REPUBLIC OF TÜRKİYE

ALTINBAŞ UNIVERSITY

Institute of Graduate Studies

Biomedical Sciences

SIMULTANEOUS ANALYSIS OF PHENOLIC COMPOUNDS FROM BIOLOGICAL MATRICES AND DIETARY SUPPLEMENTS BY (U)HPLC METHOD

Athar Sadeq Mohammed Hasan AL-KHIDHRI

Master's Thesis

Supervisor

Asst. Prof. Dr. Fatma Tuba GÖZET

Istanbul, 2023

SIMULTANOUS ANALYSIS OF PHENOLIC COMPOUNDS FROM BIOLOGICAL MATRICES AND DIETARY SUPPLEMENTS BY (U)HPLC METHOD

Athar Sadeq Mohammed Hasan AL-KHIDHRI

Biomedical Science

Master's Thesis

ALTINBAŞ UNIVERSITY

2023

The thesis titled SIMULTANEOUS ANALYSIS OF PHENOLIC COMPOUNDS FROM BIOLOGICAL MATRICES AND DIETARY SUPPLEMENTS BY (U)HPLC METHOD prepared by ATHAR SADEQ MOHAMMED HASAN AL-KHIDHRI and submitted on 18/04/2023 has been **accepted unanimously** for the degree of Master of Science in Biomedical Science.

Asst. Prof. Dr. Fatma Tuba GÖZET

Supervisor

Thesis Defense Committee Members:

Asst. Prof. Dr. Fatma Tuba GÖZET	Department of Pharmacy, Altinbaş University	_____
Asst. Prof. Dr. Özge SEÇMELER	Department of Applied Sciences, Altinbaş University	_____
Asst. Prof. Dr. Günseli BAYRAM AKÇAPINAR	Department of Medical Biotechnology, Acıbadem Mehmet Ali Aydınlar University	_____

I hereby declare this thesis meets all format and submission requirements of a Master's thesis.

Submission date of the thesis to Institute of Graduate Studies: ___/___/___

I hereby declare that all information/data presented in this graduation project has been obtained in full accordance with academic rules and ethical conduct. I also declare all unoriginal materials and conclusions have been cited in the text and all references mentioned in the Reference List have been cited in the text, and vice versa as required by the abovementioned rules and conduct.

Athar AL-KHIDHRI

Signature

DEDICATION

To the best people on the earth: mom, dad, my siblings, my best friend, my second family and greatest teacher ever dr. Tuba, thank you so much for all support, love, respect and faith in me that I will reach what I want.

You all always be part of my achievement in the future.

The experimental work in this thesis has been conducted at Altinbas University Center Research Laboratory (AU-MERLAB). I also thank Nurşah Çılgın for her help in using sample preparation equipment and laboratory facilities.

PREFACE

The work described in this thesis/dissertation has been funded by Altınbaş University Office of Scientific Research Projects as part of the Project No. PB2019-ECZ2. All the experiments have been conducted at Altınbaş University Central Research Laboratory (AU-MERLAB).

ABSTRACT

SIMULTANEOUS ANALYSIS OF PHENOLIC COMPOUNDS FROM BIOLOGICAL MATRICES AND DIETARY SUPPLEMENTS BY (U)HPLC METHOD

AL-KHIDHRI, Athar Sadeq Mohammed Hasan Al-Khıdhrı

M.Sc., Biomedical Sciences, Altınbaş University,

Supervisor: Asst. Prof. Dr. Fatma Tuba GÖZET

Date: April / 2023

Pages: 87

Medicinal plants containing pharmacological active compounds are used often in the treatment and prevention of the degenerative diseases such as cancers and neurological disorders because of their antioxidant properties. As the interest to natural sources increases, the type and number of the commercial products in the form of extracts of the capsules has also been increased. On the other hand, the safety and bioactive content of these products are questioned because they are regulated under the category of dietary supplements which is not as strict as the synthetic drugs. Therefore, establishment of the chemical analysis techniques for the determination of amount of the bioactive compounds are necessary for the quality control and assurance, especially since the type and amount of the bioactive compounds depends on the species, environmental conditions and growth in the addition to the extraction technique.

The aim of this thesis is method development and validation for the simultaneous quantitative analysis of the phenolic compound in commercial sage (*Salvia officinalis L.*) capsules by Ultra High-Performance Liquid Chromatography (UHPLC) using diode array detector (DAD). 7 phenolic compounds namely chlorogenic acid (CGA), caffeic acid (CA), syringic acid (SYR), p-coumaric acid (PCA), ferulic acid (FA), luteolin 7 O glucoside (L7O) and rosmarinic acid (ROS) determined as the most abundant phenolic compounds in salvia species based on the literature research are going to be used as the biomarker for the quality indicator of the commercial capsules. Since targeted analysis has been aimed, standard solutions generated from the pure standards of the compounds have been used throughout

the experiments. Method developed in terms of the instrumental conditions such as type of mobile phase, mobile phase gradient program, mobile phase flow rate, column type and temperature and injection volume have been optimized for the chromatographic separation of the analyte molecules in simultaneous manner. In this thesis, method development for the analysis have been continued with optimization of the sample preparation of the extraction procedure of the capsule and powder samples. The most effective extraction procedure in terms of solvent types and ratios and duration of the extraction are optimized. The linear range of quantitation has been determined based on extracted amounts and absorbances. Method validation has been done based on the FDA (food and drug administration), EPA (environmental protection agency), ICH (international conference on harmonization). System suitability experiments have also been done and presented. The method validation parameters of selectivity, precision, accuracy, linearity, limit of detection and quantitation have been determined. Also, recovery has been done based on the matrices of sage capsules, powders and maltrodextrase and magnesium streate that is potentially used in the tablet formulations. Eventually, the analysis of real samples has been done to show the applicability of the method. The sample preparation for the method development and validation here in this thesis poses challenges due to optimization of the conditions for multicomponent analysis.

Keywords: Phenolic Compounds, Extraction, (U)HPLC, Sage, Method Development and Validation.

TABLE OF CONTENTS

Pages

LIST OF TABLES

Pages

LIST OF FIGURES

Pages

ABBREVIATIONS

AA	:	Acetic Acid
W	:	Water
MeOH	:	Methanol
ETOH	:	Ethanol
PBS	:	Phosphate Buffered Saline
MECN	:	Acetonitrile
PA	:	Phosphoric Acid
Chlf	:	Chloroform
AB	:	Acetate Buffer
CA	:	Citric Acid
NaH_2PO_4	:	Monosodium Phosphate
OA	:	Orthophosphoric Acid
FA	:	Formic Acid
NaH_2PO_4	:	Sodium Dihydrogen Phosphate
KH_2PO_4	:	Potassium Dihydrogenphosphate
TEA	:	Triethylamine
Ace	:	Aceton
SA	:	Sulfuric Acid
TFA	:	Trifluoroacetic Acid
ETH	:	Ether

EA	:	Ethyl Acetate
DMSO	:	Dimethylsulphoxide
DEE	:	Diethyl Ether
hx	:	Hexane
OA	:	Orthophosphoric Acid
SA	:	Sodium Acetate
CGA	:	Chlorogenic Acid
CA	:	Caffeic Acid
SYR	:	Syringic Acid
PCA	:	P-Coumaric Acid
FA	:	Ferulic Acid
L7O	:	Luteolin-7-O-Glucoside
ROS	:	Rosmarinic Acid

1. INTRODUCTION

The class of biologically active substances found in plant-based diets is called polyphenols [1,2] Polyphenolic composition of the salvia species has been analyzed because of the antioxidant compounds [3]. Quantitation of phenolic compounds in sage has been done to reveal their content accurately and determine content according to the standards and regulations of the food and pharmaceutical industry. Analysis of phenolic compounds from the salvia by HPLC has been for simply determining the phenolic content of the sage, comparison of the different sage species, the comparison of the phenolic content of the sage in other lamiacae family plants such as thyme, basil, mint, oregano and etc. In some studies, the extraction method on the content of the polyphenolic compounds have been compared. Method of extraction and quantitation of by HPLC have been aimed in some studies [4]. Conditions such as solvent type and amount is optimized. Polyphenols chemicals are category of organic molecules with phenolic structural properties. There are thousands of phenolic compounds with identified structures. Depending on their location of origin, biological purpose, and chemical make-up, polyphenols have been categorized, because of their variety and widespread distribution in plants. Also, because of their important role on human health, like the avoidance of degenerative illnesses, especially cancer, heart disease, also diabetes mellitus, osteoporosis and neurological disorders, polyphenols have received the attention of many scientists and nutrition experts. Antioxidants molecules work together against antioxidant stress by reactive oxygen species. Polyphenols are antioxidants in the human diet, antioxidants in the human diet, and growing evidence suggests they may work in ways that extend their antioxidant capabilities in vivo [1,2]. Fruits consumed on daily basis, contain high percentage of antioxidants and phytochemicals [1].

1.1 CLASSIFICATION OF POLYPHENOLS

Polyphenols contain a phenolic ring and structurally there are subtypes as phenolic acids, phenolic acids, flavonoids, stiblins and lignans [1,2]**:**

1.1.1 Phenolic Acids

There are two types of phenolic acids, which are benzoic acids and cinnamic acids [1,2]. It is not recommended that Hydroxybenzoic substances have any impact on human health due

to the rarity of it in human diets [1]**.** Phenolic acids are present in fruits and vegetables including blackberries, some of the red fruits, grains, hull and bran [1**,**2].

1.1.2 Flavonoids

Subgroups of flavonoids are flavanones, flavanols, flavonols, isoflavons, flavons, anthocyanidins and chalcones. Specifically, catechin and epicatechin are found in many plants [2] and generally flavonoids are found in leeks, broccoli, and onions, and there are more than 4000 flavonoids derived from component in plants [1]**.**

The major type of plant that isoflavons found in is the leguminous plant family, which have a great impact on health, especially in some countries that rely on them in their diets. The soy is the primary source of isoflavones contain genistein, daidzein and glycetein [1**,**2].

Fruit peels and skin contain a lot of flavones. For example, Mandarin contains a high percentage of flavones and flavone derivatives 6.5 g/L [1**,**2]**.**

Because light activates their synthesis, flavonols are abundant in fruit peels, so flavonol ratios will be different on the same plant and even on the same bit of fruit [1**,**2]. The most popular type of it: aglycones, quercetin and kaempferol, and just the last one has minimum 279 and 347 various glycosidic combinations respectively [2]**.**

Also known catechins. Epicatechin has a cis structure, whereas catechin has a trans structure and they have (+) and (–) stereoisomers. Catechins and epicatechins are present in fruits such as grapes, apple, blueberries, tea and cocoa bean. When brewed, tea flavanols can create distinct dimmers like theaflavin. When brewed, tea flavanols can create distinct dimmers like theaflavin. Catechin and epicatechin can combine to create polymers, also known as proanthocyanidins, which are broken down into anthocyanidins by an acid catalyst [2]**.**

Flavanones are mainly found in citrus fruits, such as orange with a high amount of hesperidin. The solid outer layer of fruits and in covers is rich of the flavonones, juices have content more than foods. From citrus fruits, it is known that flavanonol is taxifolin [2**,**1]**.**

Most widespread anthocyanins are anthocyanidins, cyanidin, delphinidin and pelargonidin, which are found in red, blue and purple fruits, vegetables and grains [1**,**2].

1.1.3 Other Polyphenols

Other polyphenols important for human health that found in food are resveratrol, present in grapes; ellagic acids present in berries and skins of some nuts; curcumin found in turmeric; caffeic acid, gallic acid and rosmarinic acids [2].

1.2 CHROMATOGRAPHIC TECHNIQUES

Chromatography is a word used to refer to a broad range of separation methods that divide or distribute a sample (solute) between a mobile and stationary phase. Mobile phase could be gas, liquid and supercritical, and stationary phase is fixed solid or liquid. Solute partitions between mobile phase and stationary phase. The ratio of the concentration of solute between stationary phase and the mobile phase is called distribution coefficient. Analysis techniques are progressing in a large way, and among the factors that have had a great impact is chromatography, which appeared at the end of the nineteenth century by David Mikhail Tsvet and Mikhail Tsvet. Tsvet is typically given the credit for discovering chromatography since he observed and correctly understood the chromatographic processes. Gas chromatography (GC), which uses gas mobile phase is used in the petroleum industry since 1960s. On the other hand, field of liquid chromatography has been expanded by HPLC into a useful and complex technique at the same time. Currently, chromatographic instruments or systems are being frequently used for the purpose in multiple fields including pharmaceutical, food, environmental, chemical, clinical, biomedical research and industries [5].

1.2.1 Liquid Chromatography

In liquid chromatography mobile phase is a liquid and the stationary phase is liquid or solid. Types of liquid chromatography are thin layer chromatography (TLC) and column chromatography[5].

1.2.2 Column Liquid Chromatography

Column liquid chromatography is very useful in the separation of the compounds in the mixture using stationary phase, which is also called resin, gel or packing material, packed into a column depending on the dimensions of the column and amount of the sample to be loaded, separation mode, and desired resolution. Packing could be dry or wet and wet

columns are prepared by making slurry of the adsorbent, which is poured into the column. Then sample dissolved in the mobile phase added to the top of the column and mobile phase is added for the separation takes place gravitational forces. Sometimes, low pressure pump is utilized to speed up the elution, i.e. process of movement. There are two types of elution as isocratic and gradient. While the mobile phase composition is constant in isocratic elution, it changes in gradient elution over the run. Column eluate, i.e. the separated sample is detector offline using a detector [5].

1.2.3 Mechanisms of Chromatographic Separation: Adsorption

Adsorption is one of the most used mechanisms used in chromatography that the separation depends on the intermolecular forces (electrostatic forces, van der Waals forces, hydrogen bonds, hydrophobic interactions). The stationary phase is silica, alumina, or a few other materials. Mobile phase and stationary phase compete to bind to solute [5**,**6].

There are two modes of adsorption chromatography as normal phase and reverse phase depending on the polarities of the mobile phase and stationary phase. In normal phase chromatography, the stationary phase is polar and the mobile phase is nonpolar and reverse phase chromatography stationary phase is nonpolar and mobile phase is polar. The most polar component elutes first in reversed-phase chromatography, and the elution duration increases when the mobile phase polarity is increased. It is opposite in normal phase chromatography [6].

1.3 CHROMATOGRAPHIC PARAMETERS

Dead time(t_o): The components' retention time was insufficient; it is the point at which the mobile phase (solvent) enters the column.

Retention time(tr): it is associated time for eluting a chemical from an LC column, it means (the duration of time a compound remains on the column after being injected).
Resolution: the separation of the peaks

Resolution is defined as:

$$Rs = \frac{2(tr2 - tr2)}{w2 + w1} \quad (1.1)$$

Where:

R_S: resolution

w_1 and w_2: width of peak 1 and 2, respectively

tr_1 and tr_2: retention time of the first and second peak, respectively

Selectivity: Selectivity is the separation between two peaks. Mathematically it is expressed as:

$$\alpha = \frac{t_{R2} - t_o}{t_{R1} - t_o} = \frac{t'_{R2}}{t'_{R1}} = \frac{k2}{k1} \quad (1.2)$$

Where:

a : separation factor

t'_{Rl} and t'_{R2} = adjusted retention times of components 1 and 2, respectively

K_1 and K_2 = distribution coefficients of components 1 and 2, respectively

Capacity Factor (k ′): Capacity factor is a measurement of how much time a solute spends on the stationary phase. The equation below shows a relation between chromatographic retention and capacity factors:

$$k' = \frac{t''_R}{t_0} = \frac{t_R - t_o}{t_o} \quad (1.3)$$

Theoretical Plate Number (N): This chromatographic parameter gives idea about the efficiency of the column, which is divided in the theoretical plates (N) and in each of them would experience one equilibration. Efficiency is calculated as follows:

$$N \left(\frac{tR}{\alpha}\right)^2 = 16 \left(\frac{tR}{\omega}\right)^2 = 5.5 \left(\frac{tR}{\omega_{\frac{1}{2}}}\right)^2 \quad (1.4)$$

where,

w: peak width

$w_{1/2}$: peak width at half height

Theoretical Plate Height: There is an inverse relationship between the values of H and N. Good efficiency of separation refers to small plate height values, while reducing the N results in poor separation [5].

Theoretical plate height is given by the following equation:

$$\text{HETP} = \frac{L}{N} \tag{1.5}$$

Where,

L: length of the col

1.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) typically uses equipment that is both more complex than that used in other types of chromatography. Samples are separated while pumping through a pressurized fluid and particles of stationary phase at a few micrometer size. Higher operating pressures (50 bar to 350 bar) are applied. [7,6].

