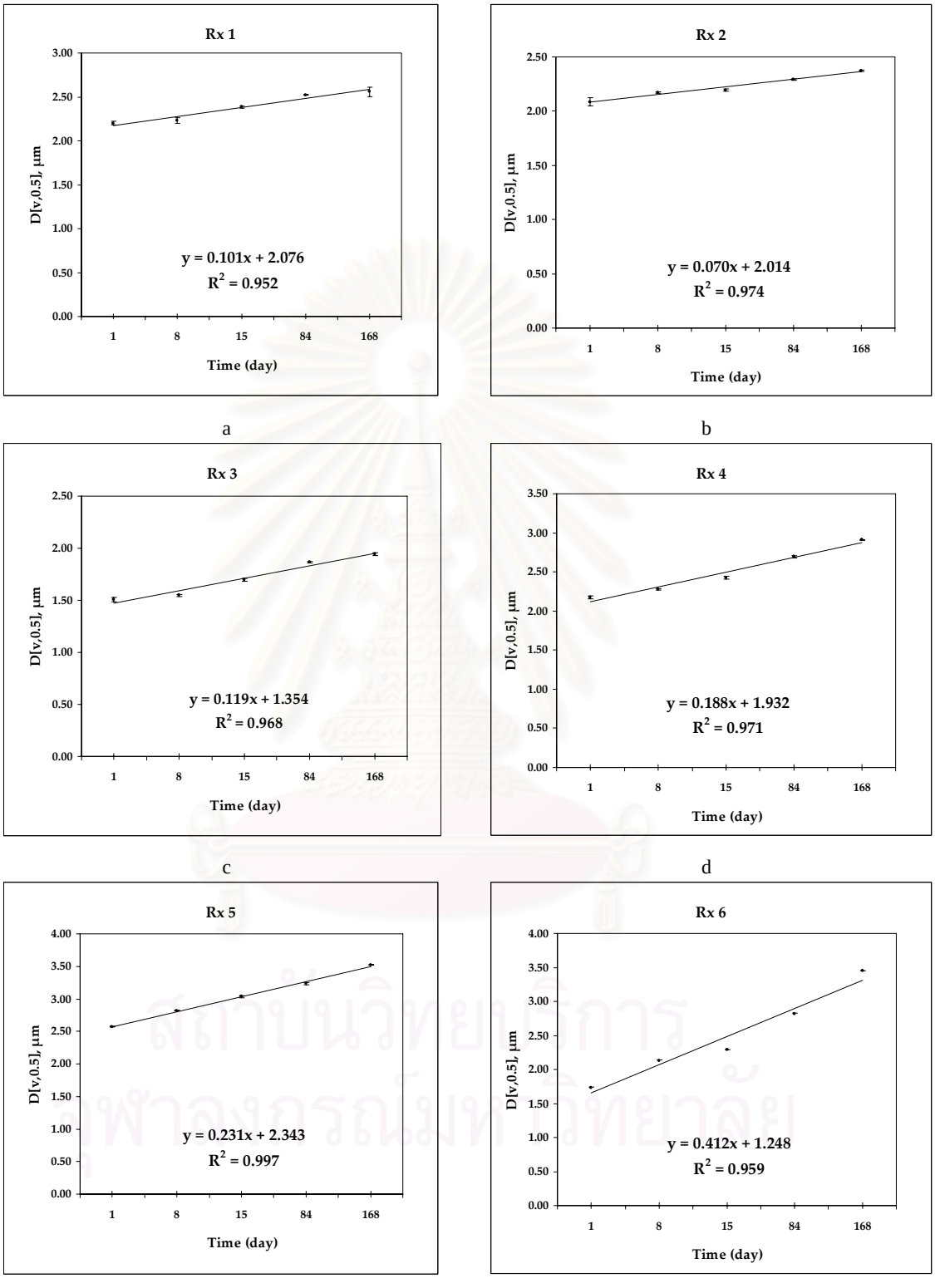
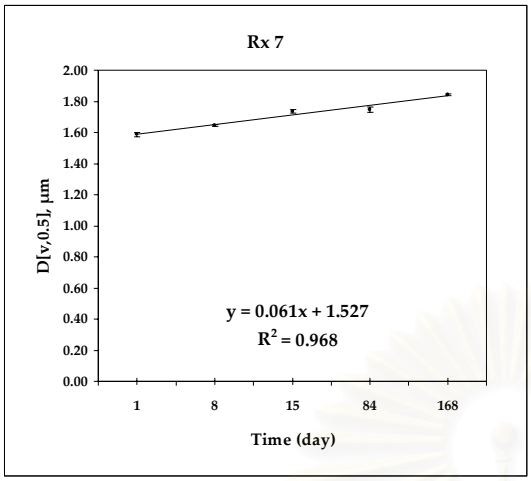
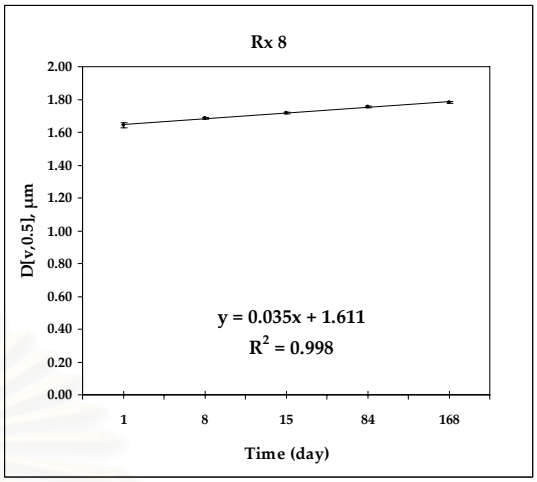


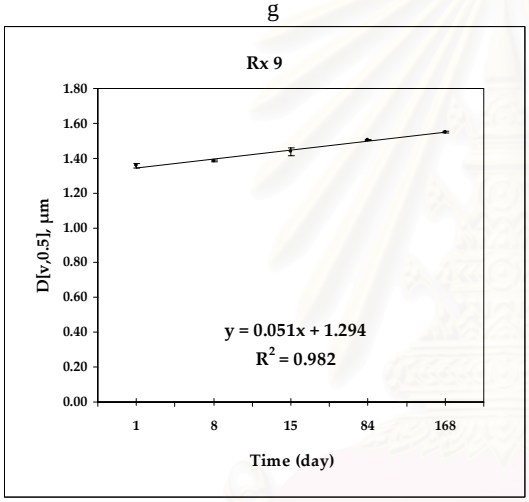
Figure 63 Average mean droplet diameter in µm (Avg. D [v, 0.5] ± S.D., n = 3), changing after 1, 8, 15 days, 3 and 6 months for Rx 1 (a), Rx 2 (b), Rx 3 (c), Rx 4 (d), Rx 5 (e) and Rx 6 (f).



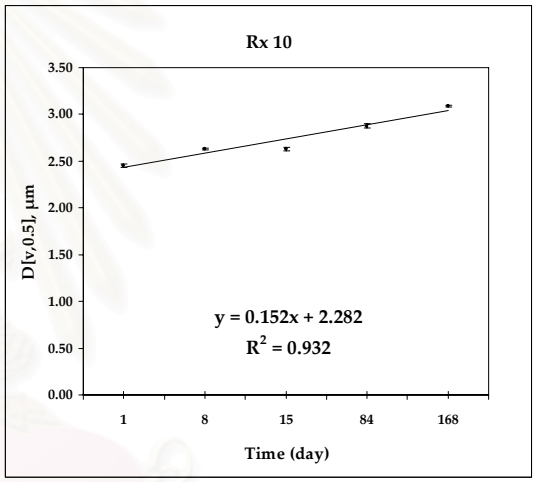
g



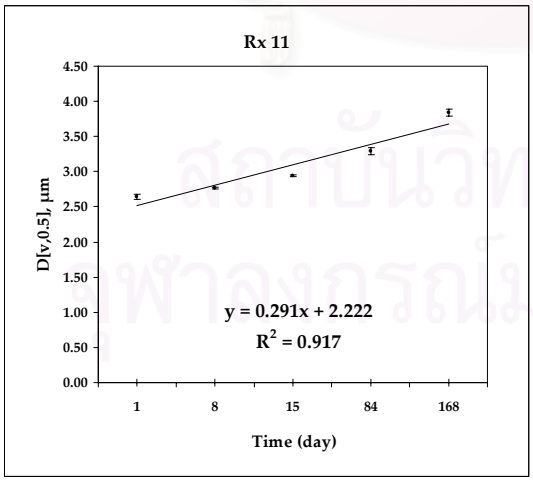
h



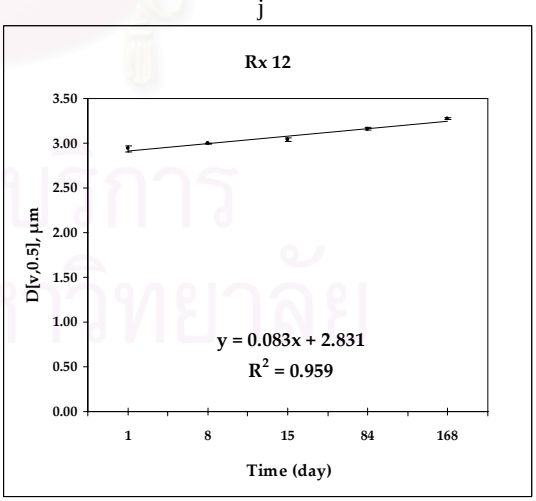
i



j

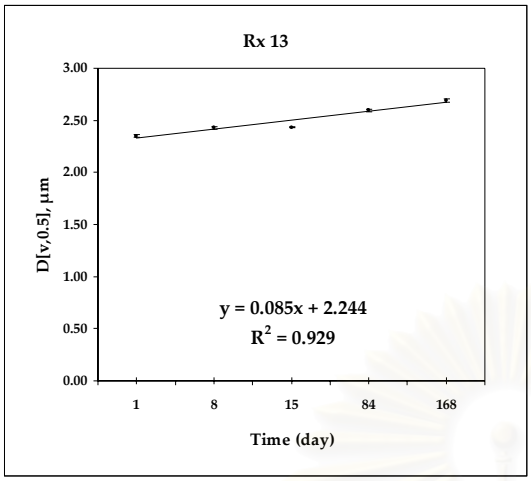


k

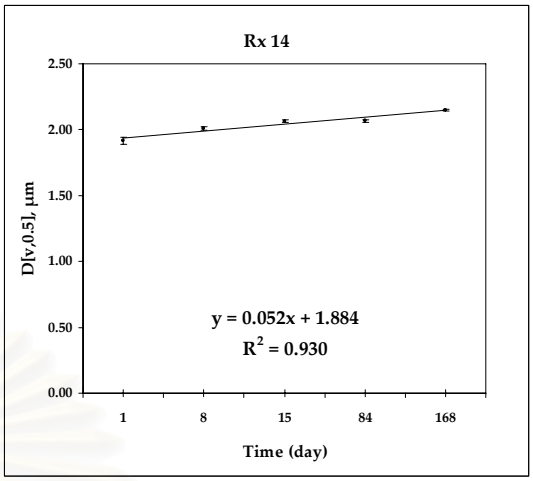


l

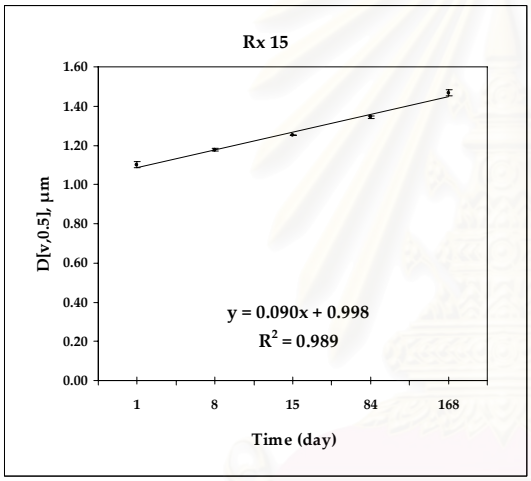
Figure 64 Average mean droplet diameter in µm (Avg. D [v, 0.5] ± S.D., n = 3), changing after 1, 8, 15 days, 3 and 6 months for Rx 7 (g), Rx 8 (h), Rx 9 (i), Rx 10 (j), Rx 11 (k) and Rx 12 (l).



m



n



o

Figure 65 Average mean droplet diameter in µm (Avg. D [v, 0.5] ± S.D., n = 3), changing after 1, 8, 15 days, 3 and 6 months for Rx 13 (m), Rx 14 (n) and Rx 15 (o).

Table 47 Estimated parameters of the linear transformation, obtained from the plots of the average mean droplet diameter in μm (Avg. D [v, 0.5] ± S.D., n = 3), as a function of time (days) for all formulations under studied.

Type of emulsifier	% of Dispersed phase	Rx. No.	Trendline		
			Slope	Intercept	R^2
3% Arlacel 83™	84% DP	Rx 1	0.101	2.076	0.952
	86% DP	Rx 2	0.070	2.014	0.974
	88% DP	Rx 3	0.119	1.354	0.968
3% Abil EM 90™	84% DP	Rx 4	0.188	1.932	0.971
	86% DP	Rx 5	0.231	2.343	0.997
	88% DP	Rx 6	0.412	1.248	0.959
3% Isolan DO™	84% DP	Rx 7	0.061	1.527	0.968
	86% DP	Rx 8	0.035	1.611	0.998
	88% DP	Rx 9	0.051	1.294	0.982
2.2% Mon:0.8% Si	84% DP	Rx 10	0.152	2.282	0.932
	86% DP	Rx 11	0.291	2.222	0.917
	88% DP	Rx 12	0.083	2.831	0.959
1% Isolan DO™	88% DP	Rx 13	0.085	2.244	0.929
2% Isolan DO™		Rx 14	0.052	1.884	0.930
4% Isolan DO™		Rx 15	0.090	0.998	0.989

The results obtained from the Malvern Mastersizer suggest that there is no significant change in the D [v, 0.5] diameter or the size distribution of the water droplets over time (throughout 6 months) as depicted in figures 63, 64 and 65. At the starting of estimated time, the Malvern results also indicates that the particle size distribution ranges from 1 to 3 μm and does not change significantly with time. The data for these figures is all included in appendix IV.

8.4.1.1 Influence of the emulsifier type

From table 47, the slopes of the glucoester, mixed, sorbitan and silicone emulsions with 88% DP were 0.051, 0.083, 0.119 and 0.412, respectively. This indicated that the changing of the mean droplet diameter after 6 months of all formulations showed the same tendency as of the apparent viscosity. This could confirm the effect of the type of emulsifier on the characteristics of CEs.

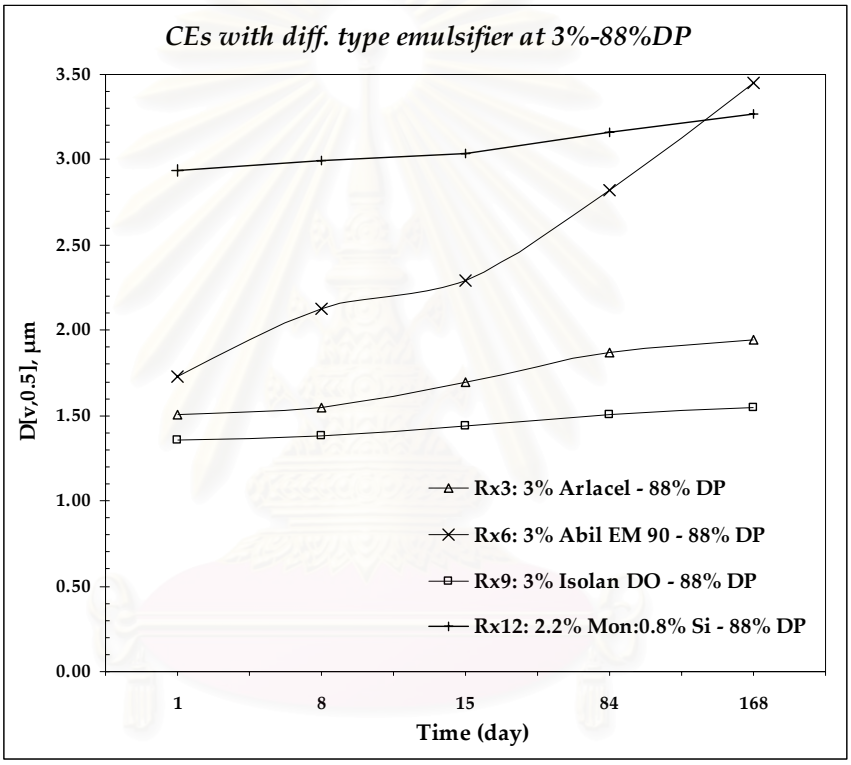


Figure 66 Average mean droplet diameter in µm (Avg. D [v, 0.5] ± S.D., n = 3), changing after 1, 8, 15 days, 3 and 6 months in CEs with 3% of sorbitan (Rx 3), silicone (Rx 6), glucoester (Rx 9) and mixed (Rx 12) emulsifiers.

The D [v, 0.5] of mixed emulsions is extremely high as shown in figure 66. Although, the D [v, 0.5] of mixed emulsions was so high, but the changing of the D [v, 0.5], after 6 months was not considerably large as like the silicone one. This might be due to the heterogeneity of silicone emulsions.

8.4.1.2 Influence of the percentage dispersed phase

The effect of % DP on the changing of the D [v, 0.5] with time are all shown in figure 67 for all type emulsifiers used under study.

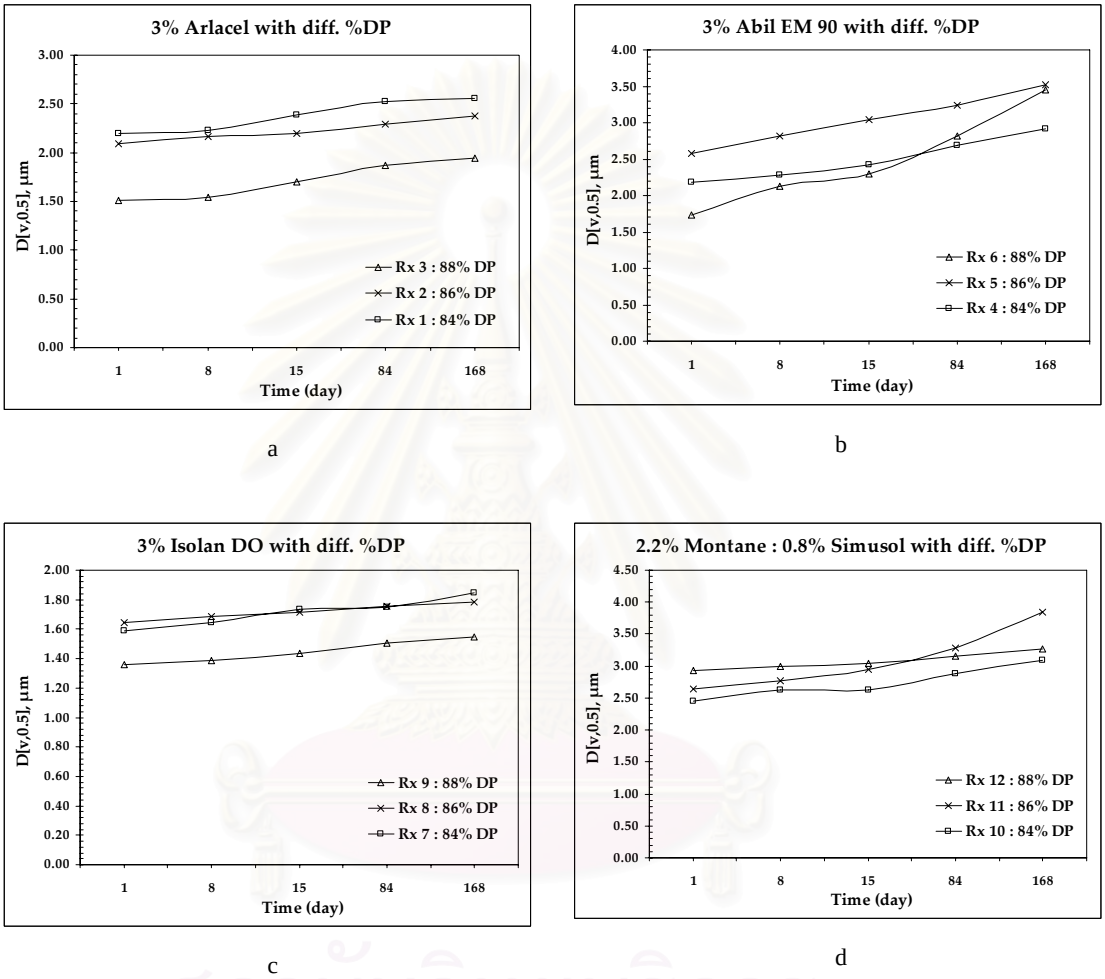


Figure 67 Average mean droplet diameter in µm (Avg. D [v, 0.5] ± S.D., n = 3), changing after 1, 8, 15 days, 3 and 6 months in CEs with 3% of sorbitan (a), silicone (b), glucoester (c) and mixed (d) emulsifiers at 84, 85 and 88% of dispersed phase.

The tendency of the effect of the % DP was not as the expected trend. In general, the D [v, 0.5] usually decreases with the % DP. It might due to the variation of the results obtained with the measurement technique employed. The Malvern only requires a very small sample of emulsion to determine the size distribution. As a result, the size distribution may not be an accurate representation of the whole emulsion particles. Any problems arising from the sampling method were minimized by taking a number of small samples and repeating the sampling and

testing procedure for each emulsion. Furthermore, the Malvern results, at the starting of estimated time also indicates that the particle size distribution ranges from 1 to 3 μm. With the same type of emulsifier used, the discrimination between the similar values, therefore, could not be obtained in practical. However, the SEM images were also combined to the size distribution data to confirm the possibility of the real situation.

8.4.1.3 Influence of the percentage of emulsifier

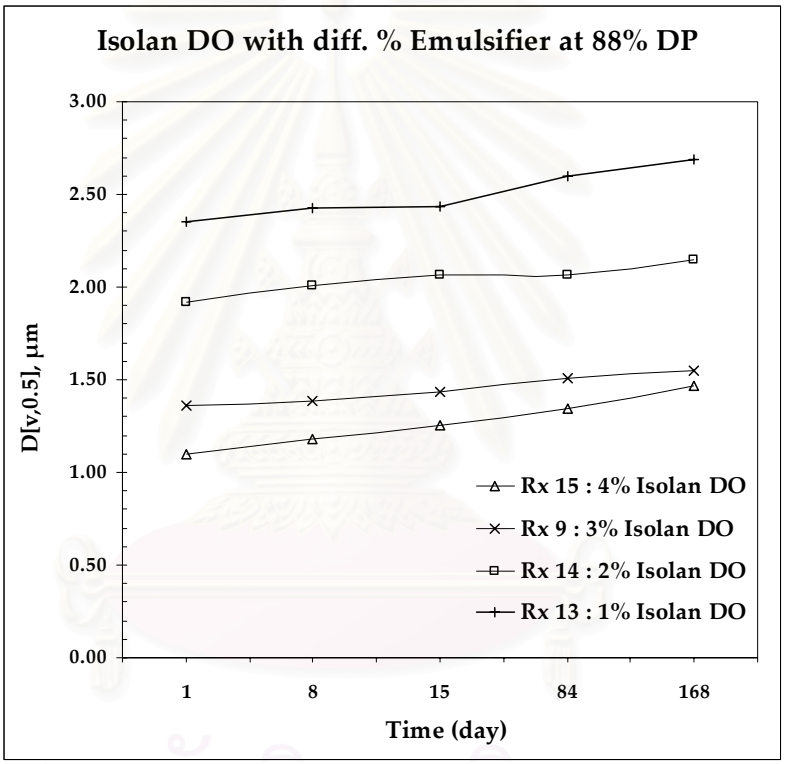


Figure 68 Average mean droplet diameter in μm (Avg. D [v, 0.5] ± S.D., n = 3), changing after 1, 8, 15 days, 3 and 6 months in glucoester emulsions with 1, 2, 3 and 4% emulsifier.

As shown in figure 68, the effect of the percentages of emulsifier concentration used on the D [v, 0.5] provides the results with tendency as following: 1% > 2% > 3% > 4%. It indicated that the higher percentages of emulsifier employed, the lesser mean droplet diameters obtained.

8.4.2 Polydispersity (Relative span)

The relative span measures the width of the droplet size distribution. Hence, a small relative span value indicates a narrow droplet size distribution.

Figure 69, 70 and 71 depict the characteristic of CEs on the polydispersity. A small relative span (a value of zero would indicate a monodisperse droplet distribution) indicates a narrow droplet size distribution.

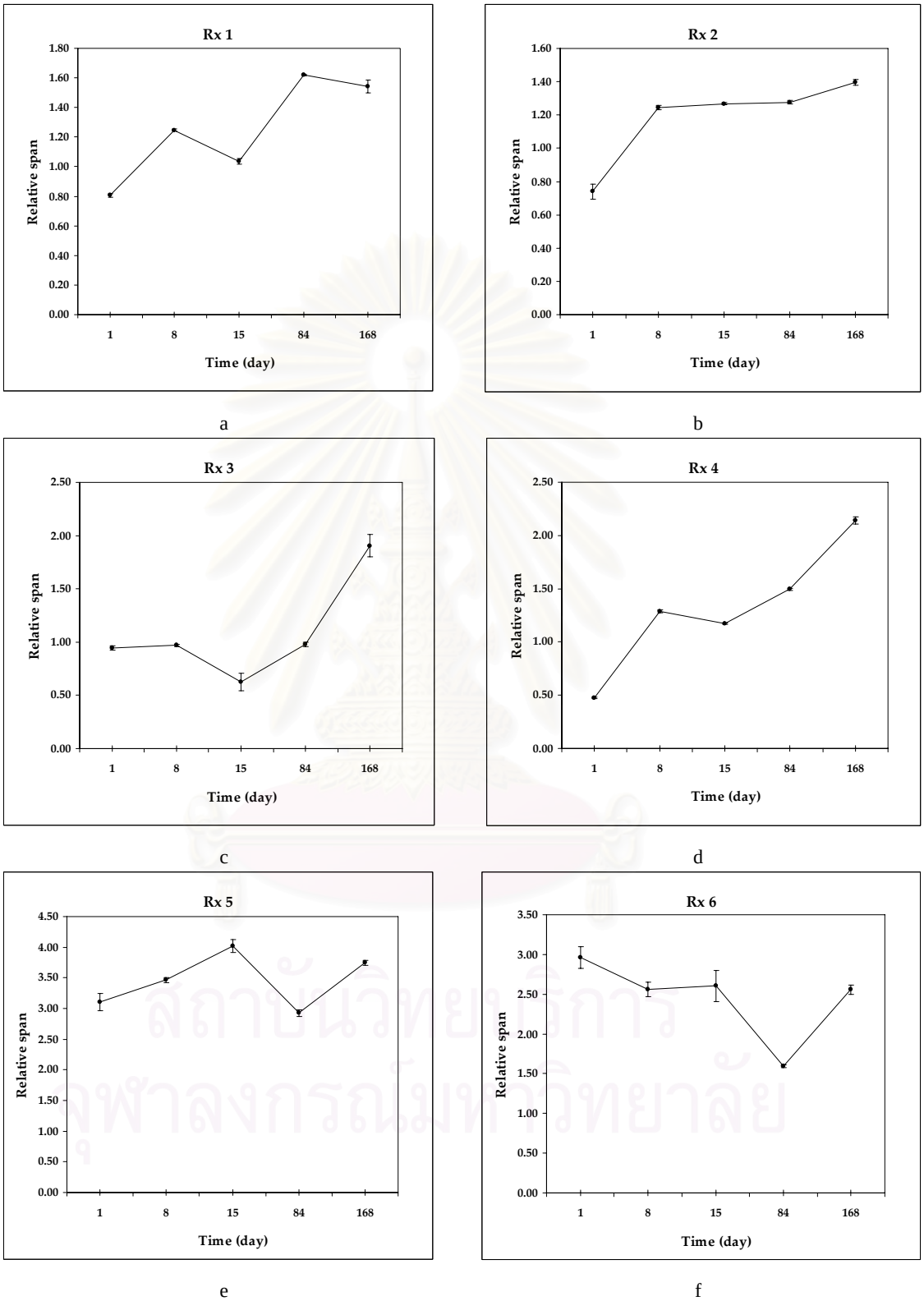
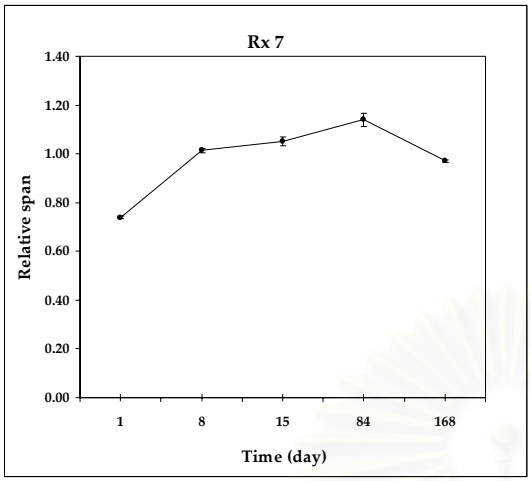
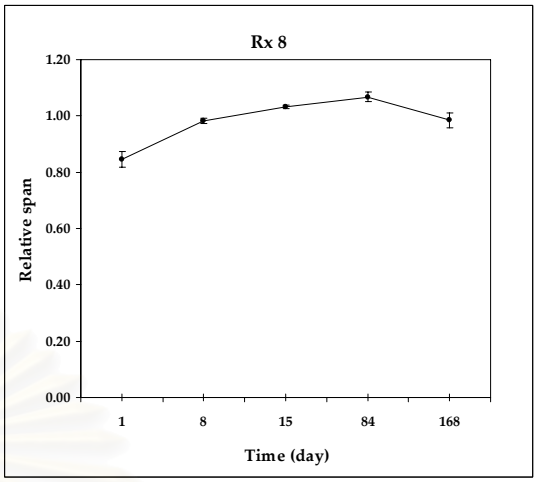


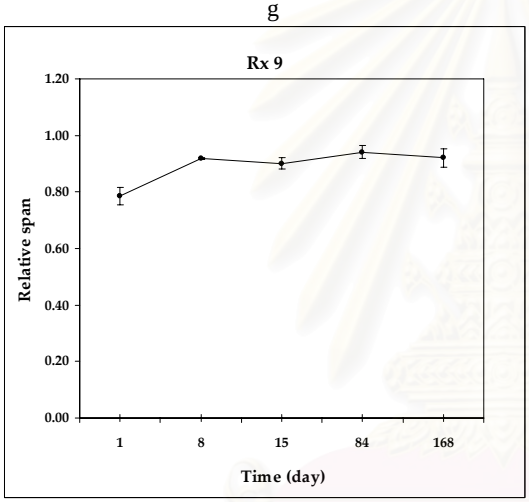
Figure 69 Average relative span (Avg. R.S. ± S.D., n=3), changing after 1, 8, 15 days, 3 and 6 months for Rx 1 (a), Rx 2 (b), Rx 3 (c), Rx 4 (d), Rx 5 (e) and Rx 6 (f).



g



h



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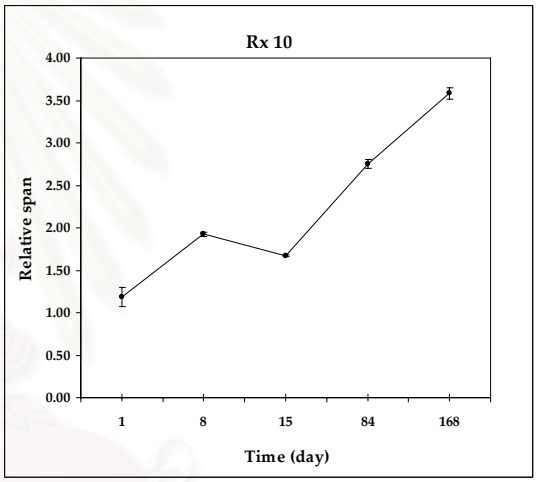

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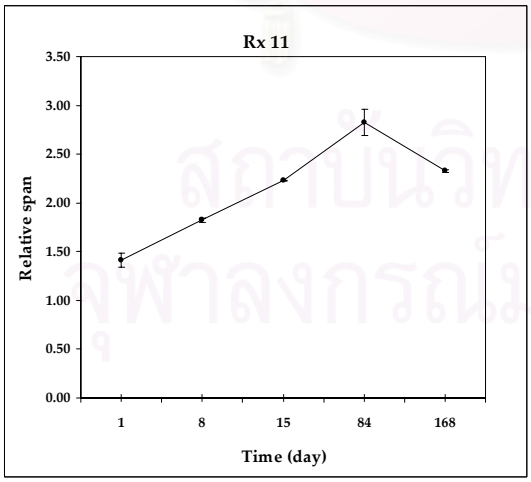

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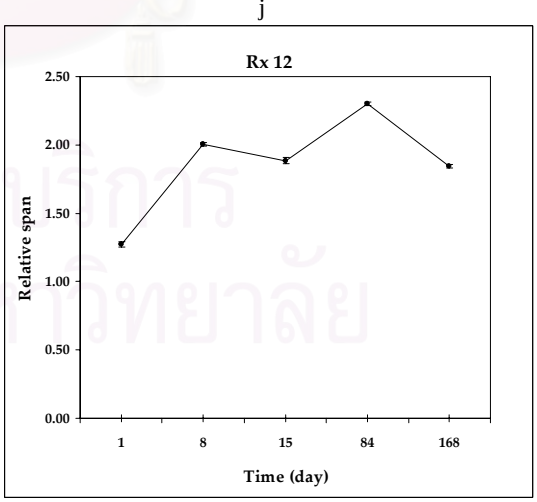
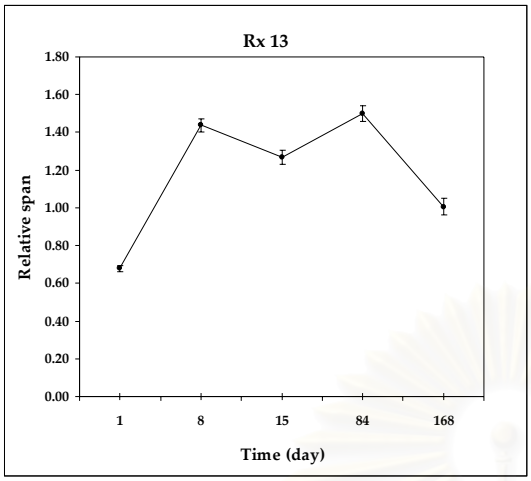


l

Figure 70 Average relative span (Avg. R.S. ± S.D., n=3), changing after 1, 8, 15 days, 3 and 6 months for Rx 7 (g), Rx 8 (h), Rx 9 (i), Rx 10 (j), Rx 11 (k) and Rx 12 (l).



