University of Alberta

Evaluation of Performance Testing and Computer Simulations for Quality by Design Approaches of Oral Dosage Forms

by

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Abstract

Performance testing and computer simulations have promising applications in Quality by Design approaches. The objectives of these studies were to investigate the performance of the disintegration test using different setups in addition to comparing the performance of the disintegration test with the rupture test using soft gelatin dietary supplements capsules. Classifying common herbs according to the Biopharmaceutical Classification System approach was also investigated using *ADMET predictor* ^{TM}. The final objective was evaluation the predictive power of computer simulations of in vitro dissolution in different media. The studies concluded that the disintegration test is robust only if firm specifications were applied. However, this test has no advantage over the rupture test. *In silico* methods can be used to classify herbs according to the BCS. Computer simulations of dissolution *in vitro* can be also a potential tool to estimate the dissolution behavior. These tools facilitate prediction of quality desired in a product.

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LIST OF ABBREVIATION

ANOVA	Analysis of variances
API	Active pharmaceutical ingredient
App	Apparatus
BCS	Biopharmaceutical classification system
BDDCS	Biopharmaceutics drug disposition classification system
BFaSSIF	Blank Fasted Simulated State Intestinal Fluid
CQAs	Critical quality attributes
Cs	Aqueous solubility
D0	Dose number
DoE	Design of experiment
EMA	European medicines agency
Exp	Expiry
FaSSIF	Fasted Simulated State Intestinal Fluid
FDA	Food and drug administration
FIP	International pharmaceutical federation
HPMC	Hydroxyl propyl methyl callous
HQ-FaSSIF	High Quality Fasted Simulated State Intestinal Fluid
HVDs	High variable drugs
ICH	International conference on harmonization
IR	Immidate release
IVIVC	In vitro/in vivo correlation
LB	Large beaker
LQ-FaSSIF	Low Quality Fasted Simulated State Intestinal Fluid
mg	Milligram
mg/mL	Milligram per milliliter
min	Minutes
mL	Milliliter
ml/min	Milliliter per minutes
mm	Millimeter

mM	Millimole
Mx	Solubility border value
Na	Sodium
NaP	Sodium based buffer
Peff	Permeability
PF	Pharmacopeia forum
pH	Acidity or alkalinity of measurement
PKa	Acid dissociation
QbD	Quality by design
QbT	Quality by testing
RPM	Ram per minute
S	Supplement
SB	Small beaker
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SUPAC	Scale up and post approval change
USP	United State Pharmacopeia
USP-NF	United State Pharmacopeia national formulary
V0	Volume of water
WHO	World health organization
%	Percent
°C	Degree centigrade

Chapter 1

Introduction

1.1 Background

Quality is the suitability of a product to its purpose. A product that is free from contamination and defects and delivers the labelled therapeutic benefits reproducibly has high quality.[1, 2] Performance, reliability, and durability are the dimensions of quality.[3] Planned quality incorporated into the product is quality by design (QbD).

1.2 The quality by design approach in pharmaceutical science

QbD pharmaceutical science was proposed by the Food and Drug Administration (FDA) and the International Conference on Harmonisation (ICH) in 2005.[4, 5] It is defined as "a systematic approach to development that begins with predefined objectives and emphasizes the product and process understanding and process control based on sound science and quality risk management".[6] This approach demands a full understanding of how a product's formulation and process impact the quality of the product.[7, 8] This understanding is implemented through the design of the experiment (DoE).[9, 10] A structured and organized method includes determining and understanding the changing critical quality attributes (CQAs) of the product (including both active ingredients and excipients), for example, physiochemical bioavailability and dissolution release profile.[11] CQAs are properties that impact consumer safety and product efficacy.[12, 13, 14, 15] These factors are studied by first varying them, identifying variability and evaluating

risk, and then determining which factors have a critical influence on product performance. A detailed description of all steps in the process and the expected outcome are required. QbD cannot completely prevent variability because variability can be due to instructions, environment, raw materials, devices, methods, manufacturing system, or personal factors.[3] QbD implies understanding sources of variability and their impact on the final product and then controlling this variability.[3] In the QbD approach objectives are defined, CQAs are identified, manufacturing is designed and developed, and sources of variation are controlled (Figure 1.1).[3, 16 ,17] The quality of the product is then determined by its performance. If QbD is followed carefully, the need for final product testing is reduced, or even eliminated.[18, 19]

Figure1.1:A quality by design approach.
*Modifed *et al.* Huang 2009

1.3 Quality by testing versus quality by design

The QbD approach is different from the traditional quality by testing approach (QbT). The QbT approach is based on testing the quality of the final product to identify any batch that varies from particular manufacturing specifications using pharmacopeia methods. In the QbT approach, a poor quality batch will be detected only after the batch was manufactured. In other words, this

approach might lead to a loss of materials, time and money. Furthermore, this approach cannot identify the root cause of variation nor can it suggest any way to prevent such events from happening again even though the sample batch undergoes extensive testing.[3] Recalling multiple products after distribution is evidence that inspection using QbT does not guarantee that batches which pass such tests are free from defects.[3] Furthermore, applying a QbT approach does not allow any flexibility in a manufacturing process.[20] Adoption of a QbD approach will overcome such problems. [16] Also, when using the QbD approach operating adjustments are not considered a change if the operating design space was approved.[16] Therefore, quality by design is useful throughout the product lifecycle.[20] Drug discovery and development is very time consuming; QbD can reduce the time required in this stage by at least one to three years and save a minimum of $102–290 million.[21] Snee et al. stated that ignoring the QbD approach is just like the tale about blind people who want to see what the elephant look like, so each one touches a different part and describes it; the blind people all came to different conclusions about the elephant's shape.[20] Process understanding gives a full image of product quality.[22, 23]

1.4 Mechanistic understanding and manufacturing control: Performance tests

Immediate release (IR) dosage forms are the most commonly used oral formulation because they have many advantages for industries and consumers. Oral preparations are convenient to administer and mostly easy to manufacture.[24]

IR dosage forms first disintegrate, dissolve, and then get absorbed by the body (Figure1.2).The first two processes can be evaluated using standardized tests. Mechanistic understanding through *in vitro* experiments allows the formulator to adapt drug release pattern for optimized absorption. These tests are also used to confirm product performance from the early development stage to the final manufacturing stage before the batch is released.[25] Disintegration tests and dissolution tests are important performance tests used for oral preparations.

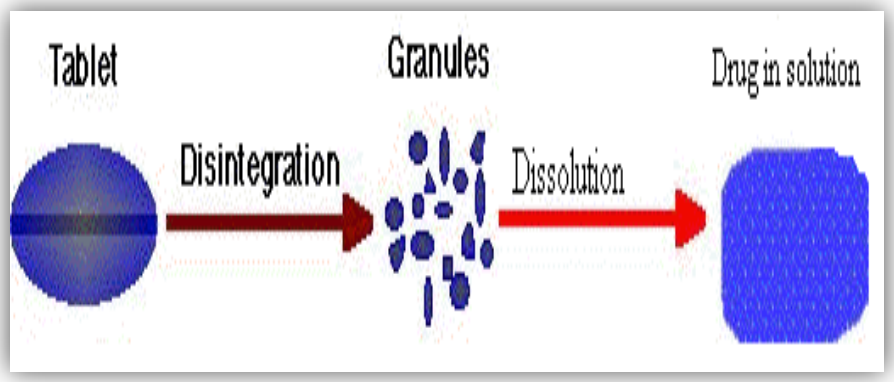

Figure 1.2: Immediate release of ingested oral dosage form: *In vivo* pathway.
*(Modified from http://www.boomer.org/c/p3/c04/c0413.html)

1.4.1 Disintegration test

A standardized disintegration test is used to verify disintegration of IR oral dosage forms under defined experimental conditions. This test is discussed in chapter 2. Disintegration has not received as much attention as dissolution testing, possibly because unlike dissolution tests, disintegration tests cannot provide specific release profiles. Disintegration tests provide the time point at which units

disintegrate. Disintegration tests in QbD may be valuable for IR dosage forms, which are prepared to have quick onset of action. Quick onset requires the drug to dissolve on disintegration; thus for such formulations the disintegration test is important. The disintegration test is believed to represent the expected *in vivo* disintegration and is used to direct IR formulation design.[26] Therefore, this test must be reliable and reproducible.

1.4.2 Dissolution test

Drug dissolution is the second step that the IR oral dosage form must undergo prior to drug absorption. The rate and extent of dissolution depends on physiochemical properties of the drug (such as pKa, solubility, and permeability) and *in vivo* physiological factors (motility rate, volume, pH, and food content).[27, 28] The *in vitro* dissolution test has many useful applications throughout the product life cycle. In the QbD approach, the dissolution test is an important tool to develop a mechanistic understanding of a desired product formulation.[29] Moreover; it might be used to simulate *in vivo* dissolution and to build a possible correlation between dissolution and availability studies. It is also used as a quality control test to check batch to batch consistency in QbT. Dissolution tests can be performed in one of seven dissolution apparatuses.[30, 31] These apparatuses are listed in the USP: basket (apparatus 1), paddle (apparatus 2), reciprocating cylinder (apparatus 3), flow through cell (apparatus 4), paddle over disc (apparatus 5), cylinder (apparatus 6), and reciprocating holder (apparatus 7) Apparatuses 1 and 2 are used mainly for IR dosage forms; apparatus 4 is useful

for products that have low solubility.[32, 33] Apparatus 3 is used usually for controlled release dosage forms. Other apparatuses are used for other dosage forms.[32] Apparatuses 1 and 2 are used frequently because they are simple and easy to assemble.[32, 33] Disadvantages of apparatuses 1 and 2 include inadequate mixing and difficulty in visually ascertaining the dosage disintegration.[34] Moreover, there are some challenges in changing the pH of the dissolution media. This can be overcome by using apparatuses 3 or 4.[35] Dissolution conditions generally follow these specification ranges: the dissolution volume is from 500–1000 ml; the pH of the media is 1–6.8 (pH 8 can be used if justification is given); the agitation speed of the apparatus 1 and 2 is usually between 50–150 RPM; the flow rate of apparatus 4 is 8–150 ml/min; and the temperature is37±0.5ºC. Dissolution conditions outside of these ranges can be applied if a scientific justification is provided.[32]

1.5 Biorelevant media

If the purpose of the test is to determine batch to batch consistency, using a simple medium might be adequate. Water or buffers are the most commonly used dissolution media. However, if the purpose is to mimic *in vivo* dissolution, biorelevant media are used in the dissolution apparatus. Simulated gastric fluid (SGF) and simulated intestinal fluids (SIF) are commonly used for oral preparations to simulate the *in vivo* conditions in regard to enzymatic activity and pH. However, the intestine, the main organ of absorption, contains around 5 mM of bile salts which work as solublizing agents. Therefore, fasted state simulated

intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) are commonly used as dissolution media.[37, 38] These media mimic physiological fluids more reliably than a simple buffer.[28, 36] This is required especially for drugs that have low solubility in aqueous media. For example, weakly acidic drugs are poorly soluble in the stomach but might show good solubility in the intestine. Release studies of poorly soluble drugs have shown that dissolution in these media is more predictive for some drugs than simple buffer media.[28, 36]

1.6 Biopharmaceutical classification systems (BCS)

The study of formulation factors that impact *in vivo* bioavailability is known as biopharmaceutics. These factors include a product's physiochemical properties, pharmacokinetics, and physiological factors at the absorption site. The BCS is a framework approach to classify products based on two biopharmaceutical factors: solubility/ dissolution rate, and intestinal permeability.[39, 40] The BCS classifies drugs into four categories as presented in Table 1.1. A drug is considered highly soluble when the highest dose dissolves in 250 ml of water over a pH range of 1.2–6.8; 250ml represents the volume of a glass of water administered with the ingested oral dosage form. The pH range represents the gastrointestinal pH in a fasting state which is: gastric pH 1.2–2.1, duodenum pH 4.9–6.4, jejunum pH 4.4–6.6, and ileum pH 6.5–7.4.[37, 41] A drug is considered highly permeable when it has 90% or more intestinal absorption. The drug is considered very rapidly dissolved if 85% of the drug dissolves in 15 minutes, and is considered rapidly dissolved if the same percentage dissolves in 30 minutes using USP apparatus 1 at an agitation speed of 100 rpm or USP

apparatus 2 at 50 rpm in a volume of 900 ml of water, USP SGF pH 4.5 buffer or USP pH 6.8 buffer or SIF.[33, 41] In the development stage, *in silico* and *in vitro* methods can be used to classify a product according to the BCS.

Table 1.1:FDA BCS drug classification with examples.*

Class	Solubility	Permeability	Examples
I	High	High	Metoprolol
II	Low	High	Mefanmic acid
III	High	Low	Rantidin
IV	Low	Low	Furosemide

*Modified from Schan et al. 2009.

1.7 Computer simulation software (*insilico*)

In silico refers to using a computer program to assess certain drug parameters using mathematical and statistical approaches.[42] Computer simulations can play an essential role in QbD and have many applications in the development stages of new drugs, particularly in the selection of promising drug candidates.[32, 43, 44] This is important since with each potential product a minimum of nine others fail. At an early stage time and money are lost if too many compounds are measured and assessed.[45, 46, 47, 48] *In silico* methods can be used to predict the physiochemical characteristics of a drug and classify it according to the BCS. *In vitro* release can be simulated *in silico* to facilitate selection of the appropriate *in*

vitro performance tests. Also, *in silico* methods can be used to link *in vitro* studies to *in vivo*performance.[49] *In silico* simulations can be used to gain a better understanding of the impact of changing raw materials and manufacturing process specifications.[23] Causes of variability and the effects of variability on the quality within a product or process can be identified using *in silico* methods. Even if not all of the predictions are perfect, they will point the developers in the desired direction and reduce time and required experiments.[50]

1.8 Objectives

The main goal of this research was to evaluate *in vitro* and *in silico* tools, which are used to define CQAs of oral dosage forms or which can be used to gain scientific knowledge about a substance's biopharmaceutical properties. The project investigates how these tools might be used in QbD through the following methods:

- Investigation of the performance of the disintegration test using different apparatus settings.
- Comparison of the performance of the disintegration test and the rupture test using soft gelatine capsules.
- Classification of common herbs according to the BCS using computer simulations.
- Evaluation of the predictive powder of DDDplus^{TM} to simulate *in vitro* dissolution profiles.

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Chapter 2

Investigation of the Disintegration Test as a Performance Test Used in Quality by Design

2.1.1 Introduction

Dietary supplements are ingested products that work as a supplement to the diet but do not represent conventional food.[1]They contain dietary ingredients such as amino acids and herbs.[1] Dietary supplements are used to improve health and can be in the form of capsules, tablets, powder, liquid, or bars.[2] The use of dietary supplements is common in the healthy population as well as in people with chronic conditions such as arthritis, cancer, and depression.[3] Dietary supplements should be appropriate for their intended use.[4] The QbD was not yet applied to dietary supplement and other natural health products. Disintegration test can be used to guide formulation scientists to design the appropriate formulation in QbD. Therefore, this test should be robust and reliable.

A disintegration test is inexpensive and easy to perform.[5] According to the USP definition, the disintegration test does not involve complete dissolution of the dosage form; disintegration is defined as "a state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disk, if used, is a soft mass having no palpably firm core".[6] The disintegration apparatus consists of a basket assembly that contains six observation cylinders (basket assembly A) or three cylinders (basket assembly B), a beaker, and a thermostat (Figure 2.1.1). Six units of the oral dosage form can be used at one time in the cylinders of

apparatus A. When the device is operated, it moves the basket assembly vertically along its axis in equal times upward and downward at a fixed speed in a beaker that contains the immersion media.

Figure2.1.1: The USP disintegration test using apparatus A.

The USP specifications of the disintegration test are described in chapter <701> which has been changed over the time. In particularly, the beaker size specifications, the height and diameters, have been changed from USP 23 to USP30. The specification range of the beaker height is as follow: In USP 23 the height of beaker was 142 -148 mm, 138 -155 mm in USP 26 and in USP 30 (138 - 160mm).[7] However, no study has investigated the impact of these changes on the disintegration test. The insider diameter has also changed from 103-108 mm in USP 26 to97-110 mm in USP 30.[7] This change was to harmonize the beaker diameter specification between European Pharmacopeia, Japanese Pharmacopeia and USP and was publish in the (ICH) Q4B - Annex 5. [7, 8, 10] No investigation has been made to see the effect of this harmonization on the performance of the disintegration test. The current harmonized specifications of disintegration include the position of basket assembly on its upward and downward stork. In

USP 27 and earlier the bottom of the basket assembly should have at least a distance of 25 mm from the bottom of the beaker in upwards movement, and the same distant from the medium level in the downwards movement .[8] After the harmonization and since USP 28 and later USP states that the basket assembly should not be totally submerged; now the distance between fluid level and bottom of the basket assembly should be at least 15 mm before the downwards stroke(Figure 2.1.2).[8] The influence of keeping, decreasing, and increasing this distance was studied by Schmidt et al., and it was concluded that the new specification should be followed strictly to get a reliable result.[8]

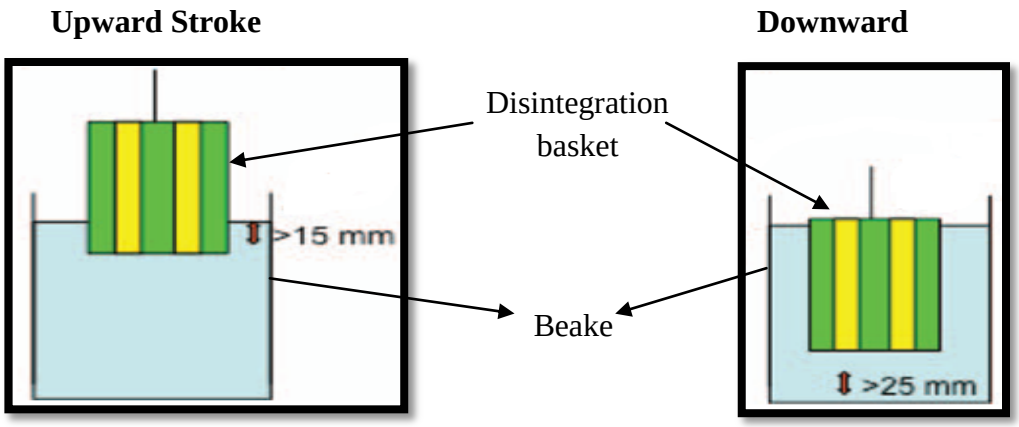

Figure 2.1.2: USP 28 and later disallow complete basket immersion.
*Modified from et al. Schmid 2010

The disintegration test is described in detail in the USP general chapter for all products <701> and in the dietary supplements chapter <2040>.[6, 9] There are some differences between the two chapters regarding disintegration test specifications (Figure 2.1.3). First, in chapter <701> the disintegration time for all dosage sizes is determined by the disintegration test using basket assembly A.[6] In

chapter <2040>, basket assembly A is used only for dosage forms that are equal or less than 18 mm in length, and basket assembly B is used for dosage forms that exceed 18 mm.[9] However, there is no existing study which describes the impact of the basket assembly type on disintegration time. Second, in chapter <701>the disintegration time for all dosage forms is determined by the disintegration test.[6] In chapter < 2040> the same requirement is applied to all dosage forms except soft shell gelatine capsules; here the disintegration time for soft shell gelatine capsules should be determined using a rupture test.[9] There are no data showing the difference between using the rupture test and the disintegration test and no reasons are given for using the rupture test for soft gelatine capsules of dietary supplement. In order to have a robust test for QbD purposes, the methods to determine disintegration should be investigated and need to be well understood. The next two studies aimed to investigate the disintegration test performance using dietary supplements tablet, HMPC hard shell capsules and soft shell gelatine capsules.

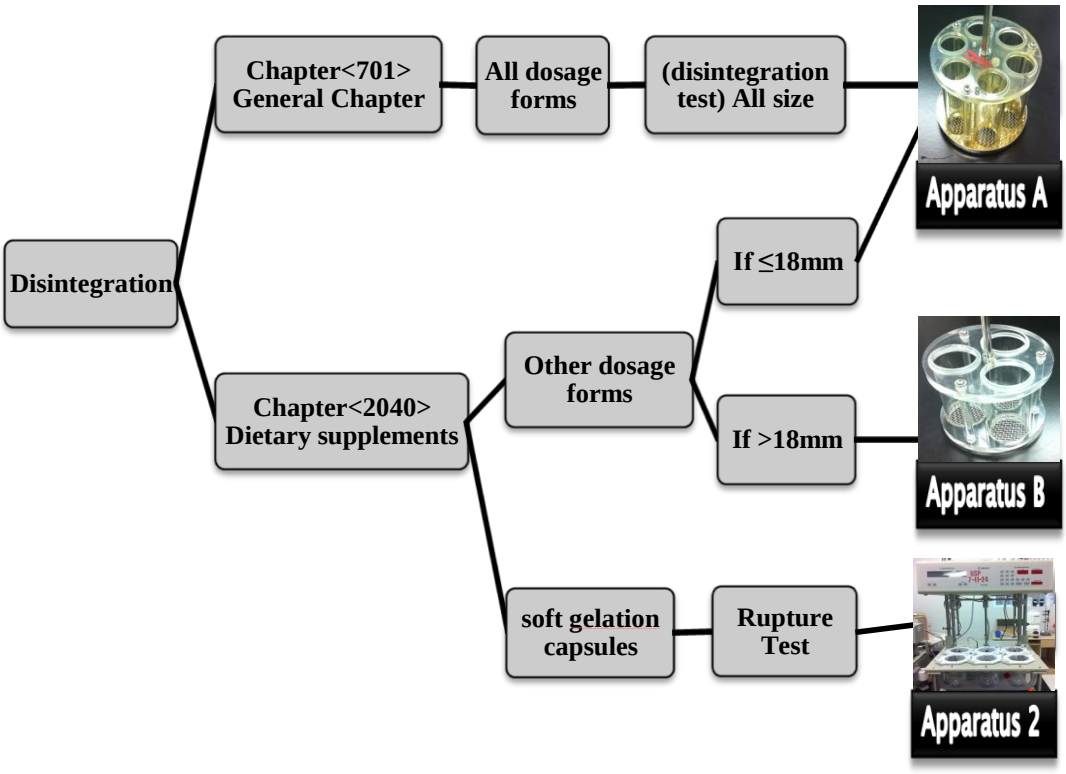

Figure 2.1.3: Schematic comparison of *USP* Chapters <2040> and <701>.

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2.2.0 Investigation of the Performance of the Disintegration Test for Dietary Supplements

2.2.1 Introduction:

The disintegration test as a performance test for immediate release oral dosage forms is receiving attention.[1] This is due to dosage form specifications for which a dissolution test might not be the best fit, as shown by Han et.al for dosage forms like liquid filled capsules or fast dissolving tablets.[2,3,4] Similarly, approaches like Quality by Design (QbD) might consider a disintegration test as the most appropriate performance test for fast dissolving BCS class 1 drugs. In QbD, scientific approaches and appropriateness decide which compendial standard will be included into the overall quality and monitoring system of a product.[1, 5]

In QbD, a disintegration test might be justified to substitute a single point dissolution test if the drug particle size ensures a sufficient rate of dissolution and disintegration is the rate-limiting step for drug release.[6] However, to be able to use the disintegration test as a performance test in QbD, its suitability as a performance test must be investigated. Today there is only very limited data available that describe the performance of the disintegration test.

USP (32) currently listed two chapters, which describe the disintegration test for dosage forms. Chapter <701> describes the general set-up with beaker specifications and basket assembly A (Apparatus A).[7] Chapter <2040> describes basket assembly B (Apparatus B) and test conditions and acceptance criteria for dietary supplements.[8] Over the years the disintegration test specifications were

changed. Donauer and Löbenberg reviewed these changes in detail.[9] Both USP chapters list test conditions for different dosage forms but there are some differences between the chapters. USP <701> uses water as the immersion medium for "hard gelatin capsules" while USP <2040> uses pH 4.5 acetate buffer for "hard shell capsules". The next difference between both chapters is that "soft gelatin capsules" are tested like uncoated tablets in USP <701> while <2040> uses a rupture test for "soft shell capsules".

Non-gelatin shell capsules are gaining importance in the pharmaceutical and dietary supplement industry. Literature reports some evidence that non-gelatin capsules made out of HPMC are sensitive to sodium and potassium ions used in dissolution media.[9] However, no data exist in literature which describes the impact of such media on the disintegration test.

USP introduced a new test requirement: "At no time should the top of the basket-rack assembly become submerged".[7] This requirement makes the use of the wire cloth for capsules unnecessary. Since the basket assembly should not be submerged, it ensures that the dosage form will not float out of the observation cylinder. The previous test condition only specified, "That at the highest point of the upward stroke the wire mesh remains at least 25 mm below the surface".[10] Schmid and Löbenberg investigated the impact of this change.[11] The study concluded that the new test conditions impact the disintegration times and the fluid level requirements must be strictly followed to obtain reliable results.
USP chapter <701> was recently harmonized under the International Conference of Harmonization (ICH) Q4B - Annex 5.[12] The harmonized guide only applies to

dosage forms under 18 mm. Since USP <701>did not specify any dosage form size limitations; it can be assumed that any dosage form, which physically fits into the observation cylinder of 20.7 to 23 mm, was tested in Apparatus A before the guideline was published.[12] In the future, dosage forms larger than 18 mm might be tested in Apparatus B as already required by USP <2040>; Size number 1 capsules are very commonly used. Their size is just above 18 mm and according to USP <2040> they have to be tested in Apparatus B. However, if their disintegration time was established in Apparatus A, it is not known if they pass the disintegration test in Apparatus B. No data exist which describes the impact of the basket assembly on the disintegration time of oral dosage forms.