For the most of HPLC separations, reversed-phase, bonded, octyl- or octyl decyl silane packings are used and such ingredients result in a brush-like, nonpolar hydrocarbon surface. As a mobile phase solvent such as acetonitrile, methanol, tetrahydrofuran and their mixtures with water are used [6]. Qualitative analysis has been done by comparison of the retention time, t′R, when comparing chromatograms produced by two various systems or columns. The retention time of many substances could be the same, this means, even though a standards and an unknown's retention times are same, the two compounds may not be the same. So, additional methods are required to verify peak identification such a diode array detector giving full absorption spectrum of the peaks and mass spectrometry distinguishes separated compounds according to m/z.

Quantitative analysis is done by taking measurements of the peak height or area, which is compared with the known concentrations of the standard solutions. Data analysis software is now used by almost all chromatography systems. Relative peak areas and relative retention times are provided. Calibration curve, i.e. the peak areas versus concentration are generated by the instrument software [5].

1.4.1 Instrumentation

The main parts of the HPLC system are injector or autosampler, pump, column and detector.

1.4.1.1 Pump

Mobile phase is pushed by the pump to the column. HPLC pumps provides constant flow rate, which does not change by the composition of the mobile phase. Binary HPLC systems contain two pumps for the delivery of two mobile phases. There are also pumps which one switch between up to four mobile phases to provide gradient elution. Pumps are also equipped with the degassing unit to remove the gasses before column [6].

1.4.1.2 Injectors

It is generally utilized to inject liquid samples. The two different types of injectors are manual and automatic.

1.4.1.3 Columns

In HPLC, the column is a crucial element and made of silica-based packing most commonly. HPLC columns are made of stainless steel, glass, or polymer such as polyetheretherketone (PEEK). Length of the columns is in the range of 30 - 250 mm with a diameter of 2.1 - 4.6 mm. The particle sizes of the columns are 3µm or 5 µm [6**,**7].

Columns are run at room temperature for some applications therefore precise temperature control of the column is not required, however, keeping the column temperature constant often results in better, more accurate chromatograms. Temperature management is seen by many chromatographers as crucial for repeatable separations. The majority of current commercial instruments have heaters that provide column temperature between room temperature and 150 ^{0}C.

1.4.1.4. Detector

HPLC detector generates an electrical signal of the eluted compounds and intensity of the signal is proportional with the concentration. Ideal HPLC detectors should have small internal volume to reduce the extra-column band spread and narrow temperature range, compact and provide a suitable flow rate. However, there is not a global detector for every compound, and choice of detectors depends on the physiochemical properties of the analyte molecules.

UV-Vis (absorption of ultraviolet or visible light) is usually used as an HPLC detector. The mercury lamps with 254 and 280 nm wavelength are frequently used by photometers for so many organic functional groups absorb in that range. Absorption is also provided by deuterium sources or tungsten-filament sources. Advanced instruments incorporate diode-array detectors that can show the full spectrum. Other detectors include electrochemical, electrical conductivity, fluorescence, refractive index and mass spectrometry with varying sensitivities [6,7]. A signal from the detector is transformed into an electrical signal by an amplifier and recorded as data points to produce chromatogram [7].

1.5 ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (UHPLC)

Ultra high-performance liquid chromatography (UHPLC) is same as HPLC, only differs in particle size of the column. Particle size of UHPLC is < 2 µm, which improves the resolving power. Columns with smaller particle size require more pressure >400 bar and pump of the instruments are also improved for generation of high pressures. Dimension of the UHPLC columns is also smaller, therefore, mobile phase consumption is less. Generally, performance of the UHPLC is better because of less band broadening. The analysis time is 10 times shorter in UHPLC, so they are high throughput. In UHPLC, UV-Vis, fluorescence, DAD and mass spectrometric detectors could be used. Dead time is reduced. In UHPLC, mobile phase should be filtered well, and clean solvents should be used [8,9].

1.6 EXTRACTION AND ANALYSIS OF POLYPHENOLIC COMPOUNDS

Samples containing polyphenols must be acquired, stored, and correctly prepared prior to extraction. To avoid the oxidation of polyphenols is used hydroxytoluene and ascorbic acid. Both filtering, and centrifugation can be used to pre-treat samples. Due to high wetness or water content promotes the enzyme activity, samples are frequently dried, it is better to stay well away of high-temperature drying because it may impact the content of polyphenols, or lyophilized before extraction to prevent degradation of natural polyphenols [2]. The most used extraction methods of plants in the laboratory are liquid-liquid or solid-liquid extraction. Rely on the solubility of the specific polyphenols, the liquid extracts may usually be partitioned using solvents like ethyl acetate [1,2]. Polyphenols are hydrophilic, therefore they are usually extracted using polar solvents such as methanol, acetonitrile, ethanol, acetone and water. Polyphenols are stable at low pH, therefore acid could be added to the

medium of extraction solvent. Phenolic compounds are released from natural sources by acid or alkaline hydrolysis [2]. Phenolic compounds are extracted from plants and food by using matrix solid-phase extraction (MSPD), supercritical fluid extraction (SFE), solid-phase microextraction (SMPE) and accelerated solvent extraction /pressurized liquid extraction (ASE/PLE).

Phenolic compounds are analyzed by HPLC using DAD and mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detectors. Separation of the phenolic compounds could be improved by using U(H)PLC (Ultra-high performance liquid chromatography) faster compared to HPLC. Identification of the polyphenols are done by comparison of the retention time of the analyte with the specific chemical. UV-Vis spectrum is obtained by using DAD detectors. Otherwise, MS or MS/MS detectors in the configuration of LC/MS/MS are used for the identification of the polyphenols [2].

1.7 ANALYSIS OF PLANTS

Metabolites produced from plants are extremely diverse lead to the generation of different species and medicinal plants containing pharmacological active compounds for the treatment of diseases [10]. The chemical analysis of the plants is also necessary for the their identification and knowing the chemical species for the generation of the experiments and accurate conclusions [11], to ensure safety of botanical dietary supplements, evaluation of the genetically modified crops and to reveal their nutritional value [10] and efficacies [10,12]. In addition, the determination of the medicinal plants is important since their complex composition may be affected by environmental factors such as harvesting, climate, season and processing conditions, traditional preparation is made by a combination of different herbs [12,13].

Dietary botanical products are chemically analyzed in terms their chemical composition for the purpose of quality control since adulteration is possible and content is affected by geographical origin and handling [14].

The chemical analysis of the herbal products has gained attention because of the inefficient quality control [15]. As well as the analysis of medicinal plants, the chemical analysis of dietary supplements derived from medicinal plants is done to for the purpose of quality control and safety since the herbs may contain carcinogenic ingredients present [16] during

manufacturing which is not done under good manufacturing practices [17]. Quality control of the commercial herbal products is made by determination of the bioactive ingredients, which are chemical markers. On the other hand, it has been reported that the most of the products analyzed do not contain the bioactive compounds indicated on the label and therefore do not meet the quality standards [17] . Furthermore, the chemical composition of the dietary supplements is necessary to adjust the doses of these medicines self-prescribed by the patients to avoid inefficiencies and more importantly overdosing, toxic and adverse effects [17]. The quality of herbal products may be varied since the regulations may be different in different countries. If the herbal products are not strictly regulated, they may not contain the bioactive compounds. The bioactive content of commercial herbal products may be variably since they are prepared by a combination of more than one herb in addition to variation contributed by the effects of the environmental factors, processing and extraction conditions, therefore, the analysis is necessary in multiple stages of production to ensure quality. Furthermore, analysis of herbal products is necessary for standardization of the materials used in clinical studies for the purpose of toxicological and pharmacological studies [13].

1.8 ANALYSIS OF PHENOLIC COMPOUNDS IN SAGE

Sage is one of the herbal products which contain phenolic compounds. Analysis of sage are done to compare the efficiency of the extraction on the phenolic compounds, comparison of the content of different species, processing and instrumental conditions.

1.8.1 Comparison of The Species

Motivation of the studies was to compare the *Salvia L*. species in terms of their phenolic composition. In a study, five bioactive phenolic compounds: ROS, CAR, CA, and salvianolic acids A and B in the 41 populations of 27 wild *Salvia* species of Iran, were identified by HPLC-UV method [18]. Phenolic profiles of different salvia species have also been studied by HPLC-UV and their antioxidant properties are compared. Such as in a paper published in 2011, phenolic profiles of the dalmatian sage in terms of the content of the phenolic compounds and the antioxidant potential are revealed [19]. In another study, seasonal variations on the phenolic compound composition of the sage has been studied using HPLC-UV. Leaves of the Dalmatian sage (*Salvia officinalis L*.) has been collected at various stages

of the growth and content of the phenolic compounds are determined [20]. Similarly, content of phenolic acids namely, FA, CA and ROS of *Salvia* species grown in China have been determined by HPLC [21]. Major phenolic compounds including gallic, vanillic, caffeic, ferulic, syringic, chlorogenic, rosmarinic, protocatechuic, and sinapic acids in sage and some other herbs such as basil, lemon, thyme, mint, oregano, rosemary were identified and quantified by HPLC [22]. Determination of the phenolic compounds including gallic acid, CGA, vanillic acid, catechin, protocatechuic acid, gentisic acid, CA, SYR, epicatechin and benzoic acid of sage and some other Mediterranean plant species grown in Jordan have been done by HPLC [23]. Phenolic compounds such as rosmarinic acid, methyl rosmarenate, caffeic acid, cinnamic acid, chlorogenic acid, and quinic acid, as well as flavonoids such as ferulic acid, apigenin, luteolin, and quercetin in methanolic extracts of thyme (*Thymus vulgaris L.*), sage (*Salvia officinalis L.*), and marjoram (*Origanum majorana L.*) were also determined by HPLC in a comparative manner [24]. Concentration of the phenolic compounds in leaves sage (*Salviae officinalis folium*) peppermint leaf (*Menthae piperitae folium*), Melissa leaf (*Melissae folium*) has been done by HPLC [25]. Phenolic compounds in sage have been identified using both DAD and MS detectors [4]. Phenolic compounds in sage and plants in the same family namely ices, rosemary, oregano, sage, basil, and thyme were identified by using LC-MS/MS. The retention times and the mass spectra of the samples are compared with the reference standards [26]. In a study published in 2017, phenolic compounds in Italian sage leaf were determined by LC-MS/MS [27].

1.8.2 Extraction of Sage

The Compounds: Eriocitrin, Luteolin 7-O-rutinoside, Luteolin 7-O-β-glucuronide, Lithospermic acid, Rosmarinic acid, Methyl rosmarinate, Carnosic acid, Caffeic acid, Salvianolic acids A and B were obtained from the extraction of Sage (*Salviae officinalis folium*) from its leaves in a study of 2007 by used (MeOH) for the extraction [25**,**18]. (Table 1.1.) While in 2011, Leaves of Sage (*Salvia officinalis L.*) have been extracted with alcoholic solvent EtOH/W and EtOH, the compounds recorded from extraction were: Gallic acid, Caffeic acid, Syringic acid, P-Coumaric acid, Trans-Ferulic acid, Rosmarinic acid, (+)-Catechin, (-)-Epi catechin, Rutin, Quercetin-40-glucoside, Quercetin, Kaempferol, Rosmarinic acid, Quercetin, Apigenin, Luteolin [19**,**28]. In 2012, a study used MeOH for extraction from aerial parts of Sage (*Salvia officinalis L.*) and the compounds observed were:

Gallic chlorogenic, Caffeic acid, Syringic acid P-coumaric acid, Luteolin acid, Quercetine acid, Ferulic acid, Rosmarinic acid, Carnosic acid. Caffeic acid, Chlorogenic acid, Carnosic acid, Ferulic acid, Gallic acid, P-coumaric acid, Quercetin, Rosmarinic acid and Syringic acid, these compounds were recorded in a study in 2010 from the extraction the Whole plant of Sage (*officinalis L.*) with MeOH (80%) [26,29]. Also in 2015, noticed these compounds: Rosmarinic a. ferulic a., caffeic a., apigenin-7-OG, quercetin-3-O-glucoside with MeOH and MeOH/W those components have been extracted from flowers, leaves and aerial parts of Sage (*Salvia miltiorrhiza, Salvia przewalskii, Salvia officinalis and Salvia deserta*) [21,30]. EtOH/W and EtOH were used to extract the phenolics from Sage (*Salvia officinalis L.*) and lemon balm, thyme, peppermint and oregano used the whole plant in 2014 and 2017 and the compounds noticed were: vanillin, gallic a., rosmarinic a., carnosic a. vanillic a. and syringic a., caffeic a., luteolin-7-O-glycoside [31,32]. The aerial parts of Sage (*Salvia officinalis L.*) extracted with Chlf and MeOH and the following compounds have been detected: quercetin, rosmarinic a., caffeic a., carnosic acid luteolin-7-glucoside and apigenin [27]. While in 2011, sage, whole plant has been extracted but with MeOH and that study recorded the compounds: chlorogenic a., rosmarinic a., gallic a., syringic a., vanillic a., caffeic a., ferulic a. and protocatechualdehyde [22]. Sage (*Salvia officinalis L.*) leaves and different parts of 9 different common edible plant species for extraction process with MeOH, the study obtained these compounds: Gallic acid, Protocatechuic acid, Chlorogenic acid, Vanillic acid, Syringic acid, Caffeic acid, Epicatechin [23]. In 2013, a study observed these compounds: rosmarinic a., caffeic a., chlorogenic a., cinnamic a., ferulic a., apigenin, luteolin, quercetin by extracting Sage (*Salvia officinalis L.*) leaves and other type of plant such as thyme and marjoram with used MeOH, EtOH, DEE, and Hx as extraction solvents [24]. Caffeic a. has been detected in MeOH extract of the leaves, stems, blossoms and roots of Sage (*Salvia officinalis, Salvia discolor* and *Salvia sclarea*) [33]. Ferulic acid, Rosmarinic acid and P-coumaric acid were recorded in 2017 from extracting the whole Salvia officinalis L. with EtOH as extraction solvent in two techniques [34]. For extraction phenolics from sage, boiling water has been used to extract from leaves of salvia or tea bag in 2011, caffeic acid, rosmarinic acid, luteolin-7-Oglucoside and carnosic acid, those are the compounds obtained from the extraction [35]. Phenolic compounds from sage have been extracted using various methodologies. Dried sample is homogenized and then put in organic solvent and then filtered [29]. Infusion is also applied by adding sample in h [1 ,2]. Polyphenolic compossible

[35] or alcoholic solvent [36,37,25, 18, 19, 28,21,32,22,23,33,34]. Extraction is facilitated using a shaker [36]. Ultrasonic extraction in an organic solvent has been extracted in less than an hour at room temperature and higher temperatures [4,21,32,34]. In microwave extraction sample is added to organic solvent [4,37,34]. While in some studies extraction is done around 1 hr [36,37,25,19,20, 28,31,23,34,35] in some other studies it is waited overnight [18,29,26,21,24,33]. When an alcoholic solvent is used, the extraction may be done at room temperature or at higher temperatures [36,37,19,20,31,34]. After adding solvents, all extracts are filtered using filters with 0.22 mm polytetra-fluoroethylene (PTFE) filters, Whatman no. 4 filter paper, 0.45 μm membrane filter, Whatman filter paper (No. 42) or a disposable polyester filter with 0.20 μm filter paper [29,4,36,25,28,26,23,24,35]. Some studies used more than one technique for extraction of plant such as [4], the methodologies that used were: Infusion (IE) which extraction was done using hot water, the second one was Ultrasound-assisted extraction (USE) as different solvents were used for extraction (W, MeOH, EtOH, Ace), while the third one was Microwave extraction (MWE), MeOH was used for extraction. Static extraction, ultrasound extraction, microwave radiation and dynamic extraction under heating and pressure with aqueous alcoholic solvents have been used for the extraction [32]. Moreover, ultrasound-assisted extraction (UAE) using EtOH as a solvent has been applied for the extraction of the phenolic compounds [34].

1.8.3 Processing and Instrumental Conditions

In studies, different extraction techniques, different species have been compared in terms of the phenolic compounds [4]. Extraction conditions such as extraction solvents (EtOH:W, MeCN:W and W), temperature and duration of the extraction on the composition of the phenolic compounds in extracts of Dalmatian wild sage (*Salvia officinalis L.*) have been investigated by HPLC-DAD [36]. In another study, microwave-assisted extraction is applied and microwave power in addition to the extraction solvent (W, EtOH or Acetone) and extraction time have been optimized and the phenolic compounds rosmarinic acid, caffeic and glycoside derivatizes of the luteolin and apigenin of dry sage (*Salvia officinalis L.*) were identified and quantified using HPLC/DAD [37]. Methanol/water (owns lowest concentration of phenolic compounds) (80:20, v/v) extracts of Salvia officinalis L. using HPLC-UV have been investigated [30]. Phenolic compounds in extracts of Lamiaceae medicinal plants, namely garden sage (*Salvia officinalis L.*), creeping thyme (*Thymus*

serpyllum L.), wild marjoram (*Origanum vulgare L.*), and common balm (*Melissa officinalis L.*) were identified and quantified using HPLC-DAD/MS/MS[32]. Polyphenol content of the extractions from sage, basil, and thyme have been determined by HPLV-UV [29]. Polyphenolic compounds by both ultrasound-assisted and microwave-assisted extractions of Sage (*Salvia officinalis L.*) dust were determined by HPLC-UV and the recovery of the extraction were compared with the those of traditional extraction techniques [34].