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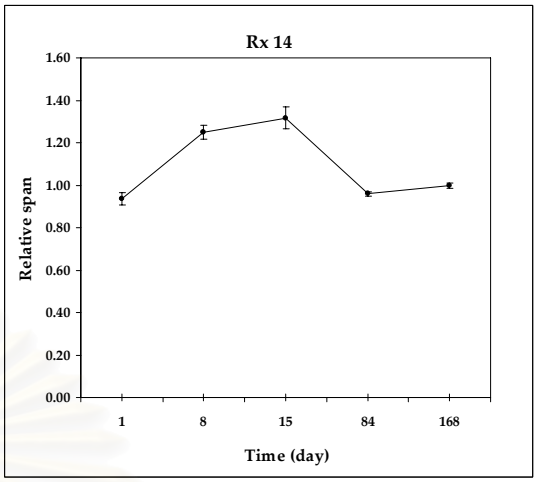

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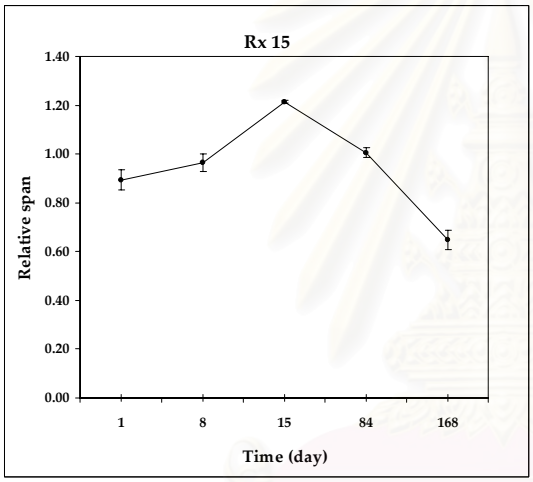


o

Figure 71 Average relative span (Avg. R.S. ± S.D., n=3), changing after 1, 8, 15 days, 3 and 6 months for Rx 13 (m), Rx 14 (n) and Rx 15 (o).

8.4.2.1 Influence of the emulsifier type

From figure 72, it showed that a clear superiority of the polydispersity of the silicone emulsions. The sorbitan and the glucoester emulsions provided the similarity of the results. Although the mixed type emulsions provided the higher value than the formers, but they were considerably lower than the silicone type.

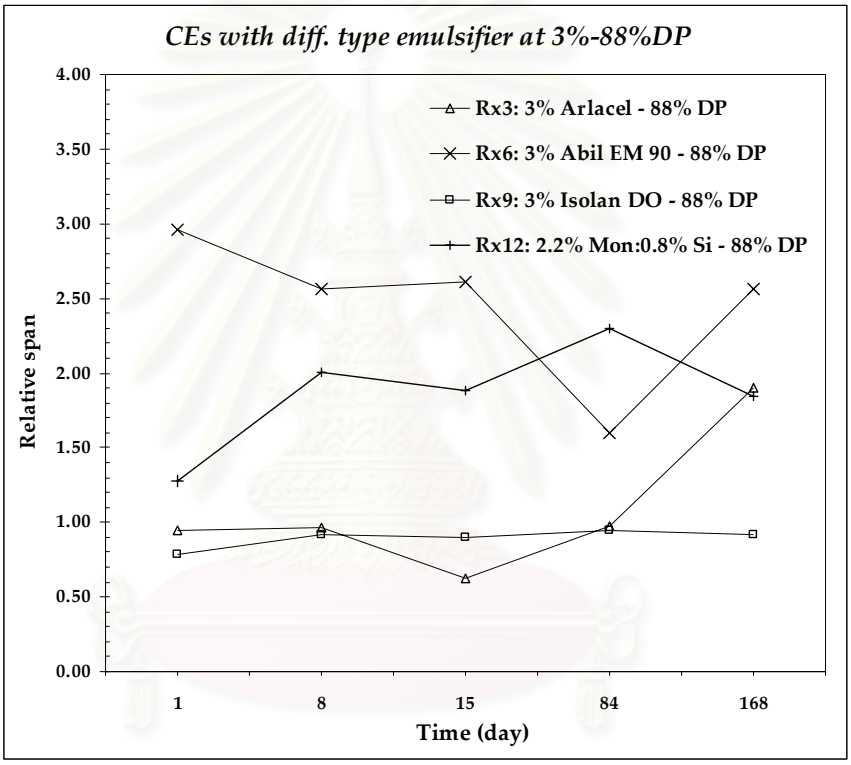


Figure 72 Average relative span (Avg. R.S. ± S.D., n=3), changing after 1, 8, 15 days, 3 and 6 months in CEs with 3% of sorbitan (Rx 3), silicone (Rx 6), glucoester (Rx 9) and mixed (Rx 12) emulsifiers.

8.4.2.2 Influence of the percentage dispersed phase

From figure 73, the sorbitan (a) and glucoester (c) emulsions with 88% DP provided the lowest of relative span. When combined with SEM images of both emulsions, it indicated that the polydispersity decreased with % DP.

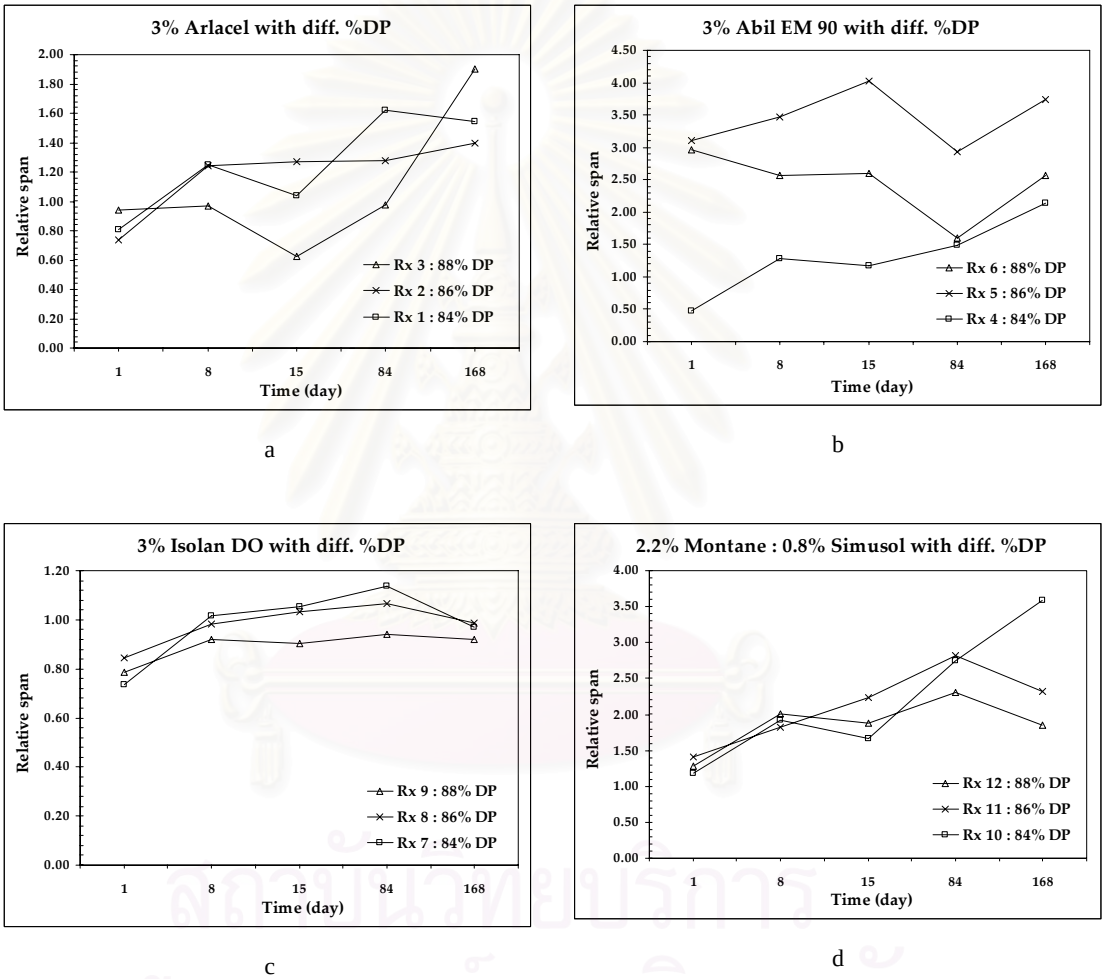


Figure 73 Average relative span (Avg. R.S. ± S.D., n=3), changing after 1, 8, 15 days, 3 and 6 months in CEs with 3% of sorbitan (a), silicone (b), glucoester (c) and mixed (d) emulsifiers at 84, 85 and 88% of dispersed phase.

For mixed emulsions (d), all ranges of % DP under studied were similar. This supported all the previous results that the increase of volume ratio of dispersed phase, did not affect significantly to the stability or other characteristics of CEs with the mixed emulsifier.

The silicone (b) emulsions provided some differences in the value of the polydispersity. From figure 73 (b), it indicated that the increase of the water amount in formulations caused to promote the heterogeneity of the silicone emulsions. This observation may be related to their achievement during fabrication of CEs, which could not perform at the high % DP.

8.4.2.3 Influence of the percentage of emulsifier

From figure 74, the polydispersity of the glucoester emulsions with four ranges of % emulsifier, were ranking from 3%, 4%, 2% and 1% emulsifier, respectively. This indicated the products of the glucoester with good homogeneity were fitted with 3% emulsifier concentration.

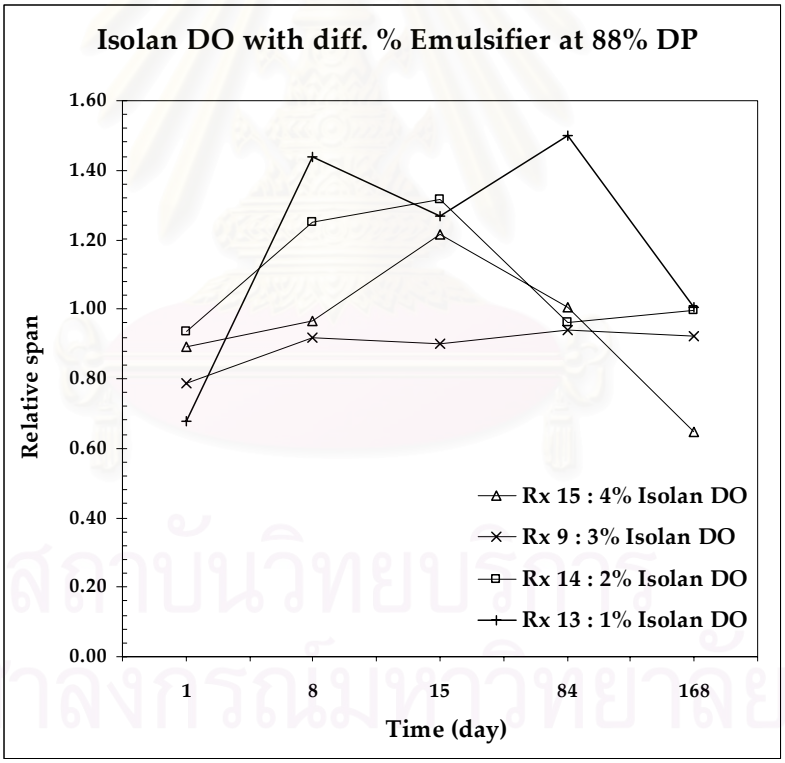


Figure 74 Average relative span (Avg. R.S. ± S.D., n=3), changing after 1, 8, 15 days, 3 and 6 months in glucoester emulsions with 1, 2, 3 and 4% emulsifier.

9. *In vitro* diffusion study

In vitro diffusion study was used as a tool for determining the effect of formulation on the release of EGCG from different formulations of CEs (table 8) with different emulsifier types, % DP and percentages of emulsifier used. The study was performed by using modified Franz diffusional cells.

With a strong centrifugal force, the emulsions obtained were separated after their formation. The aqueous phase separated from all CEs was taken the sampling of supernatant for determining the amount of EGCG, incorporated in formulation.

At the starting of the release experiment, the amount of EGCG in the donor compartment of each formulation, therefore, was determined to correct the flux values obtained from all formulations, as depicted in table 48 for all formulations.

Table 48 Actual amount of EGCG in applied dose (Q_0, µg) of all formulations.

Rx				Actual amount of EGCG in applied dose (Q_0, µg)
Emulsifier type	% Emulsifier	% Dispersed phase	Rx ID no.	
Sorbitan type (Arlacel 83™)	3%	84%	*1	2,986.60
	3%	86%	2	1,913.28
	3%	88%	3	2,848.91
Silicone type (Abil EM 90™)	3%	84%	4	1,992.05
	3%	86%	5	1,939.38
	3%	88%	6	4,415.21
Glucoester type (Isolan DO™)	3%	84%	*7	3,899.66
	3%	86%	*8	2,858.06
	3%	88%	9	4,253.92
Mixed type (Montane 481™-Simulsol 989™)	3%	84%	*10	4,595.94
	3%	86%	*11	6,468.29
	3%	88%	12	4,785.60
Glucoester type (Isolan DO™)	1%	88%	13	5,543.88
	2%	88%	14	4,769.08
	4%	88%	15	5,267.75

*; n ~ ½

The Peppas equation (Peppas, 1985) represents a general data fitting approach for drug release.

Peppas equation:

$$Q_t = kt^n \qquad \text{Eq.29}$$

Where,

Q_t is a cumulative amount of drug released per unit surface area at any time t.

k is a release rate constant incorporating structural and geometric characteristics of the controlled release formulations.

n is a release exponent, that may be used to indicate the mechanism of drug release.

This is a general semi-empirical equation, which is not based on a certain model, certain geometry or a single mechanism. It is usually used to analyze release data from the system, of which the mechanism of release is not well known or when more than one type of release may be involved (Orienti and Zecchi, 1993; Franz *et al*., 1987). Starting from the assumptions that there was no influence of the synthetic membrane on the drug release, it was expected a linear diffusion of drug molecules from the donor to the receiver compartment.

If n = 0.5, it indicates that the cumulative amount of drug released per unit surface area, Q_t, of the system is proportional to the square root of time, $t^{½}$. The release of drug in this system, therefore, assumes to be controlled by the diffusion of the drug through the system or be described by first-order kinetics.

For non-linear transformation, release data from all formulations of CEs were fitted to the Peppas equation. Each data point was a mean of three replicates. It is of interest to note that in spite of increasing number of the studies investigating drug release from emulsions, there were only a few attempts to establish an appropriate mathematical model describing drug release kinetics. After non-linear transformation using Microsoft® Office Excel 2003 program, good correlation coefficients for all formulations were obtained with $R^2 \geq 0.95$ as shown in figure 75-77 (a-o) for Rx 1-15.

9.1 Cumulative amount released (Q_t/Q_0, %·cm^{-2}) vs time (n = 3)

During the release experiment, a plot of amount released of EGCG in receiver per unit surface area available for diffusion membrane (2.271 cm^2) per one hundredth unit initial amount of drug loading, as a function of estimated time is presented in figure 75-77 (a-o) for Rx 1-15.

Estimated values of the parameter, k and n, obtained by non-linear transformation are shown in table 49 for all formulations.

The values of flux (k, %·cm^{-2}·h^{-1}) of EGCG through the polysulfone membrane from the investigated emulsions are all shown in table 49.

The cumulative amount released of EGCG at 48 h (Q_{48}/Q_0, %·cm^{-2}) from the investigated emulsions are also presented in table 49.

With n value ~ 0.5 (as shown in table 49), the linear release profile of EGCG was observed for the glucoester emulsions with 84% and 86% DP (Rx 7 and 8) and the mixed emulsions with 84% and 86% DP (Rx 10 and 11). Interestingly, drug release profile for the sorbitan one with 84% DP (Rx 1) was also linear. The mixed emulsions with 88% DP were the only one that obtained the fastest flux release with n > 0.5.

The rest of other formulations were non-linear with n < 0.5. With n value < 0.5, the non-linear trend could be attributable to the more complex distribution of EGCG between water, oil and amphiphile phases including stronger drug–vehicle interactions. Most likely, the release of EGCG from formulation Rx 2, 3, 4, 5, 6, 9, 13, 14 and 15, in which the drug was predominantly located in the water droplets, was hindered by the rigidity of interfacial film, surrounding to water droplets.

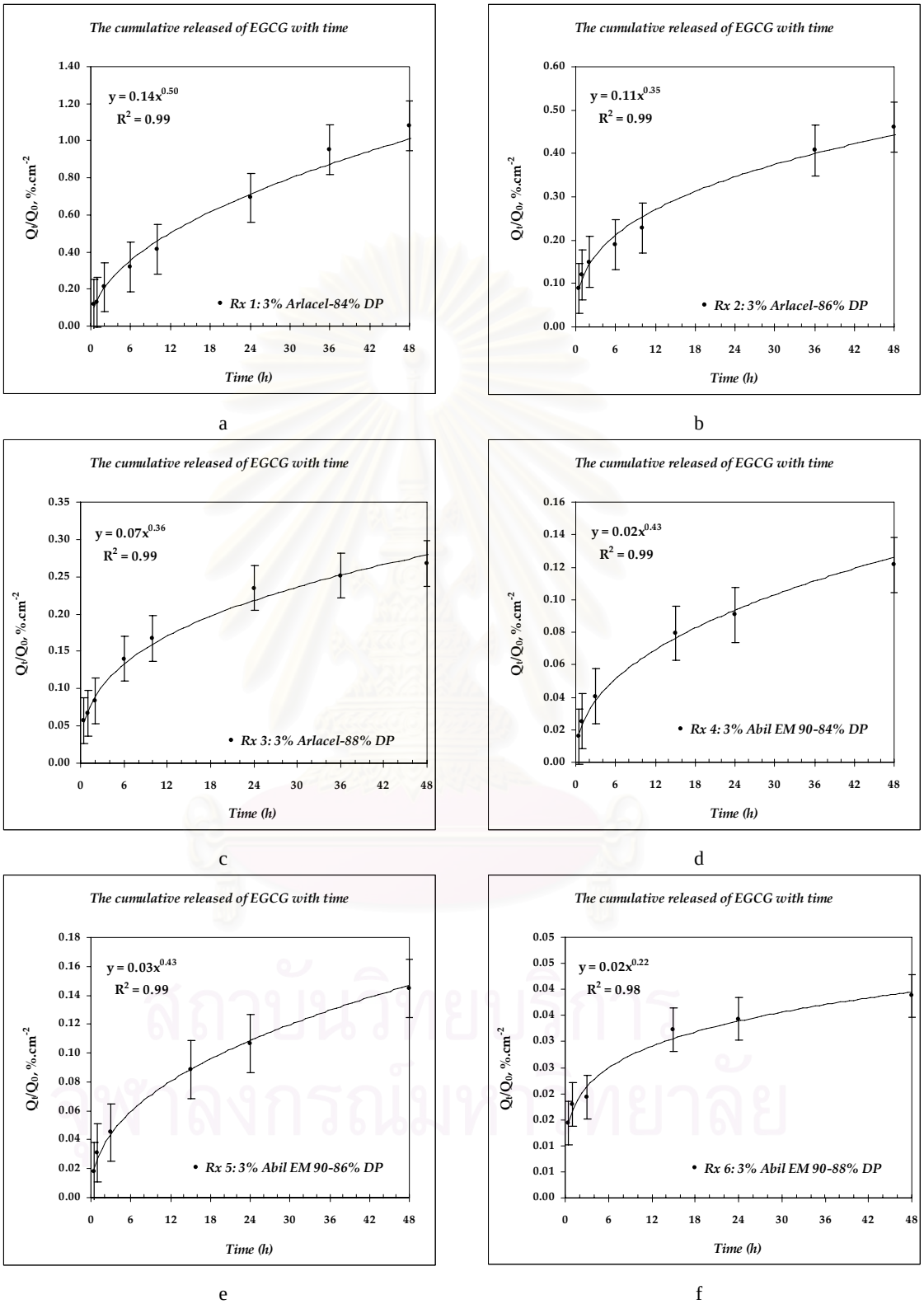
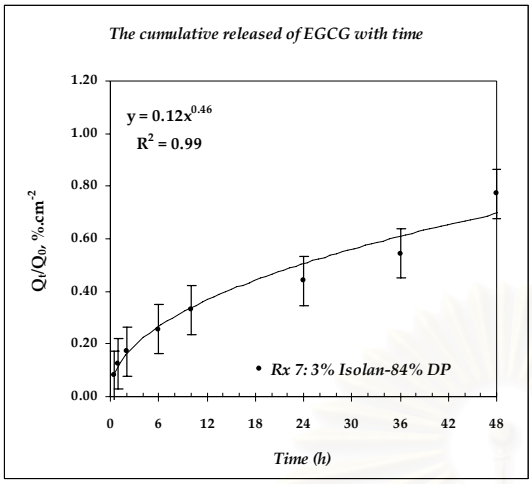


Figure 75 Cumulative amount released of EGCG per unit surface area per % unit initial amount of drug loading (%·cm^{-2}, n = 3) of different formulations of CEs, as a function of time, for Rx 1 (a), Rx 2 (b), Rx 3 (c), Rx 4 (d), Rx 5 (e) and Rx 6 (f).



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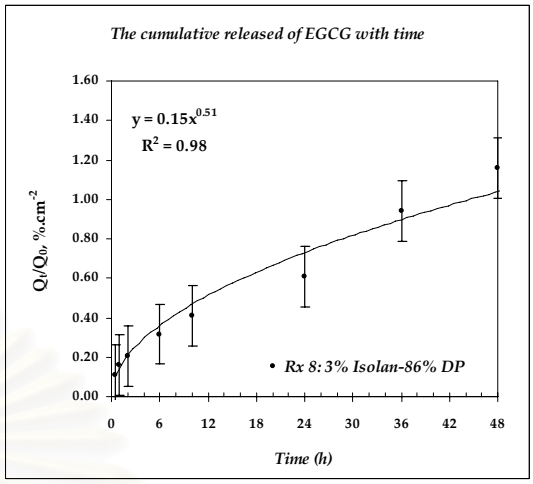

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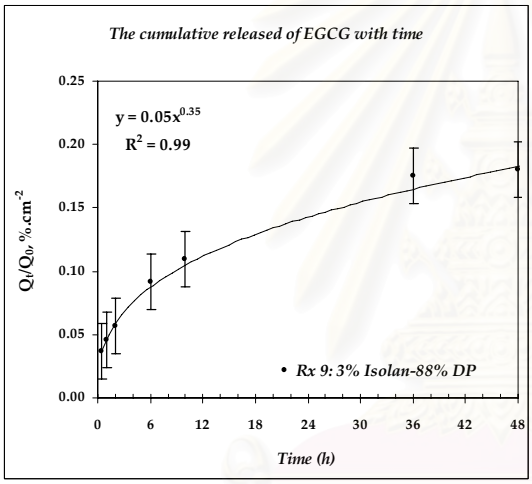

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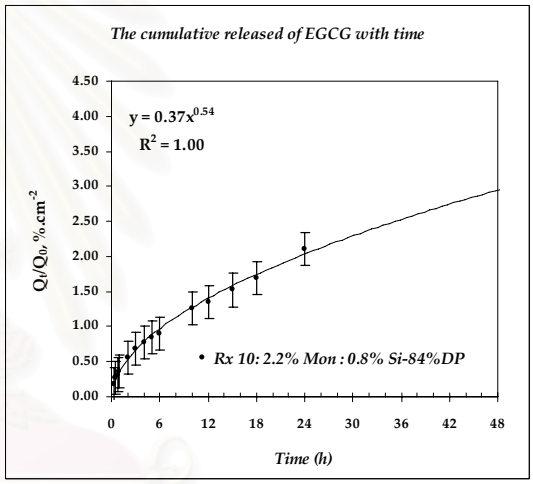

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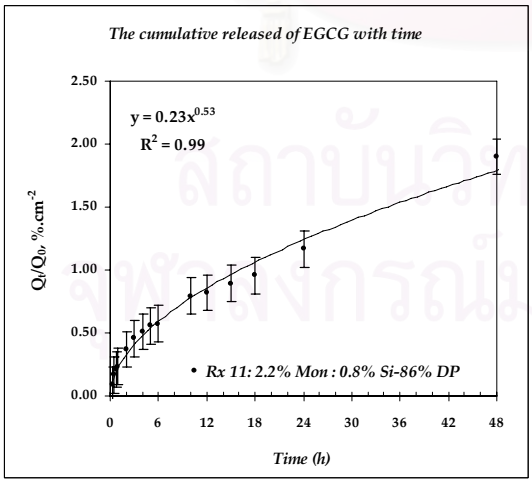

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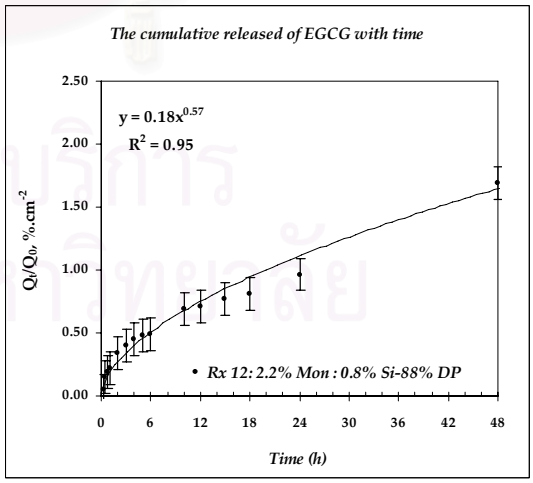

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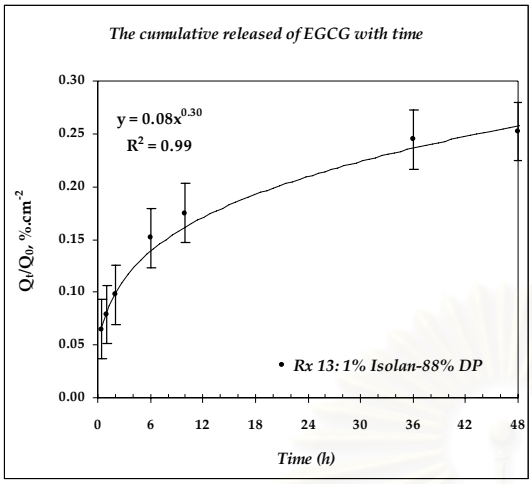
Figure 76 Cumulative amount released of EGCG per unit surface area per % unit initial amount of drug loading (%·cm^{-2}, n = 3) of different formulations of CEs, as a function of time, for Rx 7 (g), Rx 8 (h), Rx 9 (i), Rx 10 (j), Rx 11 (k) and Rx 12 (l).