As mentioned before, USP <2040> gives universal acceptance criteria for dosage forms such as tablets and capsules e.g. disintegration time less than 30 min. However, it is not known if the disintegration time will be similar if a different basket assembly is used.

In the past the USP beaker specifications were more stringent e.g. USP 24: 103 – 108 mm.[10] Now the harmonized monograph specifies the beaker size from 97 to 115 mm.[3, 12] No data exist which demonstrate that these changes do not affect the performance of the disintegration test.

The aim of this study was to systematically investigate how beaker sizes, basket assembly, use of disks and the nature of the immersion medium impact the disintegration of different commercially available dietary supplement products.

2.2.2 Method

The study investigated the influence of beaker size, apparatus, use of disks (when appropriate) and the nature of the immersion medium on the disintegration time of tablets and capsules. The disintegration times were determined for seven commercial tablets and capsule products. *Boswellia serrata* (The Vitamin Shoppe, Lot# 082658, exp 09/11), *Cinnamon* (The Vitamin Shoppe, Lot# 2036475, exp 08/12), *Ester-C* (American Health, Lot# 234536-07, exp 08/10), *Oyestercal* (Puritan's Pride, Lot# 415720-10, exp: 03/12) and *Glucosamine "Move Free"* (Schiff, Lot# S2438D4, exp 08/10) were the tablet formulations and *Chasteberry* (Solaray, Lot# 93409850807, exp 10/11) and *Zinc* (The Vitamin Shoppe, Lot# 083332, exp 07/11) were capsules products, which were investigated.

A disintegration tester (model ED-2L, Electrolab, Betatek Ontario) consisted of two stations; each was used with Apparatus A USP chapter <701> or Apparatus B as described in USP chapter <2040>. The small beaker (SB) had a nominal volume of 1,000 mL with an inside diameter of 101 ±1 mm and the large beaker (LB) had a nominal volume of 1, 500 mL and an inside diameter of 114 ±1 mm. Four different equipment configurations and 2 beaker sizes resulting in eight test conditions were investigated: A small beaker (SB) and USP Apparatus A (App A) with disk, SB App A without disk, SB USP Apparatus B (App B) with disk, SB beaker apparatus App B without disk, large beaker (LB) App A with disk, LB App A without disk, LB App B with disk and LB App B without disk. The tests were performed with 18 test units and the media employed for this study was water, for all tests.

All the equipment and beaker sizes investigated for tablets were also applied to Chasteberry capsules. Additionally, this study included the evaluation of three different media: water, USP buffer pH4.5 and USP Simulated Gastric Fluid (SGF) (n=18).

The Zinc capsules did not disintegrate within 90 minutes. Therefore, no investigation was performed without disks for this product. The disintegration time determinations were performed using SB App A, SB App B, LB App A and LB App B, all with disk. Due to the cellulose nature (HPMC) of their hard shell body, 5 different media were tested in this study: water, USP buffer pH4.5, USP SGF,USP Simulated Intestinal Fluid (SIF) a potassium phosphate based buffer and buffer pH 6.8 using sodium-phosphate(n=18).

In all cases the disintegration time was recorded as an independent variable. Next, statistical analysis was performed using two different statistics programs: Minitab 15 (MINITAB Inc.) and SPSS 17 (Statistics Grad Pack). The mean and standard deviation was calculated for all tablets and capsules. The data were analyzed using two-way ANOVA for the following factors: beaker size (small, and big) and equipment (App A with disk, App A without disk, App B with disk, App B without disk). Any value exceeding the critical value of 0.05 indicates no statistical significance. For both tablets and capsules, the ANOVA analysis was repeated using only two factors (beaker size and apparatus). The aim was to investigate the impact of the apparatus and beaker size on the performance of the disintegration test. For capsules, a further ANOVA was performed for each equipment configuration using immersion media as a factor.

For Zinc Capsules, the impact of the beaker size and apparatus on the performance of the disintegration test was investigated using disks only because the capsules did not disintegrate without disks. Tukey's test was then applied to specify which media exactly caused a statistical impact. To be able to apply ANOVA the test data must meet certain assumptions: The sample populations must be normally or approximately normally distributed. Also, the samples must be independent. Moreover, the variances of the population must be equal; this criterion was checked using Leven's (any continuous distribution) and Bartlett test (normal distribution).

2.2.3 Results

2.2.3.1 Tablets

Five tablet formulations were investigated: *Boswellia serrata*, Cinnamon, Ester-C, Oystercal and Glucosamine. Table 2.21 summarizes the mean disintegration times of all tablet products tested. The influence of the equipment configuration on the disintegration times was investigated using analysis of variance (ANOVA) and is summarized in Table 2.2.2.

Boswellia serrata tablets showed that neither beaker size (LB or SB) nor the test conditions (with or without disk) were significant in regard to the disintegration times. Only the apparatus presented statistical significance ($p = 0.030$, Table 2.2.2, Appendix A [Figure A.1, a]). Apparatus A produced lower disintegration time means compared to apparatus B (Table 2.1.1). Similarly, the interactions among these factors were not significant too. The mean disintegration

times for the eight different test conditions varied from 7.2 ± 1.5 to 8.3 ± 2.7 minutes (Table 2.1.1, Appendix A[figure A.5,a]). The test for equal variance (Levene's test, for any continuous distribution) showed no significant difference among the variances for the eight conditions studied (p= 0.170, Appendix A [figure A.3,a]). Although the difference may not be considered significant using Levene's test, SB App B, with and without disks, had the highest standard deviations: 2.4 and 2.7 minutes, respectively.

The results for *Ester-C* tablets were quite different from those observed for *Boswellia serrata* tablets. The analysis of variance for the *Ester-C* tablets showed significant differences among the conditions used. The factors (beaker size, apparatus and disk) as well as their interaction significantly influenced the disintegration times ($p < 0.01$,). (Table 2.2.2, Appendix A [Figure A.1, b]). The shortest disintegration time was obtained using SB App B with disk: 19.6 ± 1.1 minutes (Table 2.1.1). The remaining studies with disk showed disintegration times equal to 25.6, 22.7 and 24.5 minutes for LB App and B and SB App B, respectively. All studies performed without disk (SB or LB, App A and B) showed disintegration times above 31 minutes. The test for equal variance ($p = 0.161$, Levene's test, Appendix A [figureA.3, c]) revealed no significant difference among the variances although the test performed without disk using LB App A had the highest standard deviation: 5.4 minutes (Table 2.1.1). The means of the tests using disk varied from 19.6 ± 1.1 to 25.6 ± 0.9 minutes and without disk varied from 31.7 ± 1.5 to 37.6 ± 1.0 minutes clearly indicated that the use of disks decreased the disintegration times(Appendix A, Figure A.5, c). The data

showed that all selected conditions without disk would statistically result in disintegration times of more than 30 minutes and the product fails compendial acceptance criteria independent of which set-up might be used.

Similarly to the *Boswellia serrata* tablets, the analysis of variance for *Oystercal* tablets indicated that the interaction between disk, apparatus and beaker were not statistically significant for the disintegration times (Table 2.2.2, Appendix A [Figure A.1, c]). Neither were the influences of beaker, beaker disk interactions nor beaker apparatus interactions. The results clearly indicated that the conditions performed with apparatus A (App A) and with disk presented the shortest disintegration times: 5.0±0.9 and 5.1±0.8 minutes for LB App A with disk and SB App A with disk, respectively. A second group showed the following intermediate disintegration times: 5.9 ± 0.8, 6.0 ± 0.7, 6.1 ± 0.5 minutes for SB App B without disk, SB App B with disk and LB App B with disk, respectively; and a third group that presented the highest disintegration times: 6.6 ±0.7, 6.6 ± 0.5 and 6.7 ± 1.0 minutes for SB App A without disk, LB App A without disk and LB App B without disk, respectively (Table 2.1.1, Appendix A[Figure A.5,d]). The test for equal variances showed no significant differences among the conditions (Levene and Bartlett tests, Appendix A, Figure A.3, d).

The *Glucosamine tablets* followed the same pattern as observed for the *Ester-C* tablets. The analysis of variance showed that disintegration times were significantly influenced by all conditions (with disk or without disk), the beaker size, by the apparatus and their interactions, except for the beaker and disk interaction as well as the beaker, disk and apparatus interaction showing p = 0.185

and 0.231, respectively (Table 2.2.2, Appendix A [Figure A.1, d]). The disintegration times were highly influenced by the disk using SB App B or LB App B. In these conditions the difference between the disintegration times reached approximately 7.0 minutes (17.4±0.8 and 24.1±1.0 for SB App B with and without disk, respectively) (Table 2.1.1, Appendix A[Figure A.5, e]). The shortest disintegration time was 17.4±0.8 minutes observed for SB App B with disk. Although the test for equal variance showed no significant differences among the standard deviations (Levene test), the highest standard deviation was observed for LB App B with disk , 2.6 minutes (Appendix A, Figure A.3 ,e).

For Cinnamon tablets, the test for equal variances for the disintegration times in the different conditions revealed a significant difference for both tests employed: Levene (any continuous distribution) and Bartlett (normal distribution) (Appendix A, Figure A.3, b). Thus, there was a significant difference among the variances observed, for all tests. It was observed three distinct groups of standard deviation for the tests. The first one: 1.4, 1.5 and 1.3 minutes for SB App A with disk, SB App B with disk and LB App A with disk, respectively; the second group: 2.0, 2.6, 2.8 and 2.0 minutes for SB App A without disk, SB App B without disk, LB App A without disk and LB App B with disk, respectively and the highest mean and standard deviation observed: 63.2±5.2 minutes for LB App B without disk (Table 2.1.1, Appendix A [Figure A.5,b]). Only one condition met the compendia requirement (below 30 minutes): SB App B with disk (25.1±1.5minutes), (Appendix A, Figure A.1,e).This clearly showed that the current USP beaker specifications might cause a fail or pass of a batch due to the

beaker used, but not necessarily due to a batch failure. As observed for Ester-C tablets, the tests performed with disk showed lower disintegration times compared to the tests without disk. Among the conditions with disk, the test for equal variance (Appendix A, Figure A.3, b) revealed no statistical difference for the standard deviations ($p= 0.372$ and 0.320 for Levene and Bartlett tests, respectively). The two-way ANOVA, for these conditions (with disk) revealed that the disintegration time means for SB App A, SB App B, LB App A and LB App B were statistically different, highly influenced by the beaker size (SB or LB), the type of apparatus (A or B) ($p<0.001$ for the factors and their interaction).

2.2.3.2 Capsules

For capsules, the disintegration times were determined for two products: *Chasteberry and Zinc capsules*. The mean data are summarized in Table 2.2.3. For the *Chasteberry* capsules, the disintegration times were evaluated using the same eight conditions as for the tablets. Additionally the impact of different media was evaluated. The zinc capsules had a hard shell made from HPMC and therefore, the impact of sodium buffers in comparison to potassium buffers was evaluated.

The disintegration time for *Chasteberry capsules*was highly influenced by the type of apparatus, the beaker size, the type of the medium, use of disk (with or without) and by the interaction of all these factors, except for the Apparatus-Beaker, Apparatus-Medium and Apparatus-Beaker-Disk interactions with $p= 0.376$, 0.217 and 0.336, respectively (Table 2.2.4, Appendix A[Figure A.2,a]).

The shortest disintegration times were observed for SB App B with disk: 5.8±1.0, 6.7±0.7, and 6.5 ±1.0 minutes for water, pH=4.5, and SGF, respectively. The tests using LB App B with disk, the disintegration times were 9.2±3.2, 9.4±1.0 and 9.9±1.9, for water, pH=4.5 and SGF, respectively (Table 2.2.3, Appendix A[FigurA.6, a]). Clearly, the tests performed using disks showed the lowest disintegration times. Among the conditions with disk, the test for equal variance showed no statistical difference for the standard deviations (p =0.058 for Levene tests), the lowest standard deviations were for those tests performed using disks.(Appendix, Figure A.2,a)

The Zinc capsules behaved differently compared to the Chasteberry capsules. This product disintegrated under any chosen condition under the 30 minutes. The shortest disintegration time was observed for SB App B (4.0±1.0 minutes) (Table 2.2.3, Appendix A [Figure A.2,b]). This apparatus also produced very similar results for all media tested: 4.0±1.0, 4.5±1.3, 4.4±1.0, 3.8±1.3, 3.6±0.8 minutes for buffer pH 4.5, SIF, SGF, pH 6.8 Na phosphate buffer and water, respectively (Appendix A, Figure A.6,b). The test for equal variance showed no statistical difference for the standard deviations (p= 0.387 and 0.305 for Bartlett and Levene tests, respectively) among the tests performed with the media for SB App B (Appendix A, Figure A.2, b). One-way ANOVA showed that the media was not significant for the disintegration times obtained (p= 0.053). Tukey´s comparison (individual confidence level = 99.34%, data not shown) revealed that the disintegration times for all media are not statistically different when the condition SB App B was used.

2.2.4 Conclusion

The study showed that some tablet products were sensitive to the chosen test conditions and other tablets (*Boswellia serrata* and Oystercal) were not. This is somewhat surprising since Kamba et al. showed that the use of disks adds destructive forces to the disintegration test.[13] A third product (*glucosamine tablets*) only showed a partial impact of the beaker size and the equipment used on the disintegration time when no disks were used. The other tablets *(Cinnamon and Ester-C)* showed a clear impact on the disintegration time when disks were used. The results showed that these tablet products might pass or fail current USP disintegration requirements depending on which equipment configuration was chosen.

Similar results were obtained for the two capsule formulations investigated. The results showed that the equipment configuration and immersion medium used could have a significant impact on the disintegration times of these products. *Chasteberry* capsules failed to pass current USP disintegration requirements if the LB was used but passed the disintegration requirements when the SB was used. The *Zinc* capsules, which had a cellulose-based shell, were mostly influenced in their disintegration times if sodium instead of a potassium buffer was used as the immersion medium. A similar observation was reported for the dissolution behavior of capsule products with HPMC.[9]

The study clearly shows that the current beaker specifications are insufficient for the disintegration test of dietary supplements. The USP expert committee for performance testing of Dietary Supplements has used the findings

of this study to change the beaker specification in USP chapter <2040>. The changes were published in PF 35(4) (July-Aug.2009) and will become official in USP 33 2S.[7] Furthermore, the results demonstrate that the current harmonized ICH specifications for the disintegration test are insufficient to make the disintegration test into a reliable performance test for dietary supplements. The impact of the current specifications on drug products needs to be investigated especially if the disintegration test is intended as a performance test in a QbD approach for pharmaceutical dosage forms.

TABLE 2.2.1: Disintegration times (means and standard deviations) for Boswellia serrata, Cinnamon, Ester-C, Oystercal and Glucosamine tablets using small beaker (SB) and large beaker (LB), apparatus A and B and without disk (1) and with disk (2).

Tablet	Beaker	Apparatus	Condition	Mean ± sd
Ester-C	SB	A	1	34.5 ± 1.2
			2	24.6 ± 0.5
		B	1	31.7 ± 1.5
			2	19.6 ± 1.1
	LB	A	1	36.0 ± 5.4
			2	25.6 ± 0.9
		B	1	37.6 ± 1.0
			2	22.7 ± 0.8
Oystercal	SB	A	1	6.6 ± 0.7
			2	5.1 ± 0.8
		B	1	5.9 ± 0.8
			2	6.0 ± 0.7
	LB	A	1	6.6 ± 0.5
			2	5.0 ± 0.9
		B	1	6.7 ± 1.0
			2	6.1 ± 0.5
Cinnamon Extract	SB	A	1	50.1 ± 2.0
			2	33.4 ± 1.4
		B	1	51.6 ± 2.6
			2	25.1 ± 1.5
	LB	A	1	58.7 ± 2.8
			2	36.0 ± 1.3
		B	1	63.2 ± 5.2
			2	31.6 ± 2.0

Boswellia serrata	SB	A	1	7.5 ± 3.4
			2	7.4 ± 1.2
		B	1	8.3 ± 2.7
			2	7.9 ± 2.4
	LB	A	1	7.2 ± 1.5
			2	7.4 ± 1.0
		B	1	7.5 ± 2.1
			2	7.5 ± 1.3
Glucosamine	SB	A	1	25.8 ± 1.6
			2	22.7 ± 0.8
		B	1	24.1 ± 1.0
			2	17.4 ± 0.8
	LB	A	1	27.6 ± 1.4
			2	24.4 ± 0.9
		B	1	28.0 ± 1.3
			2	20.2 ± 2.6

SB = Small Beaker; **LB** = Large Beaker; **1** = without disk; **2** = with disk; **sd** = standard deviation

Table 2.2.2: P values result from ANOVA for disintegration time under the following conditions: SB App A (small beaker apparatus A) with and without disk; SB App B (small beaker apparatus B) with and without disk; LB App A (large beaker apparatus A) with and without disk; LB App B (large beaker apparatus B) with and without disk for Boswellia serrata, Ester-C. Oystercal, and Glucosamine.

Source	Boswellia serrata	Ester - C	Oystercal	Glucosamine
Beaker	0.534	<0.001	0.241	<0.001
Beaker*Disk	0.974	<0.001	0.167	0.185
Beaker*Apparatus	0.180	<0.001	0.066	0.001
Disk	0.726	<0.001	<0.001	<0.001
Disk*Apparatus	0.266	<0.001	<0.001	<0.001
Apparatus	0.030	<0.001	0.002	<0.001
Beaker*Desk*Apparatus	0.471	0.001	0.258	0.231

Table 2.2.3: Disintegration times (means and standard deviations) for Zinc and Chasteberry capsules using small beaker (SB) and large beaker (LB), apparatus A and B in different media.

Capsule	Beaker	Apparatus	Medium	Mean ± sd
Zinc Capsules	SB	A	1	9.8 ±2.9
			2	8.3 ±5.0
			3	11.6 ±3.0
			4	17.5 ±6.0
			5	9.9 ±2.7
		B	1	3.6 ± 0.8
			2	4.0 ± 1.0
			3	4.5 ± 1.3
			4	3.8 ± 1.3
			5	4.4 ± 1.0
	LB	A	1	10.0 ± 2.9
			2	11.5 ± 8.0
			3	14.0 ± 4.9
			4	20.0 ± 6.2
			5	13.3 ± 4.5
		B	1	5.2 ± 1.3
			2	6.2 ± 2.3
			3	5.0 ± 1.3
			4	7.6 ± 2.3
			5	6.3 ± 1.7
Chasteberry Extract-vegicaps	SB	A	1	32.5 ± 5.9
			2	23.6 ± 6.0
			5	28.8 ± 4.6
		A^1	1	17.1 ± 3.4
			2	14.7 ± 1.6
			5	28.8 ± 4.6
		B	1	29.9 ± 5.3
			2	25.8 ± 1.6
			5	37.6 ± 8.7
		B^1	1	5.8 ± 1.0
			2	6.7 ± 0.7
			5	6.5 ± 1.0
	LB	A	1	40.2 ± 7.3
			2	28.2 ± 1.9
			5	36 ± 4.6
		A^1	1	19.1 ± 3.3
			2	16.3 ± 1.7
			5	20.5 ± 4.7
		B	1	36.1 ± 5.3
			2	33.2 ± 3.7
			5	42.1 ± 5.9
		B^1	1	9.2 ± 3.2
			2	9.4 ± 1.0
			5	9.9 ± 1.9

SB = Small Beaker; **LB** = Large Beaker; **A** = Apparatus A without disks; **B** = Apparatus B without disks; $\mathbf{A^1}$ = Apparatus A with disks; $\mathbf{B^1}$ = Apparatus B with disks; **1**= water; **2** = buffer pH 4,5; **3** = USP Simulated Intestinal Fluid pH 6.8 (potassium buffer); **4** = pH 6,.8 Buffer (Na-phosphate) **5** = pH 1,2 (USP Simulated Gastric Fluid) ; **sd** = standard deviation

Table 2.2.4: P values of Analysis of variance for disintegration time under the following conditions: SB App A (small beaker apparatus A) with and without disk; SB App B (small beaker apparatus B) with and without disk; LB App A (large beaker apparatus A) with and without disk; LB App B (large beaker apparatus B) with and without disk and different media (water, USP Simulated Intestinal Fluid (potassium based): SIF, SIF-NaP = Sodium based buffer pH 6.8, buffer pH=4.5 and Simulated Gastric Fluid: SGF) for Chasteberry capsules.

Source	Chasteberry capsules
Apparatus	<0.001
Apparatus*Beaker	0.376
Apparatus*Disk	<0.001
Apparatus*Medium	0.217
Beaker	<0.001
Beaker*Disk	<0.001
Beaker*Medium	<0.001
Disk	<0.001
Disk*Medium	0.014
Medium	<0.001
Apparatus*Beaker*Disk	0.336
Beaker*Disk*Medium	<0.001
Apparatus*Beaker*Medium	0.001
Apparatus*Beaker*Disk*Medium	0.006

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2.3.0 Comparison of the Rupture and Disintegration Tests for Soft-Shell Capsules

2.3.1Introduction

QbD is a scientific approach that uses statistical methods for product design, quality testing, and predicting product performance from early product development to final product release.[1, 2] QbD highly depends on the appropriateness of test methods used and can only be successfully applied if a test is sensitive to the parameter that is tested.

The performance testing of soft-shell capsules is rather a challenge because the content of soft-shell capsules can vary from solids to liquids.[3] Dissolution methods used for solid oral dosage forms might not be appropriate for soft-shell capsules that have liquid or semisolid content.[4] *USP* General Chapter <701> Disintegration describes the procedure to evaluate disintegration of oral dosage forms.[5] The requirements of disintegration are met if all test units disintegrate or if not more than two units out of a total of 18 units fail to disintegrate within a predetermined time period. *USP* General Chapter <2040> Disintegration and Dissolution of Dietary Supplements uses a rupture test as performance test of soft-shell capsules.[6] In 2002 the rupture test was first published in *Pharmacopeial Previews*, then forwarded to USP's *In-Process Revision*, and in 2007 it was finally published in *USP 30–NF 25*.[7, 8] *USP 32* lists 14 monographs that use the rupture test performed in dissolution Apparatus 2 (paddle) operated at 50 rpm with 500 mL of water as the immersion medium. The test requirements are met if all capsules rupture within 15 min or if not more than 2 of the total of 18 capsules

tested rupture in more than 15 but not more than 30 min. For any other oral dietary supplement dosage form, disintegration test Apparatus A or B is used if the monograph requires disintegration.

Another difference is that for hard-shell capsules, Chapter <2040> lists USP pH 4.5 buffer as the immersion medium while Chapter <701> lists water as the default medium if a monograph does not specify any other medium.[9] *USP* Chapter <2040> also lists Apparatus B, which is intended for dosage forms greater than 18 mm in diameter. Currently there are no scientific data available that compare the performance of the rupture test with that of the disintegration test. The aim of this study was to evaluate if there are advantages in using the rupture test over the disintegration test. A series of experiments was performed and statistical analysis was used to determine differences between the tests.

2.3.2 Method

Five different soft-shell capsules were received from Banner Pharmacaps: amantadine HCl (lot No. 27060261XP), flaxseed oil (lot No. 203491-01), ginseng 100 mg (lot No.203491-01), pseudoephedrine HCl (lot No.XPP0410004B), and soybean oil (lot No. XPM0309004). These capsules were chosen based on their filling content. Flaxseed oil, ginseng, and soybean oil are filled with an oil base, and pseudoephedrine capsules are filled with a water-miscible solution. Amantadine capsules contain a suspension.

The study design compared the products as received with capsules that were treated by coating them with the liquid content of another capsule to simulate a production deficiency. This was done by pouring the liquid contents of one

capsule over the remaining capsules in a 120-mL plastic bottle. The bottle was tumbled at 50 rpm for 30 min. Then the bottle was stored until the next experiment was performed according to the testing schedule.

The capsules were incubated at room temperature and at 40 °C, and the tests were repeated after 2 weeks. At each time point, twelve capsules of each product were tested using the rupture and the disintegration tests. A disintegration tester (model ED-2L, Electrolab, Betatek Ontario) consisted of two stations; each was equipped with a basket assembly as described in *USP* Chapter <701>. The beaker had a nominal volume of 1,000 mL with an inside diameter of 101 ± 1 mm (SOTAX) and was filled with about 750 mL of immersion medium to comply with the USP requirement not to submerge the basket assembly totally at any time point. All tests were performed without disks. The rupture test was performed in 500 mL of water at 37 °C and 50 rpm using USP dissolution Apparatus 2 model 7020 (Varian, Inc.). Six capsules were tested untreated, while the other six capsules were from the treated batch. This was done to compare the sensitivity of the rupture and disintegration tests for detecting possible production errors during the manufacturing process. The uncoated capsules represent a correct batch, while the coated capsules represent a batch that has a production deficiency. The rupture time for each unit was recorded when visible leakage of the contents was shown. The criterion of the disintegration test was that the contents must be released from the capsule shells, and then the disintegration time was recorded. In each experiment, the time was recorded as a dependent variable.

The analysis of variance (ANOVA) was performed to compare the rupture and disintegration mean times for each capsule and storage condition ($p = 0.05$) using Minitab® 15 software.

2.3.3 Results

The mean and standard deviations from all conditions and capsules are shown in Table 2.3.1. The variability for the rupture and disintegration times for all capsules and test conditions are presented in Figure 2.3.1. The analysis of variance (ANOVA) was performed to compare the recorded mean times for the disintegration and rupture tests for all capsules and conditions. The *p*-values are indicated in Table 2.3.2.