Instrumental conditions for the HPLC analyses have been given in (Table 1.1). C18 column with various lengths and dimension such as 150 mm × 4.6 mm, 100 mm × 4.6 mm and 250 x 4.6 mm are utilized in the analysis of multiple phenolic compounds. Particle sizes are mostly 5 um, but columns with particle size of 2.6 µm, 3 µm, 4 µm, 7 µm are also used. Gradient elution is applied using water with 1% FA, 3 % FA, 5 % FA, MeCN, AA, TFA and MeCN, MeOH, TFA, AA, 5% FA, 3% FA as the organic solvent. Flow rate was in the range of 0.25 – 2.5 ml/min. The run time of the analyses was 5 – 97 min.

The fastest separation is achieved in 5 min using the column C18 (50 mm × 2 mm) and flow rate 1.5 mL/min for the separation of caffeic acid derivative, rosmarinic acid, apigenin-7-OG, Salvianolic acid H, Salvianolic acid I and luteolin derivatives of 7-O-rutinoside and 7-O-glucoronide. In each literature different number and sets of phenolic compounds are separated, therefore the run time has varied. While in some literature single wavelength was applied [36,25,18,20,28,21], maximum absorbances are determined in other literature [4,37,19,30,33,34].

Table 1.1: Instrumental Conditions In Analysis Of Phenolic Compounds In Sage by HPLC-UV.

Lit.	Extraction solvent / Specie / Part of the plant	Compounds	Detector Wavelength (nm)	Mobile phase / Run time/ Injection volume / Column
[36]	EtOH Ace W *Salvia officinalis* L. Dry leaves	CA ROS SYR L7O Vanillic a. Salvianolic K a. Salvianolic I a. Luteolin 7G Apigenin 7G Apigenin 7G Luteolin 3G	278	A: 3 % FA in MeCN B: 3 % FA in W 35 min. Flow rate: 0.9 mL/min. 20 µL C18 (250x4.6 mm, i.d. 5 um) 20 °C
[37]	W EtOH Ace *Salvia officinalis* L. Leaves	CA ROS ROS derivatives Luteolin Apigenin-glycosides Luteolin glycosides	278 340	A: 3 % FA in W B: 3 % FA in MeCN 35 min. Flow rate: 0.9 mL/min 20 µL C18 (250x4.6mm i.d., 5 µm) 20 °C
[25]	MeOH *Salvia officinalis* L. Leaves	ROS Eriocitrin Luteolin 7OR Luteolin 7OG Lithospermic a. Methyl rosmarinate	280	A: 5% FA in MeCN B: 5% FA in W 30 min. Flow rate: 0.9 mL/min 20 µL C18 (250 × 4.6 mm, 5 μm) 20 °C
[25]	MeOH *Salvia officinalis* L. Leaves	ROS Eriocitrin Luteolin 7OR Luteolin 7OG Lithospermic a. Methyl rosmarinate	280	A: 5% FA in MeCN B: 5% FA in W 27 min. Flow rate : 0.9 mL/min 20 µL C18 (250 × 4.6 mm , 5 μm) 20 °C
[25]	MeOH *Salvia officinalis* L. Leaves	ROS Eriocitrin Luteolin 7OR Luteolin 7OG Lithospermic a. Methyl rosmarinate	280	C: 0.2% FA in MeCN D: 0.2% FA in W 19 min. Flow rate : 0.9 mL/min 20 µL C18 (100 × 4.6 mm, 5 μm)
[18]	MeOH *Salvia officinalis L.* Leaves	ROS CAR CA Salvianolic a. A Salvianolic a. B	280	A: 0.2% (v/v) AA in W B: MeCN 55 min Flow rate: 1 ml/min 20 µL C18 (125 × 4 mm, 5 µm,)

Table 1.1: Instrumental Conditions In Analysis Of Phenolic Compounds In Sage By HPLC-UV (Table Continued).

[19]	EtOH/W *Salvia officinalis L*. Leaves	CA SYR PCA FA ROS Gallic a. (+)-Catechin (_)-Epicatechin Rutin Quercetin Kaempferol	239.28, 320.60 230.10, 272.73 232.37, 307.04 236.51, 320.12 228.55, 269.38 235.45, 326.43 230.17, 276.49 229.98, 275.36 254.22, 350.25 223.29, 251.34, 361.77 228.38, 253.30, 367.13 228.03, 265.08, 333.06	A: MeCN B: (W/AA, 99:1, v/v) 75 min. Flow rate: 1.0 mL min 20 µL C18 (250 x 4.6 mm, 5 µm) 30 °C
[20]	EtOH Gallic a. *Salvia officinalis L*. Leaves	ROS SYR PCA CA Trans-resveratrol Cis-resveratrol Astringin (+)-catechin (_)-epicatechin Kaempferol Quercetin Quercetin 4GS Luteolin Apigenin	280	A: (W/AcOH 98: 2 (v/v)) B: (MeCN/AcOH 98: 2 (v/v)) 60 min Flow rate: 1.0 ml/min 20 µL C18 (250x4.6 mm, 5 µm) 30°C
[20]	EtOH *Salvia officinalis L*. Leaves	ROS SYR PCA CA Trans-resveratrol; Cis-resveratrol Astringin (+)-catechin (_)-epicatechin Kaempferol Quercetin Quercetin 4GS Luteolin Apigenin	280	A: MeCN B: W/AcOH 99: 1 (v/v) 75 min Flow rate: 1.0 ml/min 20 µL C18 (250x4.6 mm, 5 µm) 30°C

Table 1.1: Instrumental Conditions In Analysis Of Phenolic Compounds In Sage By HPLC-UV (Table Continued).

[28]	MeOH *Salvia officinalis L*. Aerial parts	CGA CA SYR FA PCA ROS CAR Vanilic a. Luteolin Kaempferol Apigenin Coumarine Carnosol Salycilic a. Naringin Gallic acid Resorcinol Protocatechuic a. Trans-cinnamic a. Quercetin dehydrate Trans-hydroxycinnamic a. Catechin hydrate Rutine trihydrate Quercetine-3-rhamnoside Naphtoresorcinol	280	A: MeCN B: W with 0.2 % SA 28 min Flow rate: 0.5 ml/min 20 µL C18 (250×4.6-mm, 4 µm) Room temperature
[29]	MeOH *Salvia officinalis L*. whole plant	ROS CA FA CAR L7O Gallic a. Lactic a. Apigenin 7OG	320 320 280	A: 6% AA in 2 mM SA (final pH 2.55, v/v) B: MeCN 80 min. Flow rate: 1.0 mL/min 10 µL C18 (150 * 4.6 mm, 5 µm)
[21]	MeOH *Salvia miltiorrhiza* *Salvia przewalskii* *Salvia officinalis* *Salvia deserta* Flowers, leaves, aerial parts	FA CA ROS	288	A: MeCN B: W contained PA (0.02%) 45 min. Flow rate: 1 mL/min 1 µL C18 (250×4.6mm, 5µm)
[30]	MeOH/W *Salvia officinalis L*. Flowering and aerial parts	CA ROS L7O Apigenin 7OG Isorhamnetin 3ORutinoside Quercetin 3OGS	328 328 336 348	

Table 1.1: Instrumental Conditions In Analysis Of Phenolic Compounds In Sage By HPLC-UV (Table Continued).

[31]	EtOH/W (80:20) *Salvia officinalis* L. *Thymus serpyllum L.* *Melissa officinalis* L. *Mentha piperita L.* *Origanum vulgare L.* Whole plant	SYR ROS Gallic a. Vanillic Hydroxybenzoic a. M-hydroxybenzoic a. P-hydroxybenzoic a. Protocatechuic a.	280	For: rosmarinic acid, stilbenes, catechins, flavonols and flavones A: (W/AA, 98:2, v/v) B: (MeCN/AA, 98:2, v/v) 60 min. Flow rate: 1.0 mL/min C18 (250×4.6 mm, 5 mm) 20 °C
[31]	EtOH/W (80:20) *Salvia officinalis* L. *Thymus serpyllum L.* *Melissa officinalis* L. *Mentha piperita L.* *Origanum vulgare L.* Whole plant	SYR ROS Gallic a. Vanillic a. Hydroxybenzoic a. M-hydroxybenzoic a. P-hydroxybenzoic a. Protocatechuic a.	280	For: Vanillin and monomeric phenolic acids A: MeCN B: W/AA =99:1, by volume) C: MeOH 75 min Flow rate: 1.0 mL/min No mention C18 column (250×4.6 mm, 5 mm) 25°C
[32]	EtOH *Salvia officinalis L.* *Thymus serpyllum* L. *Origanum vulgare* L. *Melissa officinalis* L. Whole plant	CA ROS CAR L7O Luteolin 7OGR 5-O-Caffeoylquinic a. 3-O-Caffeoylquinic a. Protocatechuic a.		35 min C18 (250 × 2.0 mm, 5 µm)
[23]	MeOH *Salvia officinalis L.*) 9 different common edible plant species Leaves and different parts for others	CA CGA SYR Gallic a. Protocatechuic a. Catechin Gentisic a. Vanillic a. Epicatechin Benzoic a.	234	A: W: MeCN: TFA (50: 50: 0.1) B: MeCN: W: TFA (10: 90: 0.1) 85 min Flow rate: 1mL /min 20 µL C18 (250 x 4.6 mm) Room temperature

Table 1.1: Instrumental Conditions In Analysis Of Phenolic Compounds In Sage By HPLC-UV (Table Continued).

[24]	MeOH EtOH DEE hX *Salvia officinalis L.* Thyme and marjoram Leaves	ROS CA FA CGA Cinnamic a. Quinic a. Apigenin Luteolin Quercetin Methyl rosmarenate	280	95% W containing 0.05% FA v/v and 5% MeOH 10 min. Flow rate: 0.8 ml/min 50 µl C18 (250 × 4.6 mm, 5 µm) 30 ◦C
[33]	MeOH *Salvia officinalis* *Salvia discolor and Salvia sclarea* Leaves, stems, blossoms and roots	CA PCA ROS	220, 254, 280 320, 350	MeCN and 0.1% FA 50 min. Flow rate: 1.0 mL/min No mention C18 (250*4 mm, 5um) 30 ◦C
[34]	60% EtOH 40% 60%, 80% EtOH *Salvia officinalis* L. Whole plant	FA ROS PCA CA Gallic a.	256 256 308 256	A: W/FA (98/2, v/v) B: MeCN/W/FA (80/18/2, v/v/v) 97 min. Flow rate: 0.5 mL/min No mention C18 (250 × 4.6 mm, 7 µm) 30◦C

1.9 HPLC ANALYSIS OF COMMERCIAL PRODUCTS

HPLC-UV analysis of bioactive compounds in commercial herbal supplements were demonstrated in the literature. In addition to capsules, tablets, tinctures, raw plant material, herbal formulas, extract powders that are sold in herbal drug stores are analyzed [38,39,40, 41, 42, 43, 44, 45, 46, 47,48,49,50,51,52].

1.9.1 Extraction Solvents and Extraction Procedure

Most solvents that have been used for extraction procedure: MeOH, EtOH, W, AA, MeOH/W mixture with different techniques [38**,**44**,**45,53**,**46**,**43]**.** Sample preparation and extraction in the analysis of phenolic compounds from tablet and capsules are done by adding extraction solvent [38**,**39**,**45,53**,**49**,**40**,**54**,**55]**,** followed by shaking at room temperature and/or sonication around1 hr [38**,**45,53**,**49**,**40,55**],** diluting with extraction solvent [38**,**39**,**45,53**,**49**,**40**,**55]. In some studies, the sonication process took time between 15 min-2

hours with extraction solvent [43]. After the extraction process is completed, solutions are generally cooled at room temperature [43,38,45,53,54,55,43]. For some extractions water has been used as extraction solvent in some studies [54,42,43]. In some studies, the refluxing with extraction solvent is applied [39,54]. Centrifugation is applied for separation of the supernatant [45,49,42] and/or followed by extraction by filter membrane [39,38,45,49,53,40,54,42,55]. According to a literature sample added with extraction solvent is also boiled before filtration [54]. Used MeOH, EtOH, W or MeOH/W mixture as extraction solvents, samples extracted by the ultrasonic technique at a range between 15 min-1 h, then the solutions have been cooled [45,53,49,55].

1.9.2 HPLC Conditions

Since high performance liquid chromatography is a technique used for separating substances, so for doing this procedure there are some conditions it should be suitable such as mobile phases, type of column used, run time and flow rate. The value of these parameters was different from some literature to another, as it varies according to the type of compounds to be separated. Mobile phase has two sections A, B, MeCN was the most widely used as a mobile phase B [38,49,55,40,48], While in other studies MeCN was used for mobile phase A [44,53]. Also, mobile phase A was containing AA [38,39,43,48,43], additionally FA has been used for mobile phase A [45,49]. Among the solutions that were used for mobile phase A [46,42], and B [56] was MeOH. W, also has been utilized as mobile phases A and B [38,49,42]. In a study, used $Na_2H_2PO_4^{-}$ for mobile phase A and B MeCN/EtOH absolute/3% PA [40]. About column, C18 is most commonly used in various sizes (1-5 µm) [38,39, 48,44, 45,47, 49,40, 46,55,42, 43, 56, 53, 50,51,52]. As for the wavelengths that were used, single wavelength [47,46,42,43,53,50,51,52] and maximum wavelength [38,39,44,45,49,40,55,48] have been used. Simultaneous quantitation of bioactive compounds, including rosmarinic a. and salvianolic a. B in biological fluids upon administration of raw or commercial herbal supplements have also been done by HPLC-UV for pharmacokinetic or various clinical investigations [57,54,58]. Instrumental conditions did not vary after the proper sample preparation and extraction considering the biological fluids such as serum. Basically, reverse phase column, typically C18 with various dimensions and water and acetonitrile as mobile phase in gradient elution has been applied. The wavelengths selected was either at the maximum absorbance of the individual compounds [57] or at the single wavelength that all

the compounds absorb well [54]**.** Wavelength at high value of 413 nm have been selected for the analysis of around two dozen bioactive compounds including rosmarnic acid, salvionic acids A, B, C, D, daidzin and genistin [58]**.**

Table 1.2: HPLC Conditions For The Analysis Of The Commercial Supplements Of Sage And Extraction Solvents.

Lit.	**Formulation / Herb / Extraction Solvent**	**Compounds**	**λ (nm)**	**Mobile Phase / Run time/ Flow rate / Column**
[38]	Gegen–Danshen Capsules *Pueraria lobata* *Salvia miltiorrhiza* MeOH /W /AA	Salvianolic acid B Danshensu Protocatechuic aldehyde Cryptotanshinone Tanshi-none I Tanshi-none II Puerarin Daidzin Daidzein	270 270 270 270 270 250 250 250 250	A: W, 0.1% AA B: MeCN, 0.1% AA C18 (250 × 4.6 mm i.d.; 5 μm particle size)
[39]	Compound Danshen Tablets *Radix Salviae Miltiorrhizae* *Radix Notoginseng* *Borneolum syntheticum* MeOH	Caffeic a. Lithospermic a. Protocatechuic a. Protocatechualdehyde Rosmarinic a. Salvianolic a. Salvianolic a. B Salvianic a.	270 280 290 326	A: 0.1% AA in MeCN B: 5 mmol/L CA and 10 mmol/L NaH_2PO_4 W C18 (250 ×4.6 mm, 5.0 μm)
[44]	Xueshuantong capsule *Panax notoginseng* *Radix astragali* *Salvia miltiorrhizae* *Radix scrophulariaceae* EtOH	Panaxytriol Ginsenoside Rb1 Angoroside C Protocatechualdehyde Ginsenoside Rd Calycosin-7-O-b-D-GS	270 203 203 270 203 203	A: MeCN B: 0.05% OA C18 (150 x 4.6 mm, 3 μm) 25ºC
[45]	Fufang Danshen Tablet *Radix Salvia miltiorrhiza* *Panax notoginseng* MeOH	Danshensu Protocatechuic aldehyde Salvianolic acid B Cryptotanshinone Tanshinone I Tanshinone IIA Notoginsenoside Ginsenoside Rg1 Ginsenoside Rb1	 280 254 254	A: 0.1% FA:W (v/v) B: 0.1% FA: MeCN (v/v) C18 (250 x 4.6 mm, 5 μm) 30 º C
[47]	Microencapsulated *Juglans regia L.* *Salvia hispanica L.* Chlf	Carnosic a. Rosmarinic a. Carnosol Rosmanol Rosmariq-uinone Rosmarid-iphenol	232	

Table 1.2: HPLC Conditions For The Analysis Of The Commercial Supplements Of Sage And Extraction Solvents **(**Table Continued).