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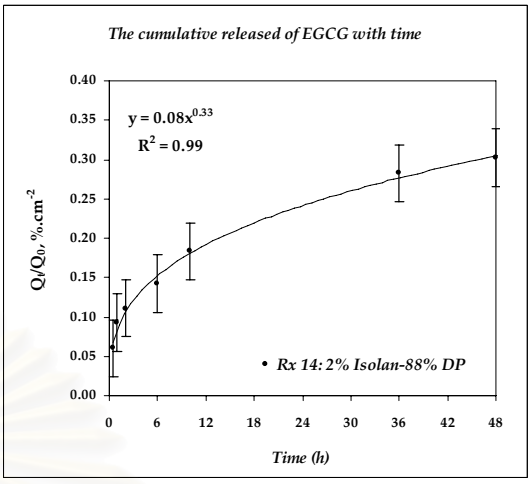

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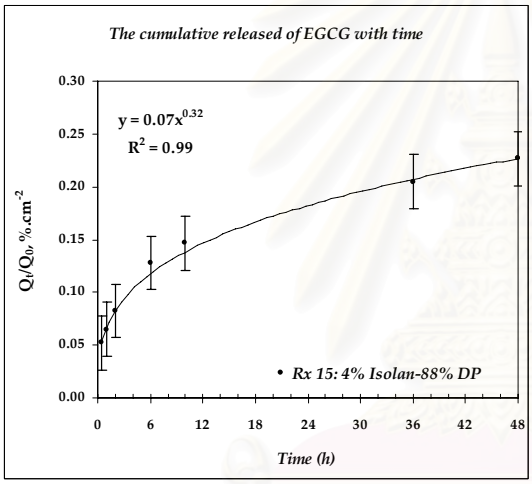


o

Figure 77 Cumulative amount released of EGCG per unit surface area per % unit initial amount of drug loading (%·cm^{-2}, n = 3) of different formulations of CEs, as a function of time, for Rx 13 (m), Rx 14 (n) and Rx 15 (o).

All types of emulsions with 88% DP, excepted mixed type, provided the slow release characteristics with non-linear profiles ($n < 0.5$).

Combined with the SEM images, the water droplet structure of these formulations were all polyhedra. Accordingly, it might be assumed that the CEs, which possessed the polyhedral structure, would allow the slow release of hydrophilic drug attributed to the self-diffusion of water molecules between adjacent droplets and stronger EGCG-water vehicle interactions.

It might indicate that the stable CEs with very high amount of water internal phase could act as the controlled-release system.

Table 49 Estimated values of flux (k, %·cm^{-2}·h^{-1}), amount released of EGCG at 48 h (Q_{48}/Q_0, %·cm^{-2}), R^2 and n for all formulations.

Rx				The estimated parameters			
Emulsifier type	% Emulsifier	% Dispersed phase	Rx ID no.	Flux (k) (%·cm^{-2}·h^{-1})	Q_{48}/Q_0 (%·cm^{-2})	n	R^2
Sorbitan type (Arlacel 83™)	3%	84%	*1	0.14	1.08	*0.50	0.99
	3%	86%	2	0.11	0.46	0.35	0.99
	3%	88%	3	0.07	0.27	0.36	0.99
Silicone type (Abil EM 90™)	3%	84%	4	0.02	0.12	0.43	0.99
	3%	86%	5	0.03	0.14	0.43	0.99
	3%	88%	6	0.02	0.04	0.22	0.98
Glucoester type (Isolan DO™)	3%	84%	*7	0.12	0.77	*0.46	0.99
	3%	86%	*8	0.15	1.16	*0.51	0.98
	3%	88%	9	0.05	0.18	0.35	0.99
Mixed type (Montane 481™-Simulsol 989™)	3%	84%	*10	0.37	3.59	*0.54	1.00
	3%	86%	*11	0.23	1.90	*0.53	0.99
	3%	88%	12	0.18	1.69	0.57	0.95
Glucoester type (Isolan DO™)	1%	88%	13	0.08	0.25	0.30	0.99
	2%	88%	14	0.08	0.30	0.33	0.99
	4%	88%	15	0.07	0.23	0.32	0.99

*; n ~ ½

9.2 Influence of the emulsifier type on release characteristics

From the n values as shown in table 56, the sorbitan (0.36), glucoester (0.35) and silicone (0.22) emulsions all performed the non-linear release characteristics of EGCG, while the silicone one was the only one that obtained the rapid release of EGCG with the n value of 0.57. Combined with the other characteristics of CEs as previously discussed, this could confirm the instability of the mixed emulsions, which caused the burst release of EGCG.

From the flux values of EGCG as shown in table 56, the order is the mixed (0.18) > the sorbitan (0.07) > the glucoester (0.05) > the silicone (0.02) emulsions with 88% DP. As well as, the Q_{48}/Q_0 values of EGCG as shown in table 56, the order was the same as the ranking of the flux values. This support to the general rule that the higher rate of release performed, the greater amount released of drug during the release experiments obtained.

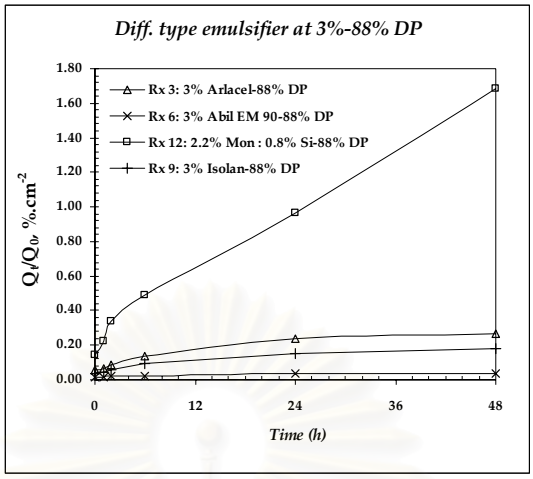


Figure 78 Effect of emulsifier type of CEs on the cumulative amount released of EGCG per unit surface area per one hundredth unit initial amount of drug loading ($\%\cdot cm^{-2}$, n = 3), as a function of time, for the sorbitan (Rx 3), the silicone (Rx 6), the mixed (Rx 12) and the glucoester (Rx 9) emulsions with 88% DP.

For mixed type, the flux values (k) and Q_{48}/Q_0 of EGCG in all range of % DP showed high extent than the other type emulsifiers. As the results, it indicated that the superiority of release characteristics of mixed type formulation might be due to the instability of these emulsions. The droplets in this system were broken rapidly with time investigated, causing of the burst release of all formulations employed with mixed emulsifier. Although the apparent viscosities of the mixed type formulations were considerably higher than the sorbitan and glucoester types, the release profiles of the mixed type were not correlated to their viscosities obtained. This should be established that the release characteristics of CEs were not only depended upon their viscosities.

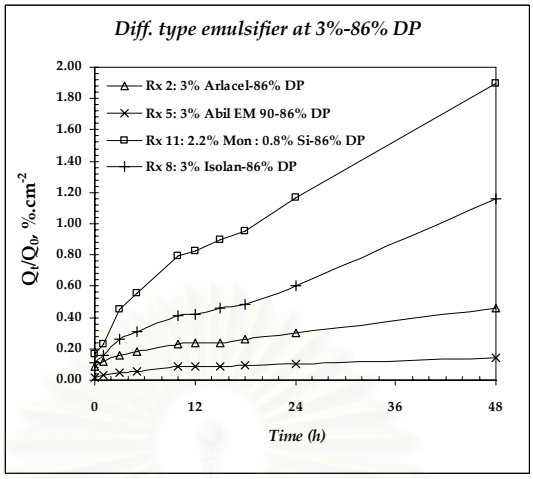


Figure 79 Effect of emulsifier type of CEs on the cumulative amount released of EGCG per unit surface area per one hundredth unit initial amount of drug loading (%·cm^{-2}, n = 3), as a function of time, for the sorbitan (Rx 2), the silicone (Rx 5), the mixed (Rx 11) and the glucoester (Rx 8) emulsions with 86% DP.

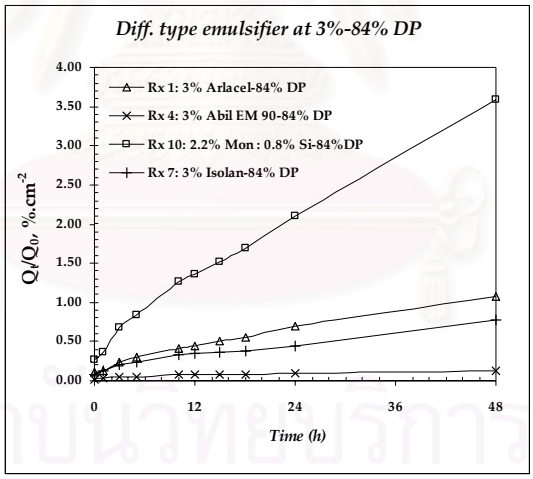


Figure 80 Effect of emulsifier type of CEs on the cumulative amount released of EGCG per unit surface area per one hundredth unit initial amount of drug loading (%·cm^{-2}, n = 3), as a function of time, for the sorbitan (Rx 1), the silicone (Rx 4), the mixed (Rx 10) and the glucoester (Rx 7) emulsions with 84% DP.

Especially for the case of very unstable CEs, it looks as if the release characteristics were mainly affected by the rate of droplets rupture due to their instability. This could confirm the results of the silicone emulsions, which showed the superiority of their apparent viscosities and long-term stability over 6 months. The

very stable silicone emulsions, therefore, provided the lowest of release characteristics, which mainly depended upon their viscosities.

9.3 Influence of the percentage dispersed phase on release characteristics

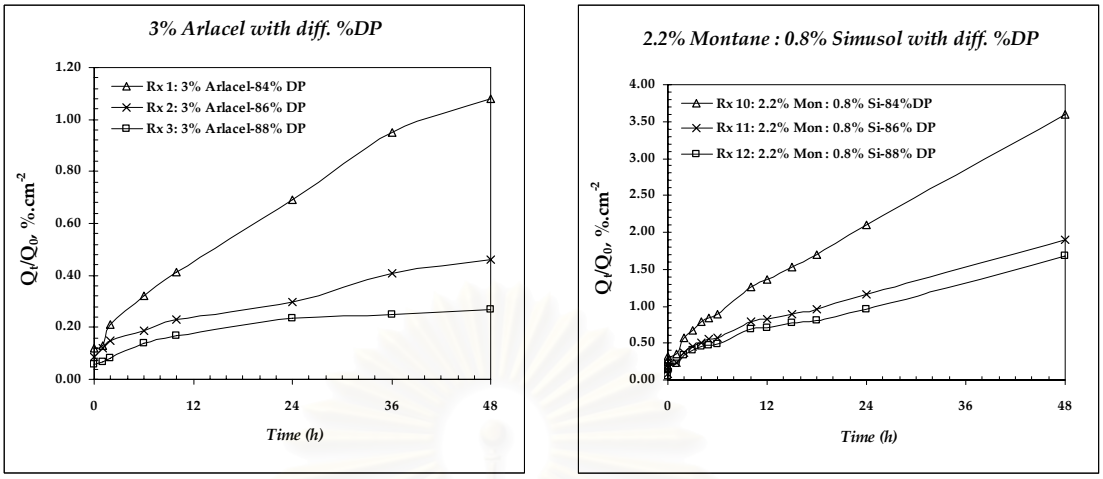
A difference in release profiles is observed with the different emulsifiers used. The release characteristics observed with different type of emulsifier showed the tendency of the different results in two behaviors. The sorbitan and mixed types showed the same tendency, and the silicone and glucoester emulsions were the same. It was not only indicated that each emulsifier type behaved in different ways, but also suggest to the similarity between the emulsifier types under studies.

The flux values, compared between 84% (Rx 1), 86% (Rx 2) and 88% (Rx 3) DP of the sorbitan emulsions decreased proportionally to the content of water phase, and the minimum of EGCG flux was obtained from CEs containing the 88% DP.

For the mixed type, the flux values, compared between 84% (Rx 10), 86% (Rx 11) and 88% (Rx 12) DP decreased proportionally to the content of water phase, and the minimum of EGCG flux was obtained from CEs containing the 88% DP. This should confirm faster release observed in these systems.

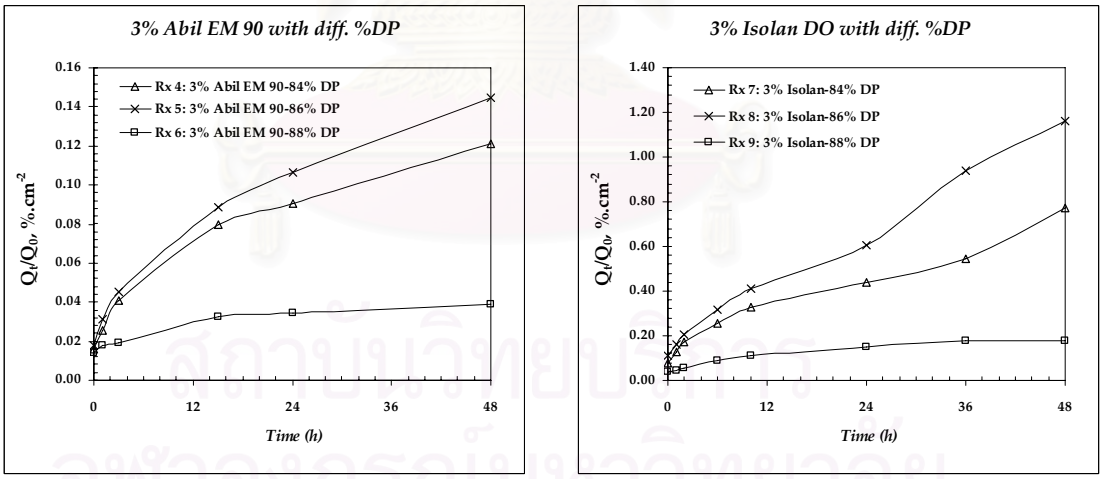
In addition, the obtained results indicate strong correlation between the % DP and the flux of EGCG in these systems. As the stability of CEs is increased with the % DP. Therefore the higher % DP employed, the lesser release of drug obtained. These observations should be the characteristics of these systems.

The flux values of the silicone and glucoester emulsions increased proportionally to the content of water phase when compared between 84% (Rx 4 and 7, respectively) and 86% (Rx 5 and 8, respectively) DP, while the minimum of EGCG flux was obtained from the silicone and glucoester emulsions containing the 88% DP (Rx 6 and 9, respectively).



a b

Figure 81 Effect of % DP on the cumulative amount released of EGCG per unit surface area per one hundredth unit initial amount of drug loading (%·cm^{-2}, n = 3), as a function of time, for the sorbitan emulsions (a) with 84% DP (Rx 1), 86% DP (Rx 2) and 88% DP (Rx 3) and for the mixed emulsions (b) with 84% DP (Rx 10), 86% DP (Rx 11) and 88% DP (Rx 12).



a b

Figure 82 Effect of % DP on the cumulative amount released of EGCG per unit surface area per one hundredth unit initial amount of drug loading (%·cm^{-2}, n = 3), as a function of time, for the silicone emulsions (a) with 84% DP (Rx 4), 86% DP (Rx 5) and 88% DP (Rx 6) and for the glucoester emulsions (b) with 84% DP (Rx 7), 86% DP (Rx 8) and 88% DP (Rx 9).

Although, the obtained results did not indicate strong correlation between the % DP and the flux of EGCG in these systems. It still allowed the retaining of drug released for the CEs with 88% DP. This might due to the very high extent of viscosity obtained from these emulsions at 88% DP.

9.4 Influence of the percentage of emulsifier on release characteristics

There was no difference between the release profiles of the glucoester emulsions with 1%, 2% and 4% emulsifier concentration. A tendency of faster release for 1%, 2% and 4% glucoester was suggested by the results obtained as shown in figure 83.

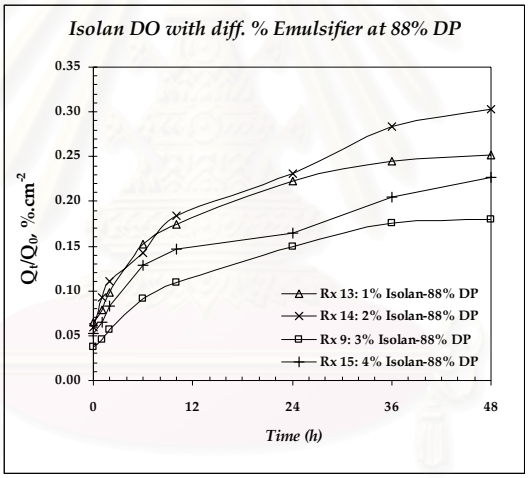


Figure 83 Effect of % emulsifier on the cumulative amount released of EGCG per unit surface area per one hundredth unit initial amount of drug loading (%·cm^{-2}, n = 3), as a function of time, for the glucoester emulsions with 1% (Rx 13), 2% (Rx 14), 3% (Rx 9) and 4% (Rx 15) of emulsifier, fixed at 88% DP.

As the most retaining of EGCG released from the CEs with 3% glucoester, this might due to the good stability of the 3% glucoester emulsions during the release experiments.

CHAPTER V

CONCLUSION

Major constituents found in green tea, known as GTCs and caffeine were extracted from ground powder of green tea leaves (*Camellia sinensis* plant). The extraction of GTCs and caffeine was performed by using 80±1 °C DI water with and without solvent extraction using DCM (procedure A and B, respectively), and then dried by a freeze-drying technique. FD-GTE obtained from the both extraction procedures were determined their GTCs and caffeine contents by using RP-HPLC analysis. The analysis of major constituents in FD-GTE samples included of four principal compounds of catechins, i.e. EGC, EGCG, EC, ECG and caffeine. A total yield of 34.1267% (w/w) of dried tea leaves with total GTCs ~ 8.8883% and caffeine ~ 2.0451% (w/w), based on dried tea leaves could be obtained from procedure A. Whereas, procedure B provided the total yield of extract at approximately 34.3067% (w/w) of dried tea leaves with total GTCs ~ 9.9119% and caffeine ~ 0.1646% (w/w), based on dried tea leaves. The total yields of FD-GTE obtained from both extraction procedures are not significantly different. Without significant effect on the total yields of FD-GTE, the DCM could be effective solvent for removing caffeine from the hot water extraction of green tea leaves.

The CEs with DP of 84, 86 and 88% w/w were prepared with 3% of four different emulsifiers (sorbitan, silicone, mixed and glucoester). Approximately 1, 2, 3 and 4% of emulsifier concentration were also conducted with one emulsifier system (glucoester) at a fixed percentage of DP (88% DP).

FD-GTE composed of GTCs together with caffeine from the previous study was incorporated to all formulations and only EGCG constituent was determined as a model drug substance. Fifteen formulations of CEs containing approximately 2.25% (w/w) of FD-GTE were manufactured to study the effect of formulation parameters on the *in vitro* release of EGCG on synthetic polysulfone membrane.

It is well known that formulation parameters can play a role in the release of an active from emulsions (Kundu *et al*., 1993). The characterization will allow making correlation between the formulation parameters of CEs and the diffusion

profiles of EGCG. Stability, viscosity, droplet diameter and shape can be important parameters controlling the release of EGCG from CEs.

The stability of CEs is influenced by various factors, e.g. emulsifier type, water content and concentration of emulsifier employed (Kunieda *et al.*, 1987; Chen and Ruckenstein, 1991; Pons *et al.*, 1992). For this study, differences in the stability, as well as in viscosity, droplet diameter and shape were observed for the emulsions produced with the four emulsifier types.

The silicone emulsifier, which is a silicone polymer, produces emulsions with a very strong interfacial film due to steric crowding and obtains more stable products. In contrast, the production of the mixed system obtained the less stable products, probably due to the presence of stearic acid and beeswax in the emulsifier commercial mixture. The two other emulsifiers used (sorbitan and glucoester) are small molecules, which allow the production of elastic interfacial films. The cosmetic qualities of these products are superior to the two previous ones but due to a lack of rigidity of their interfaces, these systems are less stable.

The emulsifier type can have an influence on the droplet diameter and the apparent viscosity. However, the difference in the droplet diameter among the different surfactants is not as marked as the difference in the viscosity. The apparent viscosity can vary as much as by a factor of 5. For example, the glucoester emulsions at 88% DP have an apparent viscosity of 7 Pa.s, compared with the silicone emulsions at 88% DP with an apparent viscosity of 35 Pa.s.

Differences in the release profile from these CEs are expected since the surfactants used do not have the same structure.

Emulsion stability or interfacial film properties could play an important role in the release process. They could explain the differences observed in the release profiles of EGCG between the stable and unstable CEs. There are essentially two mechanisms, whereby the DP may cross the film from one droplet to a neighboring one.

The first is through the rupture of the film and the subsequent coalescence of the droplets. Since the sorbitan, silicone and glucoester emulsions are stable for several months. This is, therefore, not a mechanism that is effective in these systems as the film rupture would have to occur during the *in vitro* release studies (over 48 h). However, this suggestion could be support to the mixed emulsions that allow less

stable products over weeks. Among the four emulsifiers (fixed at 88% DP), only the mixed emulsions gave a considerable higher release of EGCG after 48 h. The waxes, presenting in the surfactant mixture of the mixed emulsifier render the oil film very rigid and probably hinder a deformation of the film. However, this hypothesis needs to be verified.

The second mechanism involves a molecular transport of water across the film and/or a transport of water mediated by the reversed micelles present in the continuous phase, and this is clearly the mechanism for the sorbitan, silicone and glucoester emulsions. Although, the 88% DP emulsions have a thin interfacial film due to the shape of the droplets (higher surface area), which, therefore, could be a smaller barrier for the passage of EGCG in comparison with the 84 and 86% DP emulsions. With 88% DP, all CEs, excepted for only the mixed one obtain the very slow release profiles. This might due to the equilibrium of water flux across the oil film may retard the diffusion of drug molecule, EGCG in this case, and the release profiles obtained from these systems, therefore, tend to be non-linear with n values < 0.5 (n ~ 0.3).

However, other parameters varied between these emulsions like the amount of water (which can affect the amount of solubilized EGCG) and the shape of the droplets.

It can be noticed that they have a structure composed of polyhedral water domains surrounded by a thin layer of oil and surfactant. This is expected since the volume fraction in these cases is superior to 74%.

For the sorbitan and glucoester emulsions, the less concentrated (84 and 86% DP) emulsions have a structure composed of spherical droplets adjacent to each other. As the % DP is increased, reaching to 88% DP, the droplet structure deforms from a spherical to a polyhedral geometry, in case of the sorbitan and glucoester. In contrast, the silicone and mixed emulsions do not obtain the polyhedral structure for whatever % DP under studies.

The structure of the droplet probably plays a role in the diffusion of EGCG inside the CEs. Polyhedral CEs slow the release of EGCG when compared with spherical CEs. It is interesting to note that all CEs that possess the polyhedral droplets

obtain the retaining release profiles with n values of about 0.3, whereas the spherical CEs provide the rapid release characteristics with n values of around 0.5.

In addition, the influence of the viscosity and the droplet diameter on the release rate has been found to be negligible for a fixed % DP. The sorbitan, silicone and glucoester gave the lesser release rate for a fixed at 88% DP. These results are surprising since the CEs made with these four emulsifier systems are very different when one looks at their apparent viscosity, and to a lesser extent, their droplet diameter. A considerable difference in the flux was expected, at least between the emulsions, which have very different apparent viscosities.

However, it was demonstrated that differences in viscosity and droplet diameter of the glucoester emulsions (fixed with the 88% DP) with 1–4% of emulsifier do not change the flux of EGCG from these CEs. This indicated that the droplet diameter and the viscosity of emulsions have no direct influence on the release kinetic of an active.

In contrast, diffusion of EGCG from CEs has been found to be highly dependent on the stability of CEs due to rigidity of interfacial films of water droplets. The stability of CEs increases with the % DP. In contrast, the flux of EGCG decreases with the % DP. The droplet diameter decreases and the apparent viscosity increases with the % DP. With 88% DP, the droplet shape of all types, excepted for the mixed type provided the polyhedral structure of water droplets. The shape of the droplets goes from spherical to polyhedral as the % DP is increased. The polyhedral structure obtained at 88% DP might play major role on their extreme viscosities and stability. However, the flux could be correlated either with the apparent viscosity or with the rigidity of interfacial films at a fixed % DP.

The data obtained from different % emulsifier (1%, 2%, 3% and 4%) showed only negligible differences in the fluxes.

As confirmed by the SEM images of the CEs with different % emulsifier under studies, the release characteristics obtained from these systems correlated with their spherical structures observed. All of them show linear release profiles with n values about 0.5. This indicated that the release characteristic of CEs mostly depend on the shape of water droplets in the systems.

The CEs can be of interesting vehicle for the controlled delivery of EGCG, hydrophilic compounds. The flux of EGCG from these emulsions is mostly influenced by the shape but not directly by viscosity, droplet diameter, surfactant type or its concentration. The structure of the droplets, which change from sphere to polyhedra as the % DP is increased, seem to play an important role in the release process, the polyhedral shape decreasing the flux of EGCG. The non-linear profiles obtained with all the stable CEs under study, excepted for the mixed emulsions might be interested for further study.

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APPENDICES

APPENDIX I

STATISTICAL ANALYSIS

1. Statistical analysis of percentages of yield of freeze-dried green tea extract from two different extraction procedures

In study I, statistical analysis was done by student's t-test independence analysis (SPSS 14.0 for Windows). Values of $P < 0.05$ were considered statistically significant.

The independent-samples t test procedure compares means for two groups of cases. The mean values and other descriptive statistics for the two groups are displayed in the group statistics table in table I-1.

This table displays the number of cases, mean value, standard deviation, and standard error for the test variable(s) within categories defined by the grouping variable.

Since the independent samples t test procedure compares the two group means, it is useful to know what the mean values are. Means of the percentage of the total yield of FD-GTE, obtained from procedure A and B were 34.1267 and 34.3067, respectively. From the table I-2, the mean difference value indicated that means of procedure A was less than procedure B for approximately 0.1800. Standard deviations of the percentage of the total yield of FD-GTE, obtained from procedure A and B were 0.1845 and 0.7447, respectively. Standard errors of the mean of the percentage of the total yield of FD-GTE, obtained from procedure A and B were 0.1065 and 0.4299, respectively.

From the results of Levene's test for equality of variances in table I-2, the significance value was greater than 0.05 (sig. > 0.05). Accordingly, the results that assumed equal variances for both groups were utilized. It showed that there was not significantly difference between groups at significant level of 0.05.

A high significance value for the t test (greater than 0.05) indicates that there was not a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference contained zero, this, therefore, indicated that the difference was not significant. Accordingly, the percentage of the total yield of FD-GTE, obtained from the procedure A was not significantly different from the one obtained from the procedure B (sig. > 0.05).

Table I-1 Descriptive statistics of percentages of total yield of FD-GTE, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Yield of FD-GTE	Procedure A	3	34.1267	.1845	.1065
	Procedure B	3	34.3067	.7447	.4299

Table I-2 Statistical analysis of percentages of total yield of FD-GTE, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Yield of FD-GTE	**Equal variances assumed**	6.64	**.0615**	-.4064	4	**.7053**	-.1800	.4429	-1.4098	1.0498
	Equal variances not assumed			-.4064	2.245	.7200	-.1800	.4429	-1.9000	1.5400

Table I-3 Summarized results of statistical analysis of percentages of total yield of FD-GTE, obtained between extraction procedure A and B.

% Yield of FD-GTE	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	34.1267	0.1845	-0.4064*	4	**0.7053**
Extraction procedure B	2	34.3067	0.7447			

*, statistical significant level at 0.05.

2. Statistical analysis of percentages of individual catechins, total catechins and caffeine in freeze-dried green tea extract, obtained from two different extraction procedures

In study II, statistical analysis was done by student's t-test independence analysis (SPSS 14.0 for Windows). Values of $P < 0.05$ were considered statistically significant.

The independent-samples t test procedure compares means for two groups of cases. The mean values and other descriptive statistics for the two groups are displayed in the group statistics table in table I-4, I-7, I-10, I-13 and I-16 for individual EGC, EGCG, EC, ECG and caffeine, respectively. In addition, the group statistics table in table I-19 shows the mean values and other descriptive statistics for the two groups of total GTCs, including EGC, EGCG, EC and ECG.

These tables display the number of cases, mean value, standard deviation, and standard error for the test variable(s) within categories defined by the grouping variable.

2.1 Statistical analysis of percentages of EGC in freeze-dried green tea extract, obtained from two different extraction procedures

Since the independent samples t test procedure compares the two group means, it is useful to know what the mean values are. Means of the percentage of EGC content, obtained from procedure A and B were 6.8340 and 7.1740, respectively. From the table I-5, the mean difference value indicated that means of procedure A was less than procedure B for approximately 0.3400. Standard deviations of the percentage of EGC content, obtained from procedure A and B were 0.0181 and 0.0386, respectively. Standard errors of the mean of the percentage of EGC content, obtained from procedure A and B were 0.0128 and 0.0273, respectively.

From the results of Levene's test for equality of variances in table I-5, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of EGC content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-4 Descriptive statistics of percentages of EGC content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of EGC in FD-GTE	Procedure A	2	6.8340	.0181	.0128
	Procedure B	2	7.1740	.0386	.0273

Table I-5 Statistical analysis of percentages of EGC content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of EGC in FD-GTE	Equal variances assumed	2821676882481 9520.00	**.0000**	-11.2763	2	.0078	-.3400	.0302	-.4697	-.2103
	Equal variances not assumed			-11.2763	1.4194	**.0232**	-.3400	.0302	-.5366	-.1434

Table I-6 Summarized results of statistical analysis of percentages of EGC content, obtained between extraction procedure A and B.

% Content of EGC in FD-GTE	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	6.8340	0.0181	-11.2763*	1.4194	**0.0232**
Extraction procedure B	2	7.1740	0.0386			

*, statistical significant level at 0.05.

2.2 Statistical analysis of percentages of EGCG in freeze-dried green tea extract, obtained from two different extraction procedures

Means of the percentage of EGCG content, obtained from procedure A and B were 11.6425 and 13.2580, respectively. From the table I-8, the mean difference value indicated that means of procedure A was less than procedure B for approximately 1.6156. Standard deviations of the percentage of EGCG content, obtained from procedure A and B were 0.0274 and 0.0488, respectively. Standard errors of the mean of the percentage of EGCG content, obtained from procedure A and B were 0.0194 and 0.0345, respectively.

From the results of Levene's test for equality of variances in table I-8, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of EGCG content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-7 Descriptive statistics of percentages of EGCG content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of EGCG in FD-GTE	Procedure A	2	11.6425	.0274	.0194
	Procedure B	2	13.2580	.0488	.0345

Table I-8 Statistical analysis of percentages of EGCG content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of EGCG in FD-GTE	Equal variances assumed	4140933 5666269 58.00	**.0000**	-40.8422	2	.0006	-1.6156	.0396	-1.7857	-1.4454
	Equal variances not assumed			-40.8422	1.5725	**.0023**	-1.6156	.0396	-1.8388	-1.3923

Table I-9 Summarized results of statistical analysis of percentages of EGCG content, obtained between extraction procedure A and B.

% Content of EGCG in FD-GTE	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	11.6425	0.0274	-40.842*	1.5725	**0.0023**
Extraction procedure B	2	13.2580	0.0488			

*, statistical significant level at 0.05.

2.3 Statistical analysis of percentages of EC in freeze-dried green tea extract, obtained from two different extraction procedures

Means of the percentage of EC content, obtained from procedure A and B were 1.9834 and 2.1460, respectively. From the table I-11, the mean difference value indicated that means of procedure A was less than procedure B for approximately 0.1626. Standard deviations of the percentage of EC content, obtained from procedure A and B were 0.0083 and 0.0050, respectively. Standard errors of the mean of the percentage of EC content, obtained from procedure A and B were 0.0059 and 0.0036, respectively.