2.3.3.1 Amantadine Soft-Shell Capsules

Amantadine capsules showed the highest variability among all capsule products for the rupture test (Figure 2.3.1a). For this product, the shortest recorded disintegration and rupture times were 9.3 ± 1.0 and 8.3 ± 0.9 min, respectively.

Differences between the uncoated and coated conditions ($p = 0.00$, $\alpha = 0.05$) were detected with the disintegration test (Table 2.3. 2). However, the analysis of variance did not reveal any statistical differences in the mean times among the storage conditions using both tests (*p*-values of 0.57 and 0.89, $\alpha = 0.05$) (Table 2.3.2). Additionally, it was revealed that the rupture test was not faster than the disintegration test, but both test durations were similar (Table2.3.1).

2.3.3.2 Flaxseed Oil Soft-Shell Capsules

Figure 2.3.1b reveals significant differences between the disintegration and the rupture test times for flaxseed oil capsules. The shortest test times were 7.7 ± 0.5 and 0.8 ± 0.4 min for the disintegration and the rupture tests, respectively (Table 2.3.1). The analysis of variance for the rupture test shows that the mean times for coated/uncoated and the storage conditions are statistically different (p = 0.00, α = 0.05) (Table2.3. 2).

For the disintegration test, no statistical differences were observed for the coated/uncoated and the storage condition mean times (p-values of 0.86 and 0.27, respectively,α = 0.05) (Table2.3. 2). For this product, the rupture test was able to differentiate the tested conditions, but no meaningful tendencies were observed between them. Furthermore, the rupture test was faster than the disintegration test.

2.3.3.3 Ginseng Soft-Shell Capsules

Figure2.3.1c shows the mean and the standard deviation of ginseng capsules for the disintegration and the rupture tests. The shortest disintegration and rupture times were 8.2 ± 0.8 min (coated capsules after 2 weeks at room temperature) and 3.4 ± 1.8 min (uncoated capsules after 2 weeks at 40 °C), respectively (Table2.3. 1). For the rupture test, analysis of variance shows no statistically significant differences in the recorded mean times among the storage conditions (p = 0.38, α = 0.05) or the coated and uncoated conditions (p = 0.34, α = 0.05) (Table 2.3.2). However, the analysis reveals that the interaction between these factors (storage and coated/uncoated conditions mean times) was statistically significant

(p = 0.00, α = 0.05) (Table2.3. 2). For the disintegration test, the storage and the uncoated/uncoated conditions presented significant differences (p = 0.00, α = 0.05). Despite its higher mean times, the disintegration test seems to have better discriminating properties.

2.3.3.4 Pseudoephedrine HCl Soft-Shell Capsules

Figure 2.3.1d shows the mean and standard deviations for pseudoephedrine capsules. The shortest disintegration and rupture times were 5.9 ± 0.6 and 1.9 ± 0.5 min, respectively, both for capsules stored at 40 °C for two weeks (Table 2.3.1). The rupture and the disintegration tests showed no significant statistical differences for the uncoated/uncoated mean time conditions (p-values of 0.89 and 0.36 for the rupture and disintegration tests, respectively, α = 0.05). However, both tests presented significant differences for the storage conditions (p = 0.00, α = 0.05) (Table 2.3.2). From the statistical analysis, the disintegration and the rupture tests seem to have similar discriminating properties. However, the rupture test was faster (Table 2.3.1).

2.3.3.5 Soybean Oil Soft-Shell Capsules

Figure 2.3.1e shows that the disintegration and rupture times (mean and standard deviation) for soybean oil capsules were clearly different. The shortest disintegration and rupture times were 7.6 ± 0.6 and 0.9 ± 0.2 min, respectively (Table2.3.1). However, both tests presented statistically significant differences for the uncoated/coated mean time conditions (p = 0.00, α = 0.05). The disintegration

test was also able to differentiate the storage conditions (p = 0.00) (Table 2.3.2). Thus, for this product, the rupture test had the shortest test duration, but the disintegration test was able to reveal the differences among the storage and the uncoated/coated conditions.

2.3.4 Discussion

The introduction of the rupture test in the *USP* as a performance test for dietary supplement soft-shell capsules triggers the question of what are the advantages of this test over the disintegration test. In the future, can the rupture test, if advantageous, also replace the disintegration test for pharmaceuticals?

The present work is the first study to address this question scientifically. Disintegration is defined by *USP* as "that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disk, if used, is a soft mass having no palpably firm core".[5] According to this definition, the rupture of a soft-shell capsule fulfills the endpoint criterion of the disintegration test if the capsule content is semi-solid or liquid. For these products, the endpoint is the same for the rupture test and the disintegration test.[5, 6] However, in practice we observed that it was much easier to detect capsule rupture in a dissolution apparatus than capsule disintegration in the disintegration tester because of the basket assembly and its constant up and down movements. Therefore, this study defined the disintegration endpoint as the time needed to release the entire capsule contents from the shells. This endpoint could be visually determined by observing the empty shells on the screen, whereas the moment of the shell opening was not

easily observed. Therefore, the rupture test was faster than the disintegration test, except for amantadine capsules.

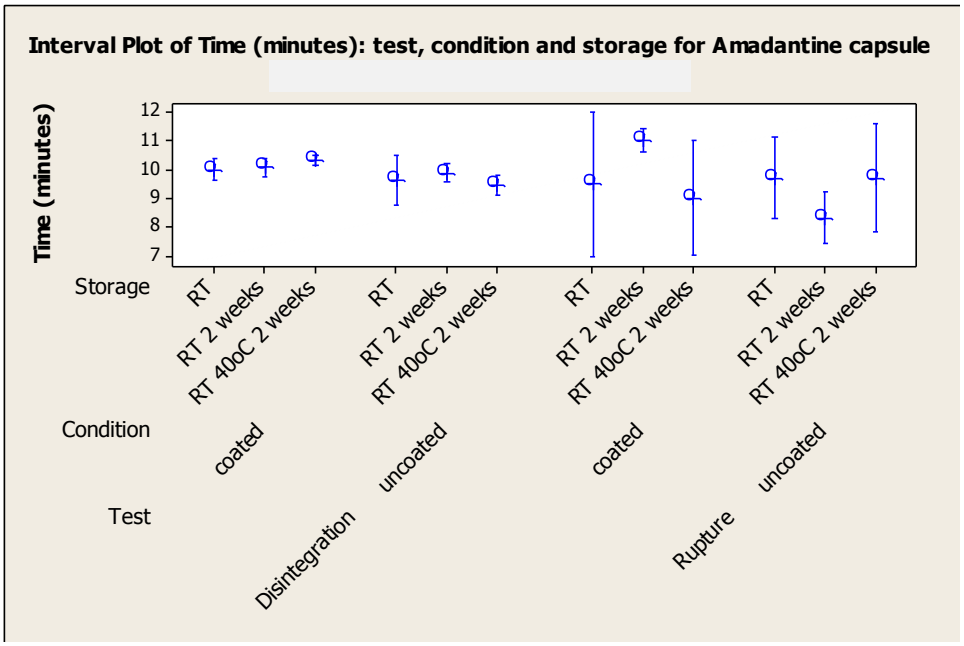
As shown, the standard deviations of the rupture test were sometimes higher than the standard deviations of the disintegration test and vice versa. Moreover, although we hypothesized that the rupture test may show better performance as a quality control tool for soft-shell capsules as compared with the disintegration test, from a broad statistical perspective, none of the tests showed an advantage over the others. The rupture and disintegration apparatus were only sensitive to some test conditions depending on the individual product. The disintegration or rupture of a soft-shell capsule is controlled by its shell.[3] Therefore, product-specific factors such as shell composition, gelatin age, and fill content will impact the performance of a soft-shell capsule. The study shows that both tests were suitable as universal performance tests for soft-gel capsules.[10] However, the ability of both performance tests to detect differences was product-specific.

2.3.5 Conclusion

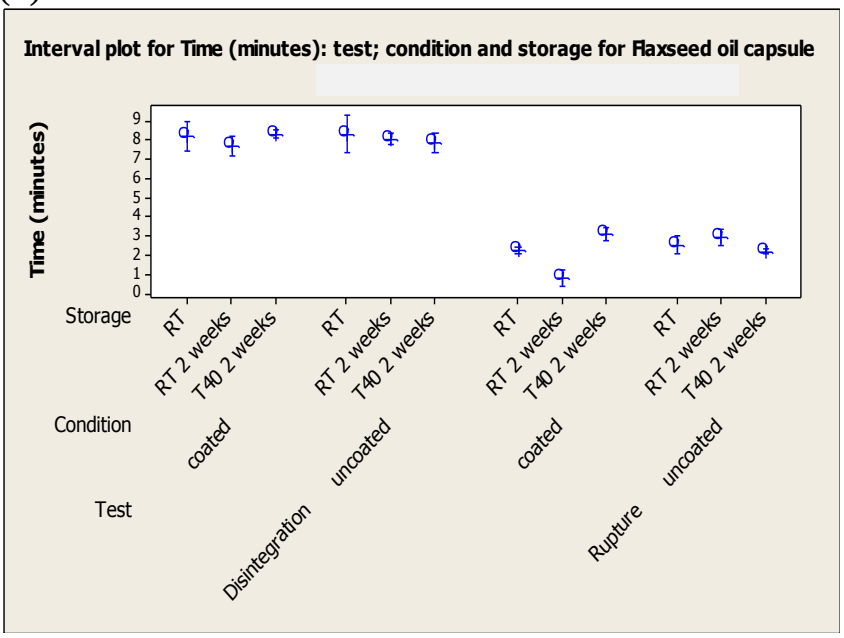
Statistical analysis comparing the rupture and the disintegration tests proved that both tests are comparable and equally sensitive in detecting simulated production deficiencies or storage conditions of soft-shell capsules. The only statistically significant difference between the rupture and disintegration tests was the time needed to reach the defined endpoint. The rupture test needed less time than the disintegration test. However, because its endpoint determination depends on an observation, it might not be easily automated. The study showed the case-by-case sensitivity of both performance tests for discriminating between test

conditions. Products that are developed in a QbD approach should be investigated by both test methods to determine which performance test is more sensitive to the specific product characteristics.

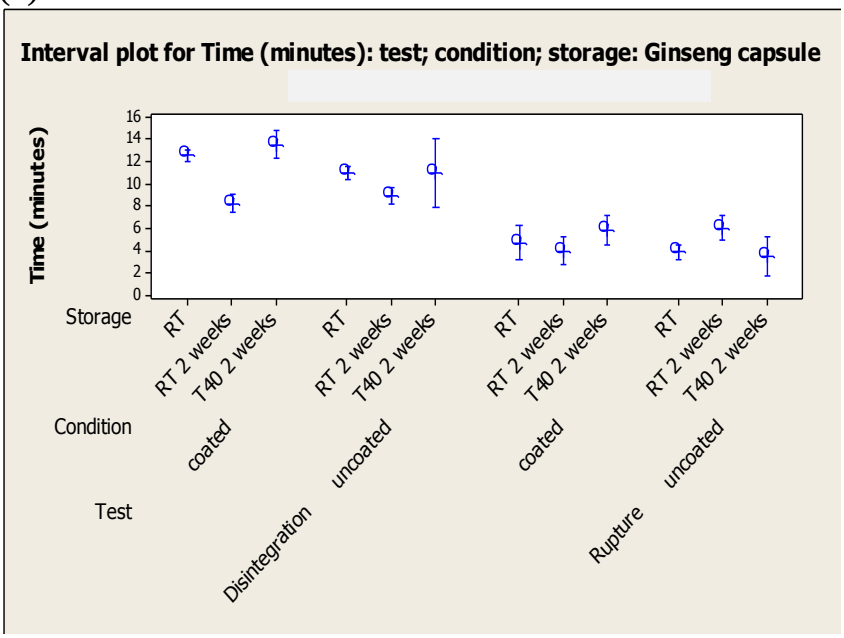
(a)



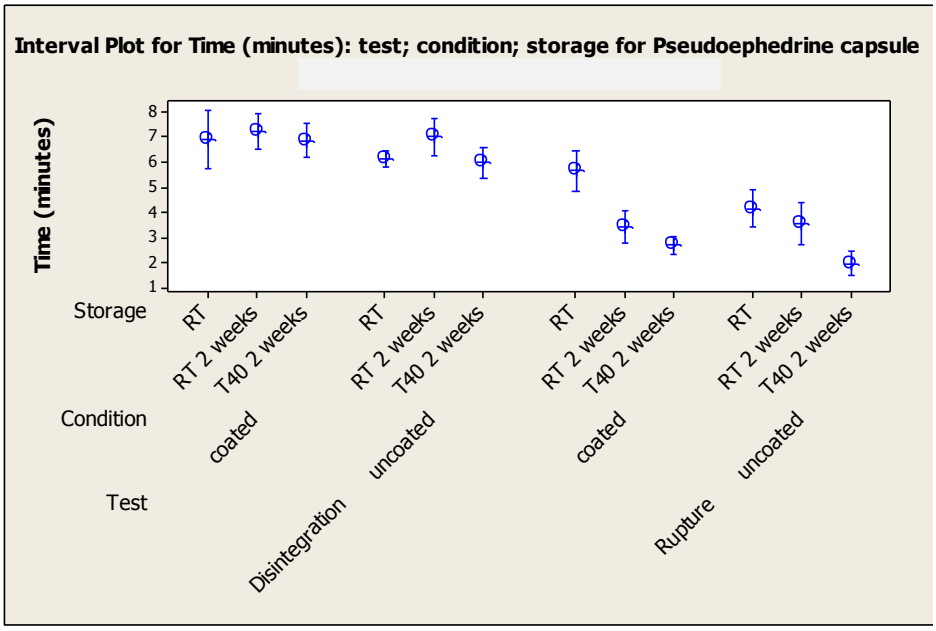
(b)


Interval plot for Time (minutes): test; condition and storage for Flaxseed oil capsule
Time (minutes)
Storage
RT
RT 2 weeks
T40 2 weeks
Condition
coated
uncoated
Test
Disintegration
Rupture

(c)


Interval plot for Time (minutes): test; condition; storage: Ginseng capsule
Time (minutes)
Storage
RT
RT 2 weeks
T40 2 weeks
Condition
coated
uncoated
Test
Disintegration
Rupture

(d)



(e)

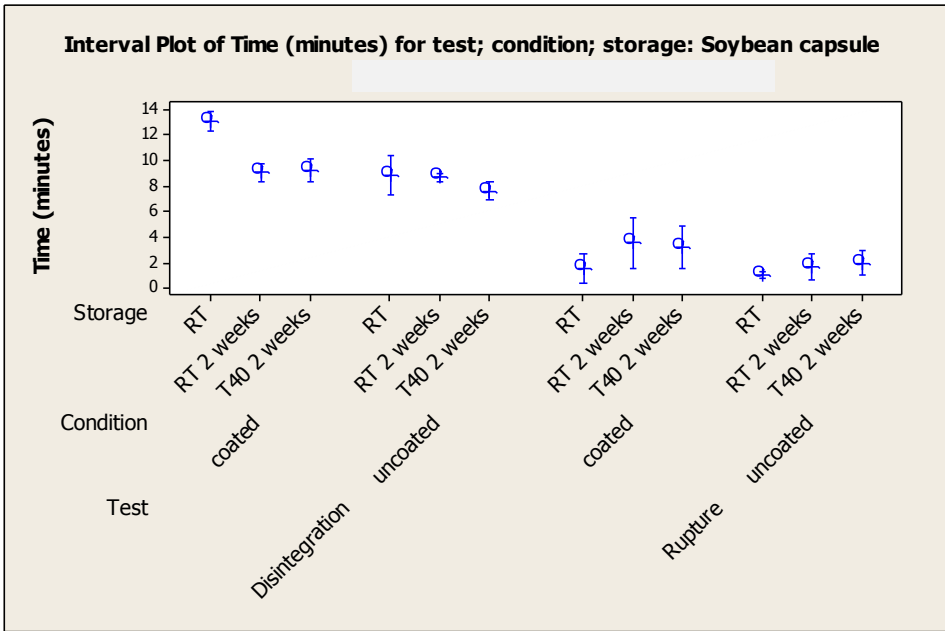


Figure 2.3.1:Interval plot of the disintegration and the rupture times (min) for coated and uncoated capsules of (a) amantadine, (b) flaxseed oil, (c) ginseng, (d) pseudoephedrine, and (e) soybean oil under different storage conditions: RT (room temperature), RT after 2 weeks, and 40 °C after 2 weeks.

Table 2.3.1:Disintegration and Rupture Times (min) for Amantadine, Flaxseed Oil, Ginseng, Pseudoephedrine, and Soybean Oil Capsules under Different Storage Conditions

Capsule	Test	Condition	Storage Condition		
			RT	RT after 2 weeks	40 °C after 2 weeks
Amantadine	Disintegration	coated	9.7 ± 0.3	10.0 ± 0.3	10.3 ± 0.15
		uncoated	9.3 ± 1.0	9.9 ± 0.3	9.4 ± 0.3
	Rupture	coated	9.5 ± 2.4	10.9 ± 0.4	8.9 ± 1.9
		uncoated	9.7 ± 1.3	8.3 ± 0.9	9.8 ± 1.8
Flaxseed Oil	Disintegration	coated	8.2 ± 0.7	7.7 ± 0.5	8.3 ± 0.2
		uncoated	8.3 ± 0.9	8.1 ± 0.3	7.9 ± 0.5
	Rupture	coated	2.2 ± 0.2	0.8 ± 0.4	3.1 ± 0.3
		uncoated	2.5 ± 0.4	2.9 ± 0.4	1.9 ± 0.4
Ginseng	Disintegration	coated	12.1 ± 0.5	8.2 ± 0.8	13.5 ± 1.3
		uncoated	11.0 ± 0.6	8.9 ± 0.7	10.9 ± 2.9
	Rupture	coated	4.7 ± 1.5	3.9 ± 1.2	5.8 ± 1.3
		uncoated	3.8 ± 0.6	5.9 ± 1.1	3.4 ± 1.8
Pseudoephedrine	Disintegration	coated	6.9 ± 1.1	7.2 ± 0.7	6.8 ± 0.6
		uncoated	6.1 ± 0.3	7.0 ± 0.7	5.9 ± 0.6
	Rupture	coated	5.6 ± 0.7	3.4 ± 0.6	2.6 ± 0.3
		uncoated	4.1 ± 0.7	3.5 ± 0.8	1.9 ± 0.5
Soybean Oil	Disintegration	coated	13.0 ± 0.7	9.0 ± 0.7	9.2 ± 0.8
		uncoated	8.8 ± 1.4	8.6 ± 0.3	7.6 ± 0.6
	Rupture	coated	1.5 ± 1.1	3.5 ± 1.9	3.1 ± 1.6
		uncoated	0.9 ± 0.2	1.6 ± 0.9	1.9 ± 0.9

RT: room temperature

Table 2.3.2: *P*-values from Analysis of Variance for All Products Versus the StorageRT, RT after 2 Weeks, 40 °C after 2 Weeks) and the Uncoated/Coated Conditions

Capsule	Test					
	Disintegration			Rupture		
	Storage	Uncoated/Coated	Interaction	Storage	Uncoated/Coated	Interaction
Soybean Oil	0.00	0.00	0.00	0.86	0.00	0.45
Ginseng	0.00	0.02	0.02	0.38	0.34	0.00
Amantadine	0.57	0.00	0.15	0.89	0.27	0.03
Flaxseed Oil	0.27	0.86	0.20	0.00	0.00	0.00
Pseudoephedrine	0.00	0.36	0.04	0.00	0.87	0.04

RT: room temperature; $p < 0.05$ (significant, $\alpha = 0.05$)

2.3.6 References

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A version of this study has been published in Dissolution Technology, February, 2011; 18(1): 21-15

2.4.1Chapter 2 summary

QbD approaches require a reliable test to study the mechanistic behaviour of formulation factors. The disintegration test is one of these tests and is especially useful for highly soluble drugs. It is also commonly used as an initial test for formulations in product development. USP describes the test conditions, but they have changed over time. In order to be sure of the test reliability under these specifications at different apparatus setups, a series of two studies has been performed using dietary supplements.

In the first study the disintegration times of seven dietary supplement products in form of tablets and capsules were recorded. The time recoded using the following condition: small and large beaker size, baskets assembly A and B, with and without a disk. In addition the study investigated the impact of the immersion medium on the disintegration times of dietary supplements capsules made of HPMC. The study showed that the current apparatus specifications are not sufficient to ascertain that test results reflect product performance rather than apparatus set up conditions. In sufficient specifications can impact both, quality by design and quality by testing. Using these findings, new USP specifications for beaker size and basket assembly were defined. They will be published in USP 33 2S. The new specification states that "the differences between the diameter of the plastic plates, which hold the tube in vertically position, and inside diameter of the beaker should not be more than 6 $mm^{2"}$ (Figure 2.4). These new specification will reduce variability in disintegration test which will make it a more reliable performance test.

The study also showed that HPMC capsules are sensitive to sodium ions in the disintegration media. USP buffer pH 6.8 prolong led the disintegration time for HPMC capsules compared to water and SIF, which contained potassium instead of sodium. This finding can expand the mechanistic understanding of the performance of HPMC capsules when the disintegration test and different media are used.

The next study was to compare the performance of disintegration test with the rupture test. Soft gelatine dietary supplements capsules were used for this investigation. The rationale for the study was that no justification was given by USP why to use the rupture test as an alternative performance test over the disintegration test. Therefore, five products (dietary supplements) were chosen based on their content. To simulate gelatine cross-linkage the capsules were manipulated and incubated at different storage conditions. The aim was to compare the discriminative power of the disintegration and the rupture test. A coating of the capsule shells was used to simulate a production deficiency. The study showed that the disintegration and the rupture test were equally sensitive/ insensitive to detect production errors or the impact of storage conditions. The results were product specific and not apparatus specific. This is surprising because all capsule shells are made from gelatine. It was expected that the different apparatuses would pick up the manipulations and storage conditions but no clear tendency was found. Therefore, soft-shell capsules that are subject to a QbD approach should be tested with both rupture and disintegration test to determine which performance test is the most appropriate for a specific product.

The aim in quality by design is to understand the mechanisms of a formulation process**.** A deep understanding of these tests with scientific justification is essential in order to rationalize test selection.These studies were undertaken to evaluate the performance of the disintegration test and to gain more understanding about the test itself, which can provide essential knowledge to built-in the quality.

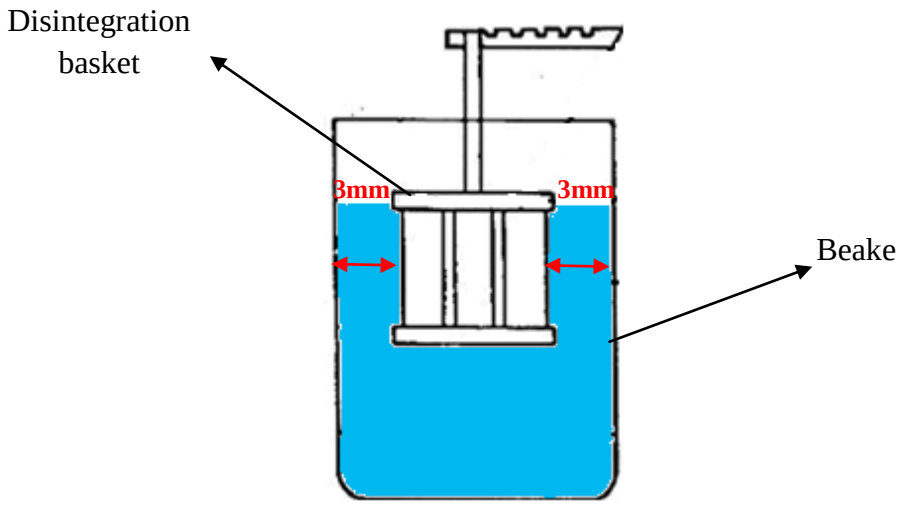

Figure 2.4: New specification of beaker size in USP disintegration test

*****Modified from http://www.hc-sc.gc.ca/dhp-mps/prodpharma/applic-demande/guide-ld/method/images/timetab_e-f1.gif

Chapter3

Provisional Biopharmaceutical Classification of Some Common Herbs Used in Western Medicine

3.1 Introduction

Introduction. For any orally administered drug product, the main parameters controlling rate and extent of absorption are aqueous solubility and gastrointestinal permeability. [1] The Biopharmaceutical Classification System (BCS) introduced by Amidon et al.[1] Classifies drugs into four classes according to these two parameters using the highest therapeutic dose within the physiologically relevant pH ranges of pH 1.2 to 6.8:

- Class I – high solubility, high permeability
- Class II – low solubility, high permeability
- Class III – high solubility, low permeability
- Class IV – low solubility, low permeability.

The FDA adapted the BCS for regulatory and scientific purposes.[2] Based on the BCS classification, waivers for *in vivo* bioequivalence testing of immediate-release oral solid dosage forms of Class 1 drugs can be granted if dissolution testing can demonstrate that two products are similar *in vitro.* The term biowaiver is defined by the World Health Organization (WHO) as approving a generic medicine based on strictly defined dissolution criteria relating to the active pharmaceutical ingredient (API) as a surrogate measure for *in vivo* bioequivalence

testing.[3] The concept of biowaivers can be traced back to the guidance document issued by the US Food and Drug Administration in 2000. Biowaivers for Class III drugs with very rapid dissolution properties and low permeability are also scientifically justified and have recently been recommended [4, 5] including the recent EMA European Guidance. [6]

The BCS concept would also be valid for herbal medicines. Since herbals usually contain more than one defined substance, the BCS classification might be more complex compared to conventional/orthodox medicines which contain one or a few combinations of APIs in a defined matrix of excipients. However, its usefulness might not be as obvious as for orthodox medicines. Herbal medicines are unregulated in many regions of the world or considered as dietary supplements as in the United States. Other countries such as Europe and recently Canada have special regulations for traditional medicines, which require regulatory approval.[7, 8] A BCS classification of herbals has therefore different implications in different regions of the world. From a scientific point of view the BCS classification can be used to set *in vitro* quality standards for products. For example an herb with highly soluble phytochemical components might only pass a disintegration test while a herbal dosage form containing a poorly soluble component should pass a dissolution test to demonstrate that its content is appropriately released. [9] The principal here is that while statistically proven clinical efficacy is often lacking and can be difficult and expensive to prove, including the difficulty of defining the product components, insuring batch to batch dissolution over time, can insure the therapeutic consistency over time.