[49]	Qing-Hua-Yu-Re-Formula *Radix rehmanniae recen* *Salvia miltiorrhiza* Bge *Cortex moutan* *Rhizoma coptidis* *Radix paeoniae rubra* *Euonymus alatus*	Danshensu Paeoniflorin Acteoside Lithospermic a. Salvianolic a. B Salvianolic a. C	280 254 320 254 254 280	A: W containing 0.1% FA B: MeCN C18 (4.6 ×150 mm, 5 µm) 35°C
[40]	Danshen Tablet *Radix Salviae miltiorrhizae* *Radix Notoginseng* *Borneolum syntheticum* MeOH	Lithospermic a. Salvianolic a. A Salvianolic a. B Salvianic a.A sodium Rosmarinic a. Notoginsenoside R1 Ginsenoside Rb1 Ginsenoside Rg1	203 228 254 270 286	A: aqueous phase composed of 30 mmol/L H_3PO_4 and 30 mmol/L $Na_2H_2PO_4$ B: MeCN/EtOH absolute/3% PA (123:15:12, v/v/v) C18 (250×4.6 mm, 5 µm) 35°C
[46]	Raw plant material (Chinese herbal drug store) Radix *salvia miltiorrhiza* W	Tanshinone	270	Mixture of MeOH and W (75:25, *v*/*v*) C18 (150 mm×4.6 mm id, 5 µm) 25 °C
[55]	Fufang Danshen Tablet *Salvia miltiorrhiza* *Panax notoginseng* MeOH	Protocatechuic aldehyde Notoginsenoside R1 Ginsenoside Rg1 Salvianolic a. B Ginsenoside Rb1 Cryptotanshinone Tanshinone IIA	281 203 203 281 203 270 270	A: aqueous PA (0.1%, v/v) B: MeCN C18 (250 × 4.6 mm, 5um) 30°C
[42]	Fufang Danshen Pian Tablet *Salvia miltiorrhiza* EtOH	Danshensu Protocatechualdehyde Salvianolic acid B	500 500 500	A: MeOH B: W containing 0.01% H_3PO_4 at pH 2.6 C18 (250 x 4.6 mm, 5 µm) 30º C

Table 1.2: HPLC Conditions For The Analysis Of The Commercial Supplements Of Sage And Extraction Solvents **(**Table Continued).

[43]	Fufang Danshen Dripping Pill Fufang Danshen Tablets *Salvia miltiorrhiza* *Panax notoginseng* *Cinnamomum caphora* MeOH / W	Cryptotanshinone Tanshinone I Tanshinone IIA Protocatechuic aldehyde Salvianolic a. B	280 280 280 280 280	A: 0.5% AA in MeCN B: 0.5% AA in W C18(100 cm×8mm, 4µm) Room temperature
[48]	whole V. agnus-castus L. fruits *Vitex agnus-castus* *Lamiaceae fruits* MeOH	Agnuside isovitexin casticin 5-hydroxykaempferol-3,6,7,4_-tetramethylether Vitetrifolin D	210 260	A: 0.5% AA:W(v/v) B: MeCN C18 (50 × 2.1 mm, 1.8 µm)
[50]	Tinctures *Salvia officinalis L.* Valeriana *officinalis* L. EtOH/W (Mixture)	Cineole Borneol α and β_thujones	254 254 254	MeCN/W C18 (250 × 4 mm, 5 µm)
[51]	*Salvia miltiorrhiza* extract powder *Salvia miltiorrhiza* MeOH	Cryptotanshinone Tanshinone I Tanshinone IIA	270 270 270	MeCN/W (45:55 v/v%) C18 (5 µm, 4.6 × 100 mm) 30º C
[52]	Compound salvia tablets Compound salvia dropping pills *Salvia miltiorrhiza* MeOH	Tanshinol Protocatechualdehyde Salvianolic acid B Cryptotanshinone Tanshinone Ginsenoside Rgl Ginsenoside Rbl	281 281 281 281 281 281 281	A: 0.1% PA aqueous solution B: MeCN C18 (5µm, 250mm × 4.6mm) 30°C

1.10 AIM of THE STUDY

The aim of the study is:

a. Develop and UHPLC method for the separation of the phenolic compounds: rosmarinic acid (ROS), caffeic acid (CA), chlorogenic acid (CGA), syringic acid (SYR), ferulic acid (FA), p-coumaric acid (PCA), luteolin-7-O-glucoside (L7O) that are found in sage (*Salvia officinalis*),
b. Optimize the instrumental conditions such as column type, column temperature, mobile phase type, and gradient and etc.,
c. Do the quantitative analysis of the separated compounds using DAD detector at specific wavelengths for each of the phenolic compounds,
d. Method validation of the quantitation method in terms of selectivity, linearity, limit of detection, limit of quantitation, precision and accuracy,
e. Optimize the sample preparation/extraction of the commercial products as capsule and powder of sage,
f. Apply the method in analysis of commercial sage products.

2. MATERIALS AND METHODS

This chapter involves the description of materials used with their specifications, in addition, the methods used for extracting of the samples, the conditions of the experiment will be explained in detail. All of the experiments are performed at Altinbas University Central Research Laboratory (AÜ-MERLAB).

2.1 MATERIALS

Gallic acid, Maltodextrin, Quercetin (grade for pharmaceutical secondary standard), Chlorogenic acid, Ferulic acid, p-Coumaric acid (grade for primary reference standard), were purchased from Sigma-Aldrich. (+)-Catechin (≥99.0%) (grade for analytical standard), Butylhydroxytoluene (≥ 99.0%) (pharmaceutical primary standard), Acetaldehyde diethyl acetal ((≥99.0%), Carnosic acid (≥95.0% (HPLC) (grade for analytical standard), Luteolin 7-glucoside, Syringic acid, Rosmarinic acid were (grade for analytical standard) and purity ≥98% (HPLC), MAGNESIUM STEARATE EXTRA PURE DAB, PH. E UR., B. P., PH. FRANC.(≥40% stearic acid basis (GC), ≥90% stearic and palmitic acid basis, 4.0-5.0% Mg basis (calc on dry sub.) also obtained from Sigma-Aldrich. Rutin trihydrate (≥94.0% (HPLC) (grade for analytical standard), manufacture was MilliporeSigma™ Supelco™. While Methanol, TRIFLUOROACETIC acid, Acetonitrile, Ethanol, Methanol, purity: (≥99.0%) and Ethyl acetate, Ph. Eur., BP, NF (≥ 99.5%) were purchased from ISOLAB. Also, CHROMAFIL® syringe filters (Filter diam.: 13mm, Pore size: 0.45 µm) was gained from CHROMAFIL Xtra. As for ELDIVEN NITRILE, the material was made from: Nitrile, while the powder Content was powder free.

Various brands of commercial sage capsules and powder are obtained from pharmacies and natural product suppliers in Turkey. Identification of the plant are based on the contents determined by the producers. The content of some products contain salvia and extracts of other plants. All of the test materials were kept at room temperature. The content of the brand name product is listed as extracts of sage, plantago media, oregano, st. John's Wort, cordyceps and grape. Ingredient of Brand 2 is given as salvia extract, bitter melon extract, myrtle extract and senna. Brand 3 is given as sage/*salvia officinalis*, lady's mantle /alchemilla vulgaris and fumeroot/fumaria officinalis. Content of the sage powder is given as sage extract. All these products are sold as sage mixture. Latin name of sage is *salvia officinalis.*

Instrument composed of Agilent 1290 Infinity II Vial Sampler, 1260 Infinity II DAD, Agilent Infinity 1260 MCT and 1290 Infinity II Flexible Pump modules present in Altinbas University Central Research Laboratory. Maximum UV absorbances of the molecules are determined by DAD of the HPLC Instrument. HPLC data are collected and analyzed by using the Agilent Open Lab CDS software system.

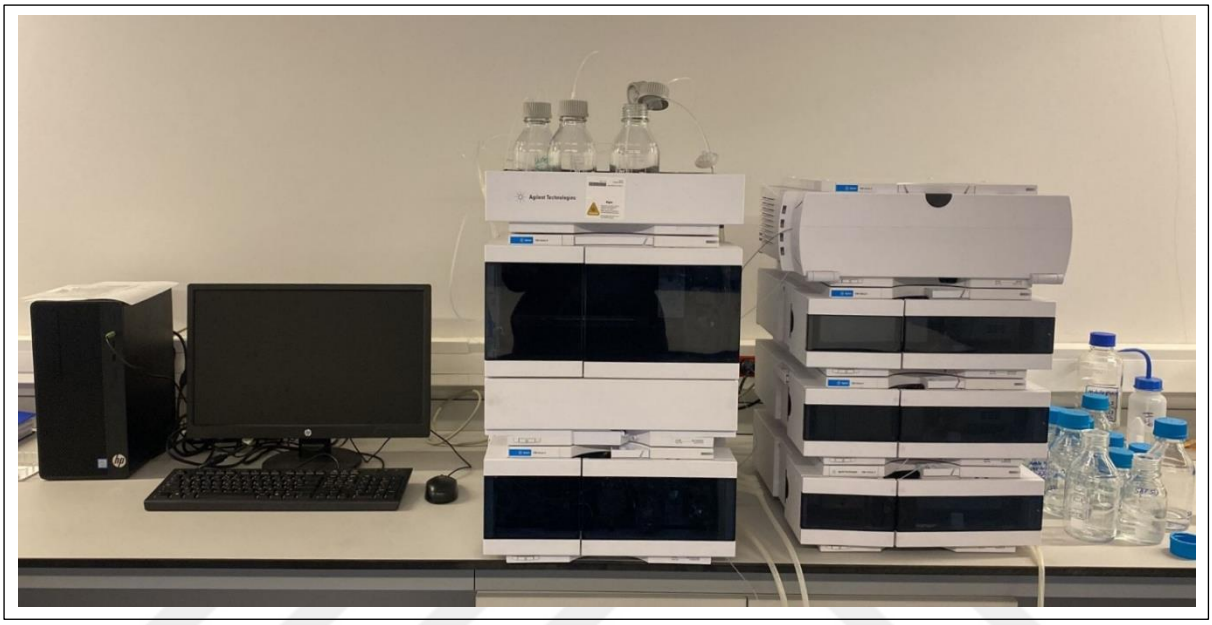

Figure 2.1: HPLC Instrument Used For The Experiment.

2.2 PREPERATION OF SOLUTIONS AND SAMPLES

Solutions which are equivalent or similar to real samples are used in method validation experiments. In this section, solution preparation is covered.

2.2.1 Pure Standards

Pure standards of the phenolic compounds are stored at appropriate temperatures (room temp., -4 ^{0}C and -20 ^{0}C) recommended by the chemical company and away from light.

2.2.2 Stock Solution Preparation

Stock solutions of phenolic compounds have been prepared by dissolving pure standards of phenolic compounds in 100% EtOH, MeOH:H_2O (6:4, v/v) or MeOH:H_2O (1:1, v/v). The stock solution concentration has been adjusted according to the sample available and signal generated based their absorptivity and their presence in the sample determined during

method development. Butylhydroxytolune (BHT) is used an internal standard (IS) and prepared by dissolving BHT in 100% EtOH. Stock solutions have been portioned into 1.5 ml centrifuge tubes and stored at -80 ^{0}C until use for the long term. Samples used daily are stored at - 20 ^{0}C away from sunlight.

2.2.3 Preparation of Calibration Solutions

Preparation of the calibration curve solutions are optimized. Stock solutions are mixed and then diluted to generate the calibration curve solutions with MeOH:H_2O (v/v, 2:1). The volume of the mixture stock solution has been optimized based on the concentration of each analyte and number of levels generation. Volume of the calibration solutions have also been optimized according to volume of the HPLC vials and for leading the minimal loss of chemicals. Stock solutions and calibration solutions are kept at -20^{0}C and protected from light by covering aluminum folio [59].

2.3 RESOLUTION AND SYSTEM SUITABILITY TESTS

Resolution test has been done by application of the different column temperatures 30, 35 and 40, TFA acid concentration of the mobile phase 0.1% and 0.05% and fixed flow rate of 0.5 ml/min or variant flow rate that has changed between 0.5 ml/min to 0.6 ml/min has been applied as indicated in Table 2.3. When one parameter has changed the other parameters are kept constant.

Capacity factor (k'), N (theoretical plate number), T(tailing) and Asy (Asymmetry factor) are evaluated for the system suitability tastings by injecting one of the calibration standard solution. Solutions are injected into the HPLC system after the equilibration (i.e. constant pump pressure conditions and background signals are obtained).

2.4 METHOD VALIDATION

Method validation is done by following the guidelines from AOAC (Association of Official Analytical Chemists) [60], USP (United States Pharmacopeia) [61], IUPAC (International Union of Pure and Applied Chemistry) [62], EPA (Environmental Protection Agency) [63] and FDA (Food and Drug Administration) [64].

2.5 SELECTIVITY

Selectivity has been done by comparing the mixture of pure standard solution, extracted capsule and extracted capsule spiked with the pure standard have been run. Capsule is extracted using the procedure in Table 2.1. Capsule is spiked with the pure standard is prepared by mixing the capsule extraction the pure standard mixture in equal volumes.

2.6 LINEARITY

Best concentration level for each of the compounds and the dilutions have been determined based on the extracts, peak areas and the limit of quantitation values. The calibration curve of each phenolic compounds are expressed be expressed by an equation of linear Curve: y=ax+b, where y: peak area (ratio of the peak areas of analyte to internal standard), x: concentration, b: intersection. All calculations are done according to the Agilent Open Lab CDS software program. Calibration by direct calibration and internal standard addition are evaluated. Calibration solutions are prepared on the different days and run on different days, solutions are also injected on different days and the same day to validate the linearity.

2.7 LOD (LIMIT OF DETECTION) AND LOQ (LIMIT OF QUANTITATION)

LOD is the minimum amount of analyte that can give a signal but cannot measured. LOQ is the minimum amount of analyte which can be quantified by precision and accuracy. LOD and LOQ are obtained by measurement of standard solutions with lowest concentrations 7 times according to the method validation of environmental protection agency (EPA) [59]. Standard deviation is calculated for each of the analyte in the standard mix. LOD is 3 times the standard deviation and LOQ is 10 times the standard deviation.

2.8 PRECISION

Precision is the consistency of the measured values in the presence of uncertainties in the laboratory. Accuracy in analytical method validation is how close the measured value to the accepted or true value. Within-day (repeatability) and between day (intermediate), precision and accuracy experiments have been done. Pure standard solutions of the phenolic compounds at three levels are measured in 3 days.

2.9 RECOVERY

In this study, the recovery (R) is also validated. Recovery has been evaluated by comparison of the sample, sample spiked with the pure standards and pure standards. Extractions are performed according to Table 2.1 upon the results of the extraction.

2.10 APPARATUS

The devices that have been used in this project are centrifuge—Cryste Separation Technology / Varispin 12R Multi Centrifuge, orbital shaker—Maxi Lab Biotechnology / MS3-MaxiOrbit 300, ultrasonic bath—ISOLAB Laborgerate GmbH, Rotator—Biosan Multi Bio RS-24, Vortex mixer—DAIHAN Scientific, Water distiller—Sartorius Arium Comfort in AÜ-MERLAB.

2.11 EXTRACTION

Extraction of the commercial capsules have been done following a similar procedure as described in literature [59]**.** 10-20 capsule are mixed for the generation of homogenous mixture. Capsule mixture is transferred to 50 ml conical centrifugal tubes and added with extraction solvent. The desolvation of the samples are facilitated by using rotator and the orbital shaker. Supernatant is separated by using centrifuge. Supernatant is filtered using the filter membrane (Figure 2.2).

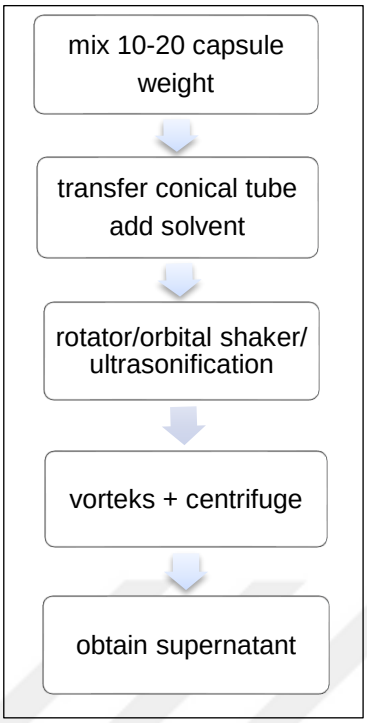

Figure 2.2: Sample Preparation And Extraction.