From the results of Levene's test for equality of variances in table I-11, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of EC content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-10 Descriptive statistics of percentages of EC content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of EC in FD-GTE	Procedure A	2	1.9834	.0083	.0059
	Procedure B	2	2.1460	.0050	.0036

Table I-11 Statistical analysis of percentages of EC content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of EC in FD-GTE	Equal variances assumed	2973136 9529337 97.00	**.0000**	-23.7619	2	.0018	-.1626	.0068	-.1920	-.1332
	Equal variances not assumed			-23.7619	1.6486	**.0044**	-.1626	.0068	-.1990	-.1262

Table I-12 Summarized results of statistical analysis of percentages of EC content, obtained between extraction procedure A and B.

% Content of EC in FD-GTE	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	1.9834	0.0083	-23.7619*	1.6486	**0.0044**
Extraction procedure B	2	2.1460	0.0050			

*, statistical significant level at 0.05.

2.4 Statistical analysis of percentages of ECG in freeze-dried green tea extract, obtained from two different extraction procedures

Means of the percentage of ECG content, obtained from procedure A and B were 3.1041 and 3.5603, respectively. From the table I-14, the mean difference value indicated that means of procedure A was less than procedure B for approximately 0.4562. Standard deviations of the percentage of ECG content, obtained from procedure A and B were 0.0092 and 0.0666, respectively. Standard errors of the mean of the percentage of ECG content, obtained from procedure A and B were 0.0065 and 0.0471, respectively.

From the results of Levene's test for equality of variances in table I-14, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A high significance value for the t test (greater than 0.05) indicates that there was no significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference contained zero, this, therefore, indicated that the difference was not significant. Accordingly, it could not conclude that the percentage of ECG content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. > 0.05).

Table I-13 Descriptive statistics of percentages of ECG content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of ECG in FD-GTE	Procedure A	2	3.1041	.0092	.0065
	Procedure B	2	3.5603	.0666	.0471

Table I-14 Statistical analysis of percentages of ECG content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of ECG in FD-GTE	Equal variances assumed	9277902.83331740.00	**.0000**	-9.5948	2	.0107	-.4562	.0475	-.6608	-.2516
	Equal variances not assumed			-9.5948	1.0381	**.0611**	-.4562	.0475	-1.0104	.0980

Table I-15 Summarized results of statistical analysis of percentages of ECG content, obtained between extraction procedure A and B.

% Content of ECG in FD-GTE	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	3.1041	0.0092	-9.5948*	1.0381	**0.0611**
Extraction procedure B	2	3.5603	0.0666			

*, not statistical significant level at 0.05.

2.5 Statistical analysis of percentages of caffeine in freeze-dried green tea extract, obtained from two different extraction procedures

Means of the percentage of caffeine content, obtained from procedure A and B were 5.9924 and 0.4796, respectively. From the table I-17, the mean difference value indicated that means of procedure A was less than procedure B for approximately 5.5128. Standard deviations of the percentage of caffeine content, obtained from procedure A and B were 0.0334 and 0.0052, respectively. Standard errors of the mean of the percentage of caffeine content, obtained from procedure A and B were 0.0236 and 0.0037, respectively.

From the results of Levene's test for equality of variances in table I-17, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of caffeine content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-16 Descriptive statistics of percentages of caffeine content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of caffeine in FD-GTE	Procedure A	2	5.9924	.0334	.0236
	Procedure B	2	.4796	.0052	.0037

Table I-17 Statistical analysis of percentages of caffeine content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of caffeine in FD-GTE	Equal variances assumed	938719046716847000.00	**.0000**	230.7742	2	.0000	5.5128	.0239	5.4100	5.6156
	Equal variances not assumed			230.7742	1.0491	**.0021**	5.5128	.0239	5.2409	5.7847

Table I-18 Summarized results of statistical analysis of percentages of caffeine content, obtained between extraction procedure A and B.

% Content of caffeine in FD-GTE	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	5.9924	0.0334	230.7742*	1.0491	**0.0021**
Extraction procedure B	2	0.4796	0.0052			

*, statistical significant level at 0.05.

2.6 Statistical analysis of percentages of total green tea catechins in freeze-dried green tea extract, obtained from two different extraction procedures

Since the independent samples t test procedure compares the two group means, it is useful to know what the mean values are. Means of the percentage of total GTCs content, obtained from procedure A and B were 26.0449 and 28.8923, respectively. From the table I-20, the mean difference value indicated that means of procedure A was less than procedure B for approximately 2.8474. Standard deviations of the percentage of total GTCs content, obtained from procedure A and B were 0.0511 and 0.1706, respectively. Standard errors of the mean of the percentage of total GTCs content, obtained from procedure A and B were 0.0362 and 0.1207, respectively.

From the results of Levene's test for equality of variances in table I-20, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of total GTCs content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-19 Descriptive statistics of percentages of total GTCs content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of total GTCs in FD-GTE	Procedure A	2	26.0449	.0511	.0362
	Procedure B	2	28.8923	.1706	.1207

Table I-20 Statistical analysis of percentages of total GTCs content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of total GTCs in FD-GTE	Equal variances assumed	.	.	-22.6075	2	.0020	-2.8474	.1259	-3.3893	-2.3055
	Equal variances not assumed			-22.6075	1.1781	**.0169**	-2.8474	.1259	-3.9754	-1.7194

Table I-21 Summarized results of statistical analysis of percentages of total GTCs content, obtained between extraction procedure A and B.

% Content of total GTCs in FD-GTE	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	26.0449	0.0511	-22.6075*	1.1781	**.0169**
Extraction procedure B	2	28.8923	0.1706			

*, statistical significant level at 0.05.

3. Statistical analysis of percentages of individual catechins, total catechins and caffeine in dried tealeaves, obtained from two different extraction procedures

In study I, statistical analysis was done by student's t-test independence analysis (SPSS 14.0 for Windows). Values of $P < 0.05$ were considered statistically significant.

The independent-samples t test procedure compares means for two groups of cases. The mean values and other descriptive statistics for the two groups are displayed in the group statistics table in table I-22, I-25, I-28, I-31 and I-34 for EGC, EGCG, EC, ECG and caffeine, respectively. In addition, the group statistics table in table I-37 shows the mean values and other descriptive statistics for the two groups of total GTCs, including EGC, EGCG, EC and ECG.

These tables display the number of cases, mean value, standard deviation, and standard error for the test variable(s) within categories defined by the grouping variable.

3.1 Statistical analysis of percentages of EGC in dried tealeaves, obtained from two different extraction procedures

Since the independent samples t test procedure compares the two group means, it is useful to know what the mean values are. Means of the percentage of EGC content, obtained from procedure A and B were 2.3323 and 2.4612, respectively. From the table I-23, the mean difference value indicated that means of procedure A was less than procedure B for approximately 0.1289. Standard deviations of the percentage of EGC content, obtained from procedure A and B were 0.0062 and 0.03861, respectively. Standard errors of the mean of the percentage of EGC content, obtained from procedure A and B were 0.0044 and 0.0094, respectively.

From the results of Levene's test for equality of variances in table I-23, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of EGC content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-22 Descriptive statistics of percentages of EGC content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of EGC in dried tealeaves	Procedure A	2	2.3323	.0062	.0044
	Procedure B	2	2.4612	.0132	.0094

Table I-23 Statistical analysis of percentages of EGC content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of EGC in dried tealeaves	Equal variances assumed	2417851 6392293 700.00	**.0000**	-12.4996	2.00	.0063	-.1289	.0103	-.1733	-.0845
	Equal variances not assumed			-12.4996	1.4135	**.0203**	-.1289	.0103	-.1966	-.0612

Table I-24 Summarized results of statistical analysis of percentages of EGC content, obtained between extraction procedure A and B.

% Content of EGC in dried tealeaves	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	2.3323	0.0062	-12.4996*	1.4135	**.0203**
Extraction procedure B	2	2.4612	0.0132			

*, statistical significant level at 0.05.

3.2 Statistical analysis of percentages of EGCG in dried tealeaves, obtained from two different extraction procedures

Means of the percentage of EGCG content, obtained from procedure A and B were 3.9732 and 4.5484, respectively. From the table I-26, the mean difference value indicated that means of procedure A was less than procedure B for approximately 0.5752. Standard deviations of the percentage of EGCG content, obtained from procedure A and B were 0.0093 and 0.0168, respectively. Standard errors of the mean of the percentage of EGCG content, obtained from procedure A and B were 0.0066 and 0.0119, respectively.

From the results of Levene's test for equality of variances in table I-26, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of EGCG content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-25 Descriptive statistics of percentages of EGCG content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of EGCG in dried tealeaves	Procedure A	2	3.9732	.0093	.0066
	Procedure B	2	4.5484	.0168	.0119

Table I-26 Statistical analysis of percentages of EGCG content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of EGCG in dried tealeaves	**Equal variances assumed**	3535761662046681.00	**.0000**	-42.4026	2	.0006	-.5752	.0136	-.6335	-.5168
	Equal variances not assumed			-42.4026	1.5660	**.0022**	-.5752	.0136	-.6521	-.4982

Table I-27 Summarized results of statistical analysis of percentages of EGCG content, obtained between extraction procedure A and B.

% Content of EGCG in dried tealeaves	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	3.9732	0.0093	-42.4026*	1.5660	**.0022**
Extraction procedure B	2	4.5484	0.0168			

*, statistical significant level at 0.05.

3.3 Statistical analysis of percentages of EC in dried tealeaves, obtained from two different extraction procedures

Means of the percentage of EC content, obtained from procedure A and B were 0.6769 and 0.7362, respectively. From the table I-29, the mean difference value indicated that means of procedure A was less than procedure B for approximately 0.0594. Standard deviations of the percentage of EC content, obtained from procedure A and B were 0.0028 and 0.0017, respectively. Standard errors of the mean of the percentage of EC content, obtained from procedure A and B were 0.0020 and 0.0012, respectively.

From the results of Levene's test for equality of variances in table I-29, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of EC content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-28 Descriptive statistics of percentages of EC content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of EC in dried tealeaves	Procedure A	2	.6769	.0028	.0020
	Procedure B	2	.7362	.0017	.0012

Table I-29 Statistical analysis of percentages of EC content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of EC in dried tealeaves	Equal variances assumed	4230300 3019165 10.00	**.0000**	-25.9210	2	.0015	-.0594	.0023	-.0692	-.0495
	Equal variances not assumed			-25.9210	1.6624	**.0036**	-.0594	.0023	-.0714	-.0473

Table I-30 Summarized results of statistical analysis of percentages of EC content, obtained between extraction procedure A and B.

% Content of EC in dried tealeaves	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	0.6769	0.0028	-25.9210*	1.6624	**.0036**
Extraction procedure B	2	0.7362	0.0017			

*, statistical significant level at 0.05.

3.4 Statistical analysis of percentages of ECG in dried tealeaves, obtained from two different extraction procedures

Means of the percentage of ECG content, obtained from procedure A and B were 1.0593 and 1.2215, respectively. From the table I-32, the mean difference value indicated that means of procedure A was less than procedure B for approximately 0.1622. Standard deviations of the percentage of ECG content, obtained from procedure A and B were 0.0031 and 0.0228, respectively. Standard errors of the mean of the percentage of ECG content, obtained from procedure A and B were 0.0022 and 0.0162, respectively.

From the results of Levene's test for equality of variances in table I-32, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do

no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A high significance value for the t test (greater than 0.05) indicates that there was no significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference contained zero, this, therefore, indicated that the difference was not significant. Accordingly, it could not conclude that the percentage of ECG content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. > 0.05).

Table I-31 Descriptive statistics of percentages of ECG content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of ECG in dried tealeaves	Procedure A	2	1.0593	.0031	.0022
	Procedure B	2	1.2215	.0228	.0162

Table I-32 Statistical analysis of percentages of ECG content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of ECG in dried tealeaves	Equal variances assumed	.	.	-9.9484	2	.0100	-.1622	.0163	-.2323	-.0920
	Equal variances not assumed			-9.9484	1.0371	**.0590**	-.1622	.0163	-.3525	.0282

Table I-33 Summarized results of statistical analysis of percentages of ECG content, obtained between extraction procedure A and B.

% Content of ECG in dried tealeaves	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	1.0593	0.0031	-9.9484*	1.0371	**.0590**
Extraction procedure B	2	1.2215	0.0228			

*, statistical significant level at 0.05.

3.5 Statistical analysis of percentages of caffeine in dried tealeaves, obtained from two different extraction procedures

Means of the percentage of caffeine content, obtained from procedure A and B were 2.0451 and 0.1646, respectively. From the table I-35, the mean difference value indicated that means of procedure A was less than procedure B for approximately 1.8805. Standard deviations of the percentage of caffeine content, obtained from procedure A and B were 0.0114 and 0.0018, respectively. Standard errors of the mean of the percentage of caffeine content, obtained from procedure A and B were 0.0081 and 0.0013, respectively.

From the results of Levene's test for equality of variances in table I-35, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of caffeine content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-34 Descriptive statistics of percentages of caffeine content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of caffeine in dried tealeaves	Procedure A	2	2.0451	.0114	.0081
	Procedure B	2	.1646	.0018	.0013

Table I-35 Statistical analysis of percentages of caffeine content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variables	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of caffeine in dried tealeaves	Equal variances assumed	1007016 2336279 990.00	**.0000**	230.8361	2	.0000	1.8805	.0081	1.8454	1.9156
	Equal variances not assumed			230.8361	1.0482	**.0021**	1.8805	.0081	1.7876	1.9734

Table I-36 Summarized results of statistical analysis of percentages of caffeine content, obtained between extraction procedure A and B.

% Content of caffeine in dried tealeaves	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	2.0451	0.0114	230.8361*	1.0482	**.0021**
Extraction procedure B	2	0.1646	0.0018			

*, statistical significant level at 0.05.

3.6 Statistical analysis of percentages of total green tea catechins in dried tea leaves, obtained from two different extraction procedures

Means of the percentage of total GTCs content, obtained from procedure A and B were 8.8883 and 9.9119, respectively. From the table I-38, the mean difference value indicated that means of procedure A was less than procedure B for approximately 1.0237. Standard deviations of the percentage of total GTCs content, obtained from procedure A and B were 0.0175 and 0.0585, respectively. Standard errors of the mean of the percentage of total GTCs content, obtained from procedure A and B were 0.0124 and 0.0414, respectively.

From the results of Levene's test for equality of variances in table I-38, the significance value was lower than 0.05 (sig. < 0.05). Accordingly, the results that do no assume equal variances for both groups were utilized. It showed that there was significantly difference between groups at significant level of 0.05.

A low significance value for the t test (less than 0.05) indicates that there was a significant difference between the two group means. Moreover, it also assured that the confidence interval for the mean difference did not contain zero, this, therefore, indicated that the difference was significant. Accordingly, the percentage of total GTCs content, obtained from the procedure A was significantly lower than the one from the procedure B (sig. < 0.05).

Table I-37 Descriptive statistics of percentages of total GTCs content, obtained from extraction procedure A and B.

Group Statistics

Dependent variables	Extraction procedure	N	Mean	Std. Deviation	Std. Error Mean
% Content of total GTCs in dried tealeaves	Procedure A	2	8.8883	.0175	.0124
	Procedure B	2	9.9119	.0585	.0414

Table I-38 Statistical analysis of percentages of total GTCs content, obtained between two groups of different extraction procedure: Levene's test for equality of variances and t-test for equality of means.

Independent Samples Test

Dependent variable	Assumptions	Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
% Content of total GTCs in dried tealeaves	Equal variances assumed	8071325 3282224 700.00	**.0000**	-23.6941	2	.0018	-1.0237	.0432	-1.2095	-.8378
	Equal variances not assumed			-23.6941	1.1766	**.0160**	-1.0237	.0432	-1.4115	-.6358

Table I-39 Summarized results of statistical analysis of percentages of total GTCs content, obtained between extraction procedure A and B.

% Content of total GTCs in dried tealeaves	*N*	*Mean*	*Std. Deviation*	*t*	*df*	*P*
Extraction procedure A	2	8.8883	0.0175	-23.6941*	1.1766	**.0160**
Extraction procedure B	2	9.9119	0.0585			

*, statistical significant level at 0.05.

APPENDIX II

APPARENT VISCOSITIES OF CONCENTRATED W/O EMULSIONS WITH DIFFERENT FORMULATIONS

Table II-1 Average apparent viscosity (ή) of CEs containing FD-GTE with different formulation parameters (mPa.s.) (Avg. ή ± S.D., n = 3) after 1, 8, 15, 22 days and 1 month.

Rx. No.	Estimated time intervals														
	Day 1			Day 8			Day 15			Day 22			Month 1		
	Mean Viscosity (mPa.s)			Mean Viscosity (mPa.s)			Mean Viscosity (mPa.s)			Mean Viscosity (mPa.s)			Mean Viscosity (mPa.s)		
	Average	±	S.D.	Average	±	S.D.	Average	±	S.D.	Average	±	S.D.	Average	±	S.D.
Rx 1	3,637.00	±	0.00	3,417.18	±	0.00	3,529.25	±	0.00	3,321.16	±	0.00	3,433.12	±	0.00
Rx 2	4,993.00	±	0.00	4,977.60	±	0.00	5,089.66	±	0.00	4,737.54	±	0.00	5,353.63	±	0.00
Rx 3	10,275.00	±	0.00	8,698.59	±	0.00	10,010.98	±	0.00	9,094.70	±	0.00	8,234.40	±	0.00
Rx 4	14,044.00	±	0.00	13,259.81	±	0.00	14,212.10	±	0.00	11,939.46	±	0.00	11,355.23	±	0.00
Rx 5	15,292.00	±	0.00	16,300.62	±	69.30	16,132.61	±	0.00	13,019.74	±	0.00	13,395.78	±	0.00
Rx 6	46,644.00	±	0.00	19,657.51	±	0.00	68,886.67	±	0.00	23,822.62	±	0.00	36,129.63	±	0.00
Rx 7	2,713.00	±	0.00	2,817.02	±	0.00	3,169.15	±	0.00	2,576.96	±	0.00	3,841.02	±	0.00
Rx 8	3,673.00	±	0.00	3,885.31	±	0.00	4,249.44	±	0.00	3,537.22	±	0.00	4,585.22	±	0.00
Rx 9	6,314.00	±	0.00	6,417.98	±	0.00	6,289.98	±	0.00	5,337.70	±	0.00	7,081.89	±	0.00
Rx 10	4,706.00	±	0.00	3,897.31	±	0.00	3,529.25	±	0.00	3,297.15	±	0.00	4,201.12	±	0.00
Rx 11	5,941.00	±	0.00	4,377.44	±	0.00	3,345.16	±	0.00	3,057.09	±	0.00	4,729.26	±	0.00
Rx 12	5,953.00	±	0.00	5,217.66	±	0.00	5,457.73	±	0.00	4,737.54	±	0.00	5,881.57	±	0.00
Rx 13	4,633.00	±	0.00	4,257.41	±	0.00	4,113.37	±	0.00	3,777.28	±	0.00	4,561.22	±	0.00
Rx 14	4,033.00	±	0.00	4,497.47	±	0.00	4,377.44	±	0.00	4,137.38	±	0.00	4,993.33	±	0.00
Rx 15	9,074.00	±	0.00	7,618.30	±	0.00	7,618.30	±	0.00	6,778.08	±	0.00	7,802.08	±	0.00

Table II-2 Average apparent viscosity (ή) of CEs containing FD-GTE with different formulation parameters (mPa.s.) (Avg. ή ± S.D., n = 3) after 2, 3, 4, 5 and 6 months.

Rx. No.	Estimated time intervals					
	Month 2	Month 3	Month 4	Month 5	Month 6
	Mean Viscosity (mPa.s)	Mean Viscosity (mPa.s)	Mean Viscosity (mPa.s)	Mean Viscosity (mPa.s)	Mean Viscosity (mPa.s)
	Average ± S.D.	Average ± S.D.	Average ± S.D.	Average ± S.D.	Average ± S.D.
Rx 1	3,033.06 ± 0.00	2,969.06 ± 0.00	2,945.02 ± 0.00	2,824.99 ± 0.00	2,945.02 ± 0.00
Rx 2	4,593.47 ± 0.00	4,649.50 ± 0.00	4,385.41 ± 0.00	4,385.41 ± 0.00	4,145.34 ± 0.00
Rx 3	8,314.46 ± 0.00	7,410.24 ± 0.00	6,906.08 ± 0.00	6,906.08 ± 0.00	6,185.89 ± 0.00
Rx 4	10,595.07 ± 0.00	9,210.72 ± 0.00	8,746.57 ± 69.30	7,746.30 ± 0.00	7,506.24 ± 0.00
Rx 5	10,715.10 ± 0.00	9,090.69 ± 0.00	7,746.30 ± 0.00	7,146.14 ± 0.00	6,385.94 ± 69.30
Rx 6	21,037.86 ± 0.00	18,573.22 ± 0.00	16,148.54 ± 207.90	14,708.16 ± 0.00	13,187.75 ± 69.31
Rx 7	2,312.86 ± 0.00	2,368.90 ± 0.00	2,224.83 ± 0.00	2,224.83 ± 0.00	2,144.81 ± 69.30
Rx 8	3,165.09 ± 0.00	3,209.12 ± 0.00	3,065.06 ± 0.00	3,065.06 ± 0.00	3,185.09 ± 0.00
Rx 9	5,313.66 ± 0.00	4,889.57 ± 0.00	5,105.60 ± 0.00	5,345.66 ± 0.00	4,985.57 ± 0.00
Rx 10	2,672.96 ± 0.00	2,488.93 ± 0.00	2,464.90 ± 0.00	2,464.90 ± 0.00	2,264.84 ± 69.30
Rx 11	3,153.09 ± 0.00	2,969.06 ± 0.00	3,065.06 ± 0.00	2,945.02 ± 0.00	2,584.93 ± 0.00
Rx 12	4,713.50 ± 0.00	4,409.44 ± 0.00	4,265.38 ± 0.00	4,385.41 ± 0.00	3,905.28 ± 0.00
Rx 13	3,273.12 ± 0.00	3,209.12 ± 0.00	3,065.06 ± 0.00	3,185.09 ± 0.00	2,945.02 ± 0.00
Rx 14	3,873.28 ± 0.00	3,569.22 ± 0.00	3,425.15 ± 0.00	3,465.16 ± 69.30	3,305.12 ± 0.00
Rx 15	6,345.94 ± 0.00	6,221.92 ± 0.00	5,969.83 ± 0.00	6,425.95 ± 0.00	5,585.73 ± 0.00

APPENDIX III

SCANNING ELECTRON MICROSCOPES OF CONCENTRATED W/O EMULSIONS WITH DIFFERENT FORMULATIONS

DAY 1

CEs containing FD-GTE Rx no. 1 (3% Arlacel - 84% DP)

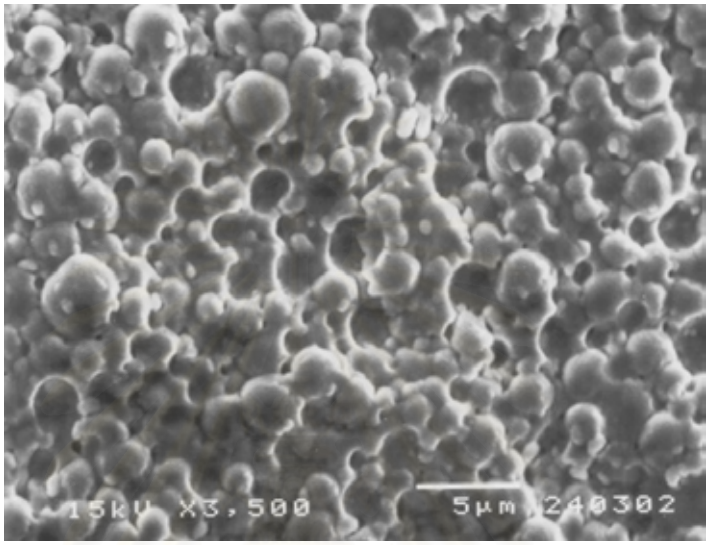


Figure III-1 SEM image of CEs containing FD-GTE Rx no.1, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 2 (3% Arlacel - 86% DP)

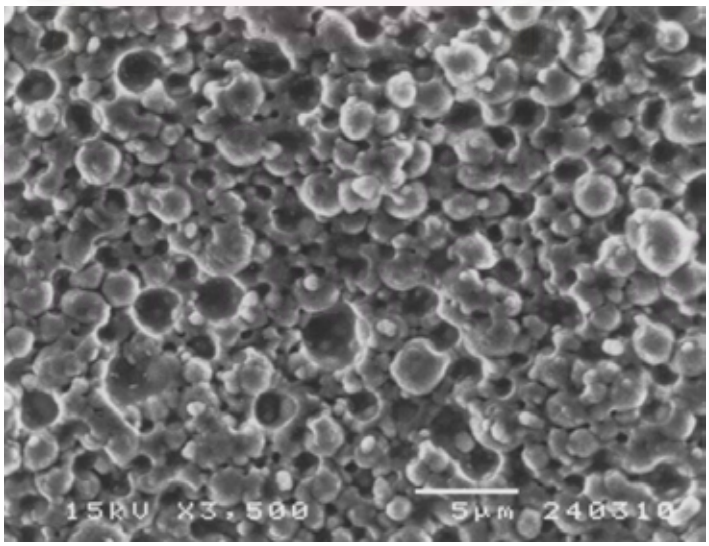


Figure III-2 SEM image of CEs containing FD-GTE Rx no.2, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 3 (3% Arlacel - 88% DP)

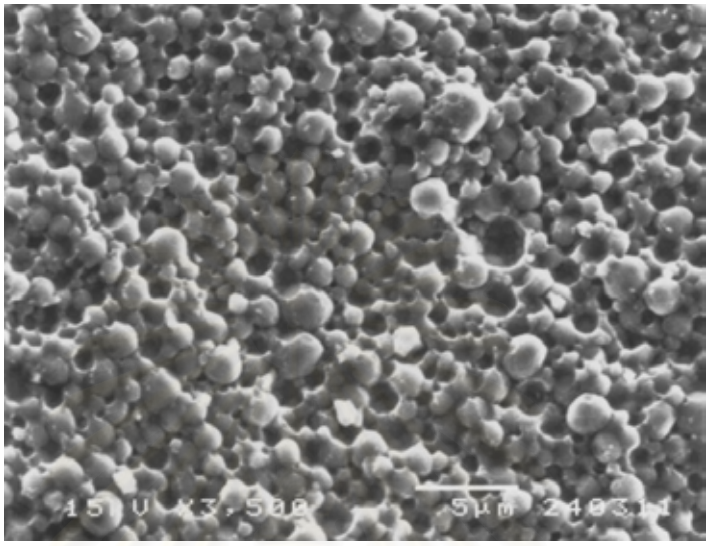


Figure III-3 SEM image of CEs containing FD-GTE Rx no.3, magnification x 3,500, scale bar 5 μm (day 1).

CEs containing FD-GTE Rx no. 4 (3% Abil EM 90 - 84% DP)

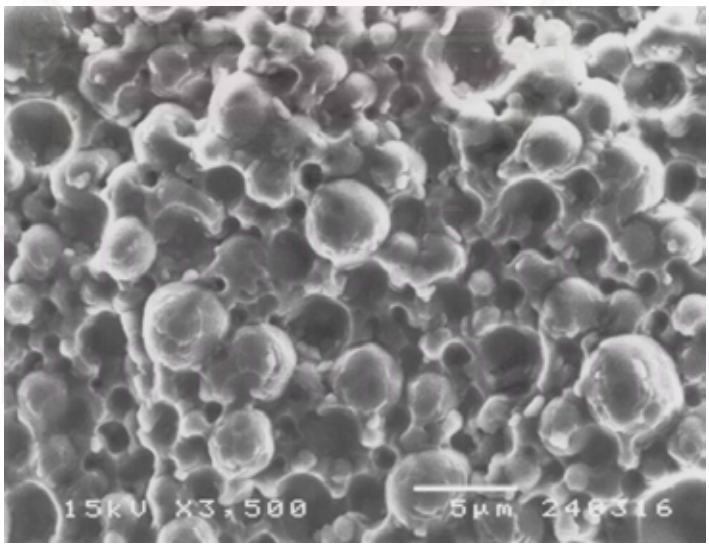


Figure III-4 SEM image of CEs containing FD-GTE Rx no.4, magnification x 3,500, scale bar 5 μm (day 1).