The challenge in contrast to chemically defined drug products is that the biopharmaceutical quality of herbal medicines is often not well documented and must be applied to the complex composition of a herbal preparation.[10, 11] The European Pharmacopoeia and the International Pharmaceutical Federation (FIP) have developed a classification system for herbals based on the information available about a herbal extract. Accordingly herbal extracts can be classified into 3 categories.

- A – standardized extracts, containing constituents solely responsible for therapeutic activity (Milk Thistle, Senna)
- B – quantified extracts, containing chemically defined constituents possessing active markers (St. John's Wort, Ginkgo)
- C – other extracts, containing no constituents documented as being determinant or relevant for efficacy or as having pharmacological or clinical relevance (Valerian)

In Europe it is recommended that products containing extracts of Type A or B, but not C, should comply with the Note for Guidance on the Investigation of Bioavailability and Bioequivalence.[10] For such herbs, the BCS and biowaivers could be used to establish therapeutic or pharmaceutical equivalence. Also the BCS could be used for post approval changes of such herbal extracts. However, the BCS classification of category C extracts could be used to demonstrate *in vitro* and most likely *in vivo* product similarity if not therapeutic equivalence as for category A extracts and their actives. [7] As further knowledge becomes

available about category C extracts, they could thus be upgraded to category B or A. [10]

The aim of the present study was to provisionally classify marker components of popular herbs according to the BCS. The classification was applied to the following twelve commonly used herbs, Cascara, Roman Chamomile, Garlic, Ginger, Ginkgo, Ginseng, Licorice, Milk Thistle, Red Clover, Senna, St. John's Wort and Valerian. A provisional BCS classification strategy for herbals according to the available information is presented.

3.2 Method

To classify herbal extracts according to the BCS, known/published marker compounds were used. These were either bioactive markers as well as chemical markers with no known pharmacological or toxicological effect. The markers were taken from the USP's Dietary Supplements Compendium book. [12] Table 3.2.1 lists the extract category assigned by the European Pharmacopoeia and the marker compounds known to be components of each of those herbal extracts.

Table 3.2.1: Categorization of the Herbal Extracts According to the European Pharmacopoeia and Marker Components According to USP Dietary Supplement Compendium.

Herb	Category according to European Pharmacopeia	Markers according to USP Dietary Supplement Compendium
Cascara	A	Cascarosides calculated as Cascaroside A
Roman Chamomile	-	Matricin, Chamazulene, Apigenin-7-glucoside, Levomenol
Garlic	-	Allicin, Alliin, ɣ-glutamyl-(S)-allyl-L-cysteine
Ginger	-	Shogaole, Gingerole, Gingerdione and volatile oil
Gingko	B	Terpenlactones(Bilobalid, Ginkgolide A, B and C), Flavonoides calculated as Flavonolglycosides with mean molecular mass of 756.7g/mol
Ginseng	C	Ginsenosides
Licorice	A	Glycyrrhizin Acid
Milk Thistle	A	Silymarin calculated as Silibinin(= Silybin A and B)
Red Clover	-	Isoflavones
Senna	A	Sennosides calculated as Sennoside B
St.John's Wort	B	Hypericin, Hyperforin, Pseudohypericin
Valeriane	C	Iridoids, Valerenic Acid

The herbal categories outlined by the European Pharmacopoeia were included for the BCS classification. For category A extracts the pharmacological active substances were used as markers. The markers for category B extracts were chosen from the quantifiable phytochemical components in those extracts for e.g. St. John´s Wort; Hyperforin, hypericine and pseudohypercine. For category C extracts chemical markers which might not represent any pharmacological activity but are main phytochemical components in the particular herb that were used. For every herb that was not classified by the European Pharmacopoeia, markers that are considered to be the active components or are the common ingredients typical for a particular herb were used. Information such as plant parts used, indications and maximum dose were collected using Health Canada's Licensed Natural Health Products Database Martindale, and the German data base Rote Liste.[13]

As there are only limited experimental data available about the biopharmaceutical properties of herbs, the *ADMET Predictor* ^{TM} (*Simulations plus, Inc.)* was used to predict those properties. Version 5.0 was used for all solubility calculations and version 2.3 for the permeability estimates. *ADMET Predictor* ^{TM} is computer software used to estimate biopharmaceutical relevant molecular descriptors. The "ADMET" acronym is commonly used in the pharmaceutical industry to indicate phenomena associated with Absorption, Distribution, Metabolism, Elimination, and Toxicity of chemical substances in the human body. The input data were "mol" files of the various chemical structures, created with Symyx Draw 3.2 (Symyx Technologies, Inc.). Using these "mol" files as input to the *ADMET*

Predictor [TM], the following parameters were estimated: pKa which is the dissociation constant, Peff which is the effective human jejunal permeability, Cs which is the physiological solubility in the pH range of 1.2 – 6.8 and calculated by the software as (S+Sp). (S+Sp) was calculated as a function of the intrinsic water solubility (mg/mL), pKa values, and solubility factor (the increase in water solubility going from neutral to the cationic or anionic species) using chemical equilibrium theory [14] (Figure 3.2.1). All the markers chemical structures are presented in Appendix B. D_0 is the dose number according to the BCS (see equation below). The criteria for P_{eff} and $C_s$are described below.

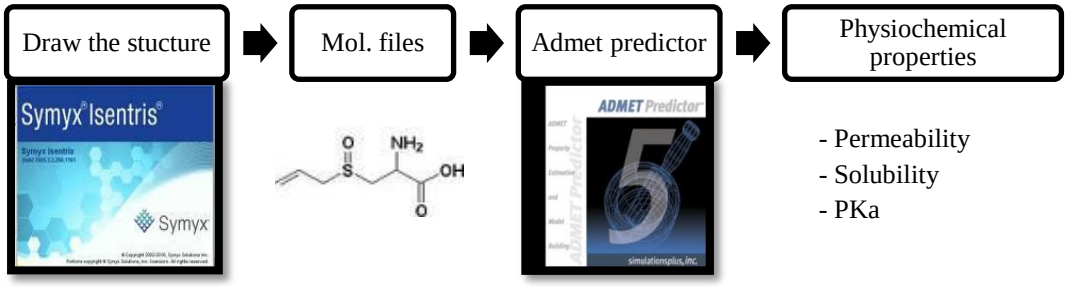

Figure 3.2.1: *ADMET Predictor* [TM] using a molecular structure as input.

3.2.1 BCS Classification Criteria

The two parameters for BCS classification are aqueous solubility at the highest therapeutic dose within the physiologically relevant pH ranges of pH 1.2 to 6.8 and gastrointestinal permeability.[1] Table 3.2.2 shows the classification criteria according to these two parameters.

Table 3.2.2: Classification Criteria for Herbs According to Their Marker Compound's Permeability (P_{eff}) and Solubility as a Function of the Dose Number (D_o).

Class	Permeability	Solubility
I	$P_{eff} \geq 1.78$	$D_0<1$
II	$P_{eff} \geq 1.78$	$D_0 \geq 1$
III	$P_{eff}<1.78$	$D_0< 1$
IV	$P_{eff}< 1.78$	$D_0 \geq 1$

3.2.2 Classification criteria according to permeability properties

P_{eff} is one major determinant of the fraction dose absorbed, and quantitatively represents the principal membrane transport coefficient of the intestinal mucosa of a substance.[15] Permeability boundaries were chosen according to the criteria proposed by Amidon et al. [1] Compounds with a higher or equal P_{eff} than metoprolol (P_{eff}=1.78) were considered highly permeable and compounds with a P_{eff} below metoprolol were considered as poorly permeable.[16]

3.2.3 Classification criteria according to dose number

Another important criteria for BCS classification is the dose number D_0 which describes the relationship between solubility and maximum dose strength according the following. Compounds with a D_o lower or equal to one are considered highly soluble. [16]

$$D° = \frac{\frac{M°}{V°}}{Cs}$$

M_0 represents the highest dose strength in mg, V_0= 250 ml (volume of water taken with the dose), and C_s is the minimum physiologic solubility in the pH range of 1.2 - 6.8 at 37˚C in mg/ml.[15] As per definition, the solubility class boundary is based on the highest dose strength of an immediate release dosage form. A drug substance is considered *highly soluble* according to the BCS when the highest dose strength is soluble in 250 ml or less.[16] The volume estimate of 250 ml is derived from typical BE study protocols that require the administration of a drug product with a glass (about 8 ounces) of water.[2] When sufficient information was available, the dose number was used for the classification. In the case when only a maximum daily dose was mentioned in literature, that value was used for the calculations**.**

3.2.4 Alternate classification system

When only limited information was available about a particular herb an alternate system was introduced: Mx represents the border value between highly soluble and poorly soluble as defined by the BCS. Mx is calculated according to the pervious equation by solving the equation towards Cs with a Dose number value of one. Any dose exceeding Mx cannot dissolve in 250 mL, which would lead to a dose number larger than 1. Mx can be used in method development and quality control to assist in choosing the right marker with sufficient solubility.

3.3 Results

3.3.1 Classification of the markers

Table 3.2.3 shows the classification of different herbs and their relevant associated marker(s) according to the BCS using permeability and dose number at defined pH values. Table 3.2.4 shows the alternate classification system.

Table 3.2.3: Permeability (P_{eff}) and Dose Number (D_0) at pH 1.2 , 4.5 and 6.8 as Criteria for the Provisional BCS Classification of Herbs with Related Makers and Known Dose Limits (M_0).

Herb	Marker	M_0 [mg]	D_0 (pH 1.2)	D_0 (pH 4.5)	D_0 (pH 6.8)	Peff	Class
Cascara	Cascaroside A	30	5.13E-04	5.13E-03	5.13E-03	0.02	3
Garlic	Alliin	27	3.05E-04	1.19E-05	1.13E-03	1.63	3
	Allicin	12	6.66E-03	6.66E-03	6.66E-03	3.3	1
Ginger	6-Gingerol	1.88	5.33E-02	5.33E-02	5.33E-02	3.13	1
	8-Gingerol	1.88	2.03E-01	2.03E-01	2.03E-01	3.17	1
	10-Gingerol	1.88	1.91E+00	1.91E+00	1.90E+00	3.22	2
	6-Shogaol	1.88	4.53E-01	4.53E-01	4.53E-01	6.84	1
	8-Shogaol	1.88	1.54E+00	1.54E+00	1.54E+00	6.14	2
	10-Shogaol	1.88	4.25E+00	4.25E+00	4.25E+00	5.51	2

	6-Gingerdione	1.88	1.79E-01	1.79E-01	1.65E-01	3.15	1
	8-Gingerdione	1.88	5.83E-01	5.83E-01	5.26E-01	3.34	1
Gingko	Bilobalide	3.84	1.35E-02	1.35E-02	1.35E-02	0	3
	Ginkgolide A	1.36	2.37E-02	2.37E-02	2.37E-02	0	3
	Ginkgolide B	1.36	9.17E-03	9.17E-03	9.17E-03	0	3
	Ginkgolide C	1.36	3.89E-03	3.89E-03	3.89E-03	0	3
	Quercetin-3-O-coumaryl-glycosyl-rhamnosid	32.4	2.04E-02	2.00E-02	4.68E-03	0.05	3
Ginseng	Ginsenoside Rb1	8.9	1.76E-01	1.76E-01	1.76E-01	0	3
	Ginsenoside Rb2	8.9	2.36E-01	2.36E-01	2.36E-01	0	3
	Ginsenoside Rc	8.9	2.36E-01	2.36E-01	2.36E-01	0	3
	Ginsenoside Rd	8.9	4.34E-01	4.34E-01	4.34E-01	0.01	3
	Ginsenoside Re	8.9	3.46E-01	3.46E-01	3.46E-01	0	3
	Ginsenoside Rf	8.9	8.46E-01	8.46E-01	8.46E-01	0.02	3
	Ginsenoside Rg1	8.9	5.23E-01	5.23E-01	5.23E-01	0.02	3

	Ginsenoside Rg2	8.9	1.67E+00	1.67E+00	1.67E+00	0.03	4
Liquorice	Glyccyrrhizic Acid	600	5.26E+00	1.00E+00	2.73E-02	0	4
Milk Thistle	Silybin A	70	4.51E-01	4.49E-01	2.11E-01	0.25	3
	Silybin B	70	4.51E-01	4.49E-01	2.11E-01	0.25	3
Red Clover	Biochanin A	30	6.32E+00	6.32E+00	3.50E+00	0.43	4
	Daidzein	30	2.91E+00	2.91E+00	2.46E+00	0.92	4
	Formononetin	30	8.76E+00	8.76E+00	8.16E+00	1.33	4
	Genistein	30	2.68E+00	2.66E+00	1.03E+01	0.27	4
Senna	Sennoside B	30	2.99E-01	1.33E-03	9.76E-01	0	3
St.John´s Wort	Hyperforin	5	8.30E+00	8.26E+00	5.83E+00	4.53	2
	Hypericin	1	1.39E+09	6.64E+07	1.06E+03	1.72	4
	Pseudohypericin	2	1.76E+08	8.88E+06	1.74E+02	0.91	4

Table 3.3.4: Alternate approach using Permeability and Solubility at pH 1.2, 4.5 and 6.8 and M_x estimates where the solubility of a marker changes form highly soluble to poorly soluble.

Herb	Marker	Peff	M_x pH 1.2	M_x pH 4.5	M_x pH 6.8
Chamomile	Chamazulene	7.89	0.2775	0.2775	0.2775
	Matricin	0.06	252.5	252.5	252.5
	Apigenin-7-glucoside	0.06	510	510	550
	Levomenol	3.3	9.075	9.075	9.075
Garlic	ɣ-glutamyl-(S)-allyl-L-cysteine	1.77	8050	3250	21000
Valerian	Didrovaltrate	0.02	25	25	25
	Isovaltrate	0.02	19.925	19.925	19.925
	Valtrate	0.02	22.225	22.225	22.225
	Valerenic Acid	2.25	23.175	54.75	5175

3.3.2 Classification of the herbs according to the BCS

The provisional BCS classification of the herbs is described below. If markers of one herb were classified as different BCS classes, then the whole herb was assigned the higher BCS class.

3.3.2.1 Class 1

No herb was classified as entirely BCS class 1.

3.3.2.2 Class 2

Rhizomes of Ginger (Zingiber officinale, Roscoe) are often used to relieve or prevent the symptoms of motion sickness.[16] There is no categorization according to the European Pharmacopoeia and a single dose of 500 mg was found in the German Database. The volatile oil content is described as 2-3 %.[13, 17, 18] Therefore, each marker in Table 3.2.3 was calculated using 1.88 mg, all markers are poorly soluble and highly permeable and thus belong to BCS Class 2 as some of the mentioned markers e.g. 8-Shogalol and 10- Shogaol in table 3 are poorly soluble.

3.3.2.3 Class 3

Ginkgo biloba L. leaves help to enhance cognitive function in an aging population and also help to support peripheral circulation. It is a category B plant according to the European Pharmacopoeia and its extracts are commonly standardized to 24% Flavone glycosides and 6% Terpene lactones with a maximum single dose of 120 mg.[19] Tebonin™ a German ginkgo medicine contains: 26.4-32.4 mg Flavonoids and 6.0-8.4 mg Terpenlactones, The Terpene lactones are further differentiated into 3,36-4,08 mg Ginkgolide A, B, C (1.36 each) and 3.12-3.84 mg bilobalide.[13] With a permeability of 0.04, 0.03, 0.02, and 0.05 respectively and a dose number smaller 1 for all markers, Ginkgo belongs to BCS class 3.

The bulb of Garlic or Allium sativum L. is traditionally used to help relieve the symptoms associated with upper respiratory tract infections and catarrhal conditions, to reduce elevated blood lipid levels and to help maintain

cardiovascular health in adults.[20] Garlic extract is not categorized by the European Pharmacopoeia; however a many garlic products are standardized on the markers Allicin and Alliin. Allicin has a permeability of 3.3 and a recommended maximum daily dose of 12 mg, leading to a dose number smaller than 1 and is therefore classified as a BCS class 1 substance .Alliin with its carboxylic acid group on the other hand also has a dose number smaller than 1 (maximum daily dose of 27mg), but a lower permeability of 1.63, thus making it a BCS class 3 substance. For the third marker given in the USP Supplement Compendium, no maximum dose strength is known. Mx is 700 mg, meaning the solubility behavior changes at a very high dose, and a P_{eff} = 2.44 so no class was assign for this marker. However, since the other two markers are well known, a BCS class can be assigned and the low permeability of one marker moves this herb into the BCS class 3.

The dried and aged bark of Cascara is traditionally used as a laxative. The maximum dose is 30 mg per day, which is also the maximum single dose, and standardization is based on the content of Hydroxyanthracene derivatives calculated as Cascaroside A.[21] The European Pharmacopoeia categorizes cascara as a category A herb extract. With a calculated P_{eff} of 0.02 and a dose number smaller than 1, Cascaroside A is classified as a class 3 substance.

Senna (Cassia senna L.) leaf dry extract is used as a laxative. It is a category A plant extract according to the European Pharmacopeia and is standardized on Sennosides calculated as Sennoside B with the highest dose strength of 30 mg per

day, which is also the maximum single dose.[22] Sennoside B with a P_{eff} of almost zero and a dose number smaller than 1 is considered a BCS class 3 herb.

Milk thistle (Silybum marianum L.) is a category A plant extract and is standardized on Silymarin calculated as Silibinin (silybin A and sylibin B). The fruits are extracted and used for hepatic protection.[23] The maximum single dose of the commercial product Legalon ® is 140 mg, therefore 70 mg was used for each active.[13] With a permeability of 0.25 and a dose number less than 1, Milk Thistle's markers belong to BCS class 3.

3.3.2.4 Class 4

Ginseng root (Panax Ginseng C. A. Mayer) is used in herbal medicine as a stimulant and as supportive therapy for the promotion of healthy glucose levels. It is a Category C plant extract but is often standardized on Ginsennosides, with a recommended single dose of 475mg ginsennosides.[13, 24] Therefore, 8.9 mg was used for each marker for all Ginsennoides. Most Ginsennoides belong to BCS class 3, except, Ginsenoside Rg2 which is BCS class 4.

The dried root of licorice or Glycyrrhiza Glabra L is used in herbal medicine as an expectorant to help relieve chest complaints, such as catarrhs, coughs and bronchitis. The maximum dose strength is 600 mg per day of Glycyrrhizic acid which is the marker for this standardized category A plant extract.[25] The low permeability and dose number higher than 1, classifies licorice as BCS class 4.

Traditionally, red clover (Trifolium pratense L.) ointments have been applied to the skin to treat psoriasis, eczema, and other rashes. Red clover also has a history

of use as a cough remedy in children.[26] Sometimes red clover is standardized to specific Isoflavone content with a maximum dose of 120 mg of Isoflavones per day; 30 mg was used for the calculations for each marker.

The aerial parts of St. John's Wort (Hypericum perforatum L.) are used for mild depression and as a sedative for relief of restlessness and nervousness.[27] It is a category B plant extract according to the European Pharmacopeia. Active ingredients are thought to be hypericin, pseudohypericin and hyperforin. Extracts are often standardized on hypericin or hyperforin with a maximum dose of 1mg and 5 mg per day, respectively.[27] The classification of these different markers vary. In newer studies on St. John's Wort, hyperforin is considered the main active ingredient. According to the classification, hyperforin is a BCS class 2 compound, with a permeability of 4.53 and a dose number higher than 1, and hypericin and pseudohypericin with a permeability of 1.72 and 0.91 and a dose number higher than 1, are BCS class 4 makers, making St. John´s Wort a BCS class 4 herb.

3.3.3 Alternative Classification

The roman chamomile or Chamaemelum Nobile L. is traditionally used to relieve mild digestive disturbances, such as nausea or dyspepsia. The flower heads are extracted [28], but chamomile extract is not categorized by the European Pharmacopoeia. As there is no information about the maximum dose of specific makers available, no class was assign for this herb. Chamazulen and Levomenol with permeability of 12 and 3.96 and Mx values of 0.278 mg, 9.075 mg .

Matricin and Apigenin-7-glucoside have a low permeability of 0.19 and 0.14 respectively and a Mx of 252.5 mg.

Valerian or Valeriana officinalis L. root is used as a sleep aid and is sometimes standardized on valerenic acid with a maximum dose of 81 mg (9 g of dried root daily standardized to 0.9% valerenic acid).[29] The European Pharmacopoeia categorizes valerian as plant C extract. Since valerenic acid is not an active ingredient, it was therefore not included in the classification, but is given as "marker" in the USP Supplement Compendium , as it is a toxic substance and therefore harmful to health. A commercial product, Baldurat® contains 650 mg valeriana extract, but no information about any other marker content could be found. Didrovaltrate, valtrate and isovaltrate have low permeability P_{eff} 0.06, 0.05, and 0.05 respectively and M_x values around only 20mg.

3.4 The classification of Aglycons

Some markers of the reported herbs have sugars attached to their molecular structure. Often it is not scientifically conclusive if the entire molecule or only the aglycon gets absorbed *in vivo* and if the entire molecule or only the aglycon represents the active molecule. This may also depend on the individual plant. In the case of quercetin, one of the markers of Gingko biloba, studies showed that this marker has poor bioavailability and no quercetin could be detected in human plasma after oral administration. [30, 31] Quercetin circulates in plasma only in its conjugated form. However, the absorption process is still poorly understood. [32] It has been suggested that the intestinal sodium-glucose co- transporter might be involved in the absorption of quercetin glycosides and Graefe et al. [33, 34] reported

that the entire molecule was absorbed by an active transport mechanism where the sugar component seems to be involved. Since these mechanisms are not conclusively known, Table 3.3.5 lists the classification of the aglycon only. As shown, the solubility behavior as well as the permeability behavior of some markers changed when only the aglycon was classified according to the BCS. Active transport was not considered for the permeability estimation.

Table 3.3.5: Different BCS Classification of the aglycons Using Permeability and Dose Number/*Solubility* at pH 1.2, 4.5 and 6.8. In bold the BCS class if the aglycon was classified differently.

Herbs	**Marker**	**D_0 pH1.2/*Mx***	**D_0 pH4.5/*Mx***	**D_0 pH6.5/*Mx***	**P_{eff}**	**BCS**
		[mg/ml]/*[mg]*			**[cm/s x10^{-4}]**	**Class**
Cascara	Cascaroside A Aglycon	3.95E-01	3.95E-01	3.90E-01	0.98	3
Chamomile	Apigenin	*1.56E+01*	*1.57E+01*	*2.75E+01*	0.57	-
	Quercetin	1.10E+00	1.05E+00	8.85E-02	0.4	**4**
Gensing	Protopanaxadiol	4.10E+02	4.10E+02	4.10E+02	1.38	**4**
	Propanaxatriol	1.60E+02	1.60E+02	1.60E+02	0.72	**4**
Senna	Sennidin B	2.69E+04	4.33E+00	1.63E+00	0.19	**4**

The hydroxyanthracene marker of senna changed from class 3 to class 4 as the solubility of the aglycon is less as shown in Table 3.3.5. Apigenin has no class assign since the highest dose is unknown. Apigenin-7glycoside is considered a potential class 3 compound. Also the aglycon of quercetin shows a different

solubility behavior and is therefore classified differently. Protopanaxadiol is the parent compound of Ginsenoside Rb1, Rb2,Rc, and Rd. Wherease, Propanaxatriol is the parent comund of Ginsenoside Re,Rg1, and Rg2. Both are changed from class 3 to class 4.

3.5 Discussion

The BCS is a framework to classify active pharmaceutical ingredients according to their solubility and permeability properties. For the 35 components considered in the 12 herbal products, the BCS Class breakdown (in terms of numbers) was 6, 5,17,7 for BCS Class I-IV respectively. Thus nearly 50 % of the components were BCS Class 3, this is somewhat higher than the percentage observed by Takagi et al.[35] BCS Class I + III (High Solubility) is 66% of all components and these would be eligible for waivers of *in vivo* bioequivalence trials today. Thus a simple dissolution test can insure therapeutic equivalence to the patient, even if definitive evidence for a clinical efficacy claim is lacking. This may be the best (and the least) we should do for consistency of herbal products.

Although herbal extracts show a complex composition of either known active compounds or chemical markers, each ingredient – active or inactive – can be classified according to the BCS system. However, the implications for this classification of herbs are different. For a category A extract with known markers, a BCS classification can be used to establish therapeutic equivalence because the well researched components responsible for an *in vivo* effect are used for the classification. For such a herb with BCS class 1 markers, dissolution testing could

be used as a surrogate for therapeutic equivalence as outlined in the biowaiver guidance [3] and as required by the EMA to establish bioequivalence between therapeutic products. [6] However, if such a herb is marketed in other regions of the world where no regulatory requirements exist, scientific approaches should be used to set quality standards. If the BCS class 1 marker shows very rapid dissolution (>85% in 15 minutes) then a disintegration test rather than a dissolution test may be scientifically justified to ensure the product performance. In the case of a longer dissolution time, we would suggest that we should require a F2 test [16] of dissolution equivalence, batch to batch, in order to insure product consistency over time. The foregoing illustrates the different utility of the BCS approach for either regulatory or scientific purposes to ensure product performance and quality to the patient. *In vivo* studies are not necessary to insure product performance to the patient.