Type of extraction solvent, ratio of organic solvent to water, volume of the solvent, duration of shaking on the efficiency of extraction are determined. 25 mg sample was used at centrifugal force of 4000 rpm 5 minutes and 30 minutes mixed using rotational mixer. Extraction solvents of MeOH, W or EtOH and their mixtures are varied. Duration of rotational mixing, shaker and ultrasonic bath has also been varied. The conditions of the experimented extraction conditions are given in Table 2.1. The extraction of the phenolic compounds is enabled using the shaker and ultrasonic bath at varying durations.

Table 2.1: Extraction Procedure.

extraction procedure	solvent V (ml)	MeOH %	H_2O %	EtOH %	shaker time (min)	Ultr. bath time (min)
1	5	60	40	0	30	0
2	5	70	30	0	30	0
3	5	30	70	0	30	0
4	5	40	60	0	30	0
5	5	90	10	0	30	0
6	5	30	40	30	30	0
7	5	40	20	40	30	0
8	5	0	60	40	30	0
9	5	60	40	0	30	0
10	4	60	40	0	30	0
11	3	60	40	0	30	0
12	2	60	40	0	30	0
13	1	60	40	0	30	0
14	5	60	40	0	0	30
15	5	60	40	0	60	0
16	5	60	40	0	0	60
17	5	60	40	0	120	0
18	5	60	40	0	0	120

3. RESULT AND DISCUSSION

3.1 METHOD DEVELOPMENT

HPLC method development for the analysis of the phenolic compounds have been done by following the separation of the compounds in the literature. Initially, the UV/Vis spectrum of the compounds are taken without a chromatographic separation on DAD detector of HPLC find out the maximum absorbance to be used quantitation. The pure standards are run in MeCN or MeCN:H_2O mixture. DAD spectra are given in Figure 3.1. The maximum absorbance of BHT, CGA, L7O, ROS, CA, FA, PCA, SYR and CAR are found at 278, 322, 336, 330, 324, 324, 310, 276 and 286 nm, respectively.

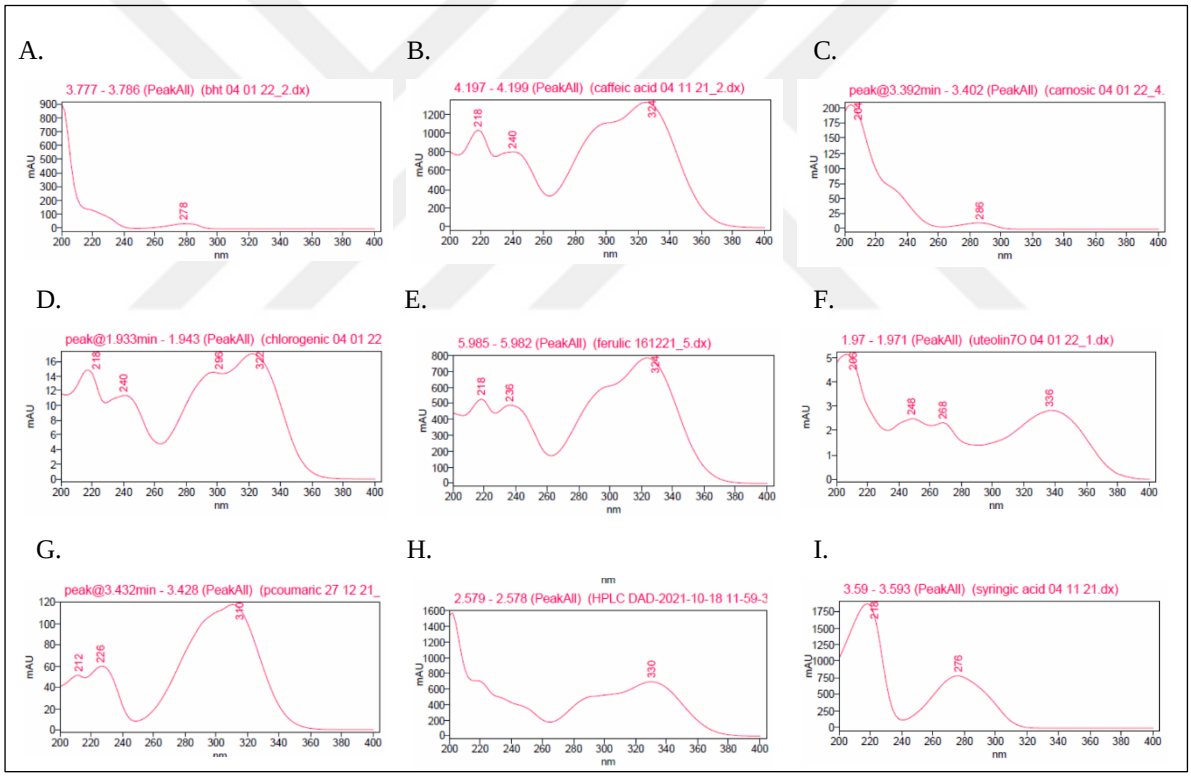

Figure 3.1: UV/Vis Spectra Of The Pure Standards Solutions A)BHT(IS) B)CA C)CAR D)CGA E)FA F)L7O G)PCA H)ROS I)SYR.

Initially, C18 column with dimensions of 250 x 4.6 mm, 5 µm was used. Then, column of C18 2.1x50 mm, 1.8 µm was examined for faster elution. 10 µl of injection volume results in overload of the column so the injection volume of 1ul is used which gave symmetrical well-defined peaks and used through the method development and validation. As a mobile

phase MeCN and H_2O has been used. Generic composition of the mobile phase as 50% MeCN and 50% H_2O initially was used to determine the relative retention times of the compounds. Only after the addition of ~0.1% TFA peaks are formed. Therefore, throughout the rest of the experiments mobile phase contained ~0.1% TFA. Then, the effect of the different composition of the mobile phase on the separation of the compounds have been explored for the separation of the analytes. Initially, H_2O:MeCN (90:10, v/v) and MeCN:H_2O (90:10, v/v) were used as mobile phase A and B, respectively. Gradient program is as follows applied: 0 min 100% A, increased to 95% B in 4 minutes and kept there up to 5 minutes, then increased to 75%B until 9 minutes and then increased up 100% B and kept constant until 12 minutes and decreased to initial conditions which is 100% A up to 12.1 minutes and flushed until 16 minutes for the stabilization of the pressure. Overlapped chromatograms taken at 278, 322, 336, 330, 324, 324, 310, 276 and 286 nm are given in Figure 3.2. The separation at two areas of the chromatograms were not efficient at these settings. One is the elution region between 2.4 - 5 min, where CGA, CA, and SYR were not separated. The second area is where the CAR and BHT peaks eluted at retention time of ~10.2 and ~10.6 minutes, respectively. Generally, the peak of presumable CAR was neither well separated nor with efficient intensity in further attempts. Other compounds namely, PCA, FA, L7O and ROS are resolved well when mobile phase B increased gradually. There is an impurity around 9.2 min. at absorption <300 nm generally and did not pose an interference for compounds at close retention times such as ROS and L7O, which have quantified at higher wavelengths. However, the peak ~9.2 min should be able to be separated either from CAR or BHT which has detection wavelength of 286 nm and 278 nm, respectively. In a further method development that is taken into consideration.

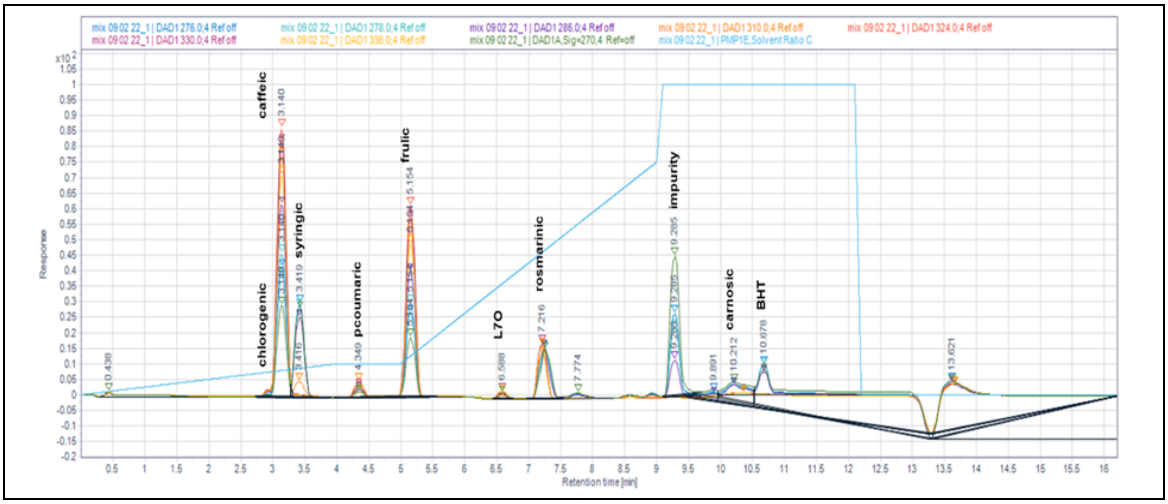

Figure 3.2: Chromatogram Of Mixtures Of Standard Solutions With Mobile Phase A H_2O:Mecn (90:10, V/V) And Mobile Phase B Mecn:H_2O (10:90, V/V), Both Have ~ 0.1%TFA. Continuous Line Shows The % Mobile Phase B.

Mobile phase A of H_2O:MeCN (95:5, v/v) and mobile phase B of MeCN:H_2O (95:5, v/v) with 0.1% TFA were used to separate CGA, CA and SYR. Gradient elution is as follows: started with 100% mobile phase A and kept there 5 minutes and then increased to 10% mobile phase B up from 5 minutes to 6 minutes and kept constant at 10% B up to 7 minutes, and increased to 80% B up to 12 minutes and then increased to 100% B from 12 minutes to 12.1 minutes and kept constant at 100% B from 12.1 minutes to 14 minutes and decreased to 0% B from 14 minutes to 14.1 minutes and run at initial conditions until 16 minutes. Elution graph is given in Figure 3.3 which differs from the elution graph in Figure 3.2. in two ways. Generally, aqueous mobile phase is run longer initially for elution of CGA, CA and SYR and organic solvent increased more slowly for better separation of CAR and BHT. While SYR is separated from CGA an CA, CGA and CA are not resolved. Other peaks are resolved.

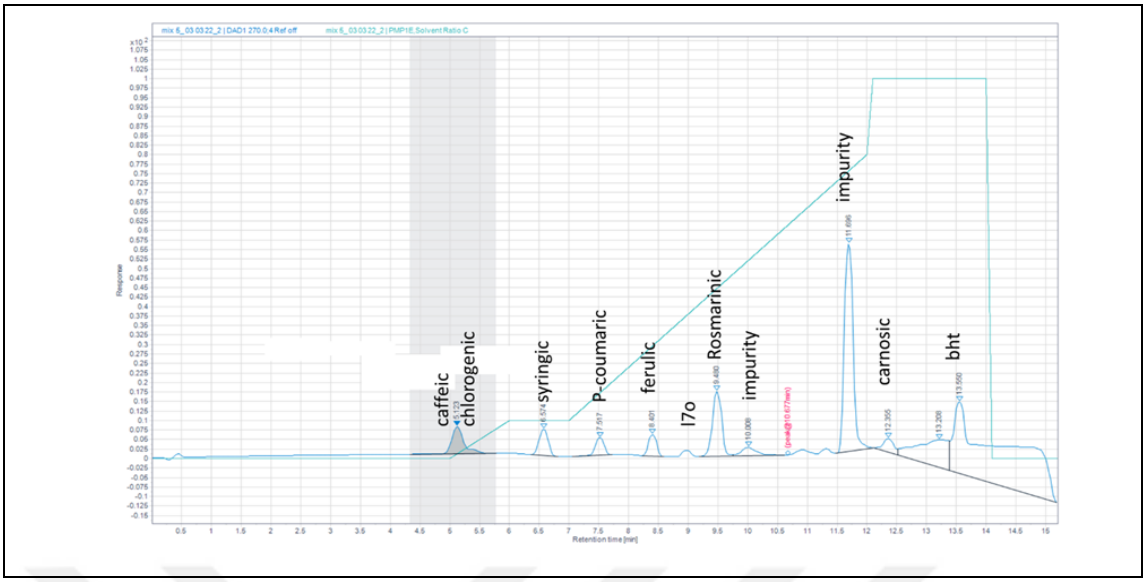

Figure 3.3: Chromatogram Of Mixtures Of Standard Solutions With Mobile Phase A Mecn:H_2O (95:5, V/V) And Mobile Phase B Mecn:H_2O (5:95, V/V), Both Have ~ 0.1%TFA. Continuous Line Shows The % Mobile Phase B.

Table 3.1: Methods For The Resolution Of CA, CGA, SYR And Other Compounds.

Time	%A	%B	Flow	Time	%A	%B	Flow	Time	%A	%B	Flow
0	96	4	0.5	0	96	4	0.7	0	96	4	0.7
8	96	4	0.5	5	96	4	0.7	5	96	4	0.7
8.1	85	15	0.5	5.1	96	4	0.6	5.1	96	4	0.6
12	75	25	0.5	8	85	15	0.6	8	85	15	0.6
13	75	25	0.5	8.1	85	15	0.6	8.1	85	15	0.6
13.1	0	100	0.5	12	75	25	0.6	12	75	25	0.6
16	0	100	0.5	13	75	25	0.5	13	75	25	0.6
16.1	96	4	0.5	13.1	0	100	0.5	13.1	0	100	0.6
18	96	4	0.5	16	0	100	0.5	16	0	100	0.6
				16.1	96	4	0.7	16.1	96	4	0.7
				18	96	4	0.7	18	96	4	0.7

Mobile phase A	100% H_2O, ~0.1%TFA
Mobile phase B	95%MeCN, 5% W, ~0.1%TFA
Injection volume	1 µL
Column	2.1x50mm, 1.8 um
Column Temp.	30 ^{0}C

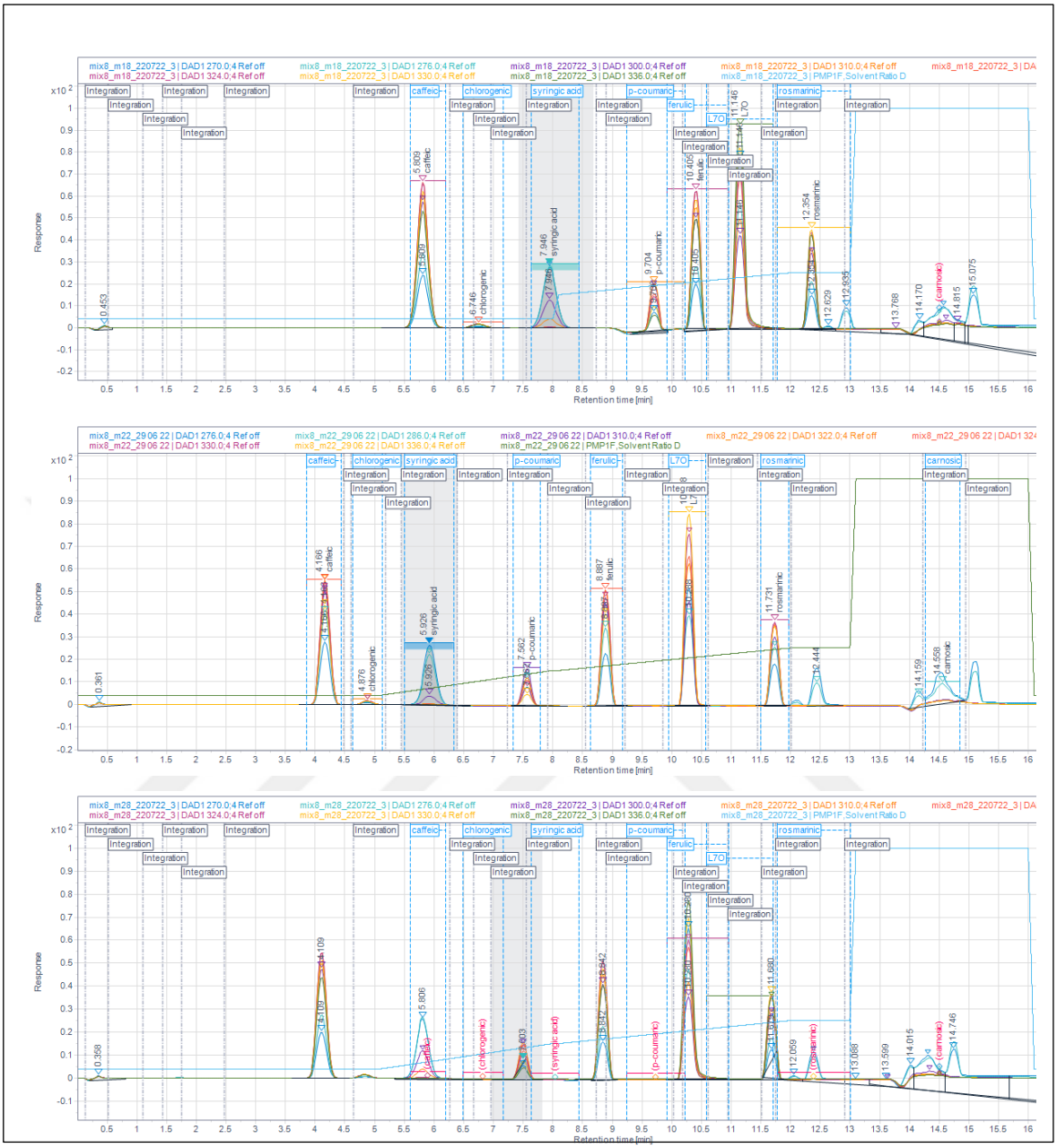

Figure 3.4: Chromatograms Of The Methods 276, 278, 286, 300, 310, 322, 324 And 336 Nm For A) Method 1 B) Method 2 C) Method 3 Given In Table 3.1. Continuous Lines Represent The Change In % Mobile Phase B.