CEs containing FD-GTE Rx no. 5 (3% Abil EM 90 - 86% DP)

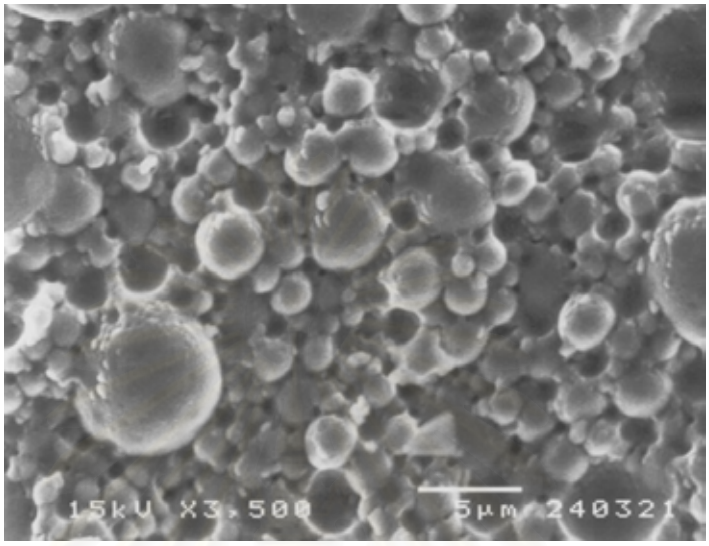


Figure III-5 SEM image of CEs containing FD-GTE Rx no.5, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 6 (3% Abil EM 90 - 88% DP)

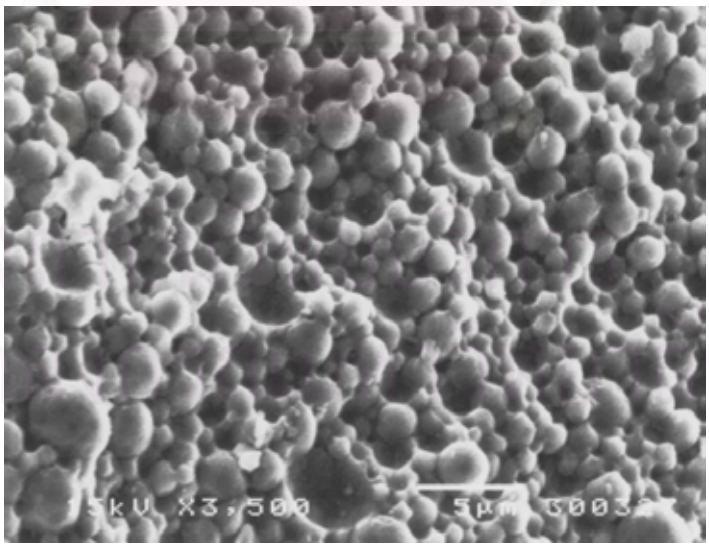


Figure III-6 SEM image of CEs containing FD-GTE Rx no.6, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 7 (3% Isolan DO - 84% DP)

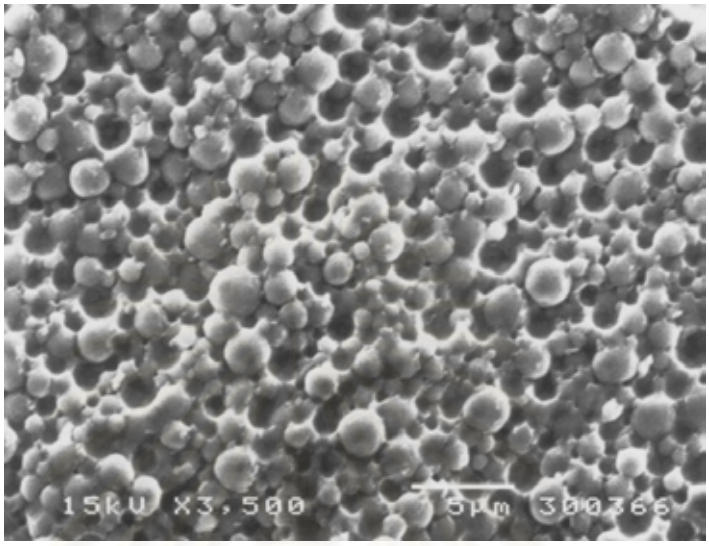


Figure III-7 SEM image of CEs containing FD-GTE Rx no.7, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 8 (3% Isolan DO - 86% DP)

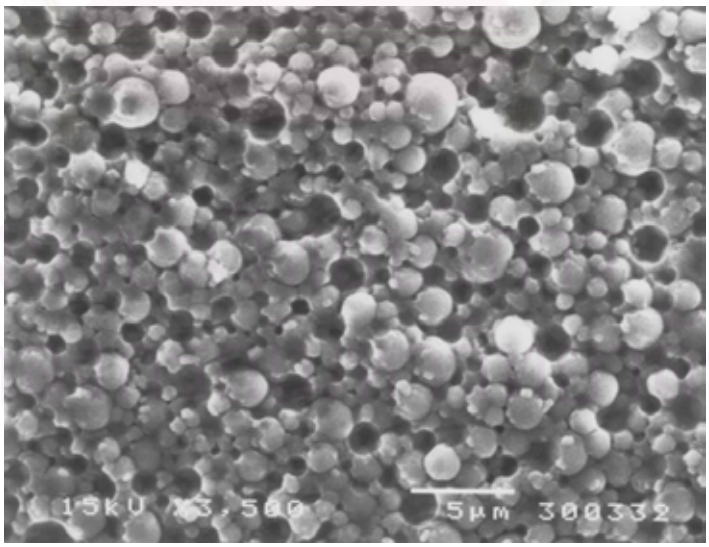


Figure III-8 SEM image of CEs containing FD-GTE Rx no.8, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 9 (3% Isolan DO - 88% DP)

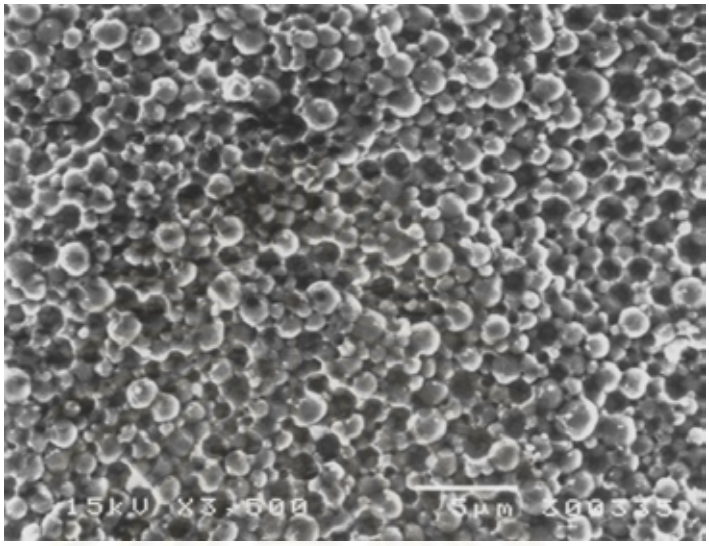


Figure III-9 SEM image of CEs containing FD-GTE Rx no.9, magnification x 3,500, scale bar 5 μm (day 1).

CEs containing FD-GTE Rx no. 10 (2.2% Montane: 0.8% Simusol - 84% DP)

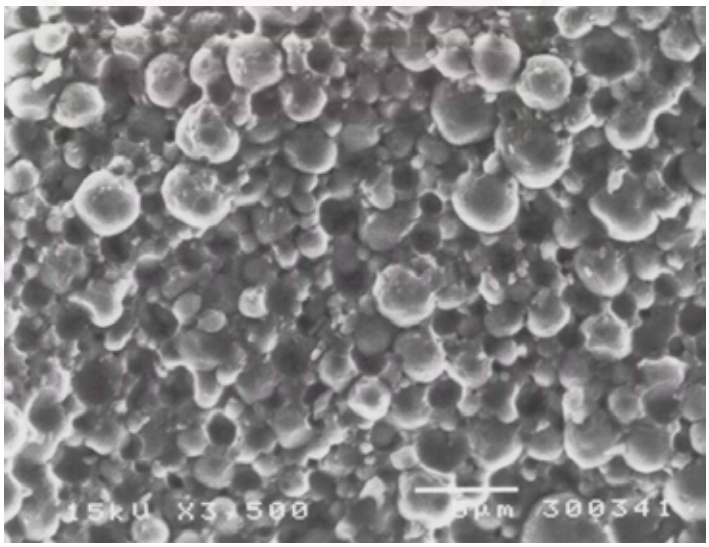


Figure III-10 SEM image of CEs containing FD-GTE Rx no.10, magnification x 3,500, scale bar 5 μm (day 1).

CEs containing FD-GTE Rx no. 11 (2.2% Montane: 0.8% Simusol - 86% DP)

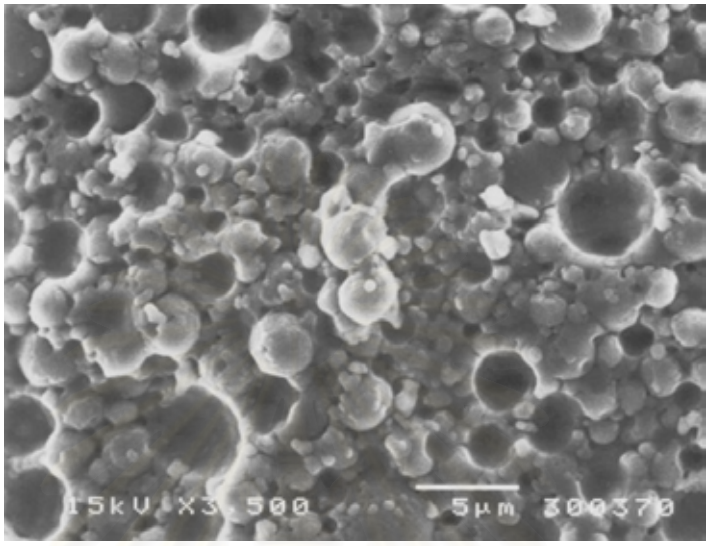


Figure III-11 SEM image of CEs containing FD-GTE Rx no.11, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 12 (2.2% Montane: 0.8% Simusol - 88% DP)

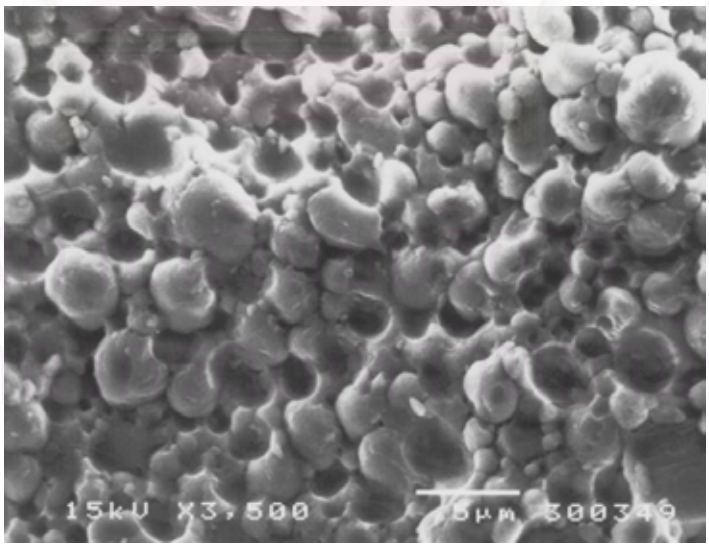


Figure III-12 SEM image of CEs containing FD-GTE Rx no.12, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 13 (1% Isolan DO - 88% DP)

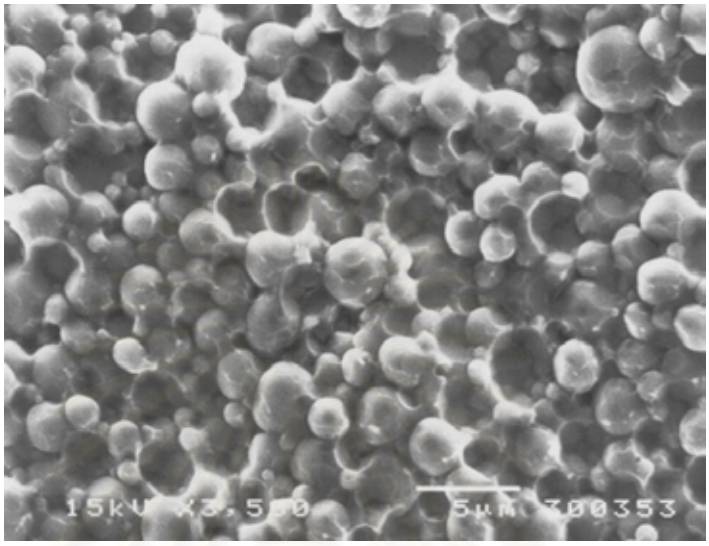


Figure III-13 SEM image of CEs containing FD-GTE Rx no.13, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 14 (2% Isolan DO - 88% DP)

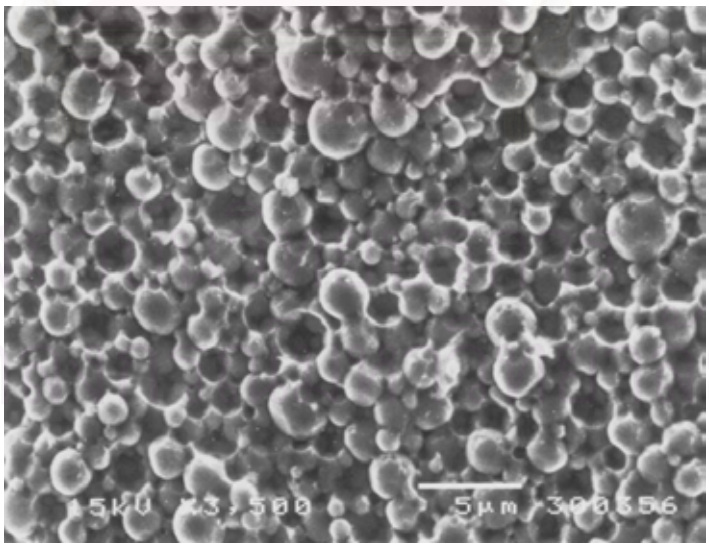


Figure III-14 SEM image of CEs containing FD-GTE Rx no.14, magnification x 3,500, scale bar 5 µm (day 1).

CEs containing FD-GTE Rx no. 15 (4% Isolan DO - 88% DP)

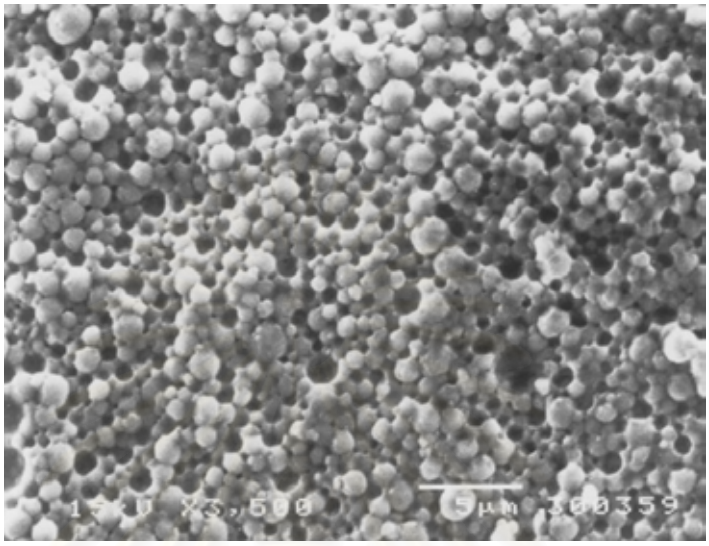


Figure III-15 SEM image of CEs containing FD-GTE Rx no.15, magnification x 3,500, scale bar 5 µm (day 1).

DAY 8

CEs containing FD-GTE Rx no. 1 (3% Arlacel - 84% DP)

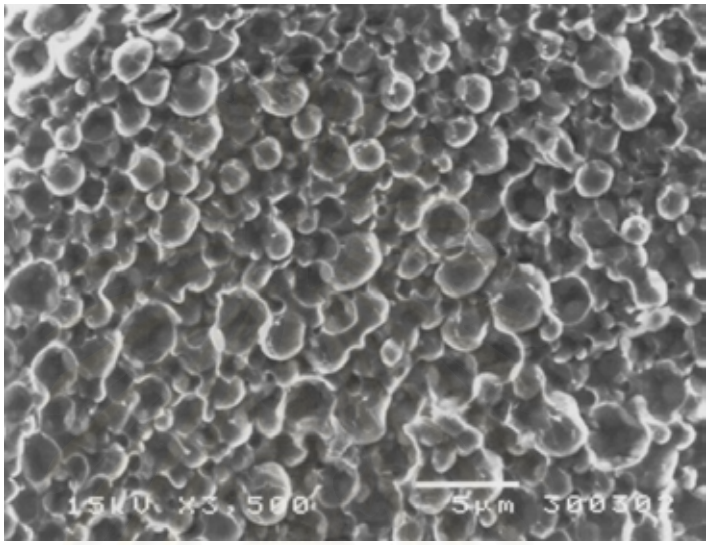


Figure III-16 SEM image of CEs containing FD-GTE Rx no.1, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 2 (3% Arlacel - 86% DP)

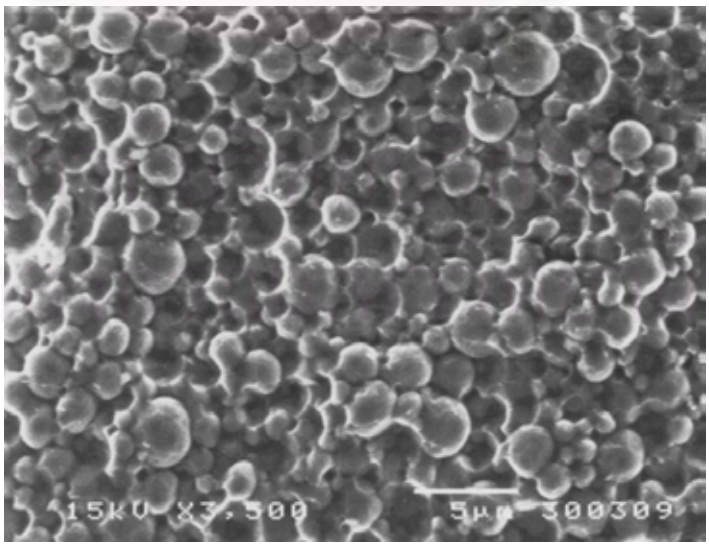


Figure III-17 SEM image of CEs containing FD-GTE Rx no.2, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 3 (3% Arlacel - 88% DP)

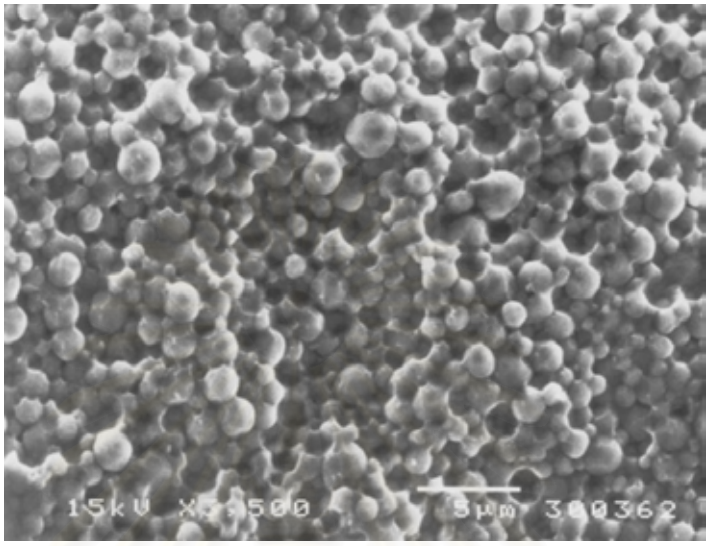


Figure III-18 SEM image of CEs containing FD-GTE Rx no.3, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 4 (3% Abil EM 90 - 84% DP)

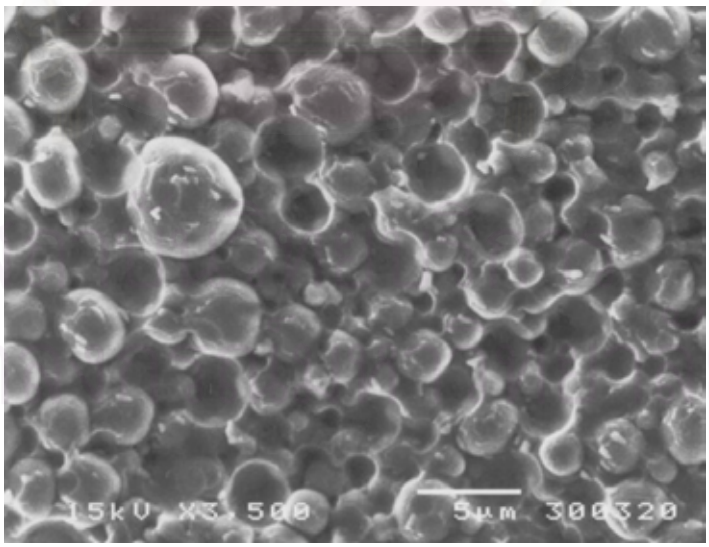


Figure III-19 SEM image of CEs containing FD-GTE Rx no.4, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 5 (3% Abil EM 90 - 86% DP)

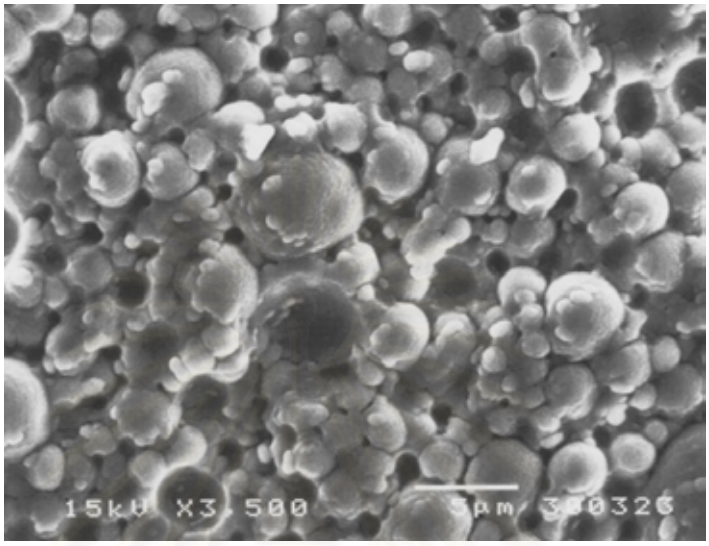


Figure III-20 SEM image of CEs containing FD-GTE Rx no.5, magnification x 3,500, scale bar 5 μm (day 8).

CEs containing FD-GTE Rx no. 6 (3% Abil EM 90 - 88% DP)

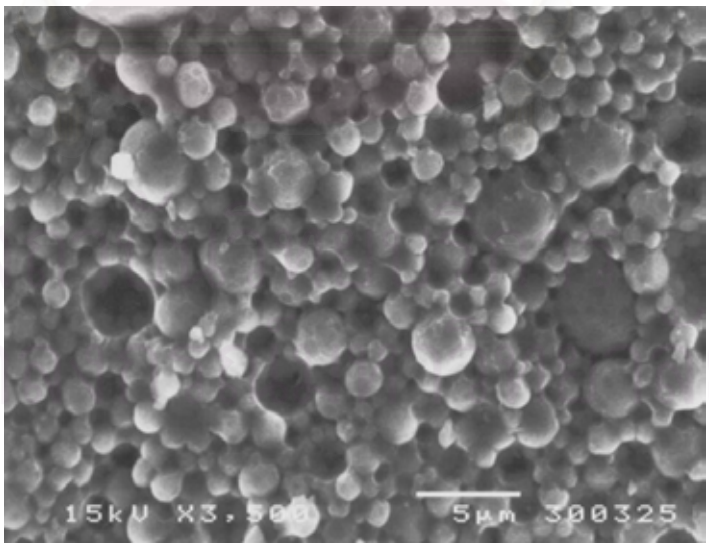


Figure III-21 SEM image of CEs containing FD-GTE Rx no.6, magnification x 3,500, scale bar 5 μm (day 8).

CEs containing FD-GTE Rx no. 7 (3% Isolan DO - 84% DP)

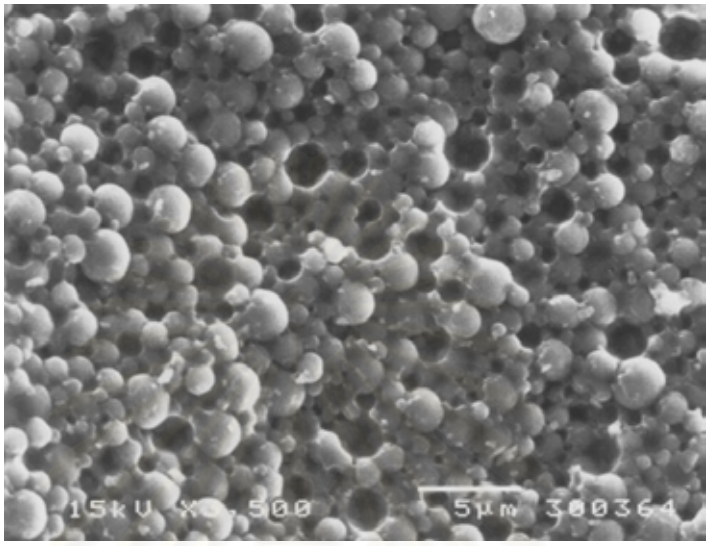


Figure III-22 SEM image of CEs containing FD-GTE Rx no.7, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 8 (3% Isolan DO - 86% DP)

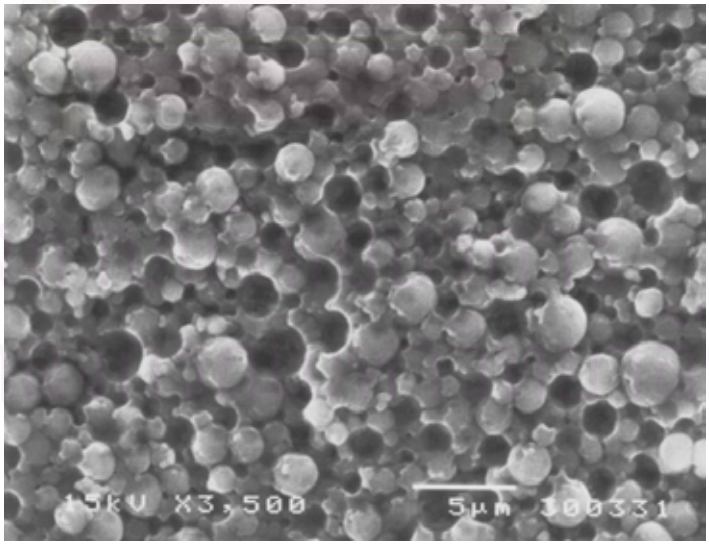


Figure III-23 SEM image of CEs containing FD-GTE Rx no.8, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 9 (3% Isolan DO - 88% DP)

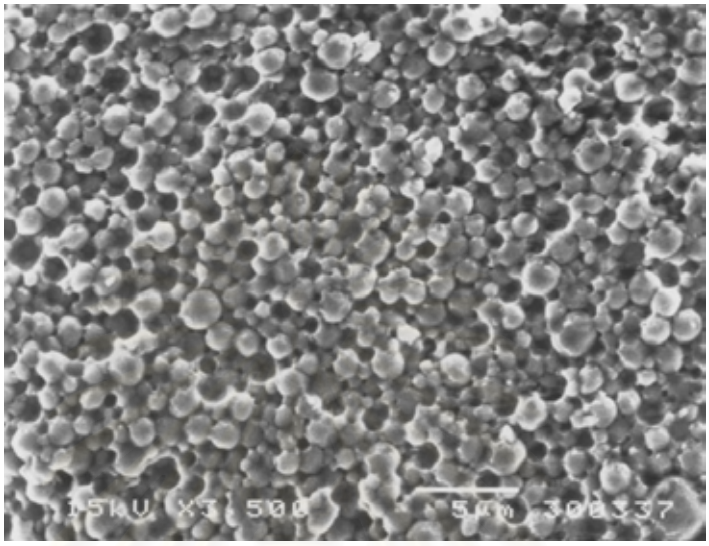


Figure III-24 SEM image of CEs containing FD-GTE Rx no.9, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 10 (2.2% Montane: 0.8% Simusol - 84% DP)

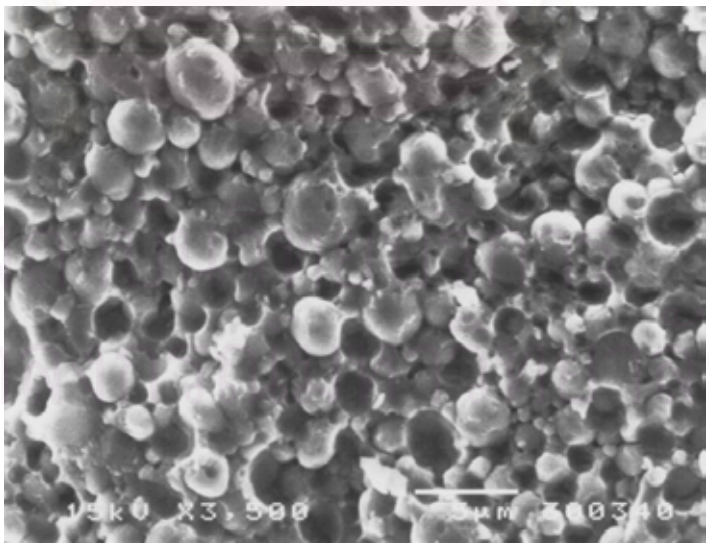


Figure III-25 SEM image of CEs containing FD-GTE Rx no.10, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 11 (2.2% Montane: 0.8% Simusol - 86% DP)

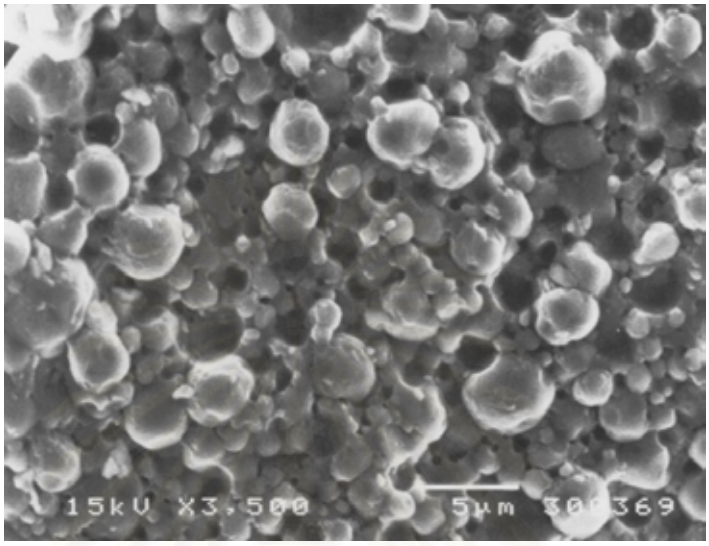


Figure III-26 SEM image of CEs containing FD-GTE Rx no.11, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 12 (2.2% Montane: 0.8% Simusol - 88% DP)

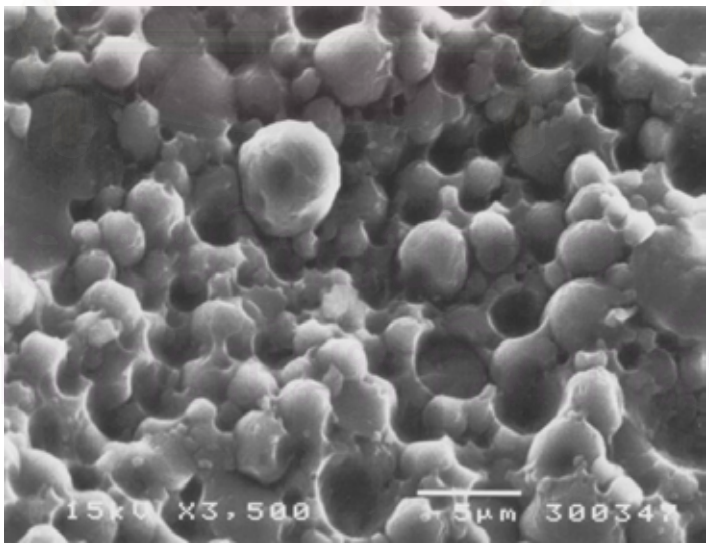


Figure III-27 SEM image of CEs containing FD-GTE Rx no.12, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 13 (1% Isolan DO - 88% DP)

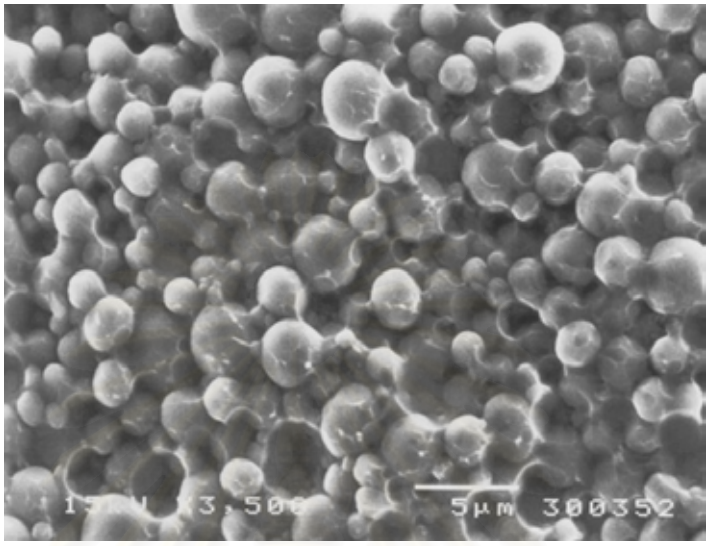


Figure III-28 SEM image of CEs containing FD-GTE Rx no.13, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 14 (2% Isolan DO - 88% DP)

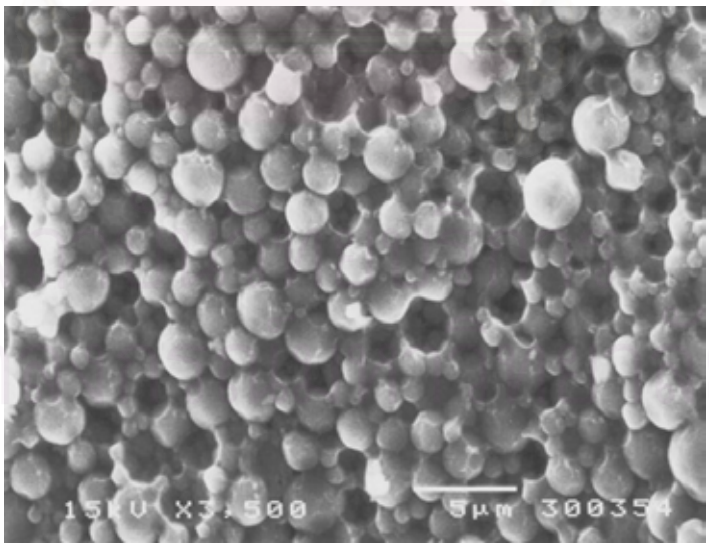


Figure III-29 SEM image of CEs containing FD-GTE Rx no.14, magnification x 3,500, scale bar 5 µm (day 8).