For category B herbs where only some of the ingredients may be used as active markers it will be challenging to establish therapeutic equivalence other than by complex clinical studies on products of identified composition. However, dissolution tests may be used to establish pharmaceutical equivalence between products and insure consistence *in vivo* performance within and between products

For category C herbs where only chemical markers are known, disintegration and dissolution tests can be used to compare products using the BCS. *In vitro* similarity can therefore be used to improve product quality and consistency, which can be seen as a major step forward in the quality control of botanical medicines.

The present study shows that a BCS classification of herbs is possible but some special considerations need to be included in the classification strategy such as category A, B or C characteristics of the markers. If available, D_0 and P_{eff} as main parameters need to be used. The alternative classification system, which is based on the solubility of individual markers, seems to be suitable for herbs where the dose is not known. The application of the solubility-based classification may be used in product development to choose a suitable marker for dissolution studies. Similarly, clinical researchers can use the classification to choose markers, which have favorable solubility and permeability properties and can be detected *in vivo*.

3.6 Conclusion

The present provisional study shows that a BCS classification of herbs is possible but some special considerations need to be included in the classification strategy. The application of biowaivers as used for drugs to establish therapeutic equivalence between products may be applied to herbs with known therapeutic markers and known dose limits; thus, minimally insuring, to the patient, that a product overtime is the 'same'. When an upper dose limit is not known or when the actives are not known, a solubility based classification of markers provides information when a marker changes from highly soluble to poorly soluble which can help chose the right marker for quality control purposes or for clinical studies.

This Provisional BCS classification of common herbs and their markers shows that more than 60% of the components of herbal products would be eligible for waivers of *in vivo* BE studies based on current regulatory standards in the US and Europe. With suitable dissolution methodology extensions product (brand) consistency over time can also be insured, thus, assuring the patient that a pharmaceutical product will perform consistently over time.

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Chapter4

Simulation of *In Vitro* Dissolution Behavior Using DDDplus™

4.1 Introduction

Drug absorption depends on physiological factors and the drug's physiochemical properties. Dissolution and absorption of a dosage depends on the solubility of the drug and the dosage form in which it is administered. It is not necessarily easy to measure a drug's solubility, especially if it is low, since it is determined by many factors (such as molecular weight and pKa).[1, 2, 3] Studies show that poor drug solubility might lead to limited drug absorption.[4] Fortunately, formulation processes can overcome this issue if the dissolution mechanism is understood.[5]

Drug dissolution is used both in the early and the late stages of drug development for many dosage forms including tablets and capsules. In the early stage of drug development the dissolution test helps the researcher to find the best formulation to tailor *in vitro* behaviour of oral dosage forms with desired performance.[6, 7] Later dissolution profiles can be used to establish an *in vitro/in vivo* correlation (IVIVC) which can reduce the need for costly bioequivalence studies.[8]

In the final stage of drug development, the dissolution test is used for quality control; that is, to manage batch to batch consistency and to detect manufacturing defects which can lead to the rejection of an entire lot. Dissolution tests are required by the U. S. Food and Drug Administration (FDA) and listed in

the United States Pharmacopeia (USP) as a performance test for quality control. The dissolution test was introduced in the USP 300 years ago and is described in detail in chapter <711> .[9, 10]

Early estimation of dissolution behavior and investigation of the influence of formulation factors on solubility is essential for pharmaceutical formulation development. Today drug development cost could reach to 1.7 million dollar.[11] Using *in silico* methods at the interface between the drug discovery and the drug development stage to estimate potential product dissolution profiles can save time material and money The objective of this study was to evaluate computer simulations that predict the *in vitro* dissolution of model drugs.

4.2 Method

4.2.1 Montelukast Sodium: Published Montelukast dissolution data was used for the simulations.[12] *In vitro* tests were performed in the USP apparatus II (paddle) at 37 ± 0.5°C in 900 mL with a rotation of 75 rpm or 100 rpm. Three different biorelevant media were used for the test: USP Simulated Intestinal Fluid (SIF), pH 6.8 buffer, Blank Fasted Simulated State Intestinal Fluid (BFaSSIF) and Fasted Simulated State Intestinal Fluid (FaSSIF). FaSSIF was used in volumes of 500 mL and 900 mL. Blank FaSSIF did not contain lecithin or sodium taurocholate. The composition of the media is presented in Table 4.1. DDDPlus™ (Dose Disintegration and Dissolution Plus), beta version 3(*Simulation Plus, Inc*.) was used to simulate the *in vitro* release of the drugs using the above mentioned dissolution test conditions. DDDPlus™ is a computer program that models and simulates the *in vitro* dissolution of drug powder or oral preparations. The

software has three main tabs: Formulation, Experimental Setup, and Simulation. In the Formulation tab, a drug's physiochemical parameters are used to run simulations as presented in Table 4.2. When input values were not available from literature, *ADMET predictor* ^{TM}, another computer program from *Simulation Plus*, was used to predict some physiochemical drug properties using the molecular structure of the molecule. In the Experimental Setup tab, the apparatus type, instrument speed, medium volume and medium type was specified. In this study, a separate database records was generated for each medium. In blank FaSSIF, the solubility enhancement factor (SEF) of the lecithin and sodium taurocholate was set to 1 since this medium had no solublization enhancer such as bile salts. In 500 mL FaSSIF at 75 rpm, the SEF was optimised, so that the software model fitted the experimental data: lecithin SEF= 36702.14; sodium taurocholate SEF= 96200.54. The same SEF values for lecithin and sodium taurocholate were used in the remaining FaSSIF media (500 mL at 100 rpm, and 900 mL at 75 rpm). The experimental data included particle size and size distribution (Figure 4.1). This distribution was used as an input in DDDplus™ to run the simulations in the simulation tab.

4.2.2 Glyburide. Published glyburide *in vitro* dissolution data was used for the simulations.[13] This published study had compared a reference product and a generic product containing the same dose of glyburide. For the purpose of this study the reference glyburide product was chosen because more information was available about this product. The study was performed in three different media:

SIF, BFaSSIF, and FaSSIF using the USP apparatus II (paddle). The experiments were carried out at 37 ± 0.5°C in 900 mL at 75 rpm at a single pH of 6.5 and using a dynamic pH range protocol with pH 6.0, 6.5, 7.0, 7.5 and 5.0. The pH conditions were changed over time to simulate the intestinal passage of the dosage form. As presented in table 3.3.1, FaSSIF contains 3mM sodium taurocholate and 0.75mM lecithin. In the experimental study, two chemical grades of sodium taurocholate and lecithin were used: low quality (crude sodium taurocholate and 60% lecithin), and high quality (97% sodium taurocholate and 99% lecithin). No option was available for different chemical grade of the sodium taurocholate and lecithin in the software. Therefore, the input to estimates the dissolution in LQ FaSSIF and HQ FaSSIF were identical.

Input parameters were applied in the same way as for montelukast using the Formulation Tab (Table 4.2). Apparatus type, instrument speed, medium volume, and medium type were used as specified in the literature.[13] In blank FaSSIF the solubility enhancement factor (SEF) of lecithin and sodium taurocholate was equal to 1; however, in FaSSIF the SEF was optimised for low quality FaSSIFand for the dynamic pH change protocol the following parameters were used: Lecithin SEF =1374.91; sodium taurocholate SEF=96200.54. The same values were applied to the remaining FaSSIF media. . To simulate the single pH dissolution experiment in the Experimental Setup tab, the experimental phase was set to pH 6.5 throughout the simulation. To simulate the dynamic pH change protocol, the experimental phase in the Experimental Setup tab was divided into 5

phases to represent the five different pH values (6.0, 6.5, 7.0, 7.5 and 5.0) as shown in Table 4.3.

After running the simulations, a regression coefficient between predicted and observed data was calculated to assess the protective power of the simulations using the statistical program SPSS 17.

4.3 Results

4.3.1 Montelukast Sodium. The predictive percent of drug dissolved in USP-SIF media was 11. 13%. The statistical analysis showed that there was a correlation between the observed in *vitro* release data and the predicted release data in all cases except for BFaSSIF (Figures 4.2). In the experimental study the measured drug release was zero in the BFaSSIF, and the predicted data was 5.9%.[12] Therefore, no correlation was found for this media. The correlations between *in vitro* release in the experimental data and the predicted values including the P values are presented in Table 4.4. The predictive percent of drug dissolved in FaSSIF 500 ml at 75 RPM and 100 RPM were almost the same 73.53% and 73.9% respectively (Figure 4.3). However, the correlation between the observed and predictive dissolution profile was highest using 75 RPM speed as shown in Figure 4.3. The highest predicted percent of drug dissolved was 89.78% in FaSSIF in 900ml at 75 RPM and the correlation between the predicted and observed *in vitro* dissolution was the highest $r^2 = 0.91$. The experimental study has shown that the highest observed percent of drug was dissolved in FaSSIF in 500ml at 100 RPM which was 88.9% [12].

4.3.2 Glyburide. The correlation between the experimental data and the predicted release profiles when using a single pH condition are shown in Table 4.4 and Figure 4.5, 4.6. Using the single pH 6.5, the highest predicted percent dissolved in 90 min. was in the FaSSIF media, 80.8% for LQ and HQ since their input parameter were the same. Using a dynamic pH change protocol increased the correlation significantly using FaSSIF media. The best correlation was found when low quality lecithin and sodium taurocholate were used as shown in Figure 4.8 and Table 4.4. The predicted percent dissolve in this media at 90 min. was 73.92%. In contrast, the correlations were insignificant in SIF and Blank FaSSIF. The predicted percent dissolve in this media at 90 min. were 62.4% for both media which is higher than the experimental dissolve percent that has seen by Wei et al. almost 34%.[13]

4.4 Discussion

Montelukast is a lipophilic drug; hence, using a solubilizing agent such as in FaSSIF improved its solubility. In contrast, no drug dissolved in blank-SIF. The predicted data in the same media also showed very limited drug solubility, which indicates that the software has a good prediction.

In the experimental study, the highest percent of dissolved drug was found in FaSSIF-500ml at 100 rpm.[12] The experimental data were used to correlate the *in vitro* dissolution with an *in vivo* profile published by Okumu et al. using Gastroplus™.[12] This software can estimate drug plasma concentrations by using

drug solubility or dissolution data for its calculations. A correlation ($r^2 = 0.83$) between the experimental data and the observed data was shown in FaSSIF-500ml at 100 rpm. In this study, the correlation between the observed and the predicted *in vitro* dissolution profiles for this media was high, $r^2 = 0.99$. The correlations of the experimental and predicted data were $r^2 = 0.79$ for FaSSIF-900ml at 75 rpm and $r^2 = 0.63$ for FaSSIF-500ml at 75 rpm. DDDplus™ was able to simulate the *in vitro* dissolution of these two media. The correlation between the observed and predictive dissolution profiles was $r^2 = 0.99$.

Glyburide is a weakly acidic drug. Studies classified glyburide as BCS class II drug, it has a low solubility and high permeability.[14, 15, 16] Therefore, studies have suggested many ways to improve glyburide's solubility, by different formulation approaches.[4, 14 ,17] The observed experimental data showed that increasing the pH increased the drug's solubility.[13] Also, using solublizing agent in the dissolution media (such as bile salt) increased the drug's dissolution.[11] The highest percentage of dissolved drug was found in experimental media LQ FaSSIF at a fixed pH; this was confirmed using DDDplus™. The regression coefficient between the observed and predicted dissolution profiles for LQ FaSSIF and HQ FaSSIFwere 0.87 and 0.83 respectively. In DDDplus™ no option is given to change the chemical grades of lecithin and sodium taurocholate. As mentioned in the method section the input was the same for the LQ and HQ FaSSIF media, but the result might suggest that the settings in DDDplus™ reflect the LQ chemical grades of lecithin and sodium taurocholate since the correlation was higher for this grade. The experimental data for glyburide had shown that low

quality FaSSIF was the best media to establish an *in vivo*/*in vitro* correlation (IVIVC).[13]

In a QbD approach, dissolution should be a reflection of *in vivo* product performance.[18, 19, 20] Using simulated *in vitro* dissolution data as an input to a program such as GastroPlus™ to build IVIVC should guide formulation scientists to define a proper formulation design.

The simulations showed that *in vitro* release of both drugs was extremely sensitive to solubility effects, which confirmed their BCS class II category.[10, 15] IVIVCs can be applied to BCS class II drugs when the *in vitro* dissolution represents the *in vivo* release.[7] Using a computer program to predict *in vitro* drug release can assist in choosing an ideal dissolution environment. By comparing the predictions of DDDplus™ with those of experimental studies, the study showed that DDDplus™ can predict dissolution of poorly soluble drugs in a variety of media. Being able to predict dissolution profiles for BCS Class II drugs, which is often more difficult than for highly soluble drugs, indicates that DDDplus™ is a powerful tool for estimating dissolution profiles, especially in the discovery phase.

4.5 Conclusion

DDDPlus™ 3, (*Simulation Plus, Inc.*) was capable in predicting the *in vitro* release pattern of montelukast sodium and glyburide. Computer simulations of the *in vitro* release of drug candidates using DDDPlus™ have the potential to estimate dissolution conditions at an early stage in the drug development.

Furthermore, such results might be used to estimate *in vivo* drug plasma levels without any *in vivo* experiments. This can assist in lead compound selection as well as in designing a dissolution test which is suitable for IVIVC or for quality control. It is expected that this will result in substantially less experiments to establish *in vitro* tests.

Table 4.1: Media composition used in the simulation.

Media	**USP SIF**	**(FaSSIF)**	**(BFaSSIF)**
Composition	(0.023 M) sodium hydroxide (0.005 M) potassium phosphate monobasic	(0.029 M) sodium phosphate monobasic (0.0095 M) sodium hydroxide (0.105 M) sodium chloride (0.0035 M) sodium taurocholate. (0.00075M) lecithin.	same composition as for FaSSIF **but without** bile salt (lecithin or sodium taurocholate)

Table 4.2: Input data used for the simulations.

Drug	Glyburide	Montelukast
Dose (mg)	3.5 [13]	10 [12]
Dosage form	Immediate release [13]	Immediate release [12]
Solubility (mg/mL)	0.043 at pH 7.4 [13]	0.0007 at pH 6.5[22]
Mean Particle radius (μm)	6.28 [13]	6.572 [10]
Particle density (g/mL)	1.38 [13]	1.2 (*ADMET Predictor* ™ 5 default setting)
Diffusion coefficient ($cm^2/s \times 10^{-5}$)	0.5878[13]	0.54(*ADMET Predictor* ™ 5)
pKa	5.5, (*ADMET Predictor* ™ 5) 11.62,10.64	2.8, 5.7 [22]
SEF of the pKa	3080 (*ADMET Predictor* ™ 5)	8090 (*ADMET Predictor* ™ 5)

Figure 4.3: Glyburide input data for dynamic pH changes.

Experiment Phase	Start Time	End Time	Medium pH
1	0	30	6
2	30	90	6.5
3	90	150	7.5
4	150	270	7
5	270	300	5

Table 4.4: P values and regression coefficients for predicted and experimental data of montelukast and glyburide.

Drug	Media	P values	Regression
Montolukast	USP-SIf	P<.001	.92
	BFaSSIF	NA	NA
	FaSSIS 500 mL at 75 rpm	P<.001	.99
	FaSSIF 500 mL at 100 rpm	P<.001	.99
	FaSSIF 900 mL at 75 rpm	P<.001	.99
Glyburide	USP-SIf at pH 6.5	P=.003	.69
	BFaSSIF at pH 6.5	P=.005	.64
	FaSSIF-LQ at pH 6.5	P<.001	.87
	FaSSIF-HQ at pH 6.5	P=.001	.83
	USP-SIf at dynamic pH	P=.19	.38
	BFaSSIF at dynamic pH	P=.19	.38
	FaSSIF-LQ at dynamic pH	P<.001	.99
	FaSSIF-HQ at dynamic pH	P<.001	.99

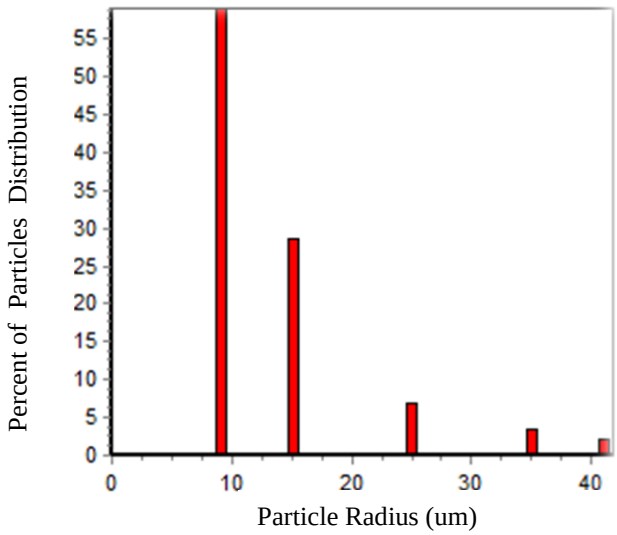

Figure 4.1: Particle size distribution of montelukast used in the simulation

*Adopted from Okumu *et al*. 2008

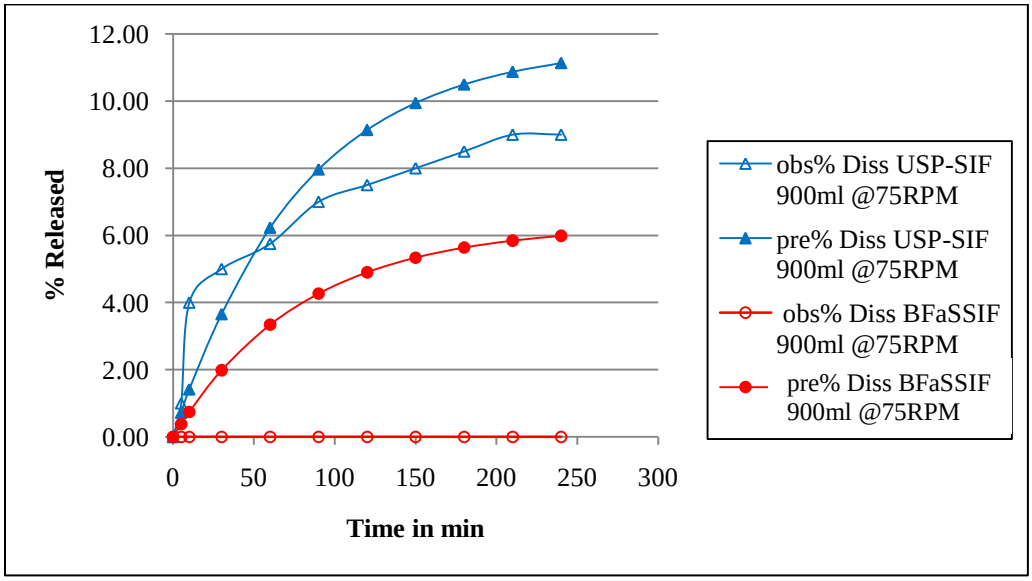

Figure 4.2: Observed (obs.) and predicted (pre.) dissolution release profile (Diss) for montelukast using USP simulated intestinal fluid (SIF) and blank fast stated simulated intestinal fluid (BFaSSIF) in 900 mL at 75 rpm using USP apparatus II at 37 C^0.

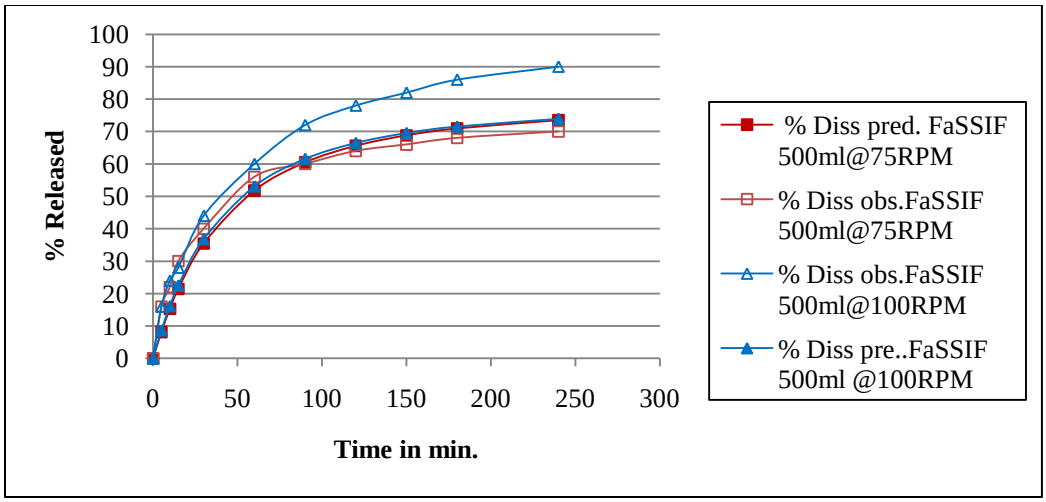

Figure 4.3: Observed (obs.) and predicted (pre.) for montelukast using fast stated simulated intestinal fluid (FaSSIF) as relative media using USP apparatus II at 37 C^0 in the following condition: (500 mL at 75 rpm, 500 mL at 100 rpm)

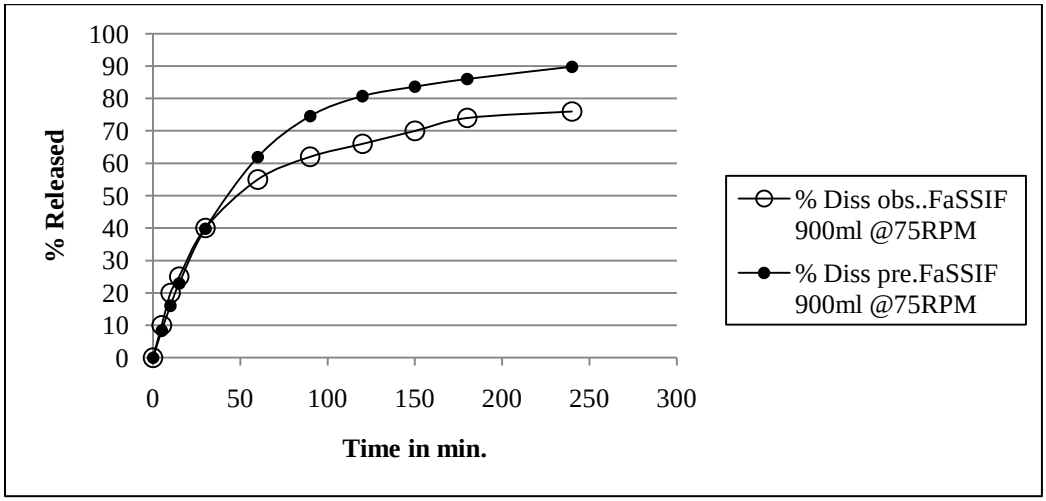

Figure 4.4: Observed (obs.) and predicted (pre.) for montelukast using fast stated simulated intestinal fluid (FaSSIF) as relative media using USP apparatus II at 37 C^0 in the
900 mL at 75 rpm.

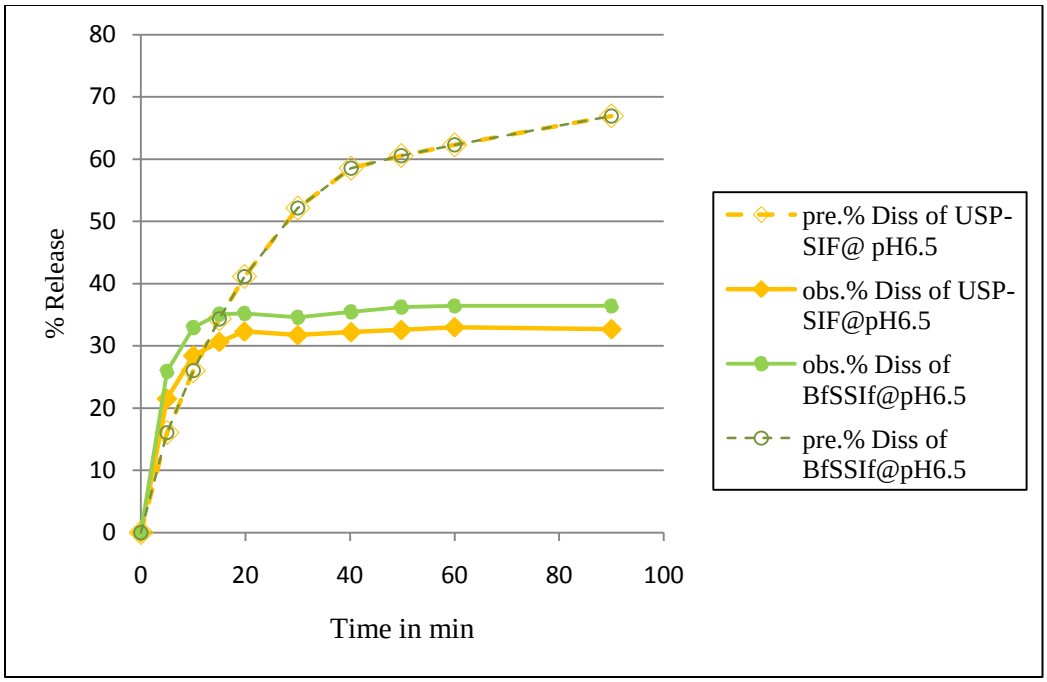

Figure 4.5: Observed (obs.) and predicted (pred.) dissolution release profile (Diss) for glyburide at pH 6.5 using USP apparatus II at 37 C^0 in 900 ml at 75 rpm in the following media: USP simulated intestinal fluid (USP-SIF), Blank fast state simulated intestinal fluid (BFaSSIF).