HPLC-UV or HPLC-DAD methods have been demonstrated in the literature. The variation of the methods is due the combination of the different phenolic compounds and the run time was very high. For example, in a study, which demonstrate the analysis of phenolic compounds, CA, SYR, PCA, FA, ROS and 6 other phenolic compounds were separated in 75 minutes [19]. Similarly, ROS, CA, SYR, PCA and CA (plus 6 other phenolic compounds)

[20]; ROS, CA, FA, CAR (plus 6 other phenolic compounds) [29]; ROS, CA and FA[21]; SYR (plus 6 other phenolic compounds) [31]; ROS and CA (plus 6 phenolic compounds) [32]; CA, CGA and SYR (plus 7 other phenolic compounds) [23]; CA, CGA and SYR (plus 7 other phenolic compounds); ROS, CA and PCA [28]; ROS, CA, FA, PCA (plus one other phenolic compound), ROS, CA, CGA, FA(plus 6 other phenolic compounds) [24] were separated in 60 minutes [20], 80 minutes [29], 45 minutes [21], 60 minutes [31]; 35 minutes [32]; 85 minutes [23]; 50 minute [33], 97 minutes [34] and 65 minutes [24], respectively. General characteristics of the methods developed in the literature is the high retention times in spite of the number of phenolic compounds separated using the columns with dimensions typical to most of the HPLC analysis of 100-250 mm length, 4.6 mm diameter and 5 um particle size. In the method presented in this thesis total run time was 18 minutes for the separation of 7 phenolic compounds and internal standard. Columns with smaller particle size are also utilized in literature and generated smaller run times. Such as ROS (plus 6 other phenolic compound) were separated in 12 minutes and 5 minutes using two different columns with smaller particle sizes [4]. Rather more significant method has been developed separation of over 20 phenolic compounds including CGA, CA, SYR, FA, PCA, ROS, CAR has been done in 28 minutes [28]. However, the resolution was not reported that 5 compounds were separated in less than 5 minutes [28].

The detection of the multiple compounds has been by monitoring one wavelength namely 278 nm [36], 280 nm [18,20,24,25,28,31], 288 nm [21], 234 nm [28] which varied depending on the number and type of phenolic compounds. In some studies multiple wavelengths as 278 nm and 340 nm [37], 280 and 320 nm [29], 336 nm, 328 nm and 348 nm [30] have been used. Compound specific wavelengths have been used in limited number of studies [4,19,34,33]. Therefore, the method presented in this thesis is also distinguished from other methods in literature not only the short run time but also maximum absorbances for each compound which improves the selectivity.

3.2 RESOLUTION TEST

Resolution of each compound for the experimental parameters of the column temperature (30 ^{0}C, 35 ^{0}C and 40 ^{0}C), mobile phase acid percentage (0.01%, 0.05% and 0.1%) and the a flow rate conditions of fixed flow rate of 0.5 ml/min and varying flow rate starting with flow rate of 0.7 and lowered up to 0.5 ml/min have been determined. Peak resolution between

each analyte are going to be calculated using the equation, $R_s = 2x\ (t_{R2} - t_{R1}) / (W_2 - W_1)$. t_2 and W_2 are the retention time and width of the second eluting peaks respectively.

$R_s = 2x\ (t_{R2} - t_{R1}) / (W_2 - W_1)$. The results are given in Table 3.2 Varying flow rate conditions have been used when changing the conditions of the temperature and mobile phase acid percentage. Mobile phase acid composition of 0.1% has been used when changing temperature and flow rate. 30 ^{0}C of the column temperature has been used when changing the acid composition and the gradient flow rate.

Table 3.2: Resolution Values For Changing Conditions Of Column Temperature, Flow Rate And % TFA Composition Of Mobile Phase.

Conditions		Resolution						
		CGA/CFC	SYR/CGA	PCA/SYR	FLC/PCA	L7O/FLC	ROS/L7O	IS/ROS
Column Temperature (^{0}C)	30	0.89	0.83	1.64	2.17	2.25	3.00	4.85
	35	0.88	0.68	1.24	2.82	2.87	1.94	3.99
	40	0.84	1.02	1.34	2.43	2.66	1.71	3.85
%TFA	0.01%	0.48	1.53	1.82	1.74	2.18	2.15	4.50
	0.05%	0.67	1.09	1.55	1.67	2.46	2.26	4.04
	0.1%	0.89	0.83	1.64	2.17	2.25	3.00	4.85
Flow rate (ml/min)	0.5	0.80	1.14	2.08	0.87	0.71	1.29	1.84
	0.5 - 07	0.89	0.83	1.64	2.17	2.25	3.00	4.85

The conditions have been evaluated altogether generating the optimum resolution for all the parameters. There was not significant difference of resolution at different temperatures. Separation of early elution compounds CGA and CA was a problem demonstrated the best resolution at 30 ^{0}C. Separation of SYR and CGA was improved at 40 ^{0}C compared to 30 ^{0}C with resolutions of 1.02 and 0.83 respectively. Separation of the PCA from SYR is better at 30 ^{0}C compared to 40 ^{0}C, with resolutions of 1.64 and 1.34. The resolutions for the late eluting compounds FER, L7O, ROS and IS are greater than 1.5 at all column temperatures. As a result, the optimum temperature was determined as 30 ^{0}C which generated better resolution for the separation of the CGA and CA, which most challenging compared to other compounds, especially at elevated concentration ranges. In addition, the around the ROS and L7O there were unknown compounds from the sample and the amounts of the ROS and L7O were greatest in the sample, and at high concentration, the peaks may penetrate so resolution

of 3 has been preferred. In terms of the % TFA in mobile phase, both 0.01% and 0.05% of acid was not generated resolutions 0.48 and 0.67, respectively for the separation of CA from CGA, and there was significant overlapping of the peaks, therefore, addition of 0.1% TFA was preferred. The resolution of the other compounds for 0.1% TFA were also acceptable. Varying flow rate between 0.7 – 0.5 ml/min has generated better resolutions compared to fixed flow rate for all compounds except separation of SYR from PCA, however, there has been hardware related issues of the high flow rates, which generated high backpressure and caused loss of connections between tubing and column, where most robust fittings are required. Therefore, when varying flow rates are used repetitive experiments over the long term should be done considering day to day variations of the hardware conditions. Indeed, it has been shown in Figure 3.5, repetitive injections at varying flow rate demonstrated a precision, whereas there was a change in the retention times when the experiments are performed in another day and generated a slight change in the flow rate. Therefore, the flow rate was fixed throughout the experiments at 0.5 ml/min and we proceeded with the Method 1 in Table 3.1. The repetitive chromatograms of Method 1 are shown in Figure 3.6.

The resolution test has been done literature as part of the system suitability and rather for the method validation parameter of the selectivity [65,66,67,68,59,69,70,71]. While the resolution of ≥ 2 or ≥ 1.5 is the acceptance criteria, observed lower values are attributed to the complexity of the sample, which contain many secondary metabolites [66,72,65]. For example, resolution <1 was obtained in analysis of flavonolignans [65]. In here, the resolution test is done for determination of the column temperature, flow rate and % TFA in mobile phase. The resolution should be determined for selectivity by analyzing real samples which contain many other compounds present.

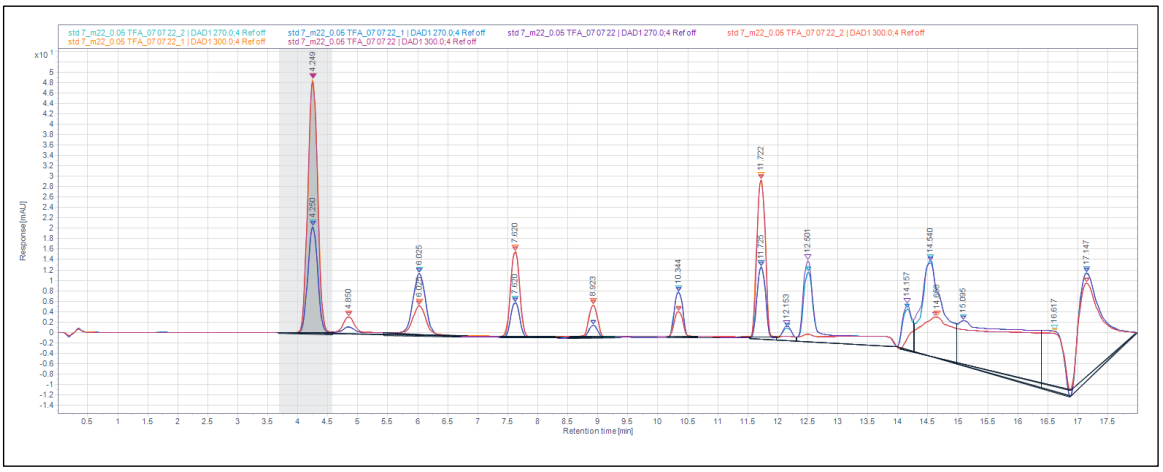

Figure 3.5: Chromatograms Of The Repetitive Runs Of The Mixture Of Standard Solutions With Gradient Flow Rate.

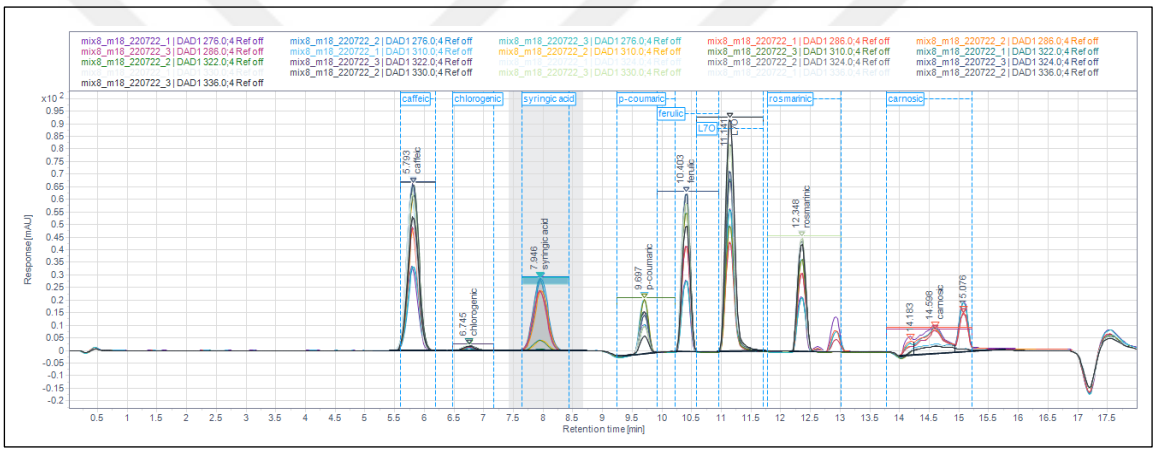

Figure 3.6: Repetitive Chromatograms Of Method 1, When 0.5 Ml/Min Flow Rate Was Used.

3.3 CHROMATOGRAPHIC SUITABILITY

Capacity factor is k' (k') ≥ 2, resolution (Rs) ≥ 1.5, (tailing) T ≤ 2 and % relative standard deviation RSD ≤ 5 % is acceptance criteria. Chromatographic suitability experiments performed by 5 repetitive injections of the standard mixture has been determined for the k' (capacity factor), T (tailing factor), N (theoretical plate number), asymmetry (Asy) have been calculated by using the following equations:

$$k' = (t_r - t_0) / t_0 \quad (3.1)$$

$$T = (w_{a5} + w_{b5})/2w_{a5} \quad (3.2)$$

$$N = 16\,(tr/w)^2 \quad (3.3)$$

$$Asy = w_{b10}/w_{a10} \quad (3.4)$$

Where,

t_r: tr: retention time

t_0 :$_0$: dead time

w: w: width of the peak

w_{a5}: first half of the peak width at the 5% of the peak height

w_{b5}: second half of the peak width at the 5% of the peak height

w_{a10}: first half of the peak width at the 10% of the peak height

$w_{b10:}$ second half of the peak width at the 10% of the peak height

The results are given Table 3.3. Capacity factor is k' (k') ≥ 2, (tailing) T ≤ 2 and % relative standard deviation RSD for the area ≤ 5 % are the acceptance criteria according to the method validation regulations [5].

Table 3.3: Mean And % RSD For The System Suitability Parameters Of Asy, T, K' And N Of The Compounds.

		RT (min)	W	Area	Asy	T	k'	N
CCA	mean	5.84	0.71	482.52	0.98	0.99	11.56	1098.67
	%RSD	0.08	10.12	0.55	1.18	0.89	0.09	18.63
CGA	mean	6.83	0.70	75.00	1.00	1.01	13.69	1531.95
	%RSD	0.09	1.55	0.74	1.39	1.16	0.10	3.08
SYR	mean	7.99	0.92	595.35	1.05	1.05	16.18	1202.57
	%RSD	0.09	2.30	0.59	3.91	3.56	0.10	5.02
PCA	mean	9.72	0.62	480.24	1.04	1.06	19.90	3887.98
	%RSD	0.02	2.12	0.52	4.57	4.32	0.02	4.17
FA	mean	10.44	0.48	692.08	1.04	1.04	21.45	7565.52
	%RSD	0.02	0.00	0.51	5.15	5.39	0.02	0.04
L7O	mean	11.20	0.76	433.95	1.04	1.10	23.08	3499.25
	%RSD	0.03	1.68	0.63	5.83	5.39	0.03	3.32
ROS	mean	12.41	0.52	485.24	1.00	1.01	25.69	9040.81
	%RSD	0.03	2.44	0.72	5.33	6.05	0.03	4.95
IS	mean	15.12	0.46	347.75	1.01	0.99	31.52	17600.60
	%RSD	0.01	1.90	1.27	4.37	4.90	0.01	3.96

Compounds are eluted with the order of CA, CGA, SYR, PCA, FA, L7O, ROS and IS between 5.84 and 15.12 min. The %RSD of the retention time were < 0.09% is within the acceptance range and indicating the repeatability of the retention time which is important for the identification and quantification of the compounds accurately. The shift in the retention

time is mostly caused by the unconditioned mobile phase in gradient elution which is prevented by going back to initial conditions at the end of the run that the mobile phase is run at composition of 96 % A and 4% B (Table 3.2.). Both T and Asy were in the range of 0.98 and 1.10 were also within the limit of the acceptance criteria. The value of the k' for the CA, earliest eluted peak was 11. 56 is way higher than 2. As it is discussed in method development CGA and CA can not be separated when mobile phase, the organic solvent concentration is increased. The only way to lower the capacity factor is to use higher flow rates by maintaining mobile phase A, aqueous phase as discussed in the Section 3.1. Method development party which can not be done since we did not have the hardware, the connection of the tubing to the column, which disintegrates at high flow rates, i.e. pressures. Theoretical plates were > 2000 for PCA, FA, L7O, ROS and IS > 2000 for CA, CGA and SYR. According to the regulations this requirement for the N> 2000 [73,74] and even higher for the UHPLC column with small particle size [75]. Therefore, the method could be improved further using higher flow rates in suitable instrument settings so that peaks with smaller widths are generated.