CEs containing FD-GTE Rx no. 15 (4% Isolan DO - 88% DP)

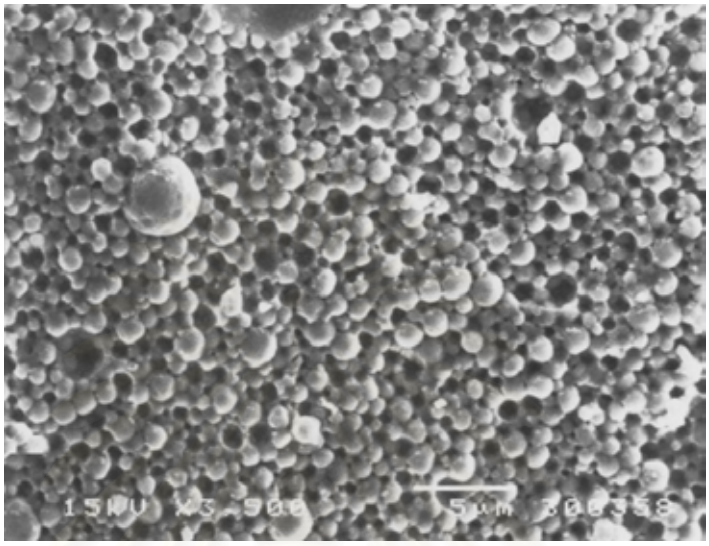


Figure III-30 SEM image of CEs containing FD-GTE Rx no.15, magnification x 3,500, scale bar 5 µm (day 8).

DAY 15

CEs containing FD-GTE Rx no. 1 (3% Arlacel - 84% DP)

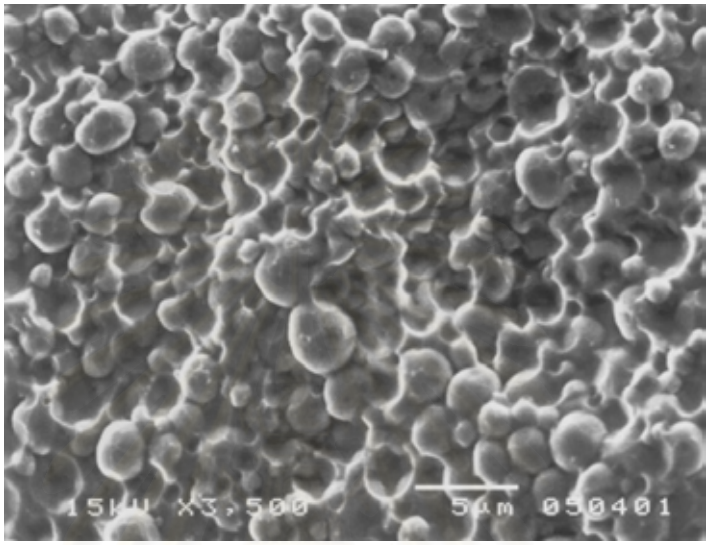


Figure III-31 SEM image of CEs containing FD-GTE Rx no.1, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 2 (3% Arlacel - 86% DP)

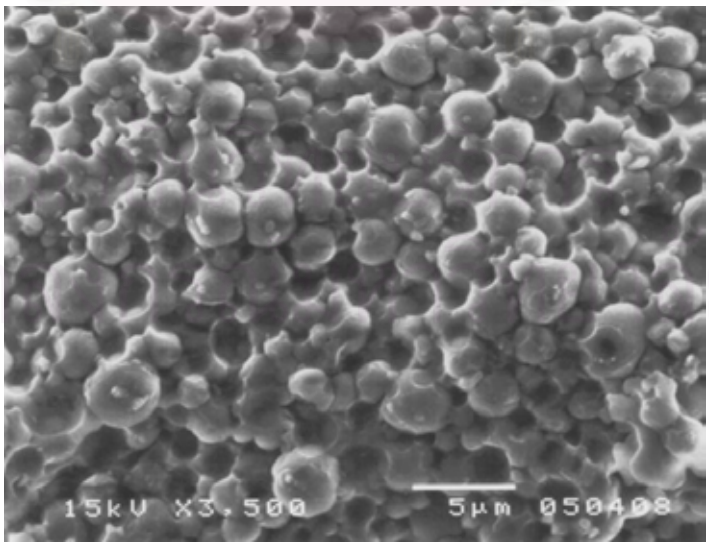


Figure III-32 SEM image of CEs containing FD-GTE Rx no.2, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 3 (3% Arlacel - 88% DP)

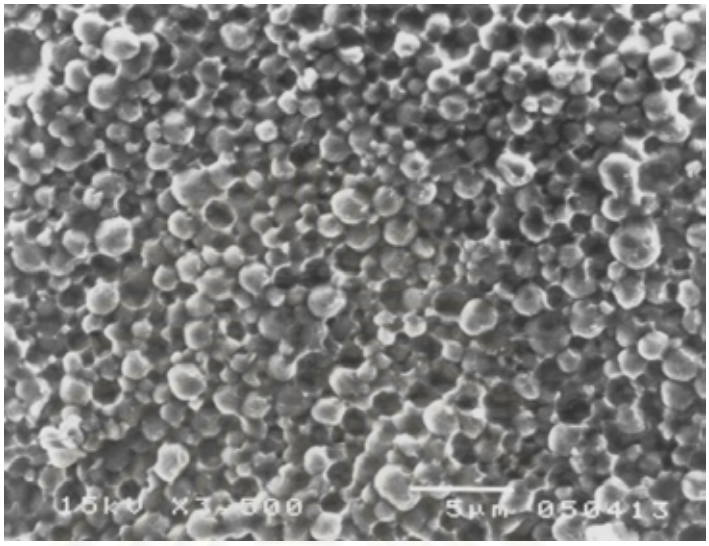


Figure III-33 SEM image of CEs containing FD-GTE Rx no.3, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 4 (3% Abil EM 90 - 84% DP)

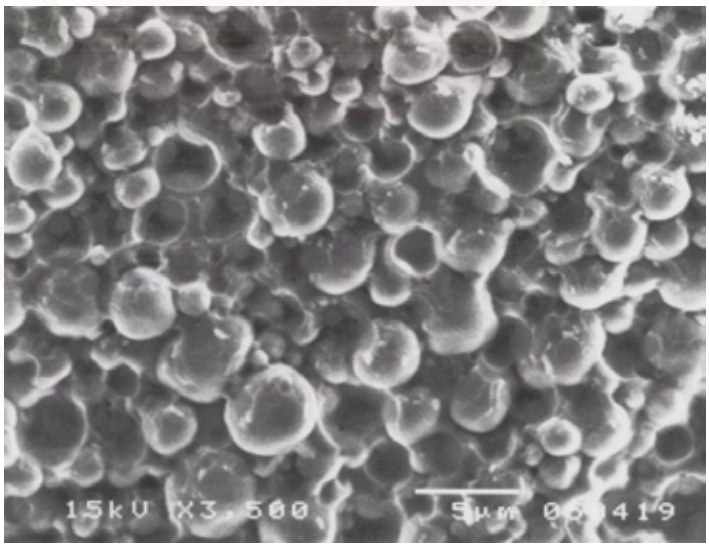


Figure III-34 SEM image of CEs containing FD-GTE Rx no.4, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 5 (3% Abil EM 90 - 86% DP)

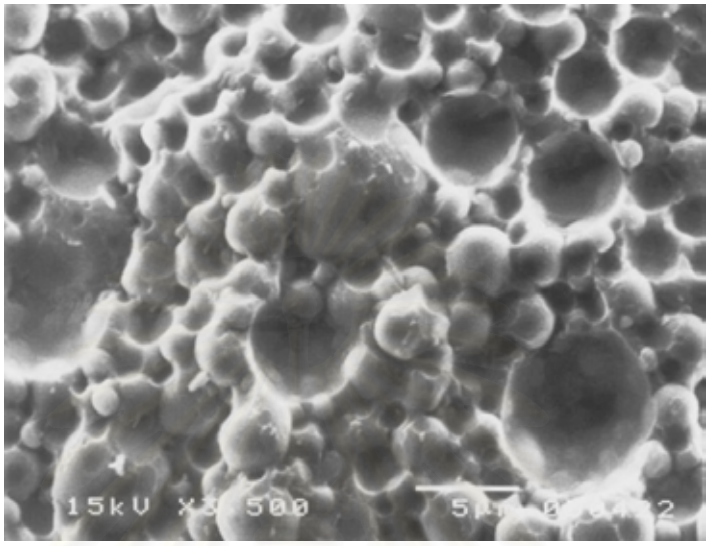


Figure III-35 SEM image of CEs containing FD-GTE Rx no.5, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 6 (3% Abil EM 90 - 88% DP)

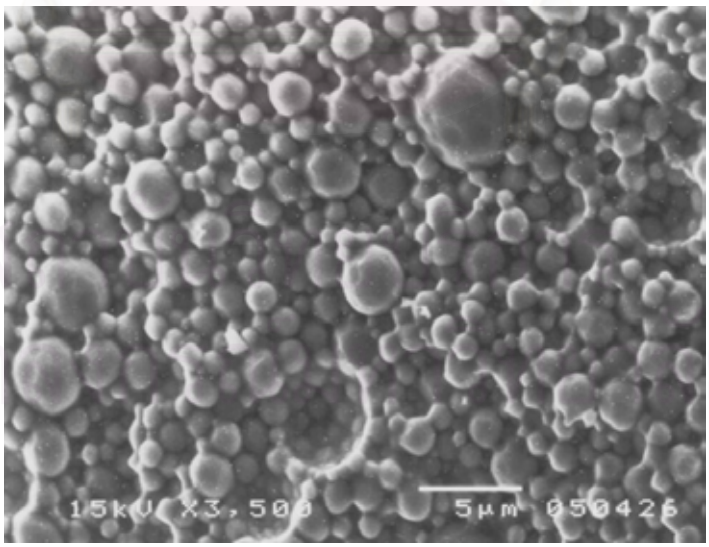


Figure III-36 SEM image of CEs containing FD-GTE Rx no.6, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 7 (3% Isolan DO - 84% DP)

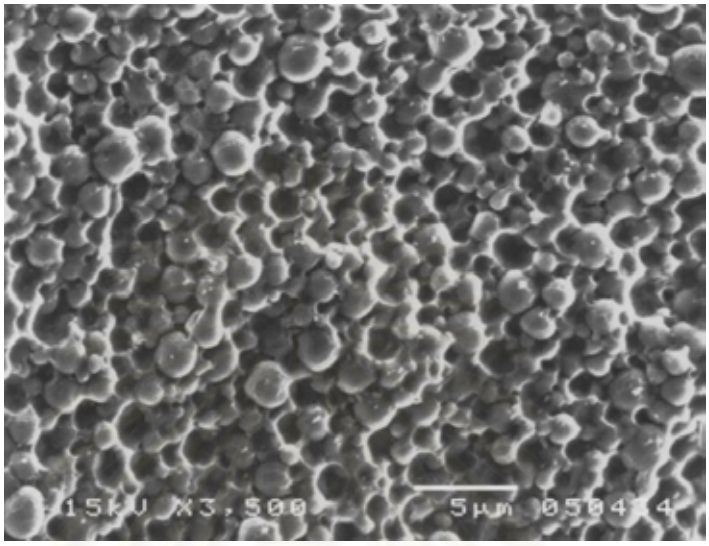


Figure III-37 SEM image of CEs containing FD-GTE Rx no.7, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 8 (3% Isolan DO - 86% DP)

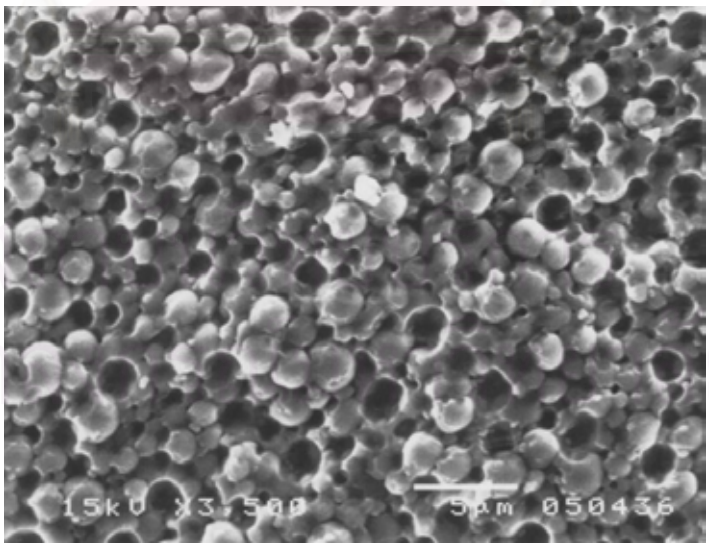


Figure III-38 SEM image of CEs containing FD-GTE Rx no.8, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 9 (3% Isolan DO - 88% DP)

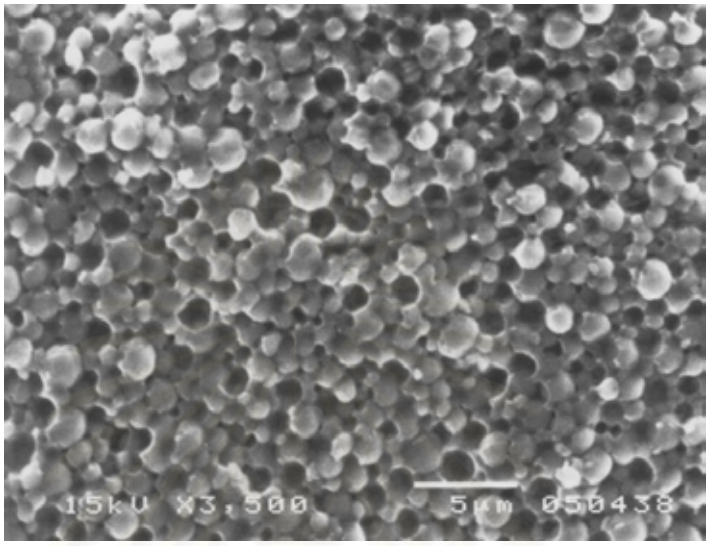


Figure III-39 SEM image of CEs containing FD-GTE Rx no.9, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 10 (2.2% Montane: 0.8% Simusol - 84% DP)

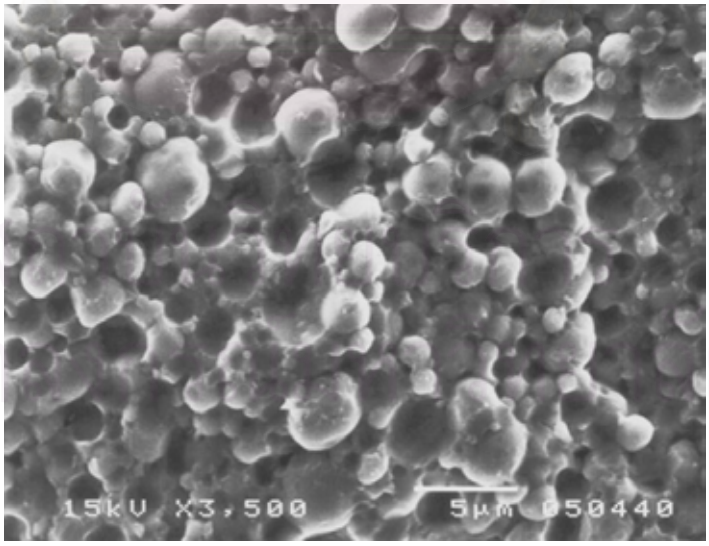


Figure III-40 SEM image of CEs containing FD-GTE Rx no.10, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 11 (2.2% Montane: 0.8% Simusol - 86% DP)

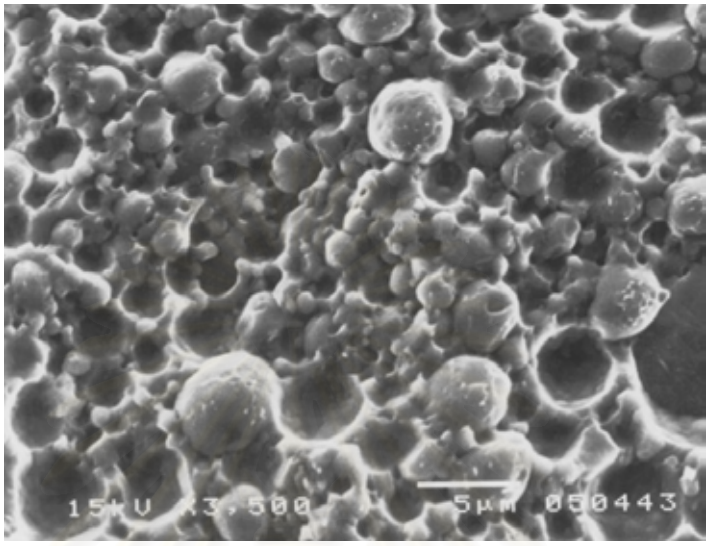


Figure III-41 SEM image of CEs containing FD-GTE Rx no.11, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 12 (2.2% Montane: 0.8% Simusol - 88% DP)

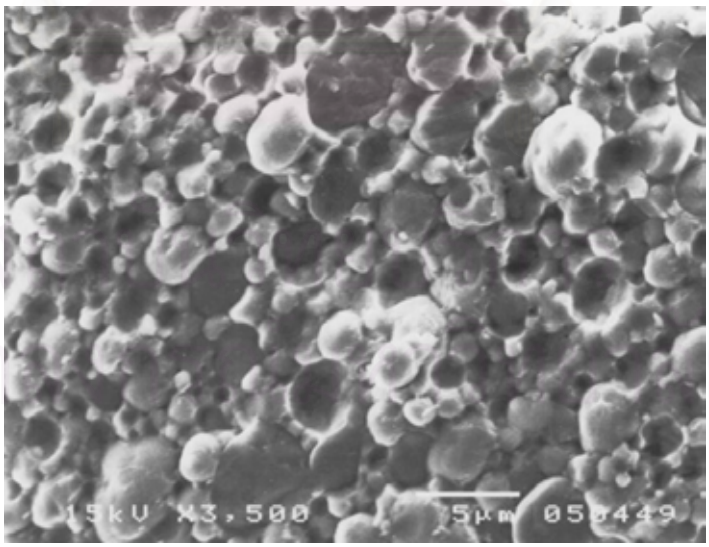


Figure III-42 SEM image of CEs containing FD-GTE Rx no.12, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 13 (1% Isolan DO - 88% DP)

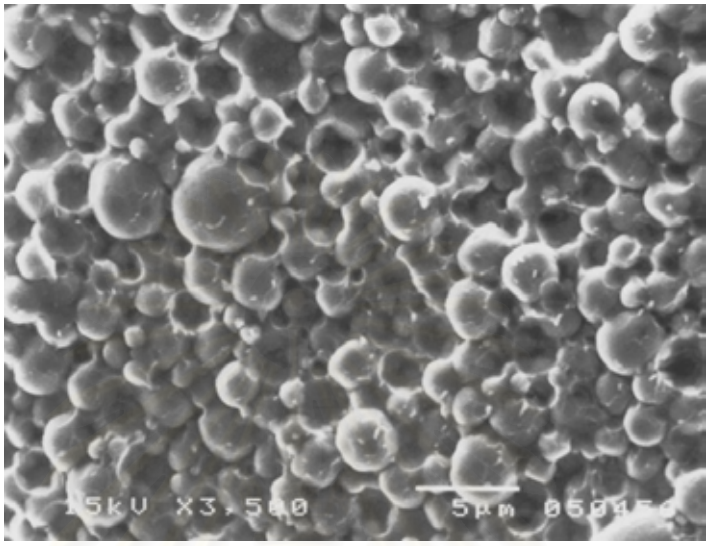


Figure III-43 SEM image of CEs containing FD-GTE Rx no.13, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 14 (2% Isolan DO - 88% DP)

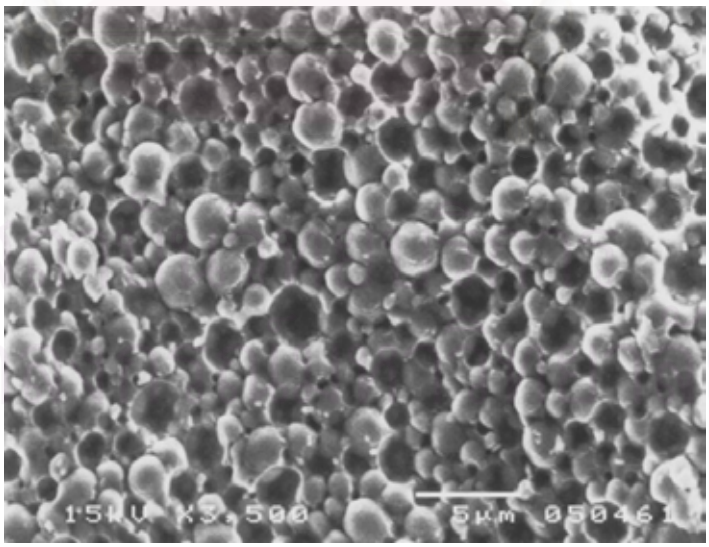


Figure III-44 SEM image of CEs containing FD-GTE Rx no.14, magnification x 3,500, scale bar 5 µm (day 15).

CEs containing FD-GTE Rx no. 15 (4% Isolan DO - 88% DP)

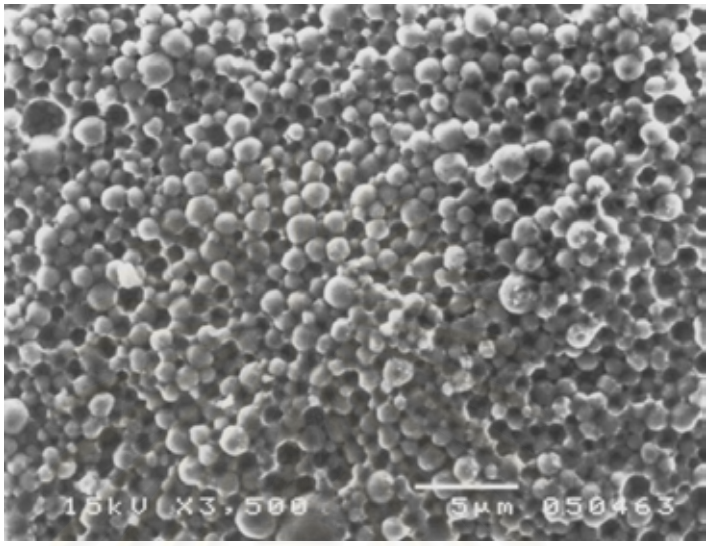
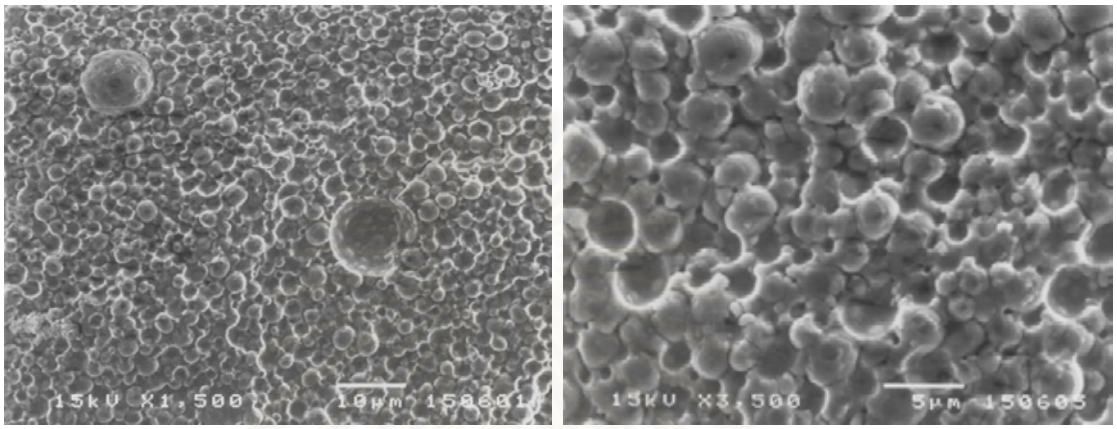


Figure III-45 SEM image of CEs containing FD-GTE Rx no.15, magnification x 3,500, scale bar 5 µm (day 15).

MONTH 3

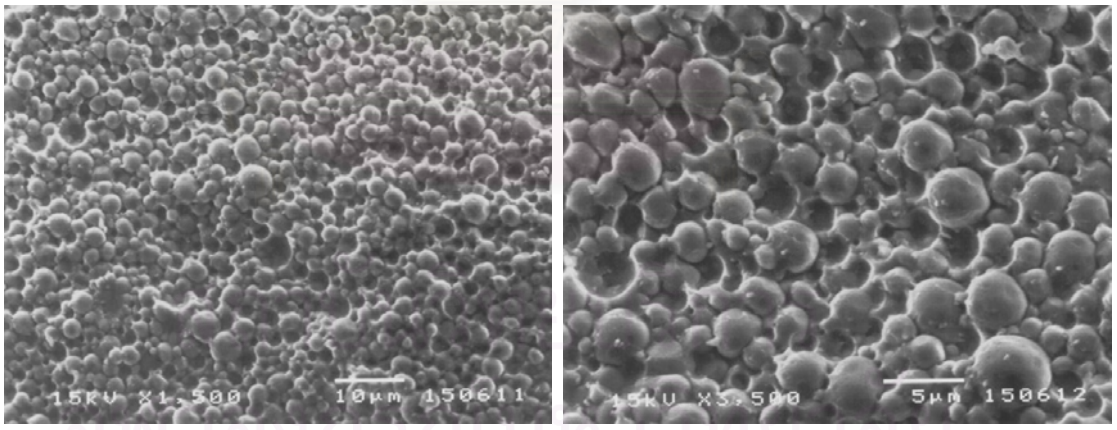
CEs containing FD-GTE Rx no. 1 (3% Arlacel - 84% DP)



(a) (b)

Figure III-46 SEM images of CEs containing FD-GTE Rx no.1; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

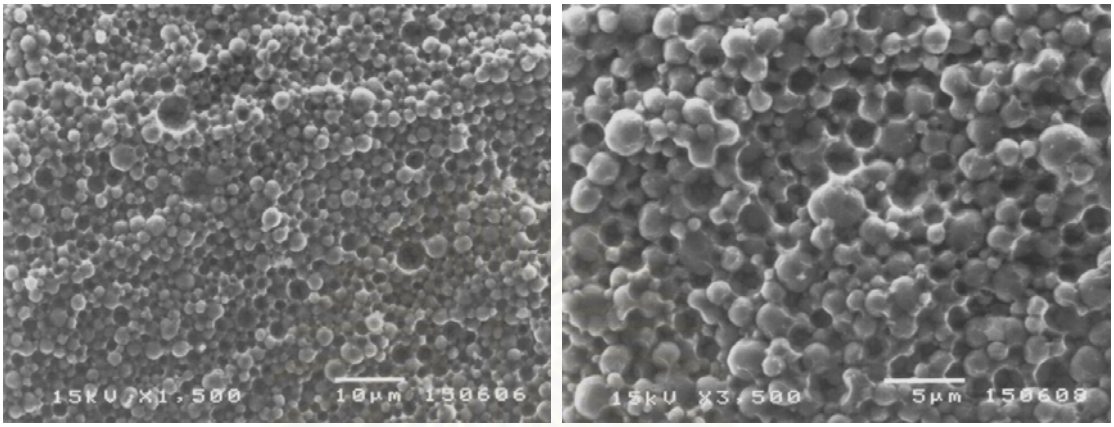
CEs containing FD-GTE Rx no. 2 (3% Arlacel - 86% DP)



(a) (b)

Figure III-47 SEM images of CEs containing FD-GTE Rx no.2; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

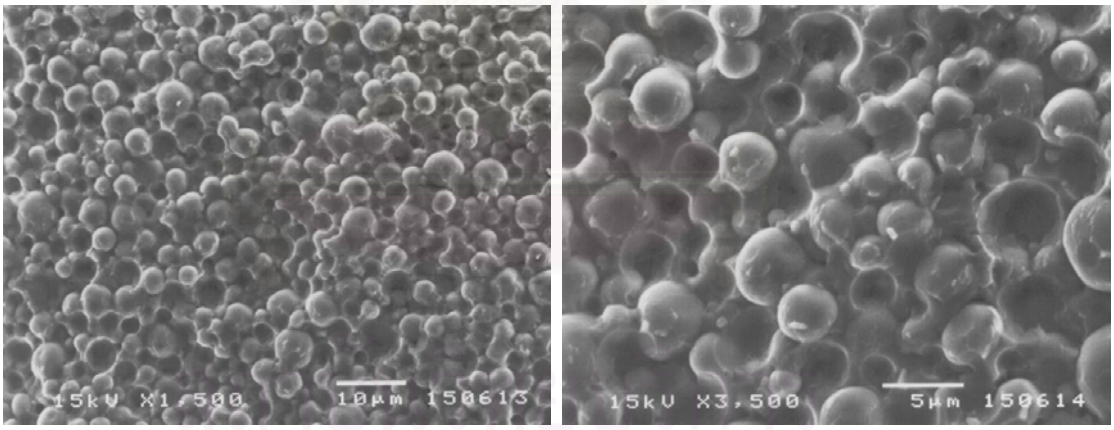
CEs containing FD-GTE Rx no. 3 (3% Arlacel - 88% DP)



(a) (b)

Figure III-48 SEM images of CEs containing FD-GTE Rx no.3; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