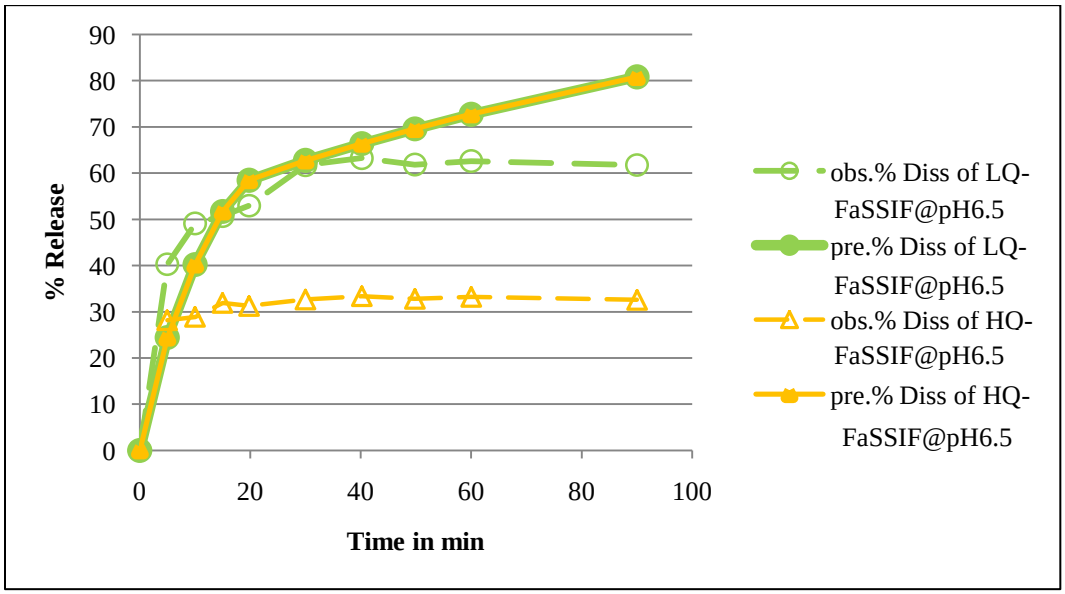

Figure 4.6: Observed (obs.) and predicted (pred.) dissolution release profile (Diss) for glyburide at pH 6.5 using USP apparatus II at 37 C^0 in 900 ml at 75 rpm in the following media low quality fast state simulated intestinal fluid (LQ-FaSSIF) and high quality fast state simulated intestinal fluid (HQ-FaSSIF).

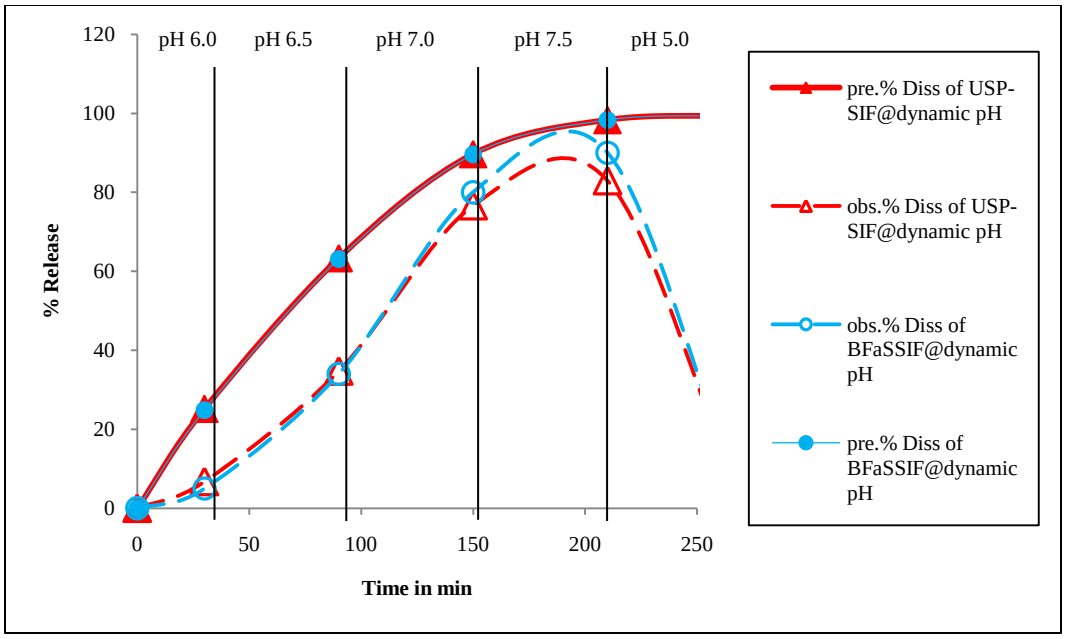

Figure 4.7: Observed (obs.) and predicted (pre.) dissolution release profile (Diss) for glyburide at dynamic pH (6.0, 6.5, 7.0, 7.5, and 5.0) changes using USP apparatus II at 37 C^0 in 900 ml at 75 rpm in the following media: USP simulated intestinal fluid (USP-SIF), Blank fast state simulated intestinal fluid (BFaSSIF)

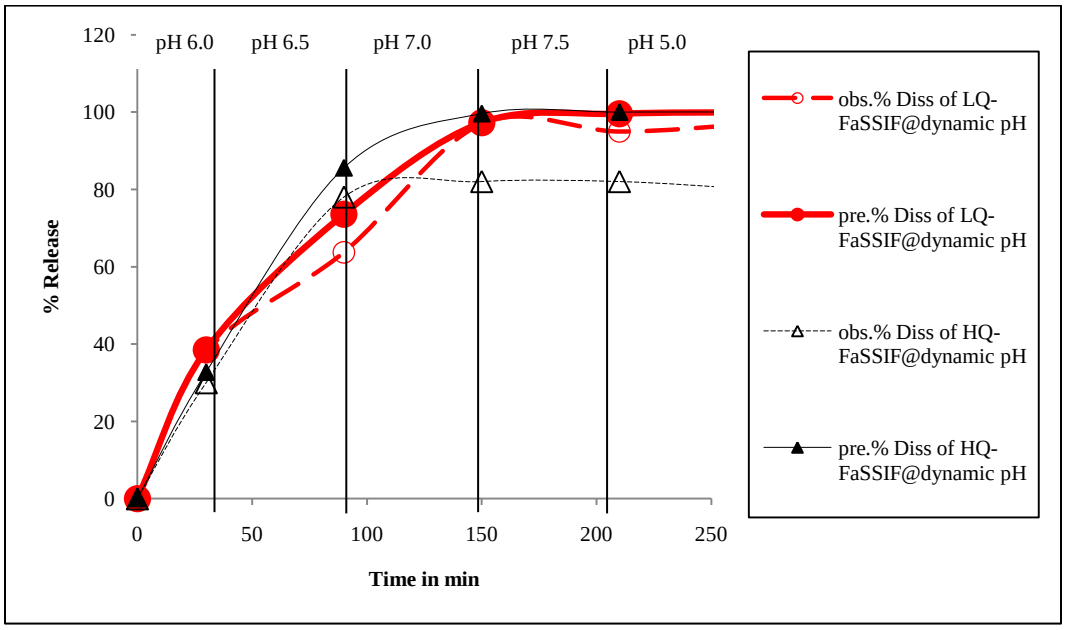

Figure 4.8: Observed (obs.) and predicted (pre.) dissolution release profile (Diss) for glyburide at dynamic pH (6.0, 6.5, 7.0, 7.5, and 5.0) changes using USP apparatus II at 37 C^0 in 900 ml at 75 rpm in the following media low quality fast state simulated intestinal fluid (LQ-FaSSIF) and high quality fast state simulated intestinal fluid (HQ-FaSSIF).

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Chapter5

Discussion, Conclusions, and Future Directions

5.1 Discussion

QbD aims to build quality into a product using predefined objectives.[1,2] To build quality into a product, a deep understanding of a product's characteristics and the manufacturing processes is necessary. *In vitro* performance tests are useful tools for understanding mechanistic factors which might impact the desired formulation. Oral preparations are the most widely used because they are convenient to administer. Disintegration is the first step in the absorption cascade of an oral dose form. The disintegration test has been used for many years. It is described in the USP as a performance test for tablets and capsules. However, no investigation of the performance of the disintegration test has been reported in the literature. Investigation of factors that influence the disintegration test is especially important if this test is used in a QbD approaches. Therefore, the influence of different conditions, tests and setups on the disintegration time of capsules and tablets containing dietary supplements was investigated. The first study (in chapter 2) concluded that the disintegration test specifications, especially the beaker size specifications, were not sufficient to make the disintegration test reliable. The USP has used these results to provide new specifications for the beaker size used in the disintegration test.[3] This specification makes the disintegration test more robust and limits variations in the disintegration time between equipment variations from different manufactures.

From a QbD perspective, *in vitro* disintegration should mimic *in vivo* disintegration.[4] In order to reach this goal; all sources of variations should known. The study has discovered that the source of variations in disintegration time was due to insufficient beaker specifications.

Another performance test used for capsules is the rupture test. In USP chapter <2040>this test is used as performance test for soft gelatine capsules, and it is listed as a specific test for this dosage form. However, no reason has been given for replacing the disintegration test for this dosage form. It was shown in chapter 2 that the disintegration test has no advantage over the rupture test as a quality control measure. The study showed that the sensitivity to detect simulated product deficiency and different storage conditions was product selective not a test selective. In QbD both tests need to be examined to determine which one is the most appropriate to the storage condition being investigated for specific product. Understanding the mechanism of the factors that influence the performance of soft gelatine capsules can help to tailor the product formulation toward predefined specifications.

The BCS is a tool that helps the developers to choose an appropriate performance test for a product.[5,6,7] The BCS, as discussed in chapter 1, classifies a compound based on two properties: solubility and permeability. This approach can be applied to any compound if therapeutic useful or toxic. It is has valuable implications in drug development, drug regulation and clinical studies. In drug development, the BCS can identify promising molecule based on their BC class. It is also a tool for decision making in early stages of drug development.[8] The BCS

can guide developers to the best formulation by determining the rate-limiting steps of molecules *in vivo* performance e.g. dissolution, dose or absorption number limited drugs. The BCS is especially helpful because an empirical approach does not permit developers to know how close the formulations are to the desired result.[9, 10] For example, if a drug is class I or class III, salt selection would be less likely to impact product dissolution. In contrast, salt selection would significantly impact drug dissolution if a product has limited solubility. However, a class II drug might require a surfactant to enhance its solubility. A class IV drug might require absorption enhancers on top of this or a prodrug approach needs to be considered, or the drug may need to be administered parenterally.[9, 10] Recently the BCS was extended by looking at a drug's metabolism. Classes I and II drugs are usually extensively metabolised; however, class III and IV drugs are expected to be eliminated mostly unchanged. This framework is called the Biopharmaceutics Drug Disposition Classification System (BDDCS).[6, 8] The BDDCS adds valuable information to the BCS by adding metabolism as an additional criterion for the permeability classification.

The BCS has been used by the FDA to waive *in vivo* bioequivalence studies for IR BCS class 1 drugs. [11] A bioequivalence study is a comparison between two drug products which have the same active ingredients, dose strength, and dosage form. For example, a generic can use a biowavier to demonstrate its therapeutic equivalence by having the same dissolution behaviour as a Comparator Pharmaceutical Product.[11] Bioequivalence can be verified by *in vitro* performance tests rather than through costly *in vivo* studies as long as the drug has

a wide therapeutic index and demonstrates rapid dissolution and is a BCS class Idrug.[12] Biowaiverscan also be applied to dose proportional products as long as the higher dose strength was used for the comparison. The world health organization (WHO) and European medicine agency (EMA) extended their biowaiver to include class III drugs which have a very rapid dissolution (85% of the drug dissolves in 15 minutes).[12] For class I drugs the gastric emptying rate controls absorption rate (Table 4.1).[13, 14] On the other hand, the dissolution rate might be the liming step for class II drugs (Table 5.1). Permeability is the rate-limiting step of class III drugs, and if a drug dissolves very rapidly then dissolution might not be the limiting factor; otherwise a clinical bioequivalence study is required to demonstrate that the two products are therapeutic equivalents. Class IV drugs are the most challenging molecules since both permeability and dissolution are limiting factors in oral drug absorption; for class IV drugs clinical studies are needed to establish therapeutic equivalence (Table 5.1). The use of biowaivers based on the BCS could save $35,000,000 per year given that 26% of IR oral dosage drug forms submitted to the FDA in last five years were class BCS class I.[12, 14] This amount could be doubled if the FDA extends its regulation to class III drugs.[12]

Drugs which have 30% or more intra- individual variations are known as highly variable drugs (HVDs).[12] Applying the knowledge of the BCS to these drugs could decrease the number of clinical studies required. For instance, if a HVD is class II, *in vitro* tests could be more appropriate than *in vivo* studies to investigate the cause of variation.[12]

Table 5.1: Rate limiting step using the BCS framework and the need of bioequivalence study.*

Class	Absorption rate control step	Bioequivalence study
Class I	Gastric motility	*In vitro*dissolution sufficient
Class II	Dissolution	*In vivo* correlation should be possible
Class III	Permeability	Dissolves rapidly (*in vivo* dissolution expected); Dissolves slowly (clinical study required)
Class IV	Case by case	Clinical study required

*Modified from Cook et al. 2008.

The BCS provides the mechanistic understanding of biopharmaceutical properties. It provides the fundamental knowledge required for QbD. QbD has not yet been applied to natural health products. Applying the BCS knowledge to herbs and other natural health products is the first step in using QbD for these products. In chapter 3 it was shown that it is possible to classify herbs according to the BCS by determining the solubility and permeability for known markers. Moreover, knowing the solubility of a marker can guide developers to choose appropriate markers that may be used *in vitro* and *in vivo*.

Using *in silico* methods to predict a drug's physiochemical properties in early discovery is crucial. At this stage, computer programs can screen molecules to eliminate unsuitable candidates; they can also be used to prioritize promising drug candidates even before their chemical synthesis.[15, 16, 17] A critical property of an oral dosage form is solubility. Solubility is the key factor for dissolution before absorption and elimination can occur.[15] Hewitt et al. showed that *ADMET Predictor* [TM] was the best of 17 programs that predicted the solubility of 122

compounds.[18] Lioan and Nicklaus showed that *ADMET Predictor* ^{TM} predicted the pK_as of 197 compounds with good accuracy.[19] Therefore, this computer program is expected to be a promising tool to predict solubility and permeability to classify natural health products according to the BCS. Many studies have shown the validity of using a computer program to determine a BCS classification for drugs.[20, 21, 22] However, till now no one had expanded this approach to include herbs and other natural health products. In most cases only very limited data is available regarding natural molecules' biopharmaceutical properties. A computer program can be extremely helpful to generate the needed database of basic BCS parameters like pH-dependant solubility and permeability for such molecules.

In silico tools have many applications in drug discovery and development, especially when a product is subject to a QbD approach. However, *in silico* models need to be evaluated to assure their validity and predictive power. DDDplus^{TM} is software from Simulation Plus for estimating the *in vitro* drug dissolution for oral dosage forms like tablets, capsules or powders. The first version was released in 2005.[23] In 2010 Simulation Plus has announced that the FDA has expanded purchases of the software licences.[24] However, no published study has evaluated the program validity. In chapter 4 an evaluation study for DDDplus^{TM} to predict *in vitro* dissolution profiles in different dissolution media and at different pH values is described. The program was able to estimate the dissolution profiles of two drugs in different media. Knowing a drug's dissolution behaviour can help to establish a suitable formulation for drug.

Using computer programs and knowledge of the BCS framework can be a road map for designing experiments in QbD without the need for expensive and time-consuming *in vitro* and *in vivo* studies.[25]

5.2 Conclusion

QbD uses statistical tools to design experiments with good process understanding to control variations in the final product. Performance tests and computer simulations are very helpful. Both need to be well evaluated so that any sources of variability or prediction errors are controlled or known. Computer programs can be used to classify natural heath products according to BCS. The BCS framework can help to choose the most appropriate performance test. These tools can provide the scientific knowledge,which is required to apply QbD to dietary supplements and natural health products.The presented studies establish the essential knowledge to build the desired quality into a product**.**

5.3 Future directions

Designing a proper formulation of dietary supplements through a design of experiment (DoE) can be done through statistical analysis. Using QbD for dietary supplements will result in robust formulations. Developers will be able to design and formulate the *in vivo* intended product performance. This will overcome undiscovered formulation problems, which today frequently occur within a product's life cycle due to a lack of process understanding or knowing the source of product variability. QbD for natural products starts with choosing a particular herb and identifying its category according to the European Pharmacopeia (category A, B and C). This is followed by a BCS classification of suitable

markers and identifying an appropriate performance test for this marker. The resulting dosage form can be then evaluated using *in silico* computer simulations. Designing a proper formulation using QbD can positively impact life cycle management of a product.

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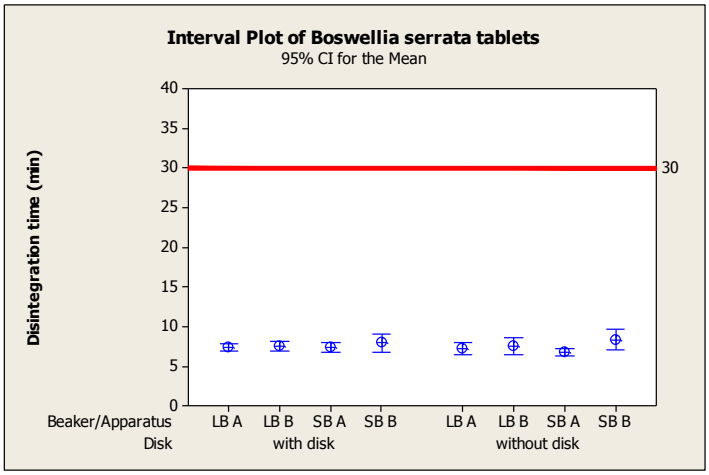
Appendix A

(Unpublished data of the disintegration study)



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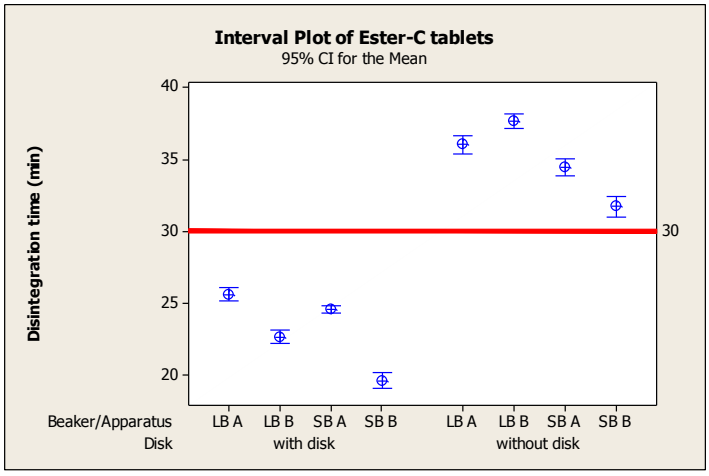
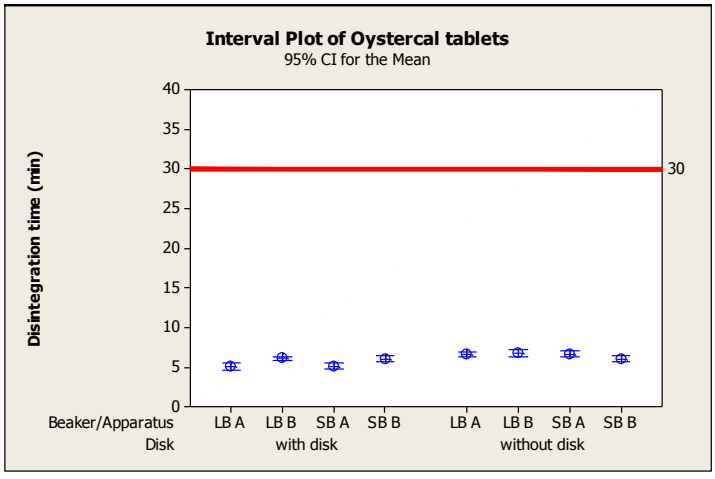
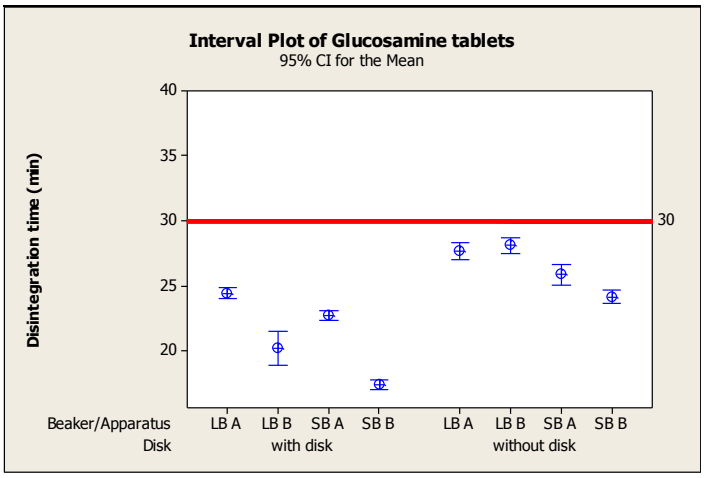


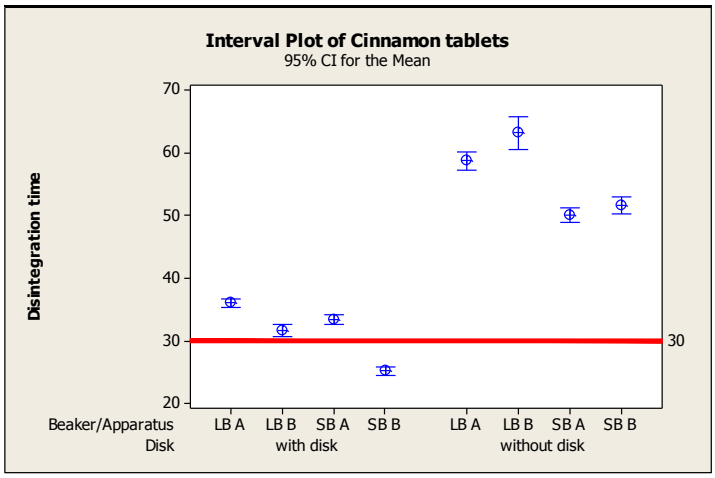
(b)



(c)

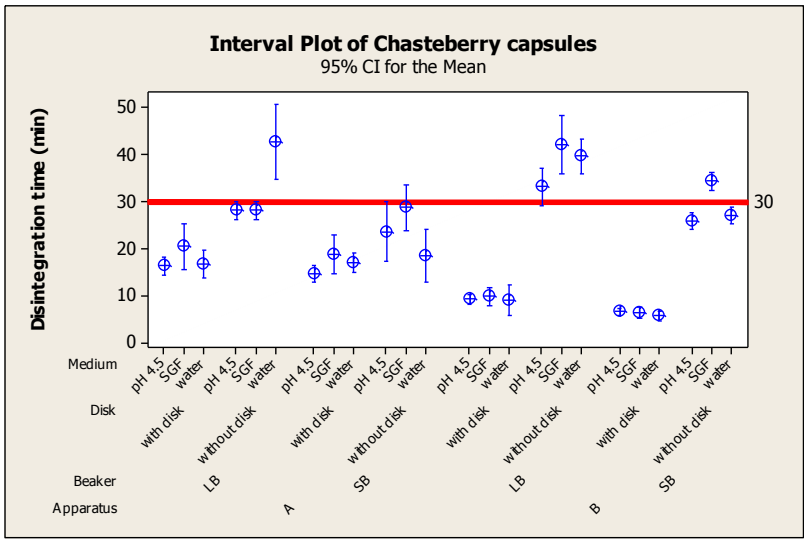


(d)

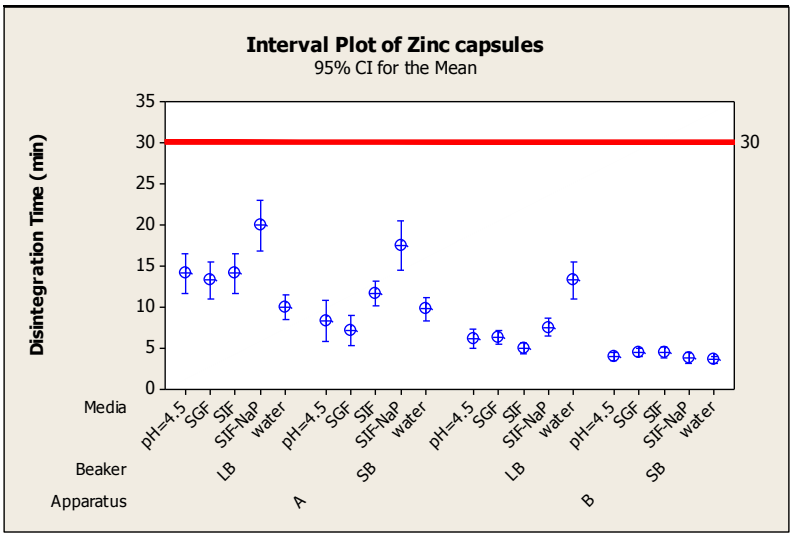


(e)

FIGURE A.1: Interval plots of the disintegration times under the following conditions: SB A (small beaker apparatus A) with and without disk; SB B (small beaker apparatus B) with and without disk; LB A (large beaker apparatus A) with and without disk; LB B (large beaker apparatus B) with and without disk for *Boswellia serrata* (a), Ester-C (b), Oystercal (c), Glucosamine (d) and Cinnamon (e) tablets. The line indicates the 30 min pass condition of USP <2040> for disintegration.