3.4 EXTRACTION

Extraction procedure of both capsule and powder samples have been optimized. Total peak areas of the analyte peaks in chromatograms are determined and used as an indicator of the effective extraction of phenolic compounds. The results of the extractions for the capsules and powder are given in Figure 3.7. In both cases the extraction procedure 13 generated the highest peak area for both capsule and powder sample. According to extraction procedure 13.25 mg sample is dissolved in 1 ml solvent and extracted 30 min using the orbital shaker. Solvent amount as 5 ml, 4 ml and 3 ml and 2 ml has also been used. The results show that the ratio of sample mass to volume of extraction solvent generated high concentration of the phenolic compounds. As the graphs of the total areas versus the extractions are investigated in close, the total peak areas increased as the volumes of the solvent decreased from 5 ml to 1 ml. This may not directly indicate that the extraction is better when smaller amount of solvent is used but the sample is richer in terms of the phenolic compound and applying as little of as small volume generates higher peak areas. Effectiveness of the solvent type and ratios were investigated through extractions procedures 1 to 8, where the MeOH, H_2O and EtOH were used in different ratios but significant difference was not noted according to the

Figure 3.7 The duration of the shaking and the effectiveness of the orbital shaking and ultrasonic bath were evaluated for extraction experiments from 14 to 18. The most significant change is the result of extraction procedure for both capsule and powder samples is the extraction number 17 where the duration using orbital shaker is 120 min. In this thesis extraction procedure of 13 where 25 mg sample is dissolved in 1 ml of MeOH: H2O and extracted in orbital shaker 30 min. On the other hand, the trends obtained are promising that further improvements are possible. Such as the different ratios and types and prolonged extraction durations using 1 ml of solvent may caught the differences which is not grabbed when high solvent volumes are used. Considering that these experiments are done during HPLC method development before method validations, the extractions can be further improved with validated method. One more note is that 50 mg sample is dissolved in 2 ml solvent in further experiments, which generated suitable sample volume for the HPLC autosampler. It has been indicated that polyphenols are extracted using MeOH, EtOH and MeCN due to their hydrophilic nature, addition of acid is to the extraction medium is suggested to stabilize the phenolic compounds [1,2] may be considered to increase the extraction efficiency. The results are parallel with literature that MeOH and MeOH:W mixture is commonly used in extraction of the phenolic compounds from sage plants [4,21, 27, 22, 23,24,25,26, 30,29,28,33].

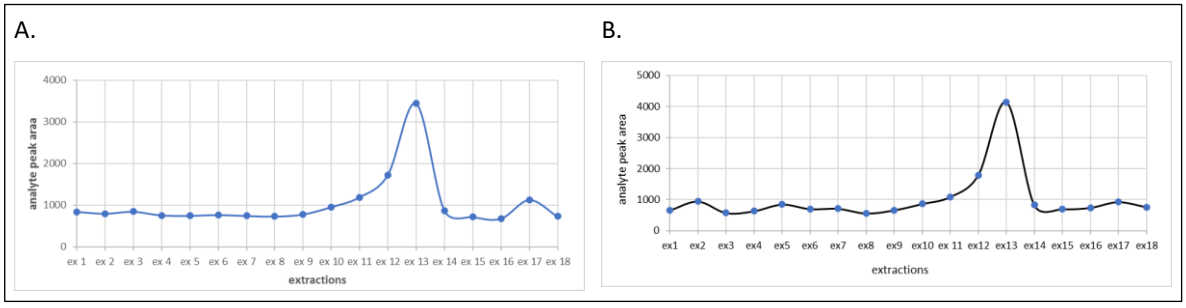

Figure 3.7: Total Peak Area Versus The Extraction Procedures For A. Capsule Samples B. Powder Samples.

3.5 METHOD VALIDATION

Method validation for Method 1 has been according to the method validation guidelines published by AOAC (Association of Official Analytical Chemists) [76], USP (United States Pharmacopeia) [61], IUPAC (International Union of Pure and Applied Chemistry) [62], EPA (Environmental Protection Agency) [63] and FDA (Food and Drug Administration) [64].

3.5.1 Selectivity

Selectivity experiments performed by comparison of the chromatograms of the pure standard solution, extracted capsule sample, and spiked extracted capsule samples and shown in Figure 3.8 A-H for capsule for the molecules CA, CGA, SYA, PCA, FA, L7O and IS. Each of the compounds are shown at their maximum absorbance selected for the quantification. Similarly, the pure standard solution, extracted powder and extracted powder spiked with the pure standard are shown in Figure 3.9 A-H. There was not interfering peak in the vicinity of the peaks eluted less than 9 min. namely CA, CGA and SYR. The chromatogram of the capsule extract is crowded with small peaks between 9 to 15 min. Many peaks arising from the capsule samples other than analyte are observed at 278 nm (Figure 3.8.H). For powder, also there was not interfering peaks in the vicinity of the peaks eluted less than 9 min (CA, SYR), while noted that number of non-overlapping peaks are appeared between 9.5 to 15 minutes Figure (3.9 A-H). At 278 nm, many peaks at 17.5 min, originating from powder samples and analyte are observed (Figure 3.9.H). The accuracy of the identification and quantification is enabled by narrowing the relative window and using signal specific identification using maximum absorbances of each peak which are above 300 nm for CA, FA, CGA, L7O, ROS and PCA.

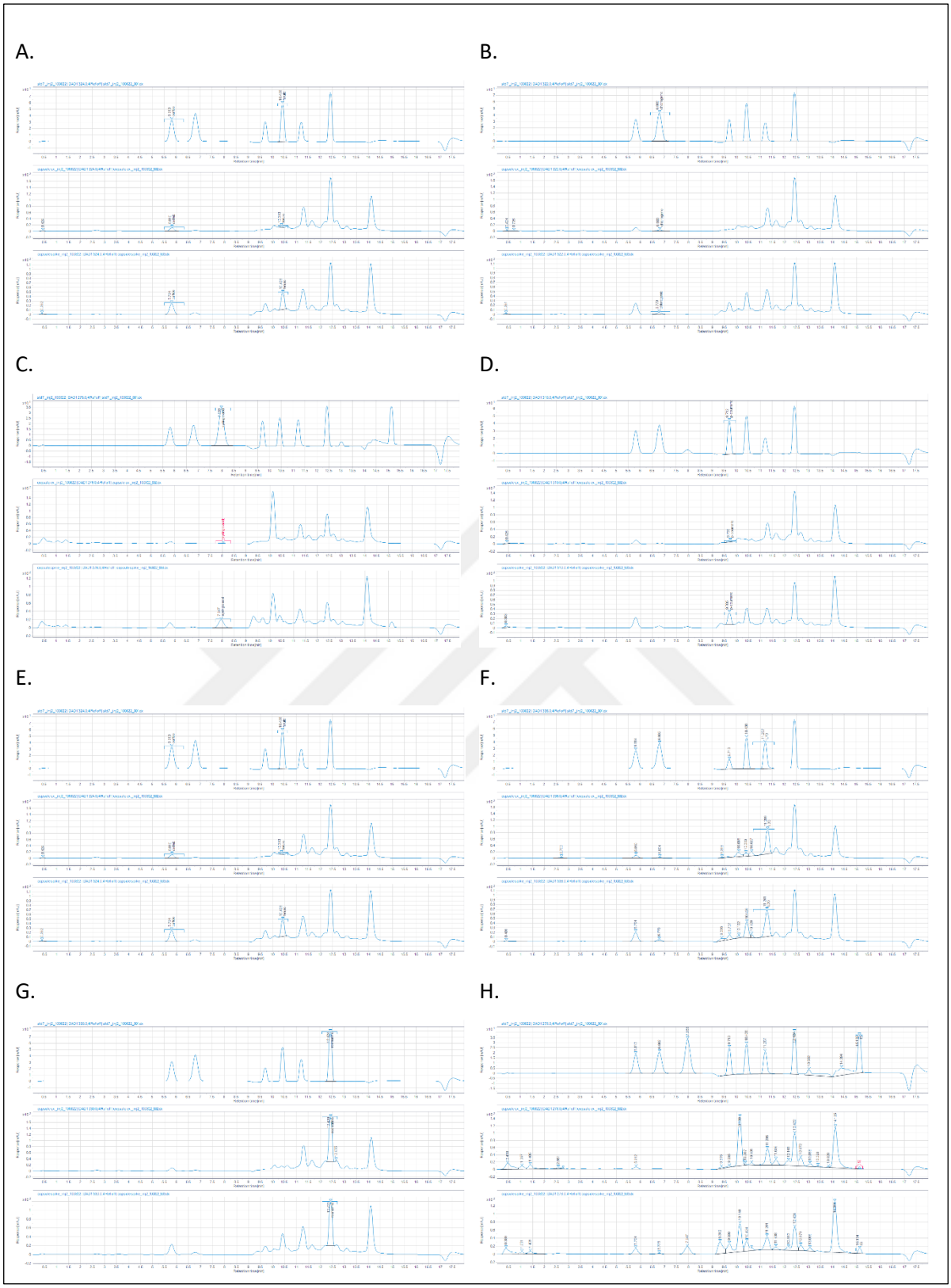

Figure 3.8: The Chromatograms Of The Standard (Top), Capsule Extract (Middle) And Standard Spiked Capsule Extract (Bottom) Of A) CA B)CGA C)SYA D) PCA E)FA F)L7O G)ROS And H)IS.

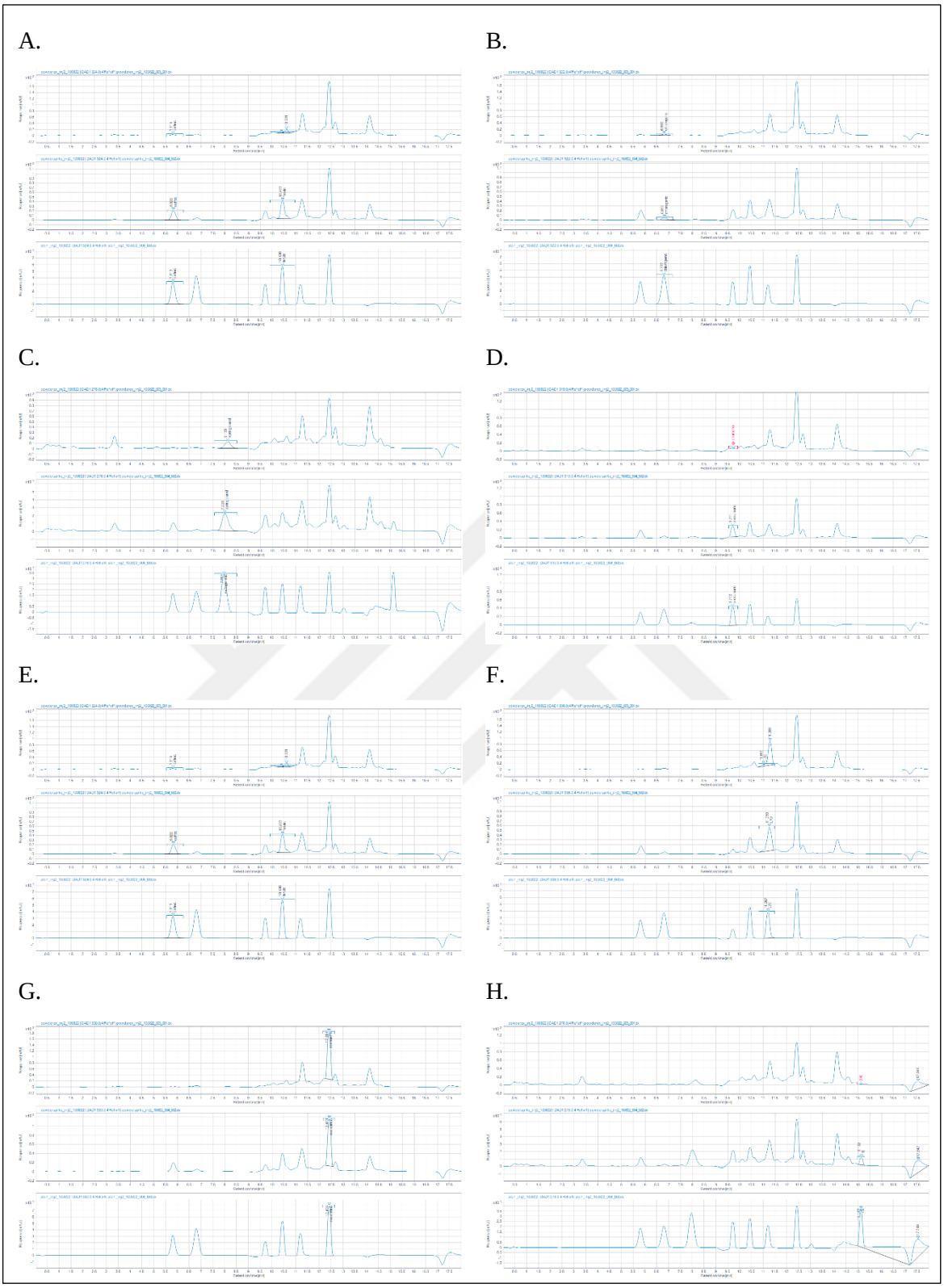

Figure 3.9: The Chromatograms Of Powder Extract (Top), Standard Spiked Powder Extract (Middle) And The Standard (Bottom), At The Absorbance Maximum Of The Compounds A)CA B)CGA C)SYA D)PCA E)FA F)L7O G)ROS And H) IS.

The selectivity of the plant samples has been done by comparison of the reference standards and the samples. Since, not all of the compounds may not present enough in the real samples [66], in this thesis, the real samples are also spiked with pure standards. The selectivity can not be tested by comparing with blank matrix, which does not exist of plants samples [69]. In some studies, the as a blank matrix another plant has been used [69]. However, it is difficult to obtain.

3.5.2 LOD and LOQ

In literature, LOD and LOQ values have been obtained using various approaches. According to USP, LOD is the concentration when S/N = 3, and LOQ is the concentration when S/N = 10. According to AOAC/IUPAC approach, blank sample is measured 7 times and mean ($\bar{x}$) and standard deviation(s) are calculated [60]. LOD and LOQ are determined according to the equations below:

$$\text{LOD: } (\bar{x} + 3s) \tag{3.5}$$

$$\text{LOQ: } (\bar{x} + 10s) \tag{3.6}$$

In literature as a blank matrix, powder of another plant is used as a blank. For example, alfalfa powder is used for the analysis of *Echinacea* samples [72]. Since, there is no available matrix for the sage capsule or powder, instead of approach by AOAC/IUPAC, determination of LOD and LOQ suggested by EPA can be used. According to EPA, solution with the lowest concentration of analyte has been measured 7 times and then the standard deviation(std) is calculated. LOD and LOQ are determined according to the equations below [70,65,66,63].

$$\text{LOD} = 3\text{std} \tag{3.7}$$

$$\text{LOQ} = 10\text{std} \tag{3.8}$$

It has been claimed that the USP method is not sufficient enough [77]. Indeed, we have found the values of LOD and LOQ irrationally low according to USP method. According to AOAC/IUPAC method, the blank signal may not generate a signal.

In this thesis, LOD and LOQ values have been calculated according to the EPA guidelines [59]. Results of 8 replications of low level of standard mixture solution, standard deviation, 3*std deviation and 10*std representing the LOD and LOQ values, respectively are given in

Table 3.4. LOD values are ranged between 2.03 -18.03 ug/ml and LOQ values are ranged between 6.77 – 60.10 µg/ml.

Table 3.4: LOD And LOQ Values In Ug/Ml. N Is Corresponding To The Different Manual Preparations Of Standard Solution Mixtures.

Compounds	1	2	3	4	5	6	7	8	LOD	LOQ
CA	3.77	4.35	4.96	5.27	3.2	4.49	4.73	4.31	1.97	6.58
CGA	10.37	12.13	13.89	14.84	8.84	11.99	12.79	11.47	5.69	18.97
SYR	7.99	9.24	10.61	11.44	6.87	9.29	9.83	8.8	4.31	14.36
PCA	9.04	10.76	12.98	14.01	7.35	-1.23	1.15	-1.37	18.77	62.56
FA	4.98	5.63	6.53	6.93	4.15	5.97	6.38	5.7	2.68	8.95
L7O	6.64	7.58	8.83	9.41	5.67	7.23	7.77	6.92	3.58	11.95
ROS	11.83	13.32	15.48	16.49	9.82	13.42	14.42	12.85	6.26	20.87

LOD and LOQ values of the bioactive compounds of some of the phenolic compounds either in raw plants or finished commercial products were generally lower than reported in this thesis [65,71,70]. Such as in analysis of phenolic compounds in tea samples, LOD for CGA, CA and SYR were determined to be less than 1 ug/ml [78]. In another study, the LOQ of CGA along with a few other phenolic compounds were < 1 ug/ml for Echinacea finished products and raw materials [70]. In UHPLC analysis of phenolic compounds in plant samples LOQ values of the CA, SYR and PCA were all < 1ug/ml [79]. In a study, LOQ values were 62.5 - 122 ug/ml for some phenolic compounds in Danshen extract [80]. Similarly, the LOQ values of the bioactive compounds in Danshen tablets by HPLC were 0.32 – 29.52 ug/ml [41].