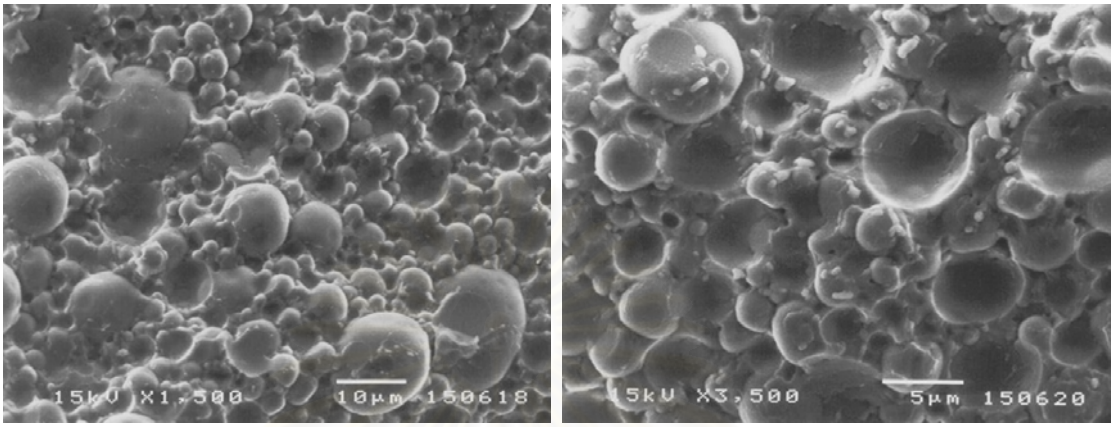
CEs containing FD-GTE Rx no. 4 (3% Abil EM 90 - 84% DP)



(a) (b)

Figure III-49 SEM images of CEs containing FD-GTE Rx no.4; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

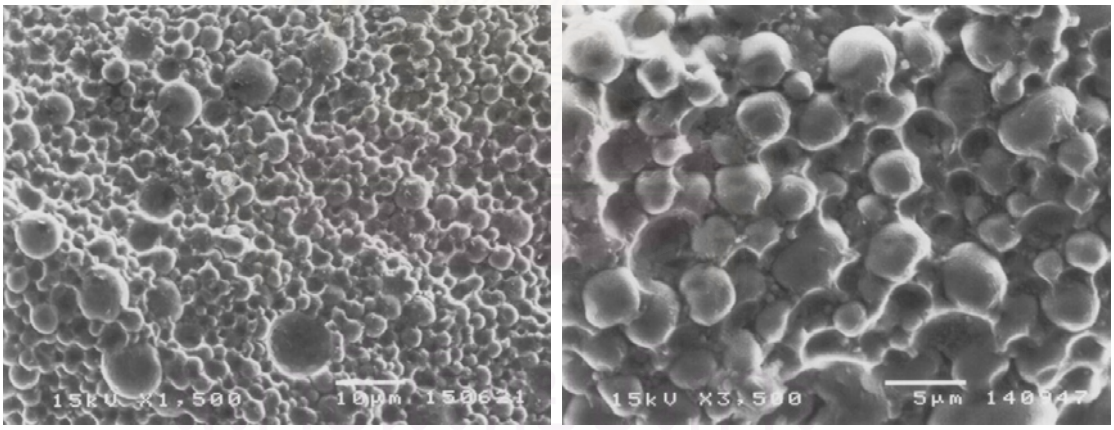
CEs containing FD-GTE Rx no. 5 (3% Abil EM 90 - 86% DP)



(a) (b)

Figure III-50 SEM images of CEs containing FD-GTE Rx no.5; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

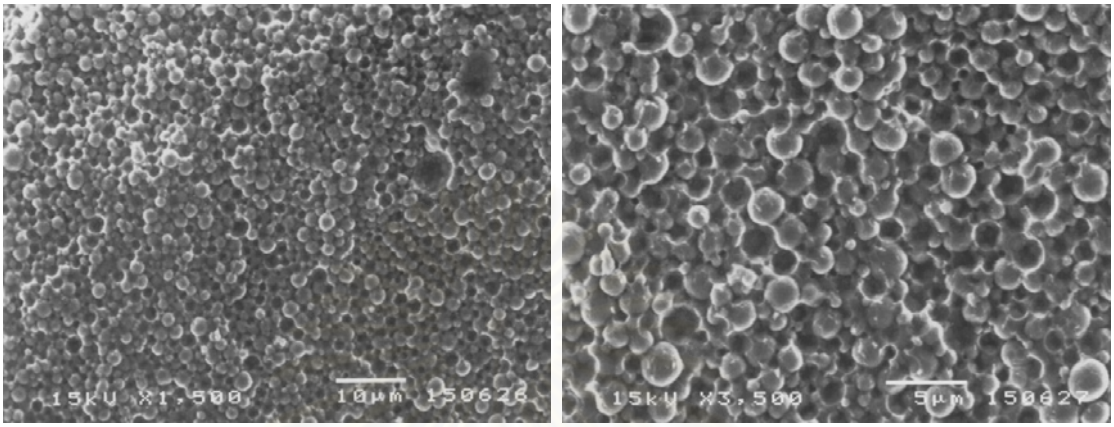
CEs containing FD-GTE Rx no. 6 (3% Abil EM 90 - 88% DP)



(a) (b)

Figure III-51 SEM images of CEs containing FD-GTE Rx no.6; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

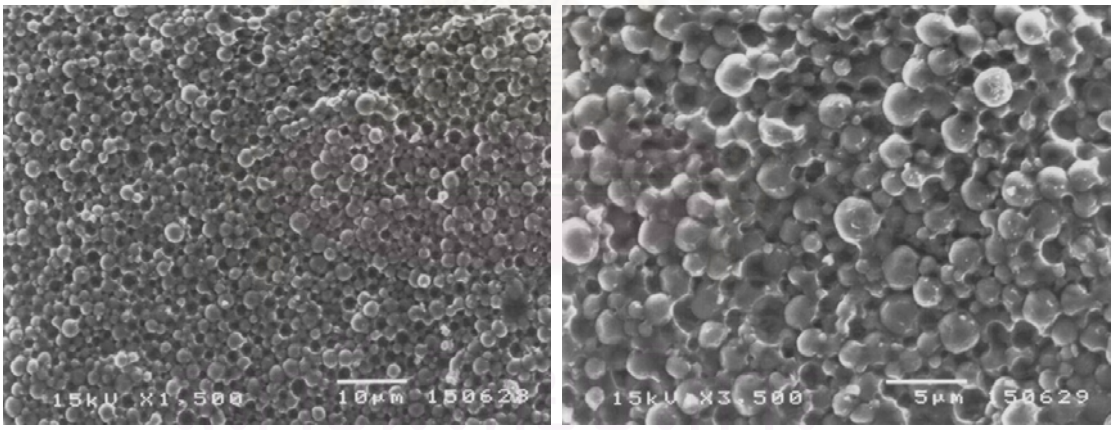
CEs containing FD-GTE Rx no. 7 (3% Isolan DO - 84% DP)



(a) (b)

Figure III-52 SEM images of CEs containing FD-GTE Rx no.7; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

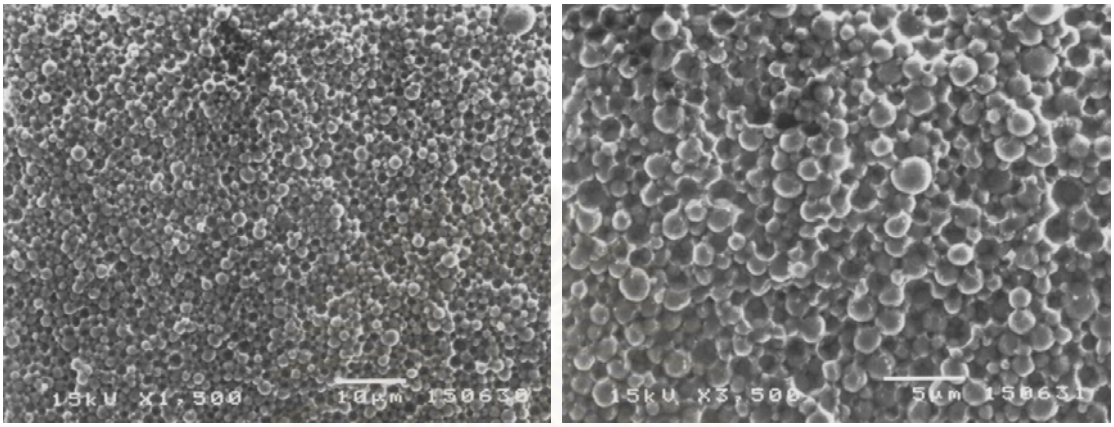
CEs containing FD-GTE Rx no. 8 (3% Isolan DO - 86% DP)



(a) (b)

Figure III-53 SEM images of CEs containing FD-GTE Rx no.8; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

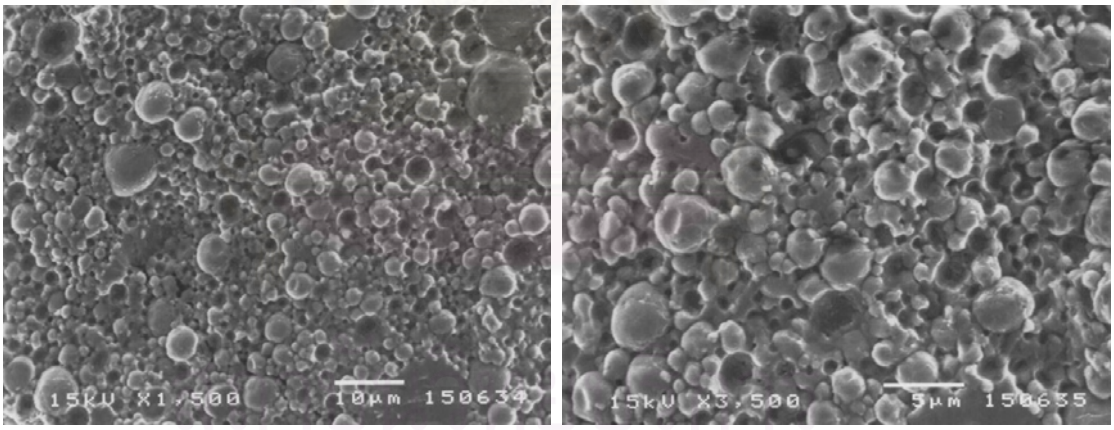
CEs containing FD-GTE Rx no. 9 (3% Isolan DO - 88% DP)



(a) (b)

Figure III-54 SEM images of CEs containing FD-GTE Rx no.9; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

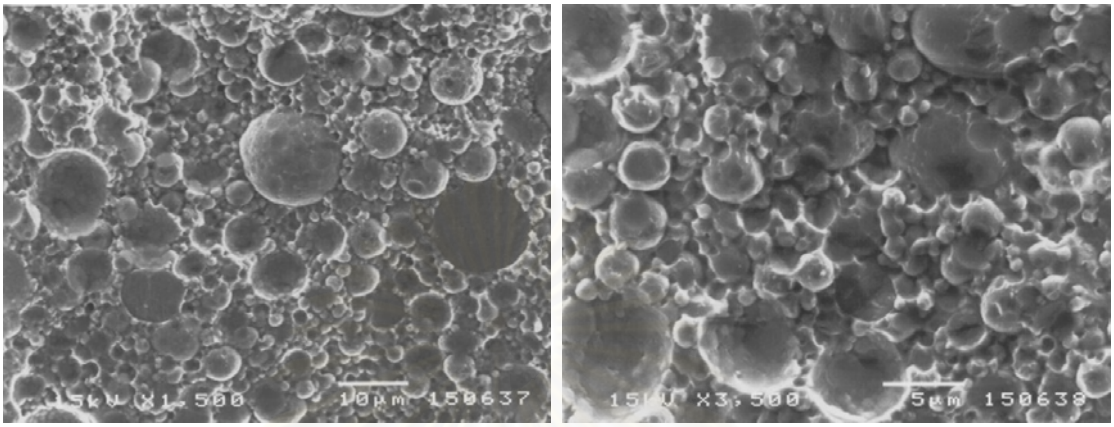
CEs containing FD-GTE Rx no. 10 (2.2% Montane: 0.8% Simusol - 84% DP)



(a) (b)

Figure III-55 SEM images of CEs containing FD-GTE Rx no.10; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

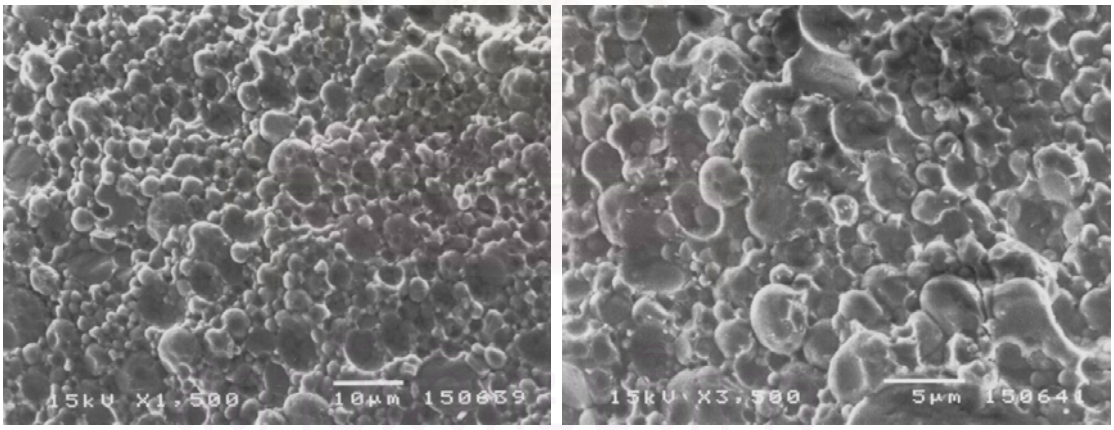
CEs containing FD-GTE Rx no. 11 (2.2% Montane: 0.8% Simusol - 86% DP)



(a) (b)

Figure III-56 SEM images of CEs containing FD-GTE Rx no.11; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

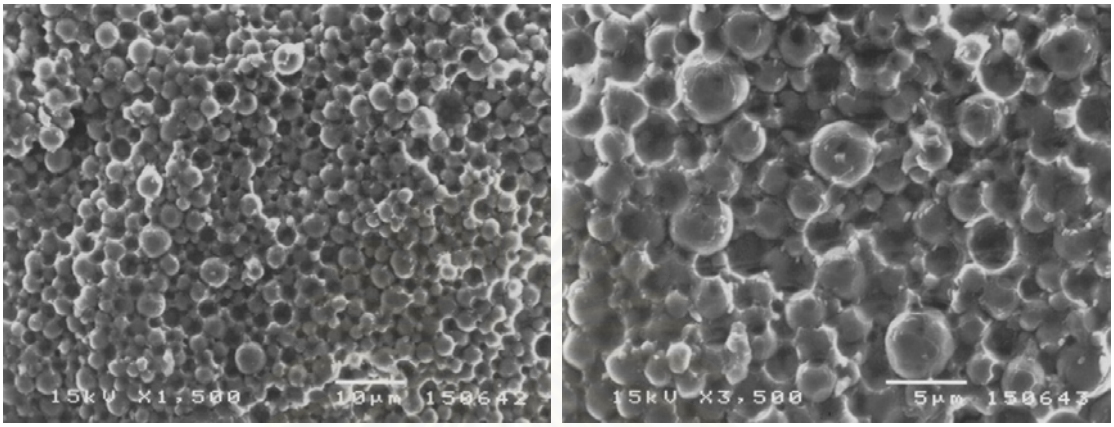
CEs containing FD-GTE Rx no. 12 (2.2% Montane: 0.8% Simusol - 88% DP)



(a) (b)

Figure III-57 SEM images of CEs containing FD-GTE Rx no.12; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

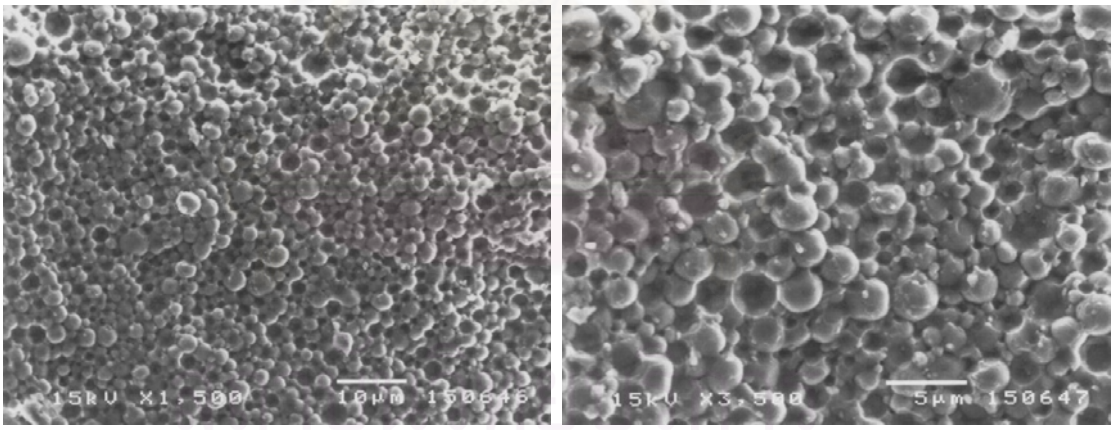
CEs containing FD-GTE Rx no. 13 (1% Isolan DO - 88% DP)



(a) (b)

Figure III-58 SEM images of CEs containing FD-GTE Rx no.13; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

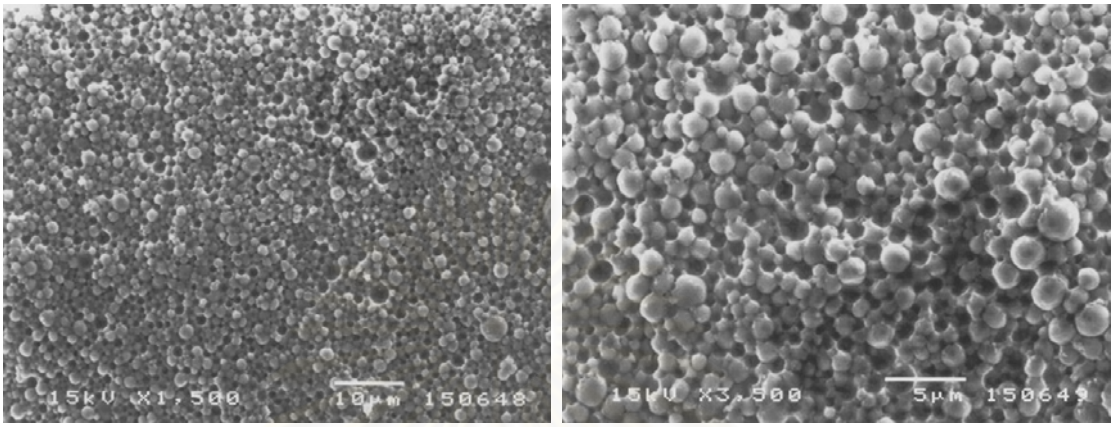
CEs containing FD-GTE Rx no. 14 (2% Isolan DO - 88% DP)



(a) (b)

Figure III-59 SEM images of CEs containing FD-GTE Rx no.14; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

CEs containing FD-GTE Rx no. 15 (4% Isolan DO - 88% DP)



(a) (b)

Figure III-60 SEM images of CEs containing FD-GTE Rx no.15; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 3).

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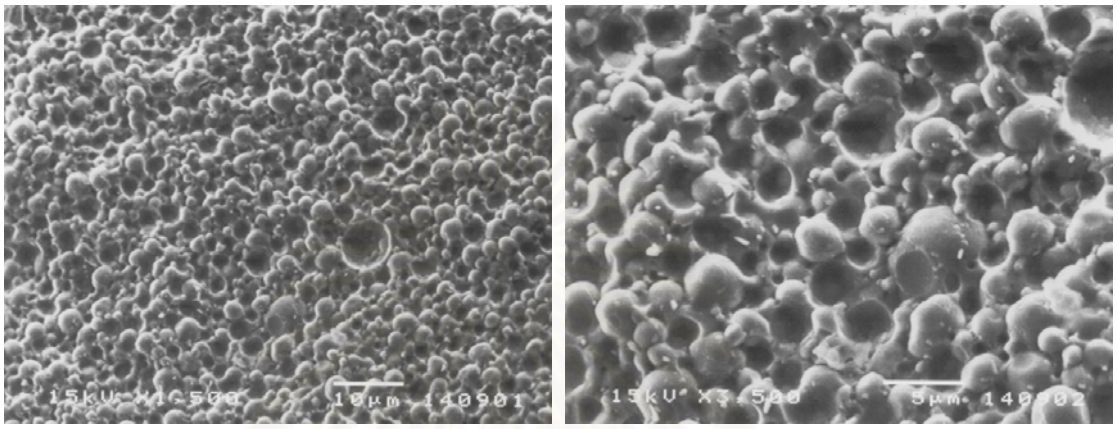
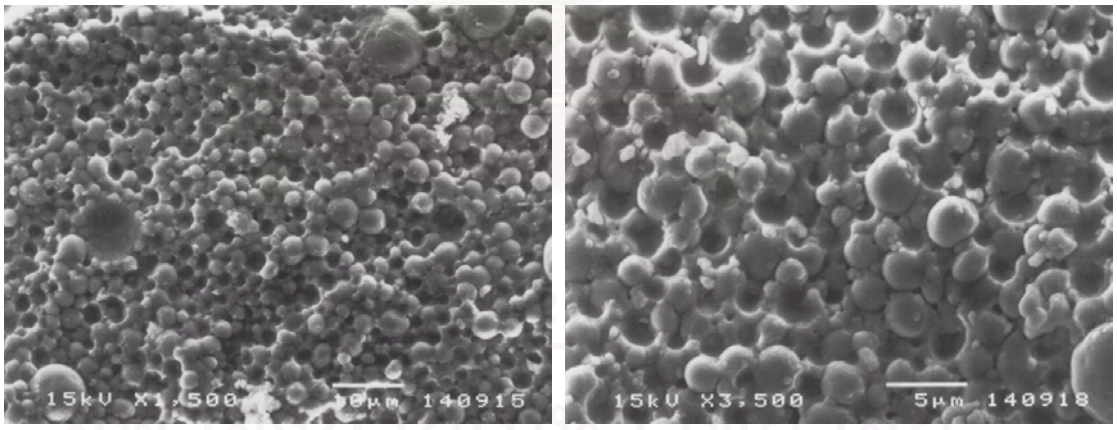
CEs containing FD-GTE Rx no. 1 (3% Arlacel - 84% DP)



(a) (b)

Figure III-61 SEM images of CEs containing FD-GTE Rx no.1; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

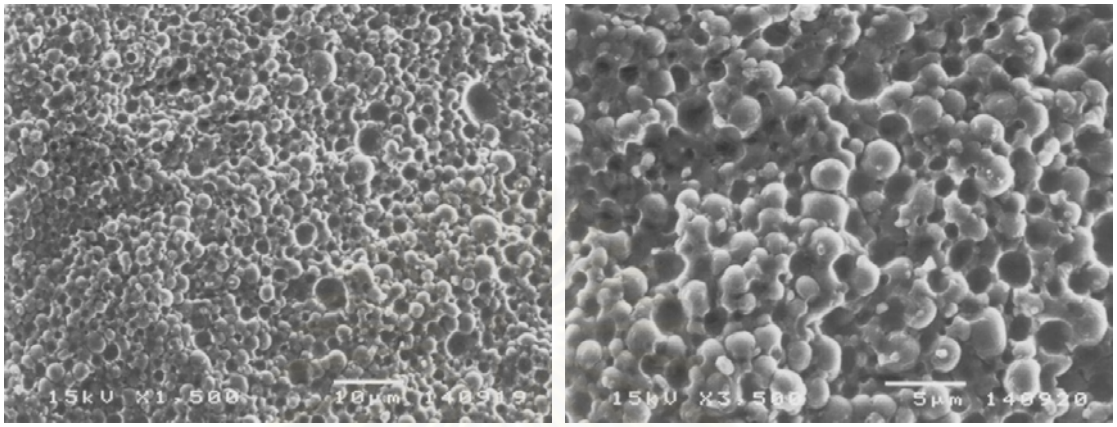
CEs containing FD-GTE Rx no. 2 (3% Arlacel - 86% DP)



(a) (b)

Figure III-62 SEM images of CEs containing FD-GTE Rx no.2; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

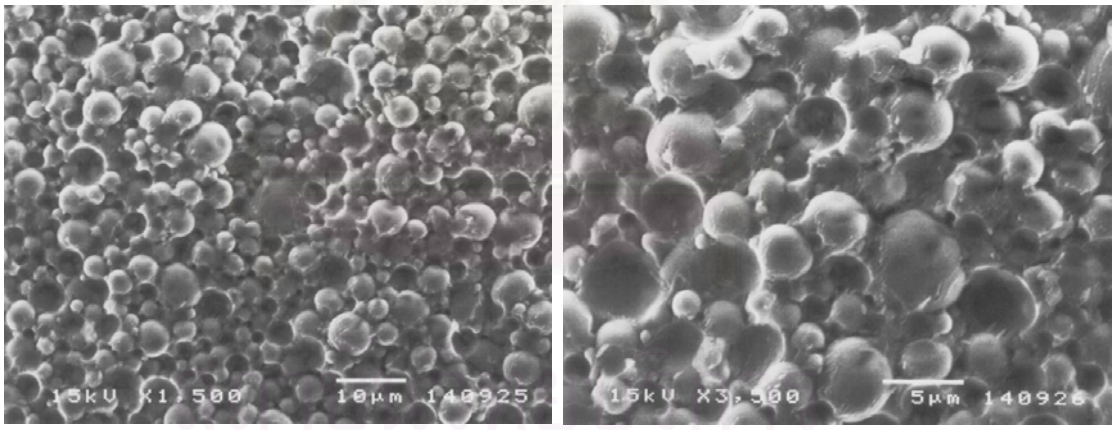
CEs containing FD-GTE Rx no. 3 (3% Arlacel - 88% DP)



(a) (b)

Figure III-63 SEM images of CEs containing FD-GTE Rx no.3; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

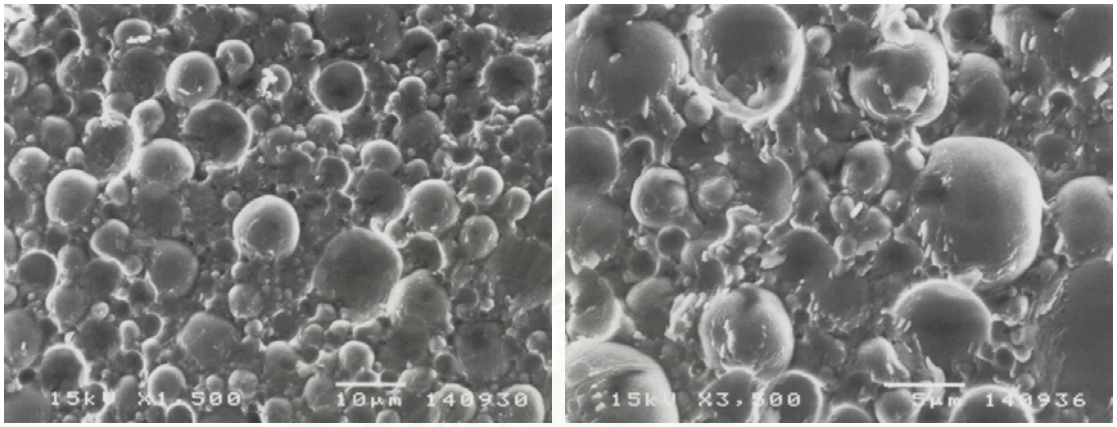
CEs containing FD-GTE Rx no. 4 (3% Abil EM 90 - 84% DP)



(a) (b)

Figure III-64 SEM images of CEs containing FD-GTE Rx no.4; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

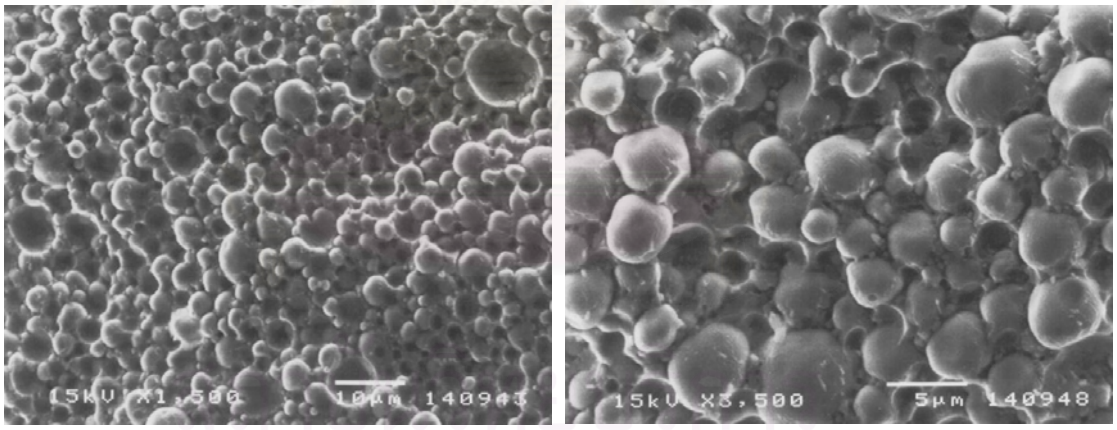
CEs containing FD-GTE Rx no. 5 (3% Abil EM 90 - 86% DP)



(a) (b)

Figure III-65 SEM images of CEs containing FD-GTE Rx no.5; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

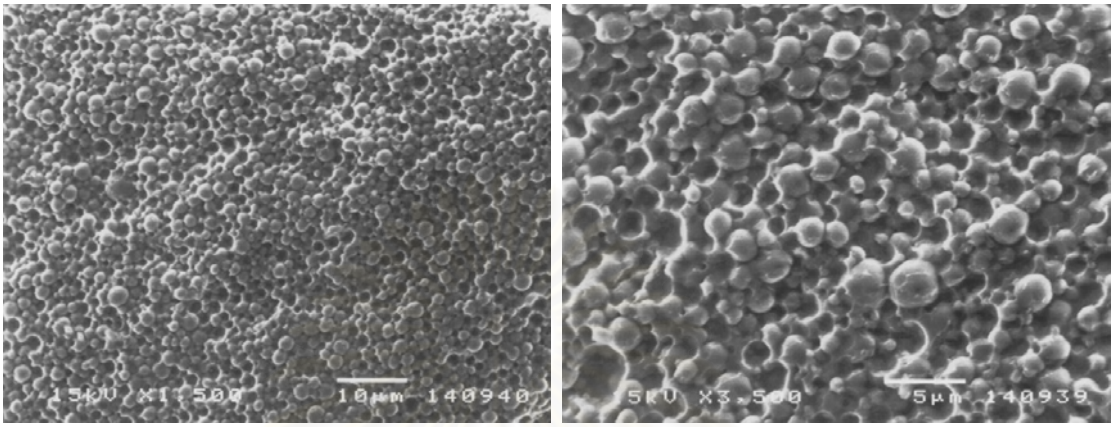
CEs containing FD-GTE Rx no. 6 (3% Abil EM 90 - 88% DP)