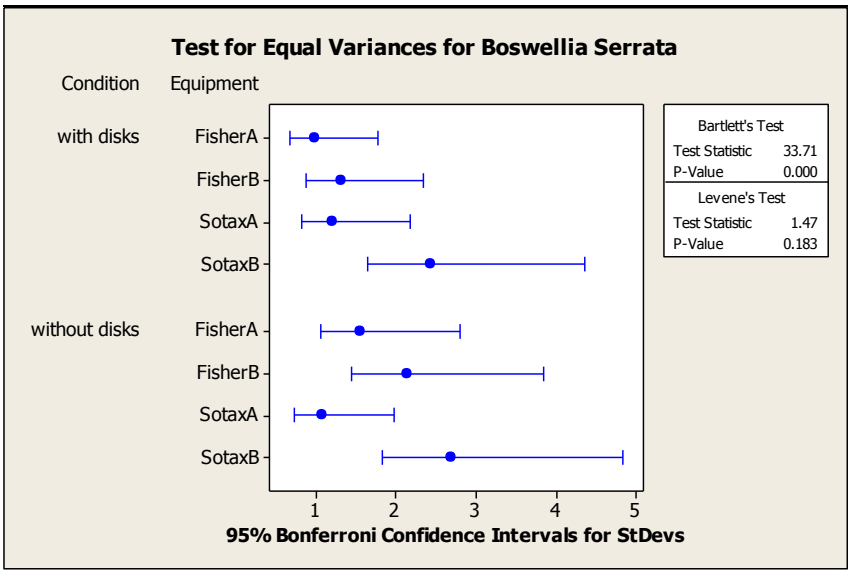


(a)

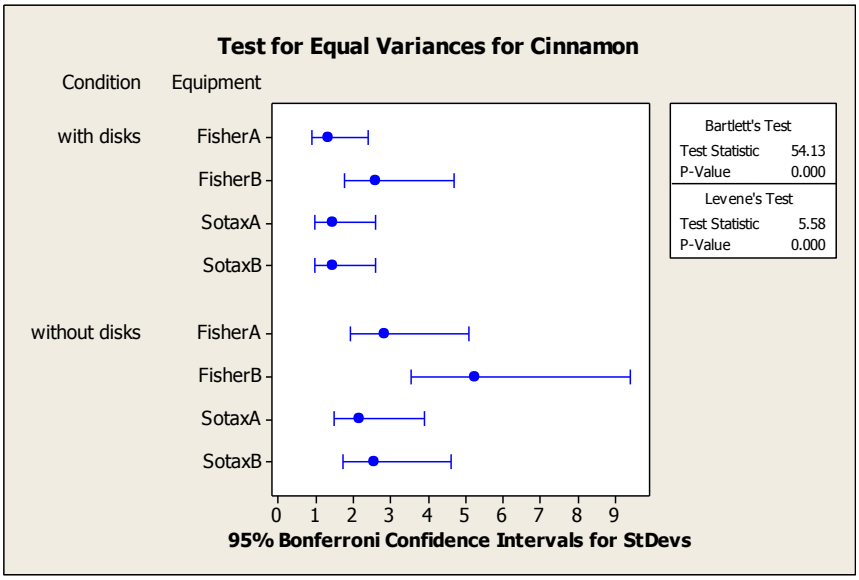


(b)

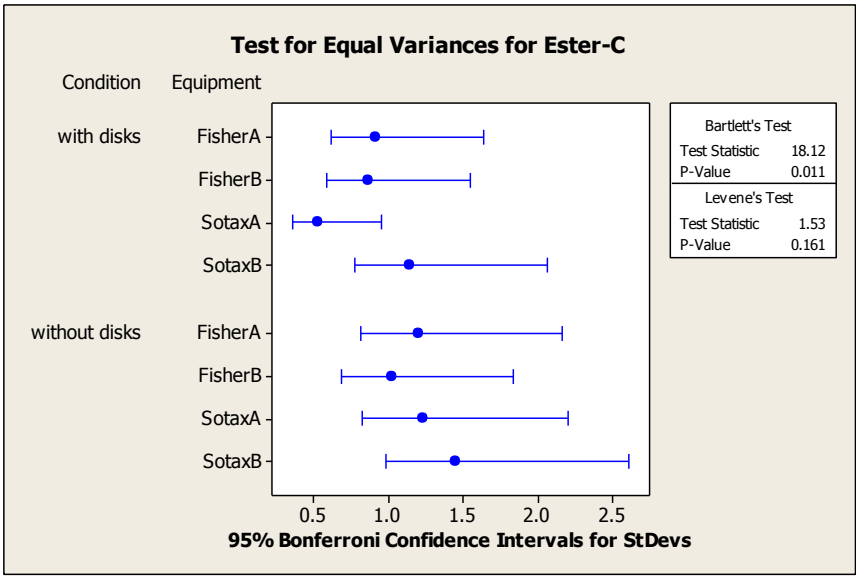
FIGURE A.2: Interval plots of the disintegration times under the following conditions for Chasteberry capsules (a): SB A (small beaker apparatus A) with and without disk; SB B (small beaker apparatus B) with and without disk; LB A (large beaker apparatus A) with and without disk; LB B (large beaker apparatus B) with and without disk and different media (water, buffer pH=4.5 and Simulated Gastric Fluid: SGF) and under the following conditions for Zinc capsules (b): SB A (small beaker apparatus A); SB B (small beaker apparatus B); LB A (large beaker apparatus A); LB B (large beaker apparatus B), with disk, and different media (water, USP Simulated Intestinal Fluid (potassium based): SIF, SIF-NaP = Sodium based buffer pH 6.8, buffer pH=4.5 and Simulated Gastric Fluid: SGF). The line indicates the 30 minutes USP <2040> acceptance criteria for disintegration.


Test for Equal Variances for Boswellia Serrata
Condition
Equipment
with disks
FisherA
FisherB
SotaxA
SotaxB
without disks
FisherA
FisherB
SotaxA
SotaxB
Bartlett's Test
Test Statistic 33.71
P-Value 0.000
Levene's Test
Test Statistic 1.47
P-Value 0.183
1
2
3
4
5
95% Bonferroni Confidence Intervals for StDevs

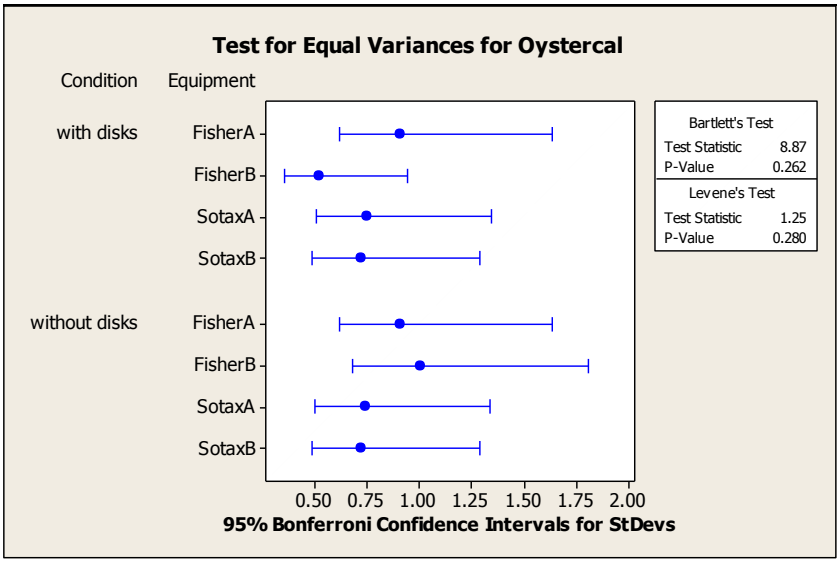
a


Test for Equal Variances for Cinnamon
Condition
Equipment
with disks
FisherA
FisherB
SotaxA
SotaxB
without disks
FisherA
FisherB
SotaxA
SotaxB
Bartlett's Test
Test Statistic 54.13
P-Value 0.000
Levene's Test
Test Statistic 5.58
P-Value 0.000
0
1
2
3
4
5
6
7
8
9
95% Bonferroni Confidence Intervals for StDevs

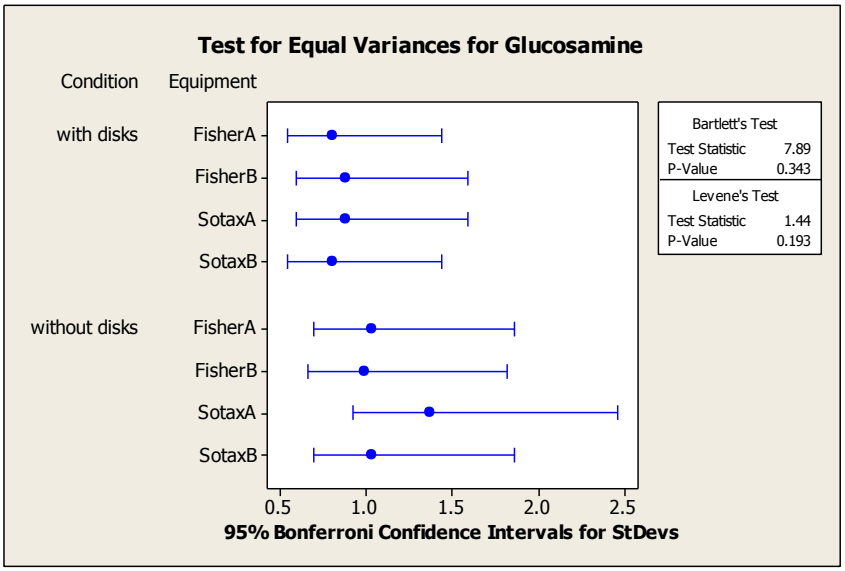
b


Test for Equal Variances for Ester-C
Condition
Equipment
with disks
FisherA
FisherB
SotaxA
SotaxB
without disks
FisherA
FisherB
SotaxA
SotaxB
0.5
1.0
1.5
2.0
2.5
95% Bonferroni Confidence Intervals for StDevs
Bartlett's Test
Test Statistic 18.12
P-Value 0.011
Levene's Test
Test Statistic 1.53
P-Value 0.161

c

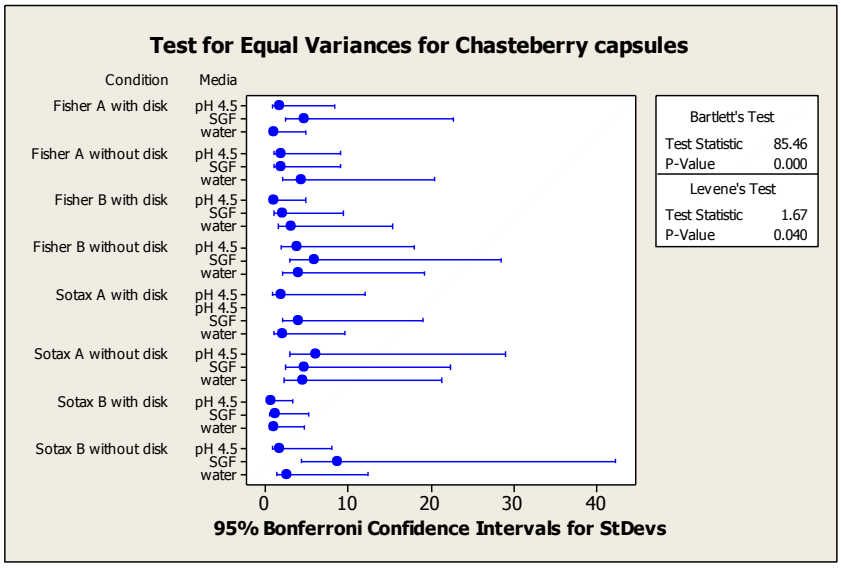

Test for Equal Variances for Oystercal
Condition
Equipment
with disks
FisherA
FisherB
SotaxA
SotaxB
without disks
FisherA
FisherB
SotaxA
SotaxB
0.50
0.75
1.00
1.25
1.50
1.75
2.00
95% Bonferroni Confidence Intervals for StDevs
Bartlett's Test
Test Statistic 8.87
P-Value 0.262
Levene's Test
Test Statistic 1.25
P-Value 0.280

d

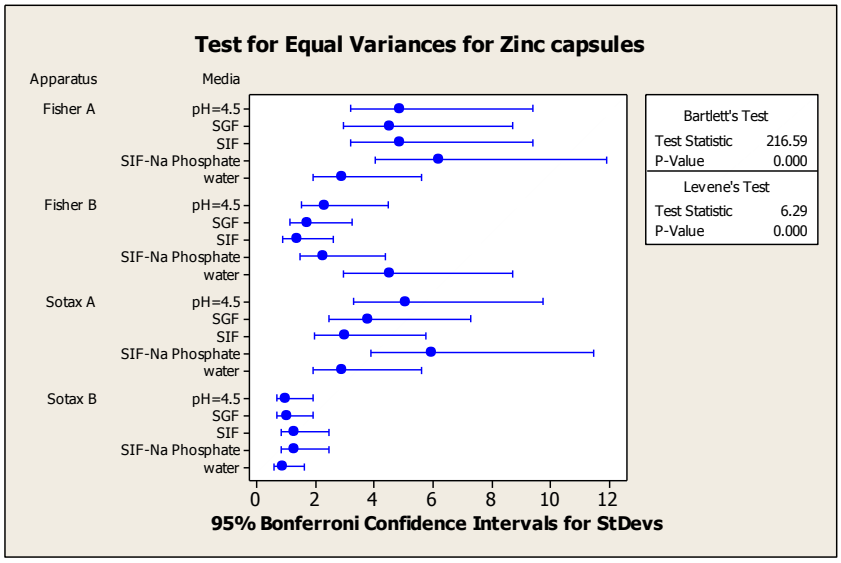


e

FIGURE A.3: Variances for tablets under the following conditions: Sotax A (small beaker apparatus A) with and without disk; Sotax B (small beaker apparatus B) with and without disk; Fisher A (large beaker apparatus A) with and without disk; Fisher B (large beaker apparatus B) with and without disk for *Boswellia serrata* (a), Cinnamon (b), Ester-C (c), Oystercal (d) and Glucosamine (e) tablets. The test for equal variances showed no significant differences among the conditions when p values of (Levene and Bartlett tests) are ≥.5

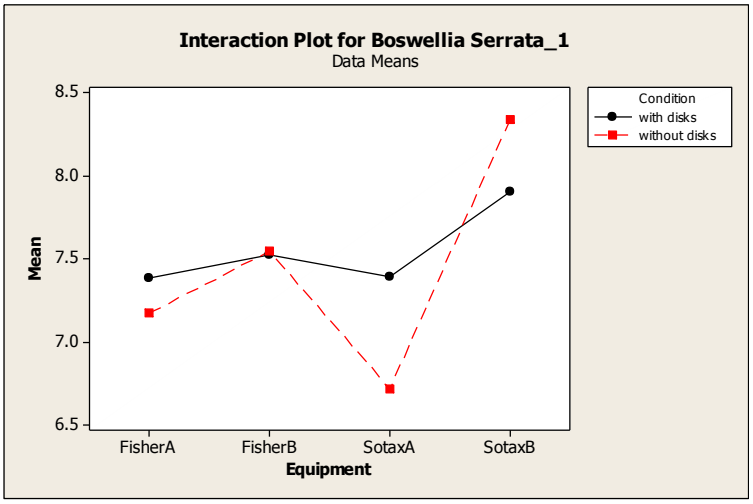


a

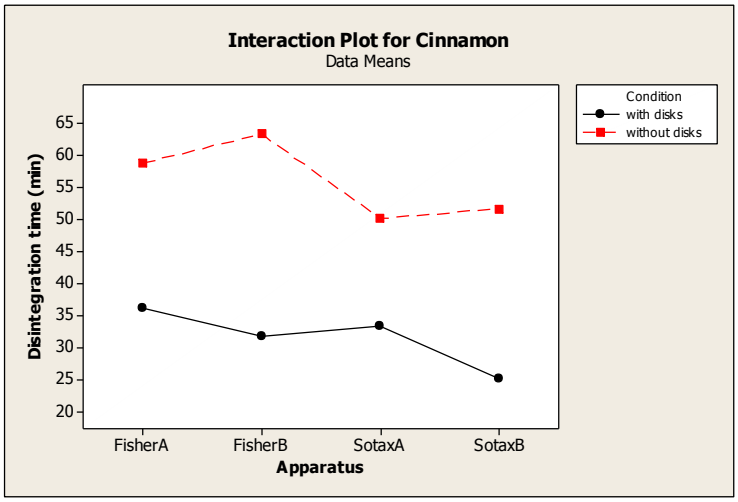


b

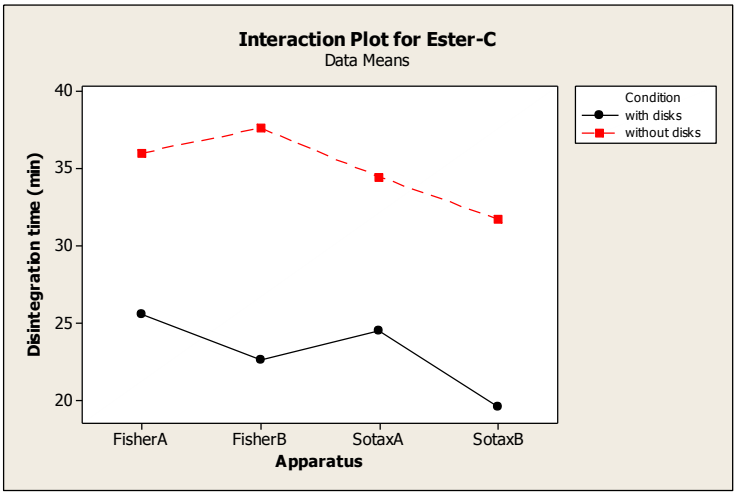
FIGURE A.4 : Variances of disintegration times for Chasteberry and Zinc capsules: Sotax A (small beaker apparatus A) with and without disk; Sotax B (small beaker apparatus B) with and without disk; Fisher A (large beaker apparatus A) with and without disk; Fisher B (large beaker apparatus B) with and without disk. The test for equal variances showed no significant differences among the conditions when p values of (Levene and Bartlett tests) are ≥.5



a


Interaction Plot for Cinnamon
Data Means
Disintegration time (min)
65
60
55
50
45
40
35
30
25
20
FisherA
FisherB
SotaxA
SotaxB
Apparatus
Condition
with disks
without disks

b


Interaction Plot for Ester-C
Data Means
Disintegration time (min)
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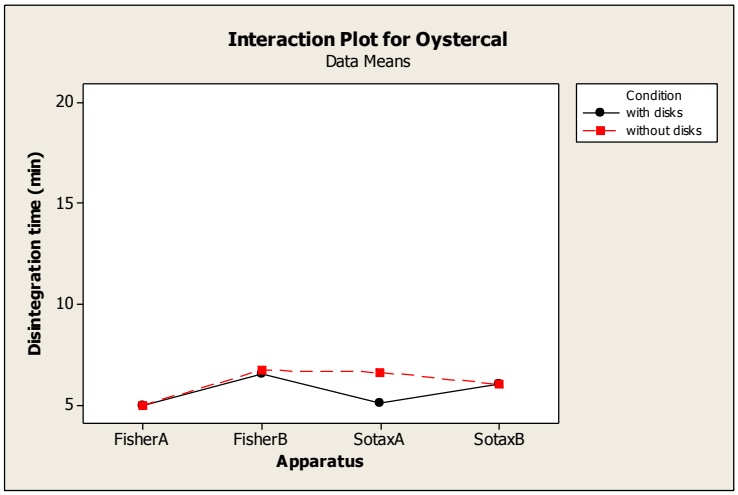
30
25
20
FisherA
FisherB
SotaxA
SotaxB
Apparatus
Condition
with disks
without disks

c


Interaction Plot for Oystercal
Data Means
Disintegration time (min)
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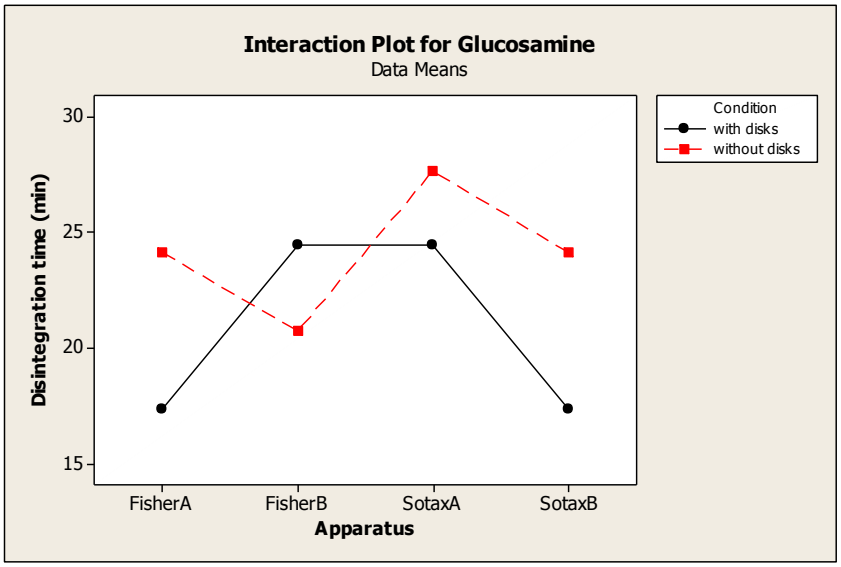
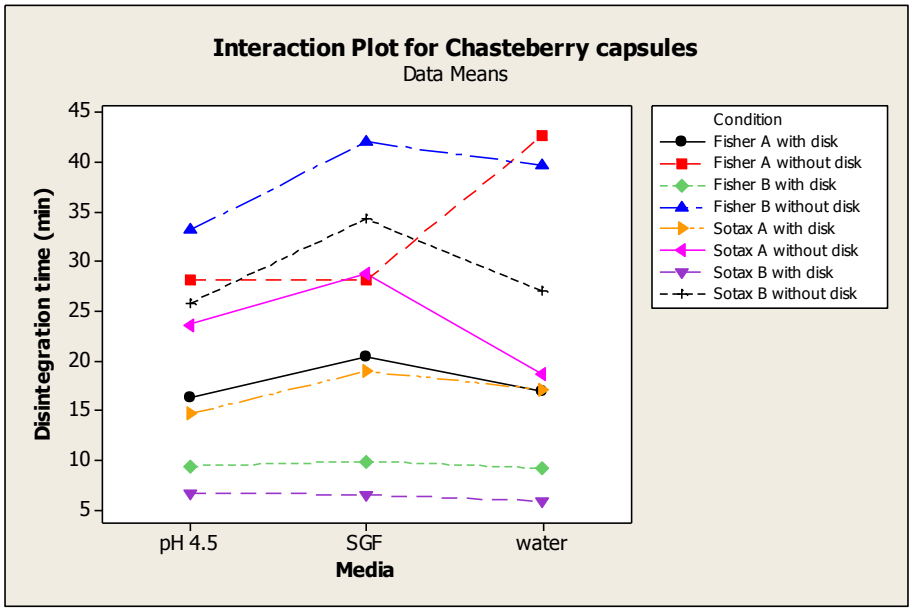
15
10
5
FisherA
FisherB
SotaxA
SotaxB
Apparatus
Condition
with disks
without disks

d

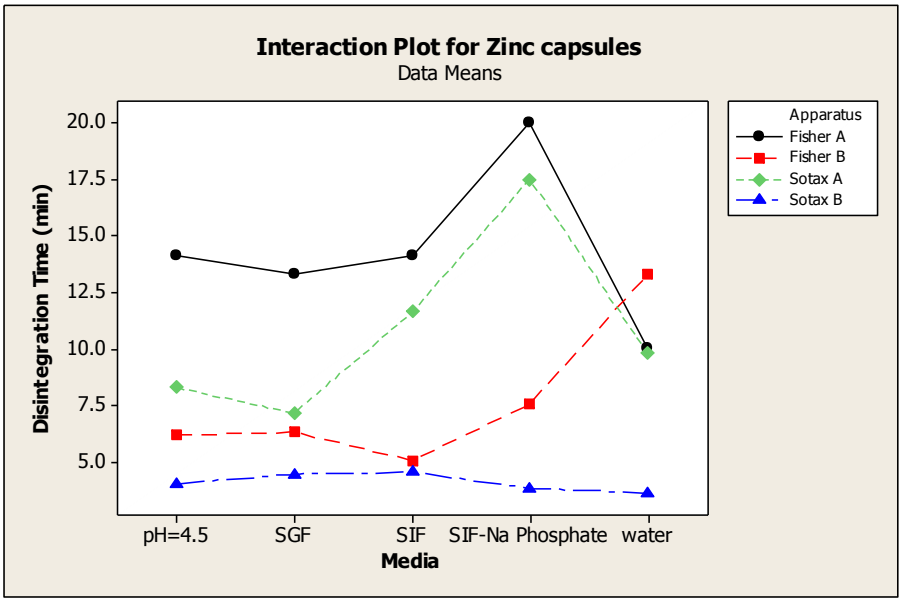


e

FIGURE A.5: Means differences for *Boswellia serrata* (a), Cinnamon (b), Ester-C (c), Oystercal (d) Glucosamine and (e) tablets under the following conditions: Sotax A (small beaker apparatus A) with and without disk; Sotax B B (small beaker apparatus B) with and without disk; Fisher A (large beaker apparatus A) with and without disk; Fisher B (large beaker apparatus B) with and without disk.