(a) (b)

Figure III-66 SEM images of CEs containing FD-GTE Rx no.6; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

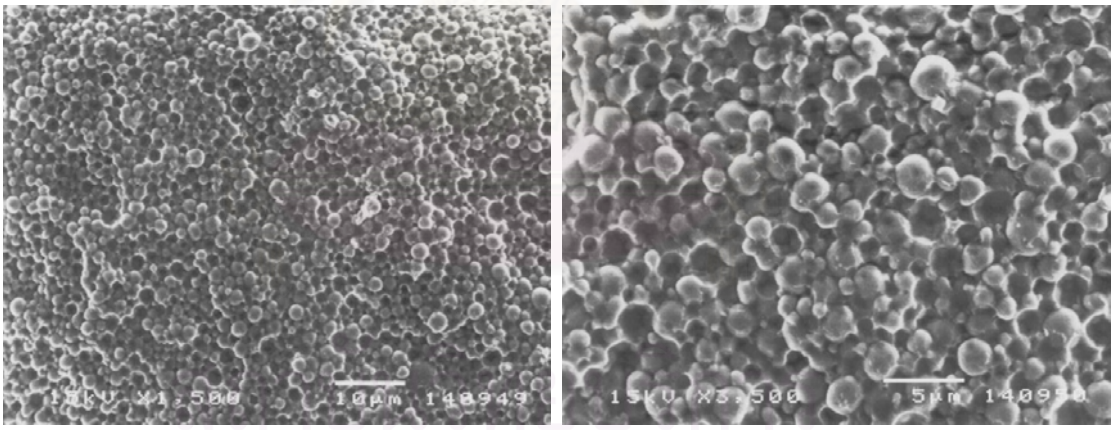
CEs containing FD-GTE Rx no. 7 (3% Isolan DO - 84% DP)



(a) (b)

Figure III-67 SEM images of CEs containing FD-GTE Rx no.7; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

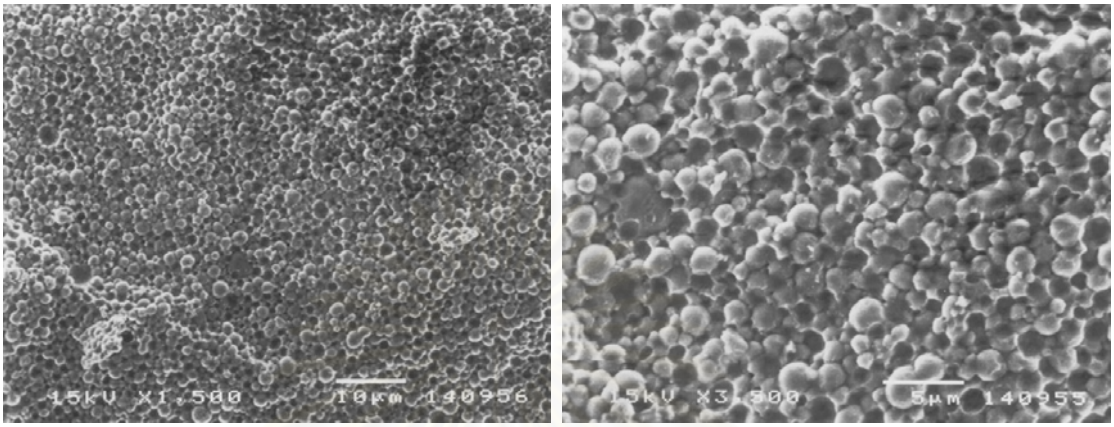
CEs containing FD-GTE Rx no. 8 (3% Isolan DO - 86% DP)



(a) (b)

Figure III-68 SEM images of CEs containing FD-GTE Rx no.8; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

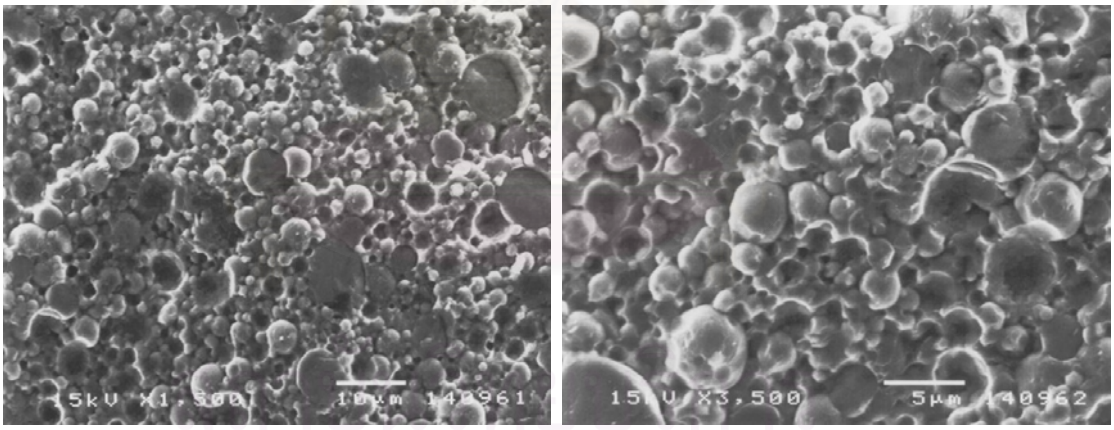
CEs containing FD-GTE Rx no. 9 (3% Isolan DO - 88% DP)



(a) (b)

Figure III-69 SEM images of CEs containing FD-GTE Rx no.9; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

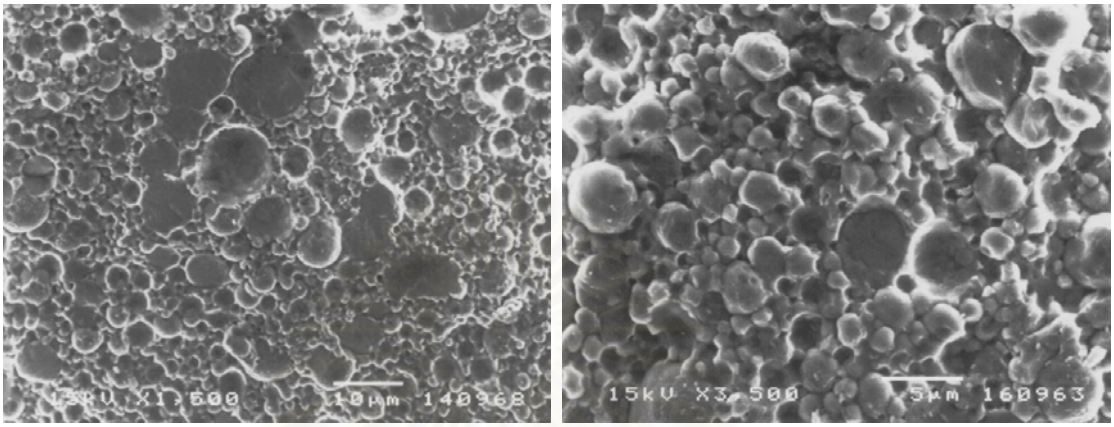
CEs containing FD-GTE Rx no. 10 (2.2% Montane: 0.8% Simusol - 84% DP)



(a) (b)

Figure III-70 SEM images of CEs containing FD-GTE Rx no.10; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

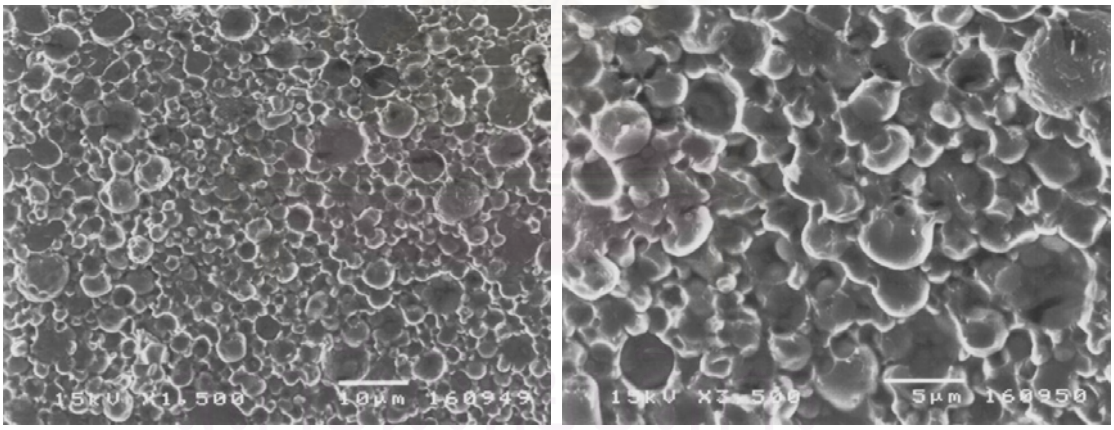
CEs containing FD-GTE Rx no. 11 (2.2% Montane: 0.8% Simusol - 86% DP)



(a) (b)

Figure III-71 SEM images of CEs containing FD-GTE Rx no.11; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

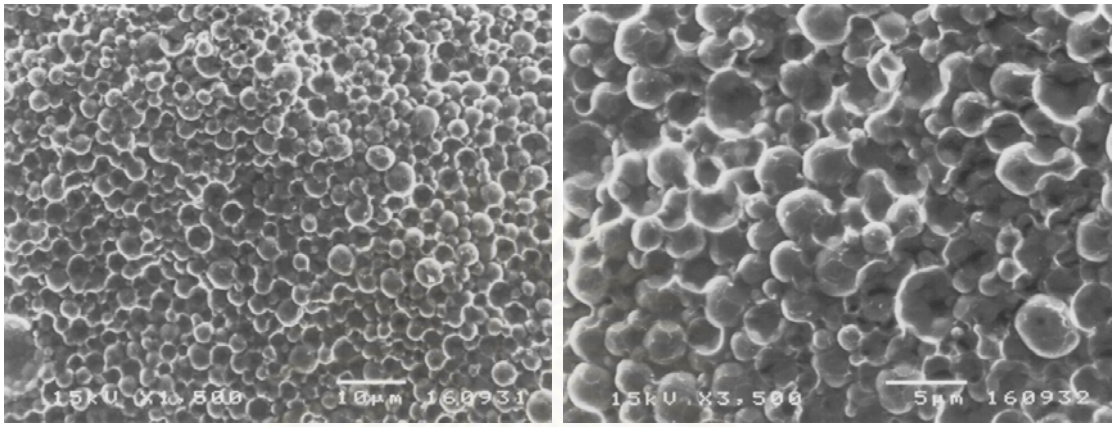
CEs containing FD-GTE Rx no. 12 (2.2% Montane: 0.8% Simusol - 88% DP)



(a) (b)

Figure III-72 SEM images of CEs containing FD-GTE Rx no.12; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

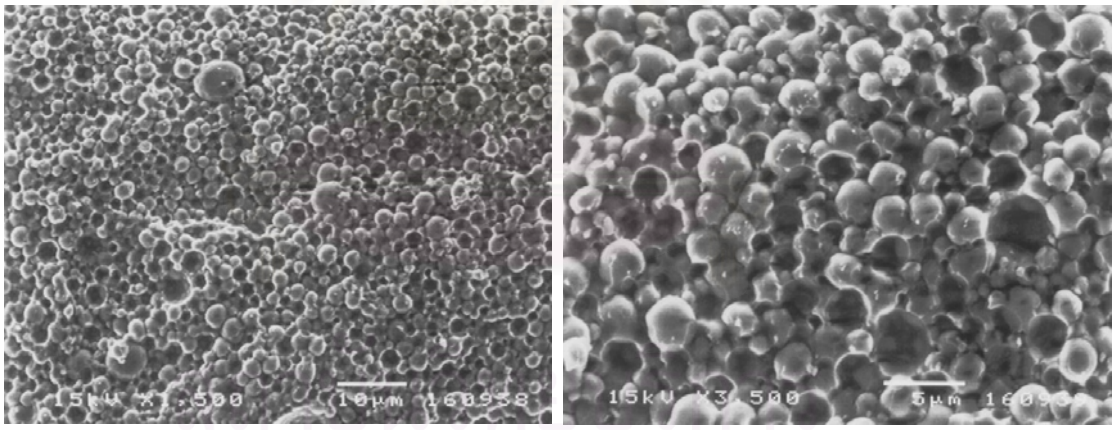
CEs containing FD-GTE Rx no. 13 (1% Isolan DO - 88% DP)



(a) (b)

Figure III-73 SEM images of CEs containing FD-GTE Rx no.13; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

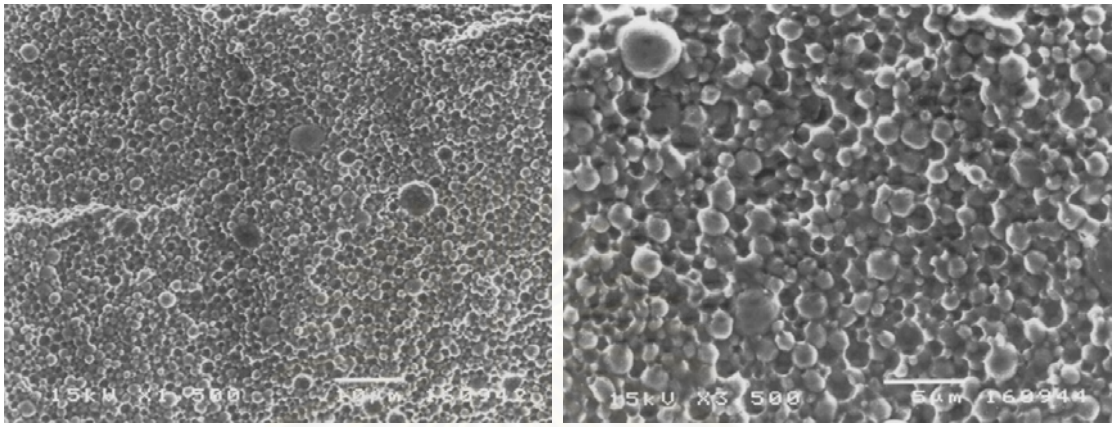
CEs containing FD-GTE Rx no. 14 (2% Isolan DO - 88% DP)



(a) (b)

Figure III-74 SEM images of CEs containing FD-GTE Rx no.14; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

CEs containing FD-GTE Rx no. 15 (4% Isolan DO - 88% DP)



(a) (b)

Figure III-75 SEM images of CEs containing FD-GTE Rx no.15; magnification (a) x 1,500, scale bar 10 µm, (b) x 3,500, scale bar 5 µm (month 6).

APPENDIX IV

MEAN DIAMETER AND DROPLET SIZE DISTRIBUTION OF CONCENTRATED W/O EMULSIONS WITH DIFFERENT FORMULATIONS

Table IV-1 D [v,0.5], D [v,0.9], D [v,0.1], D [4,3], D [3,2] and relative span of CEs containing FD-GTE with different formulation parameters (μm) (mean ± S.D., n = 3) after 1, 8, 15 days, 3 and 6 months.

Rx ID no.	The estimated time intervals	Volume median diameter (mean ± S.D., n = 3)					Polydispersity index (mean ± S.D., n = 3)
		D[v,0.5] (μm)	D[v,0.9] (μm)	D[v,0.1] (μm)	D[4,3] (μm)	D[3,2] (μm)	R.S.
Rx 1	Day 1	2.200 ± 0.020	1.430 ± 0.010	1.430 ± 0.010	2.297 ± 0.012	2.073 ± 0.015	0.807 ± 0.012
	Day 8	2.230 ± 0.035	3.860 ± 0.069	1.183 ± 0.012	2.393 ± 0.029	1.947 ± 0.023	1.247 ± 0.007
	Day 15	2.387 ± 0.012	4.213 ± 0.062	1.140 ± 0.006	2.557 ± 0.029	2.037 ± 0.006	1.037 ± 0.021
	Month 3	2.523 ± 0.006	4.997 ± 0.020	1.103 ± 0.006	2.827 ± 0.010	2.023 ± 0.006	1.619 ± 0.004
	Month 6	2.560 ± 0.053	5.073 ± 0.021	1.127 ± 0.047	2.873 ± 0.042	2.083 ± 0.059	1.542 ± 0.043
Rx 2	Day 1	2.087 ± 0.038	3.147 ± 0.131	1.290 ± 0.023	2.163 ± 0.050	1.933 ± 0.036	0.740 ± 0.044
	Day 8	2.170 ± 0.006	3.630 ± 0.015	1.210 ± 0.006	2.320 ± 0.006	1.930 ± 0.006	1.243 ± 0.012
	Day 15	2.193 ± 0.010	3.840 ± 0.006	1.140 ± 0.006	2.413 ± 0.062	1.913 ± 0.010	1.268 ± 0.006
	Month 3	2.293 ± 0.006	4.287 ± 0.023	1.067 ± 0.006	2.517 ± 0.006	1.927 ± 0.006	1.277 ± 0.011
	Month 6	2.373 ± 0.006	4.047 ± 0.015	1.380 ± 0.020	2.577 ± 0.006	2.133 ± 0.047	1.396 ± 0.018
Rx 3	Day 1	1.507 ± 0.021	2.427 ± 0.021	0.907 ± 0.006	1.863 ± 0.110	1.403 ± 0.012	0.943 ± 0.019
	Day 8	1.547 ± 0.012	2.353 ± 0.025	0.953 ± 0.006	1.613 ± 0.015	1.423 ± 0.006	0.969 ± 0.008
	Day 15	1.697 ± 0.012	3.253 ± 0.164	1.563 ± 0.010	1.947 ± 0.046	1.563 ± 0.010	0.623 ± 0.081
	Month 3	1.867 ± 0.006	2.857 ± 0.025	1.143 ± 0.006	1.943 ± 0.006	1.713 ± 0.006	0.978 ± 0.018
	Month 6	1.943 ± 0.015	4.487 ± 0.223	1.037 ± 0.000	2.987 ± 0.204	1.773 ± 0.015	1.904 ± 0.104

Table IV-2 D [v,0.5], D [v,0.9], D [v,0.1], D [4,3], D [3,2] and relative span of CEs containing FD-GTE with different formulation parameters (μm) (mean ± S.D., n = 3) after 1, 8, 15 days, 3 and 6 months.

Rx ID no.	The estimated time intervals	Volume median diameter (mean ± S.D., n = 3)					Polydispersity index (mean ± S.D., n = 3)
		D[v,0.5] (μm)	D[v,0.9] (μm)	D[v,0.1] (μm)	D[4,3] (μm)	D[3,2] (μm)	R.S.
Rx 4	Day 1	2.177 ± 0.017	4.353 ± 0.023	0.847 ± 0.000	2.413 ± 0.015	1.857 ± 0.010	0.472 ± 0.008
	Day 8	2.277 ± 0.012	4.630 ± 0.050	0.947 ± 0.006	2.563 ± 0.015	1.867 ± 0.006	1.287 ± 0.016
	Day 15	2.427 ± 0.015	4.827 ± 0.031	0.953 ± 0.010	2.697 ± 0.015	1.950 ± 0.012	1.171 ± 0.007
	Month 3	2.693 ± 0.015	4.187 ± 0.035	1.733 ± 0.006	2.847 ± 0.017	2.487 ± 0.010	1.494 ± 0.011
	Month 6	2.910 ± 0.006	4.777 ± 0.081	2.057 ± 0.006	3.323 ± 0.079	2.790 ± 0.012	2.143 ± 0.034
Rx 5	Day 1	2.573 ± 0.006	12.320 ± 0.431	0.813 ± 0.025	5.157 ± 0.078	2.157 ± 0.020	3.105 ± 0.140
	Day 8	2.817 ± 0.006	8.593 ± 0.104	1.423 ± 0.000	4.210 ± 0.046	2.393 ± 0.000	3.464 ± 0.037
	Day 15	3.037 ± 0.015	12.743 ± 0.371	1.227 ± 0.006	5.417 ± 0.121	2.437 ± 0.006	4.021 ± 0.108
	Month 3	3.237 ± 0.020	12.033 ± 0.237	0.737 ± 0.000	5.163 ± 0.080	2.050 ± 0.006	2.926 ± 0.056
	Month 6	3.520 ± 0.006	12.557 ± 0.163	1.353 ± 0.000	5.503 ± 0.040	2.643 ± 0.000	3.745 ± 0.046
Rx 6	Day 1	1.733 ± 0.010	7.077 ± 0.275	0.670 ± 0.023	3.617 ± 0.165	1.500 ± 0.015	2.962 ± 0.137
	Day 8	2.130 ± 0.012	5.017 ± 0.137	1.267 ± 0.010	3.017 ± 0.066	1.950 ± 0.012	2.560 ± 0.088
	Day 15	2.290 ± 0.000	5.677 ± 0.373	1.343 ± 0.006	3.710 ± 0.114	2.110 ± 0.000	2.605 ± 0.196
	Month 3	2.820 ± 0.010	5.323 ± 0.031	2.430 ± 0.010	3.183 ± 0.006	2.430 ± 0.010	1.596 ± 0.020
	Month 6	3.450 ± 0.000	9.867 ± 0.189	1.037 ± 0.012	5.453 ± 0.125	2.340 ± 0.017	2.559 ± 0.058

Table IV-3 D [v,0.5], D [v,0.9], D [v,0.1], D [4,3], D [3,2] and relative span of CEs containing FD-GTE with different formulation parameters (μm) (mean ± S.D., n = 3) after 1, 8, 15 days, 3 and 6 months.

Rx ID no.	The estimated time intervals	Volume median diameter (mean ± S.D., n = 3)					Polydispersity index (mean ± S.D., n = 3)
		D[v,0.5] (μm)	D[v,0.9] (μm)	D[v,0.1] (μm)	D[4,3] (μm)	D[3,2] (μm)	R.S.
Rx 7	Day 1	1.587 ± 0.015	2.657 ± 0.031	0.823 ± 0.010	1.683 ± 0.020	1.427 ± 0.015	0.738 ± 0.005
	Day 8	1.647 ± 0.006	2.597 ± 0.021	0.927 ± 0.006	1.853 ± 0.104	1.460 ± 0.010	1.014 ± 0.009
	Day 15	1.733 ± 0.012	2.830 ± 0.021	0.970 ± 0.015	1.857 ± 0.026	1.557 ± 0.017	1.052 ± 0.019
	Month 3	1.747 ± 0.015	2.667 ± 0.032	1.113 ± 0.006	1.840 ± 0.010	1.620 ± 0.010	1.139 ± 0.028
	Month 6	1.843 ± 0.006	2.667 ± 0.006	1.173 ± 0.006	1.893 ± 0.006	1.703 ± 0.006	0.972 ± 0.005
Rx 8	Day 1	1.643 ± 0.015	2.570 ± 0.026	0.893 ± 0.021	1.723 ± 0.031	1.397 ± 0.015	0.846 ± 0.028
	Day 8	1.683 ± 0.006	2.547 ± 0.006	0.997 ± 0.012	1.740 ± 0.010	1.520 ± 0.010	0.983 ± 0.009
	Day 15	1.717 ± 0.006	2.680 ± 0.006	0.997 ± 0.006	1.790 ± 0.006	1.547 ± 0.006	1.033 ± 0.006
	Month 3	1.753 ± 0.006	2.777 ± 0.036	1.007 ± 0.035	1.840 ± 0.021	1.577 ± 0.026	1.068 ± 0.017
	Month 6	1.783 ± 0.006	2.797 ± 0.042	1.040 ± 0.000	1.890 ± 0.044	1.620 ± 0.000	0.985 ± 0.026
Rx 9	Day 1	1.357 ± 0.012	2.030 ± 0.036	0.787 ± 0.021	1.390 ± 0.010	1.230 ± 0.010	0.785 ± 0.030
	Day 8	1.383 ± 0.006	2.033 ± 0.006	0.823 ± 0.006	1.413 ± 0.006	1.253 ± 0.006	0.919 ± 0.004
	Day 15	1.437 ± 0.021	2.137 ± 0.025	0.850 ± 0.015	1.497 ± 0.015	1.297 ± 0.012	0.901 ± 0.019
	Month 3	1.507 ± 0.000	2.407 ± 0.040	0.880 ± 0.006	1.597 ± 0.020	1.380 ± 0.006	0.942 ± 0.023
	Month 6	1.550 ± 0.006	2.193 ± 0.026	1.033 ± 0.017	1.587 ± 0.006	1.450 ± 0.012	0.921 ± 0.032

Table IV-4 D [v,0.5], D [v,0.9], D [v,0.1], D [4,3], D [3,2] and relative span of CEs containing FD-GTE with different formulation parameters (μm) (mean ± S.D., n = 3) after 1, 8, 15 days, 3 and 6 months.

Rx ID no.	The estimated time intervals	Volume median diameter (mean ± S.D., n = 3)					Polydispersity index (mean ± S.D., n = 3)
		D[v,0.5] (μm)	D[v,0.9] (μm)	D[v,0.1] (μm)	D[4,3] (μm)	D[3,2] (μm)	R.S.
Rx 10	Day 1	2.453 ± 0.021	4.370 ± 0.240	1.460 ± 0.053	2.727 ± 0.061	2.283 ± 0.006	1.186 ± 0.110
	Day 8	2.633 ± 0.010	5.850 ± 0.049	1.113 ± 0.010	3.123 ± 0.010	2.100 ± 0.006	1.924 ± 0.030
	Day 15	2.630 ± 0.015	5.990 ± 0.059	0.947 ± 0.006	3.110 ± 0.032	2.013 ± 0.010	1.670 ± 0.010
	Month 3	2.877 ± 0.020	9.927 ± 0.202	0.763 ± 0.006	4.520 ± 0.127	1.960 ± 0.006	2.752 ± 0.049
	Month 6	3.090 ± 0.012	9.170 ± 0.194	1.500 ± 0.006	4.513 ± 0.068	2.530 ± 0.006	3.584 ± 0.065
Rx 11	Day 1	2.643 ± 0.038	5.177 ± 0.248	1.440 ± 0.020	3.040 ± 0.095	2.403 ± 0.038	1.413 ± 0.069
	Day 8	2.763 ± 0.012	6.140 ± 0.072	1.093 ± 0.006	3.257 ± 0.021	2.150 ± 0.010	1.826 ± 0.022
	Day 15	2.943 ± 0.012	7.660 ± 0.042	0.873 ± 0.006	3.697 ± 0.017	2.000 ± 0.006	2.229 ± 0.006
	Month 3	3.287 ± 0.050	8.643 ± 0.495	1.337 ± 0.012	4.303 ± 0.164	2.483 ± 0.010	2.824 ± 0.133
	Month 6	3.837 ± 0.051	9.947 ± 0.078	1.020 ± 0.010	4.783 ± 0.047	2.383 ± 0.021	2.327 ± 0.014
Rx 12	Day 1	2.937 ± 0.035	5.600 ± 0.110	1.470 ± 0.000	3.283 ± 0.045	2.567 ± 0.015	1.274 ± 0.021
	Day 8	2.997 ± 0.006	6.960 ± 0.036	1.050 ± 0.010	3.580 ± 0.010	2.180 ± 0.010	2.005 ± 0.012
	Day 15	3.037 ± 0.020	7.710 ± 0.091	0.783 ± 0.006	3.723 ± 0.021	1.997 ± 0.010	1.885 ± 0.023
	Month 3	3.160 ± 0.015	7.390 ± 0.081	1.287 ± 0.006	3.843 ± 0.040	2.417 ± 0.015	2.302 ± 0.015
	Month 6	3.270 ± 0.010	6.677 ± 0.047	1.527 ± 0.006	3.750 ± 0.020	2.713 ± 0.006	1.847 ± 0.012

Table IV-5 D [v,0.5], D [v,0.9], D [v,0.1], D [4,3], D [3,2] and relative span of CEs containing FD-GTE with different formulation parameters (μm) (mean ± S.D., n = 3) after 1, 8, 15 days, 3 and 6 months.

Rx ID no.	The estimated time intervals	Volume median diameter (mean ± S.D., n = 3)					Polydispersity index (mean ± S.D., n = 3)
		D[v,0.5] (μm)	D[v,0.9] (μm)	D[v,0.1] (μm)	D[4,3] (μm)	D[3,2] (μm)	R.S.
Rx 13	Day 1	2.350 ± 0.012	3.760 ± 0.035	1.357 ± 0.015	2.470 ± 0.012	2.150 ± 0.006	0.679 ± 0.016
	Day 8	2.427 ± 0.010	4.473 ± 0.068	1.273 ± 0.006	2.700 ± 0.021	2.127 ± 0.000	1.438 ± 0.035
	Day 15	2.433 ± 0.006	4.307 ± 0.061	1.243 ± 0.023	2.633 ± 0.006	2.103 ± 0.015	1.269 ± 0.038
	Month 3	2.597 ± 0.012	4.647 ± 0.055	1.397 ± 0.038	2.863 ± 0.010	2.257 ± 0.038	1.499 ± 0.040
	Month 6	2.690 ± 0.017	4.303 ± 0.106	1.597 ± 0.021	2.843 ± 0.031	2.453 ± 0.006	1.006 ± 0.042
Rx 14	Day 1	1.917 ± 0.025	3.230 ± 0.086	0.993 ± 0.000	2.033 ± 0.036	1.610 ± 0.006	0.937 ± 0.028
	Day 8	2.010 ± 0.012	3.370 ± 0.055	1.153 ± 0.010	2.160 ± 0.015	1.810 ± 0.012	1.250 ± 0.033
	Day 15	2.063 ± 0.010	3.640 ± 0.057	1.100 ± 0.035	3.203 ± 0.207	1.400 ± 0.085	1.317 ± 0.052
	Month 3	2.067 ± 0.010	3.377 ± 0.000	1.133 ± 0.012	2.180 ± 0.006	1.847 ± 0.010	0.960 ± 0.010
	Month 6	2.150 ± 0.006	3.247 ± 0.006	1.360 ± 0.015	2.240 ± 0.006	1.997 ± 0.012	0.996 ± 0.013
Rx 15	Day 1	1.100 ± 0.015	1.727 ± 0.020	0.677 ± 0.020	1.317 ± 0.010	1.033 ± 0.021	0.893 ± 0.041
	Day 8	1.177 ± 0.006	1.767 ± 0.029	0.737 ± 0.006	1.283 ± 0.023	1.090 ± 0.000	0.964 ± 0.036
	Day 15	1.253 ± 0.000	1.980 ± 0.006	0.813 ± 0.000	1.340 ± 0.006	1.173 ± 0.000	1.214 ± 0.005
	Month 3	1.343 ± 0.006	2.130 ± 0.017	0.827 ± 0.006	1.530 ± 0.010	1.257 ± 0.006	1.005 ± 0.020
	Month 6	1.467 ± 0.015	2.327 ± 0.045	1.377 ± 0.006	1.570 ± 0.020	1.377 ± 0.006	0.648 ± 0.039

VITA

Miss Saranya Ukkarawittayapumi was born on July 11, 1981 in Bangkok, Thailand. She graduated with the Bachelor Degree of Science in Pharmacy from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand in 2003. After graduation, she has enrolled for the Master's Degree of Science in the Pharmaceutical Technology (International Program) at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.