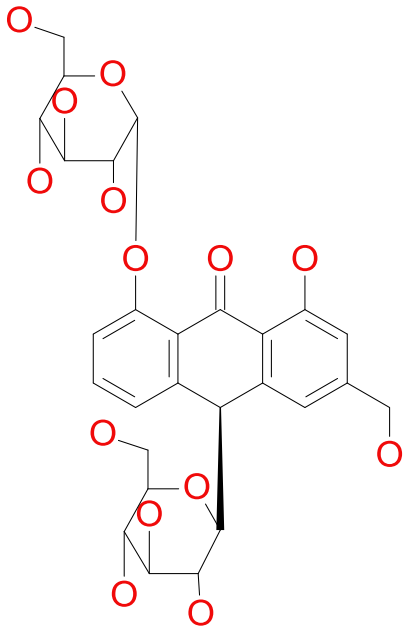
a



b

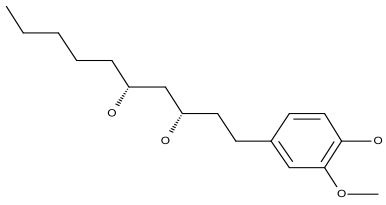
FIGURE A.6 : Means differences for Chasteberry and Zinc capsules: Sotax A (small beaker apparatus A) with and without disk; Sotax B (small beaker apparatus B) with and without disk; Fisher A (large beaker apparatus A) with and without disk; Fisher B (large beaker apparatus B) with and without disk.

Appendix B
Chemical Structures for the herb's markers

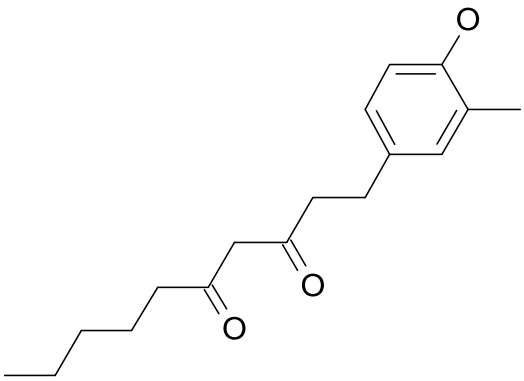


Cascaroside A

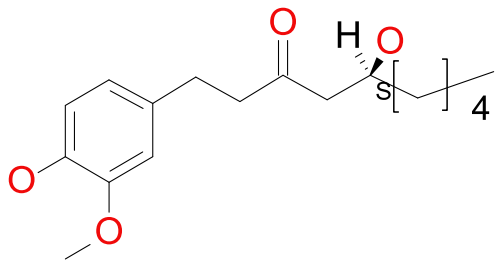
Figure B.1 Chemical structure of Cascaroside the marker of Cascara Herb



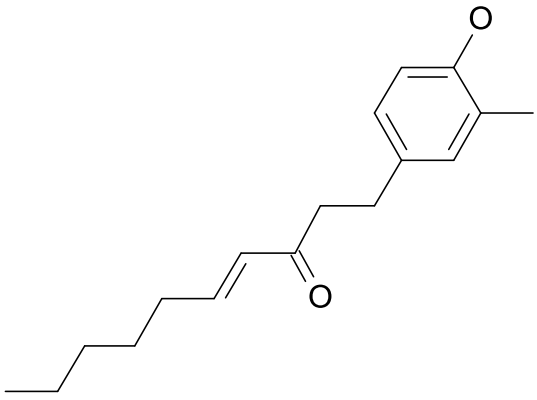
6- Gingerdiol (a)



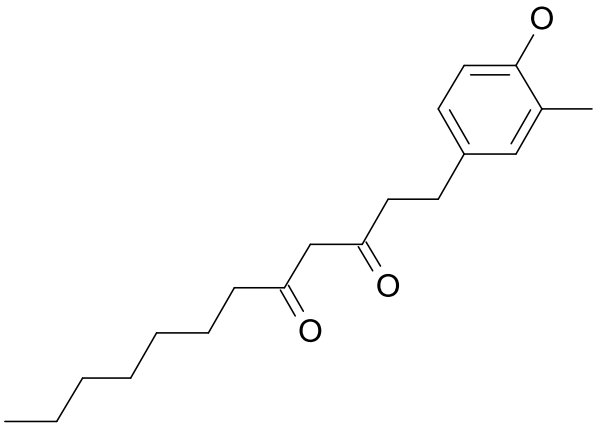
6- Gingerdione (b)



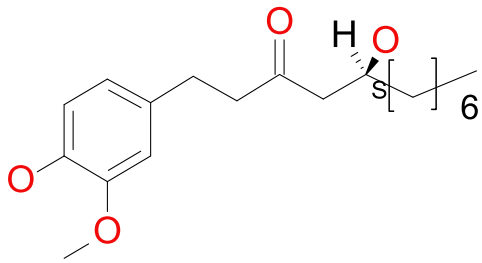
6- Gingerol (c)



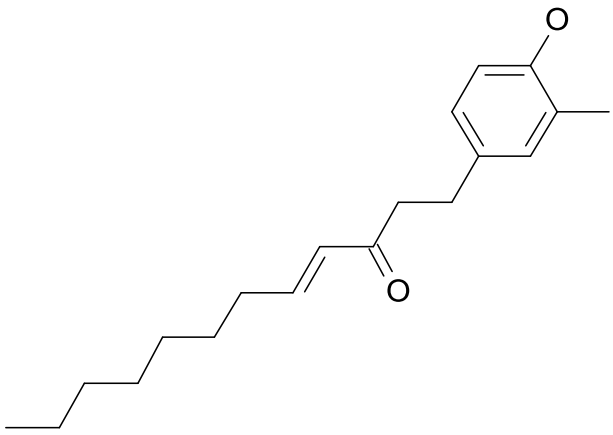
6- Shogaol (d)



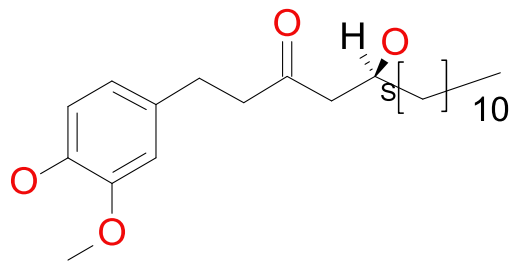
8- Gingerdione(e)



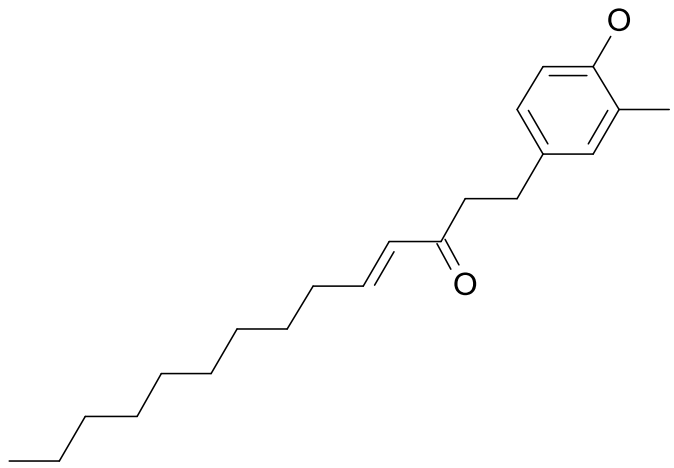
8- Gingerol (f)



8- Shogaol (g)

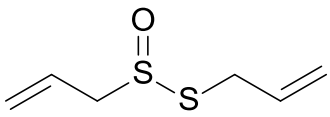


10- Gingerol (h)

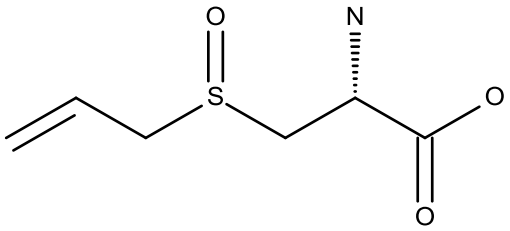


10- Shogaol (i)

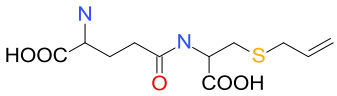
Figure B.2: Chemicals structures of 6- Gingerdiol (a) 6- Gingerdione (b) 6- Gingerol (c) 6- Shogaol (d) 8- Gingerdione(e) 8- Gingerol (f) 8- Shogaol (g) 10- Gingerol (h) 10- Shogaol (i) markers of Ginger



Allicin (a)

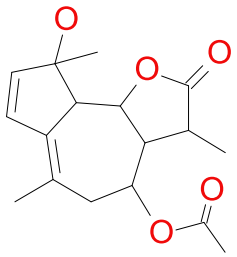


Alliin (b)

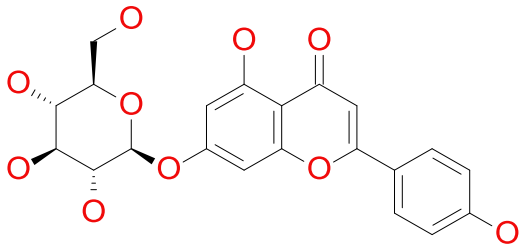


Glutamyl-S-Allyl-L-Cysteine (c)

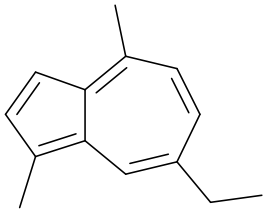
Figure B.3: Chemical structures of Allicin (a) Alliin (b) Glutamyl-S-Allyl-L-Cysteine (c) markers of Garlic



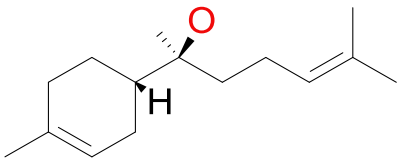
Matricin (a)



Apigenin-7-Glycoside (b)

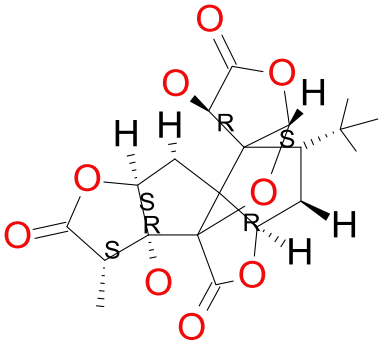


Chamazulene (C)

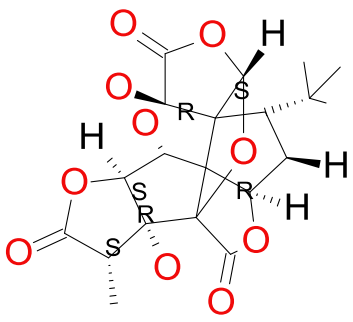


Levomenol (d)

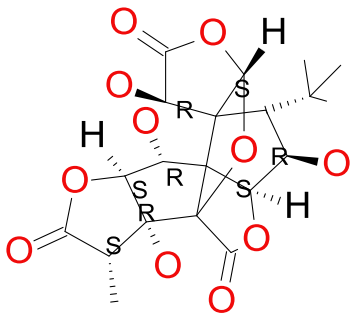
Figure B.4: Chemical structures of Matricin (a) Apigenin-7-Glycoside (b) Chamazulene (c) Levomenol (d) markers of Chamomile



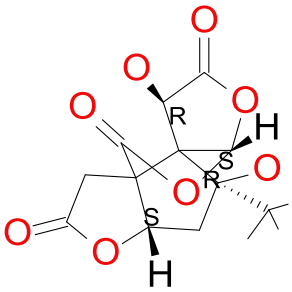
Ginkgolide A (a)



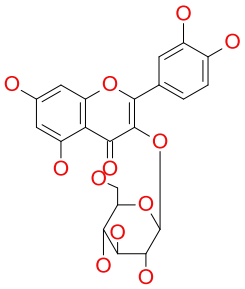
Ginkgolide B (b)



Ginkgolide C (c)

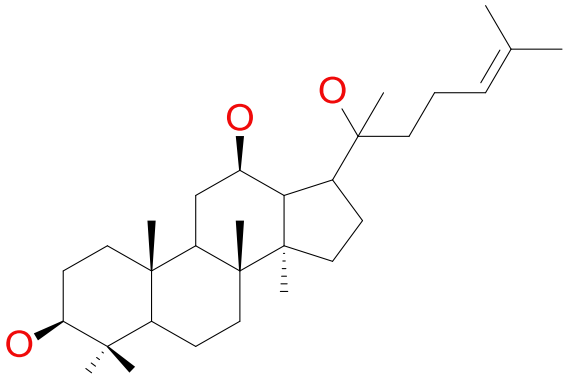


Bilobalide (d)

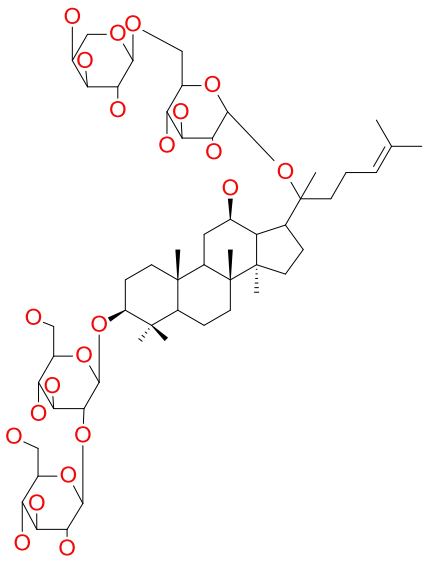


Quercetin-3-O-Glycoside (e)

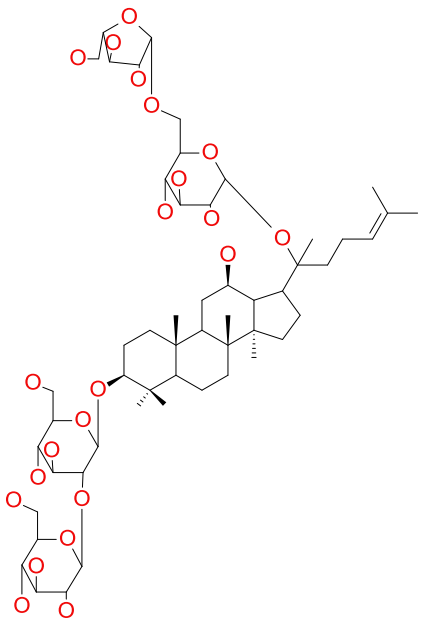
Figure B.5: Chemical structures of Ginkgolide A (a) Ginkgolide B (b) Ginkgolide C (c) Bilobalide (d) Quercetin-3-O-Glycoside (e) markers of Ginko



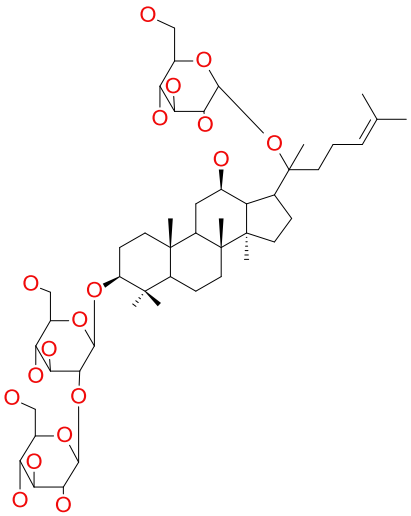
Ginsenoside RB1 (a)



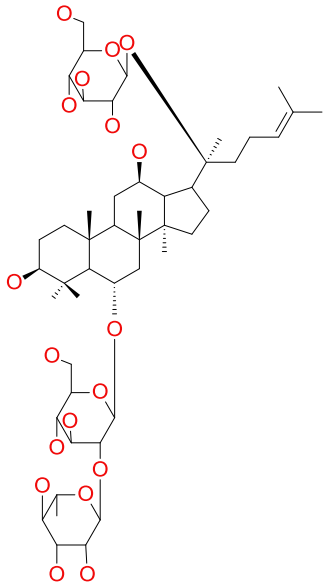
Ginsenoside RB2 (b)



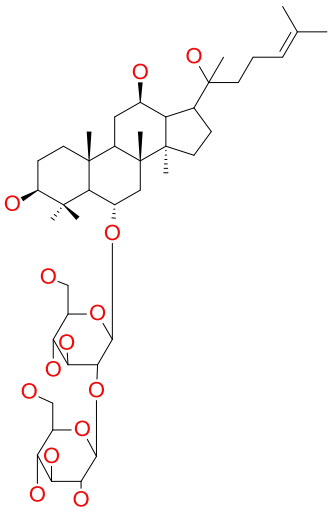
Ginsenoside RC (c)



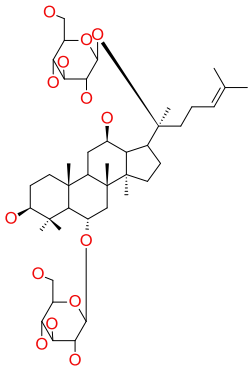
Ginsenoside RD (d)



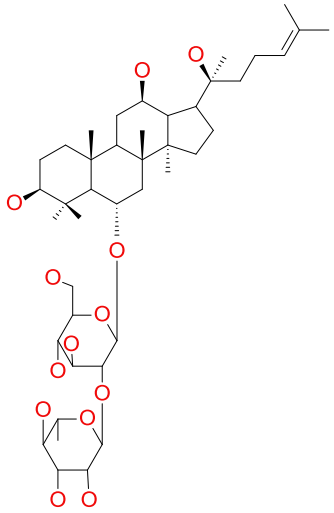
Ginsenoside RE (e)



Ginsenoside RF (f)

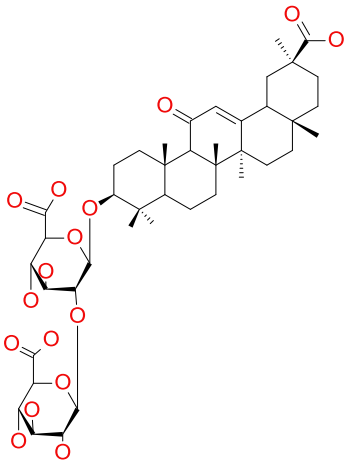


Ginsenoside RG1 (g)



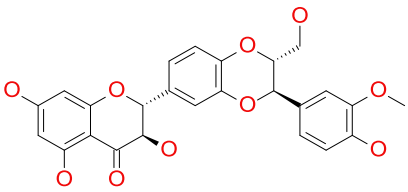
Ginsenoside RG2 (h)

Figure B.6: Chemical structures of Ginsenoside RB1 (a) Ginsenoside RB2 (b) Ginsenoside RC (c) Ginsenoside RD (d) Ginsenoside RE (e) Ginsenoside RF (f) Ginsenoside RG1 (g) Ginsenoside RG2 (h) markers of Ginseng

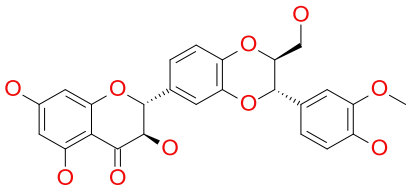


Glycyrrhizic Acid

Figure B.7: Chemical structures of GLYCYRRHIZIC ACID the marker of liquorice

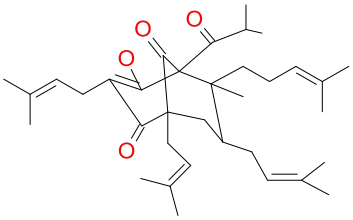


Silybin A (a)

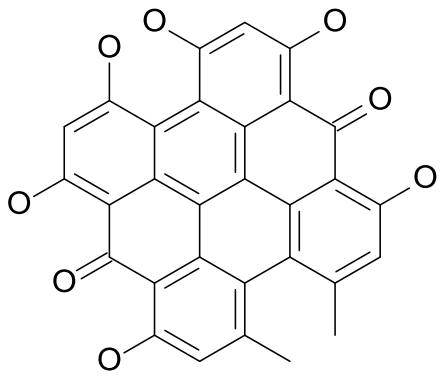


Silybin B (b)

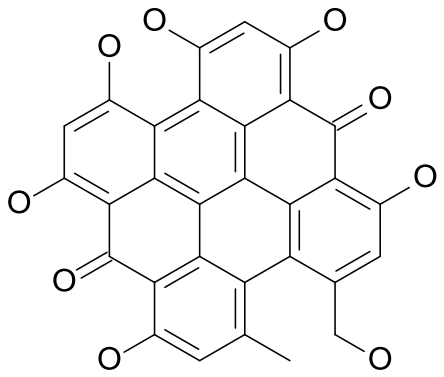
Figure B.8: Chemical structures of SILYBIN A (a) SILYBIN B (b) markers of Milk Thistle



Hyperforin (a)

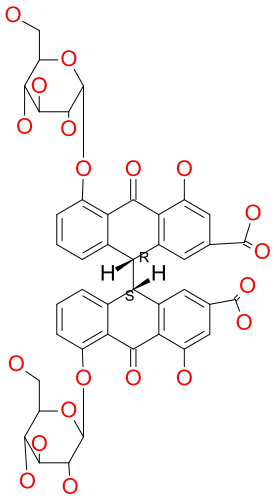


Hypericin (b)



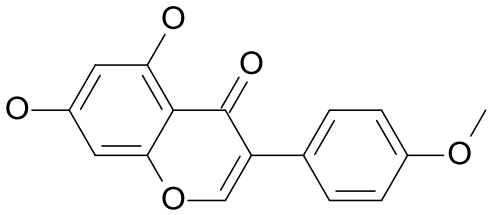
Pseudohypericin (c)

Figure B.9: Chemical structures of Hyperforin (a) Hypericin (b) Pseudohypericin (c) markers of St.John's Wort

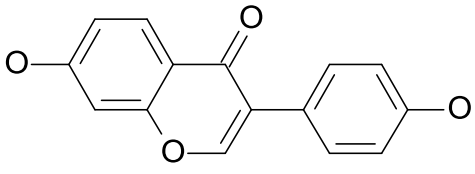


Sennoside B

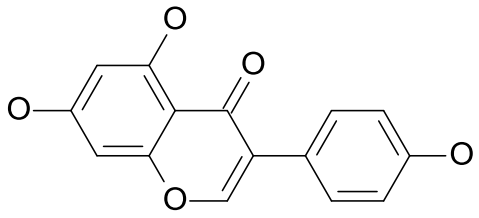
Figure B.10: Chemical structures of Sennoside B the marker of senna



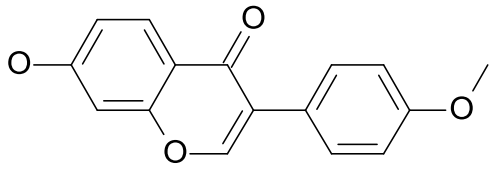
Biochanin A (a)



Daidzein (b)

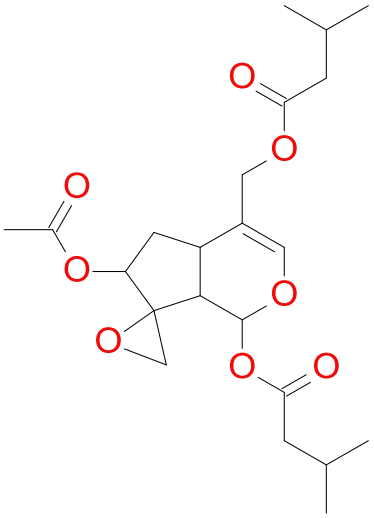


Genistein (c)

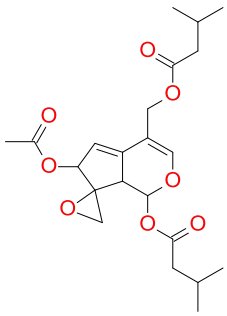


Formononetin (d)

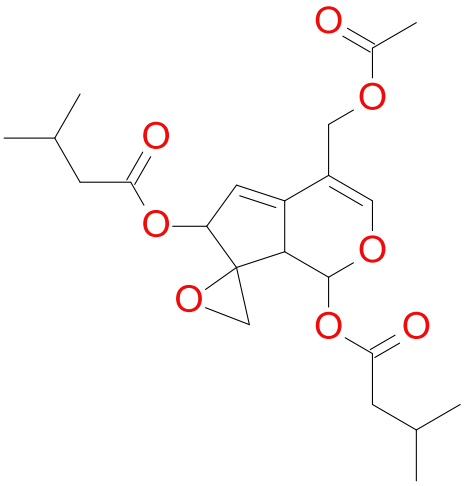
Figure B.11: Chemical structures of Biochanin A (a) Daidzein (b) Genistein (c) Formononetin (d) marker of red clover



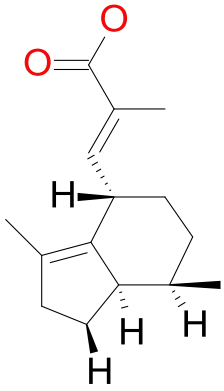
Didrovaltrate (a)



Isovaltrate (b)



Valtrate (c)



Valerianic Acid (d)

Figure B.12: Chemical structures of Didrovaltrate (a) Isovaltrate (b) Valtrate (c) Valerianic Acid (d) marker of Valerian