The values may depend on how the LOD and LOQ determination conducted. Determination of LOD and LOQ could be done based on the S/N approach by injecting very low concentration of the compounds. In addition, repeated preparations of the standard 1 could be done and injected, so that the outliers could be eliminated. Additionally, LOD and LOQ could be determined by following the ICH guidelines that uses the standard deviation of the response and intercept of 6 calibration curve by following the equations [81]:

$LOD = 3.3\ \sigma/S$

$LOQ = 10\ \sigma/S$ (S = slope of calibration curve)

In analysis of phenolic compounds from sage the LOQ values are not reported [19,20,21, 23, 24, 29,34,33,31]. In UPLC analysis of phenolic compounds including CA, CGA, FA and

ROS in chia seeds (*Salvia hispanica* L.) LOD of the compound were reported to be <1 ng/ml [82]. There is not study in the literature covering the analysis of sage capsules of the 7 phenolic compounds, CA, CGA, SYR, FA, PCA, L7O and ROS.

3.5.3 Linearity

Linearity has been evaluated by 6 calibration standards. Various concentration ranges for each compound have been tested to determine concentration range suitable for the generated signal at the maximum absorbance and the amount of the molecules presented in the extracted samples. Therefore, the extractions have been measured to get an idea about the amount of each compound. The concentration at low values is also skipped since they were not repeatable. In addition, the preparation of the calibration standards is optimized since more than one compound is measured. As a result, the concentration values given in Table 3.5 is used. Calibration standard solutions are generated by serial dilution of the standard stock solution mixtures and then finally added with 80 µl IS.

Table 3.5: Preparation Of Calibration Standards And Mixture Of Stock Solutions From Individual Stock Solutions.

analytes	conc. of stock (mg/ml)	transferred volume from stock solution	stock mix conc. (mg/ml)	Calibration standards (µg/ml)					
				std 6	std 5	std 4	std 3	std 2	std 1
CA	1.16	200	0.0464	37.12	27.84	18.56	15.467	11.6	9.28
CGA	1.705	420	0.1432	114.58	85.932	57.288	47.74	35.805	28.644
FA	0.82	400	0.0656	52.48	39.36	26.24	21.867	16.4	13.12
L7O	0.425	1000	0.085	68	51	34	28.333	21.25	17
PCA	2	800	0.32	256	192	128	106.67	80	64
ROS	2	400	0.16	128	96	64	53.333	40	32
SYR	1.04	500	0.104	83.2	62.4	41.6	34.667	26	20.8
Total volume transferred from stock solutions of each standards		3720	total volume transferred from stock solution (µl)	960	720	480	400	300	240
Volume of MeOH:H_2O (60:40) to be added to 5 ml mixture		1280	Volume of MeOH:H_2O (60:40) to be added for 1.2 ml (µl)	160	400	640	720	820	880
			IS 5000 (µg/ml)	80	80	80	80	80	80

Simple linear regression analysis has been performed to determine the equation $y = ax + b$. r^2 is the correlation coefficient. Calibration solutions are prepared on the different days and run on different days, solutions are also injected on different days and the same day to validate the linearity. The results are given in the Tables 3.6, 3.7 and 3.8. When the calibration solutions are prepared and run on different days, r^2 values are generally > 0.99 and > 0.999 on the first day and the last day (Table 3.6). This could be attributed to the day-to-day variation. Therefore, calibration solutions are prepared according to the table x from the stock solutions and kept at – 20 ^{0}C and run which is an adapted practice in quality control labs since whole sample preparations and the dilutions must be done precisely that one human error changes cause changes in concentration of all levels and the compounds. The amount of the pure standards is limited since they are expensive. Using calibration standard set on different days is also budget wise since only 1ul of the solution from 1.8 ml is used every time. The results of the calibration solutions that are run on different days are given in Table 3.7. The r^2 > 0.9999 for day 1 and day 2. On the day 3, r^2 > 0.99 except for ROS, the r^2 of which > 0.9607. When the solutions are injected everyday, r^2> 0.999 and repeatability was observed in the calibration curve equations (Table 3.8).

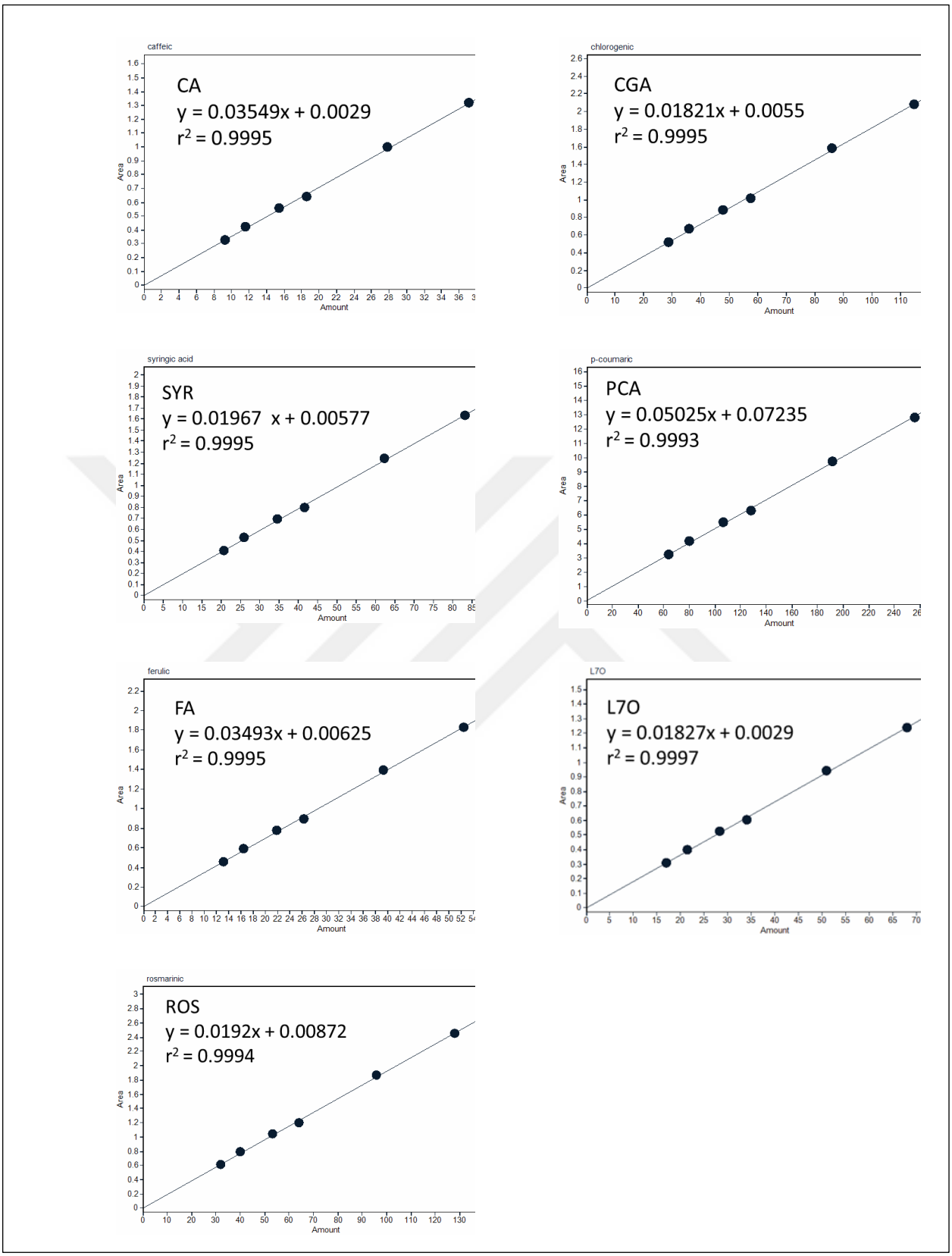

Figure 3.10: Representative Calibration Curves Of CA, CGA, SYR, PCA, FA, L7O And ROS.

Table 3.6: Linearity Data For The Calibration Solutions Prepared And Run On Different Days.

Analyte	Day 1	Day2	Day 3
CA	r^2 = 0.9999 y = 0.0340x +0.0027	r^2 = 0.9981 y = 0.0383x + 0.0100	r^2 = 0.9995 y = 0.0355x + 0.0029
CGA	r^2 = 0.9998 y = 0.0161x + 0.0071	r^2 = 0.9988 y = 0.0218x + 0.0143	r^2 = 0.9994 y = 0.0182x + 0.0056
FA	r^2 =0.9998 y =0.0320x + 0.0064	r^2 = 0.99889 y = 0.0426x + 0.0123	r^2 = 0.99942 y = 0.0349x + 0.0063
L7O	r^2 = 0.9998 y = 0.017x + 0.0035	r^2 = 0.9992 y = 0.0214x +0.0062	r^2 = 0.9996 y = 0.0183x + 0.0029
PCA	r^2 = 0.99926 y = 0.0051x + 0.0147	r^2 = 0.99857 y = 0.06312x + 0.132	r^2 = 0.99932 y = 0.05022x + 0.07271
ROS	r^2 = 0.9998 y = 0.0177x +0.0092	r^2 = 0.9986 y = 0.0237x + 0.0194	r^2 = 0.9994 y = 0.0193x + 0,0089
SYR	r^2 = 0.,9998 y = 0.0191x + 0.0049	r^2 = 0.9987 y = 0.0234x + 0.0130	r^2 = 0.9995 y = 0.0197x + 0.0064

Table 3.7: Linearity Data For The Calibration Standard Solutions Injected On Different Days.

Analyte	Day 1	Day 2	Day 3
CFC	0.9999 y=0.03403x +0.027	0.9992 y = 0.03417x – 0.0107	0.9958 y = 0.03474x – 0.0207
CGA	0.9998 y = 0.01612x + 0.00706	0.9999 y = 0.01623x + b	0.9987 y = 0.01659x -0.0219
FLC	0.9998 y = 0.03199x + 0,00642	0.9997 y = 0.03248x + 0.01209	0.9988 y =0.03291x +0,0193
L7O	0.9998 y = 0.017x + 0,00353	0.9998 y = 0.01749x + 0.00613	0.9997 y = 0.01813x +0.00319
PCA	0.9993 y = 0.00514x + b	0.9989 y = 0.00521x + b	0.9972 y = 0.00524x +0.02733
ROS	0.9998 y = 0.01766x + 0,0092	0.9991 y = 0.01733x + 0.01513	0.9607 y = 0.01678x -0.1231
SYR	0.9998 y = 0.01909x + 0,00486	0.9998 y = 0.01916x + 0.00355	0.9991 y = 0.01939x -0.0141

Tablo 3.8: Calibration Solutions Are Injected On The Same Day.

Compounds	Inj1	Inj2	Inj3
CFC	0.9991	0.99921	0.99949
	y = 0.03417x – 0.00363	y = 0.03546x + 0.00779	y = 0.03546x + 0.00294
CGA	0.99921	0.99915	0.99944
	y = 0.01755x -0.0026	y = 0.01813x + 0.01501	y = 0.01819x +0.00556
FLC	0.99901	0.99915	0.99942
	y =0.03398x – 0.003	y = 0.03477x + 0.01448	y = 0.03491x +0.00629
L7O	0.99889	0.9991	0.99956
	y = 0.01726x – 0.00406	y = 0.01818x + 0.0087	y = 0.01825x + 0.00293
PCA	0.99914	0.99901	0.99932
	y = 0.04895x + 0.01254	y = 0.05006x +0.12978	y = 0.05022x + 0.07271
ROS	0.9988	0.9991	0.99943
	y = 0.01851x – 0.01039	y =0.01909x + 0.01898	y = 0.01918x + 0.00879
SYR	0.99905	0.99912	0.99945
	y = 0.01907x – 0.00122	y = 0.01958x + 0.01373	y = 0.01967x + 0.0064

In this study, BHT is used as an internal standard and calibration with internal standard addition is adapted. IS is used to eliminate the variation in sample preparation and instrument conditions. However, it has been noted that direct calibration is also suitable. Even, in some measurements, direct calibration results are used rather than IS addition method. Peak of IS has been observed very late compared to other standards and relatively in more crowded area of the chromatogram. Therefore, there were difficulties in integration of the peak. Internal standard which elutes earlier may be more suitable. In analysis of phenolic compounds by HPLC, baicalin [83] or chrysin [84] have been used as an IS other than BHT [13]. In here, IS is not added before extraction steps, therefore, it is also suitable to use direct calibration. In UPLC analysis of the phenolic compounds including CA, CGA, FA and ROS in sage chia seeds (*Salvia hispanica* L.) were as low as 0.0004–0.1650 [82]. In analysis of phenolic compounds from sage the linearity was not reported [19,20, 21,23,24,29,34,33,31].

3.5.4 Accuracy & Precision

Accuracy and precision have been evaluated by using the solutions of known concentrations of pure standard at three levels for each compound. Both within day and between day precisions have been validated and expressed in terms of the %RSD (relative standard deviation) and %RE (relative error) for precision and accuracy, respectively.

% Relative Standard Deviation (% RSD) are calculated and acceptance criteria is ≤ %15.

$$\% \, RSD = \frac{s}{\bar{x}} \times 100 \tag{3.9}$$

Where:

s: standard deviation

$\bar{x}$: mean

Within day % RSD were in the ranges of 2.18- 4.47, 0.71-2.20 and 0.17-2.82 at low, medium and high levels, respectively for all compounds (Table 3.9.). Between day % RSD were 0.84-2.25, 5.58- 8.25 and 2.72-6.97 for low, medium and high levels, respectively for all compounds.

% RE is calculated to asses the accuracy according to the equation below:

$$\% \, RSD = \frac{(CS-CT)}{CT} \times 100 \qquad (3.10)$$

Between day %RE were 0.23 – 4.95, (-0.34) - (-7.74) and (-13.63) - (-4.72) for low, medium and high levels, respectively for all compounds (Table 3.10.). Between day % RE were (-2.65) – 2.16, 2.13 - 6.60 and (-7.81) - (-1.91) for low, medium and high levels, respectively for all compounds. There was not any trend on both %RSD and % RE values depending on the levels, but generally all values are within acceptance criteria< 15%.

Table 3.9: Within Day Precision And Accuracy. CT: Theoretical Concentration.

Low	**C_T**	**1**	**2**	**3**	**mean**	**STD**	**% RSD**	**% RE**
CFC	5.80	5.86	5.91	5.67	5.81	0.13	2.18	0.23
CGA	17.90	18.71	18.01	17.48	18.07	0.62	3.41	0.91
SYR	13.00	14.07	13.81	13.15	13.68	0.47	3.47	4.95
PCA	40.00	42.84	40.89	39.43	41.05	1.71	4.17	2.57
FLC	8.20	8.60	8.34	8.05	8.33	0.28	3.30	1.56
ROS	20.00	21.75	20.68	19.90	20.78	0.93	4.47	3.74
Medium	**C_T**	**1**	**2**	**3**	**mean**	**STD**	**% RSD**	**% RE**
CFC	18.56	16.89	17.26	17.53	17.23	0.32	1.87	-7.74
CGA	57.288	54.11	52.4	53.42	53.31	0.86	1.61	-7.46
SYR	41.6	41.56	41.13	41.69	41.46	0.29	0.71	-0.34
PCA	128	126.45	121.56	123.84	123.95	2.45	1.97	-3.27
FLC	26QA, 24	25.18	24.56	24.97	24.90	0.32	1.27	-5.37
ROS	64	63.39	60.69	61.69	61.92	1.37	2.20	-3.35
High	**C_T**	**1**	**2**	**3**	**mean**	**std**	**% RSD**	**% RE**
CFC	46.4	41.64	39.75	41.86	41.08	1.16	2.82	-12.94
CGA	143.22	126.23	125.8	126.08	126.04	0.22	0.17	-13.63
SYR	104	100.02	97.97	99.95	99.31	1.16	1.17	-4.72
PCA	320	296.94	297.15	295.07	296.39	1.15	0.39	-7.97
FLC	65.6	59.9	59.13	59.51	59.51	0.39	0.65	-10.23
ROS	160	147.47	148.38	146.33	147.39	1.03	0.70	-8.55

Table 3.10: Between Day Precision And Accuracy.

Low	**C_T**	**1**	**2**	**3**	**mean**	**std**	**% RSD**	**% RE**
CFC	5.80	5.71	5.67	5.57	5.65	0.07	1.28	-2.65
CGA	17.90	18.17	17.48	17.53	17.73	0.38	2.17	-0.99
SYR	13.00	13.12	13.15	13.59	13.29	0.26	1.98	2.16
PCA	40.00	39.16	39.43	40.83	39.81	0.90	2.25	-0.49
FLC	8.20	8.15	8.05	8.18	8.13	0.07	0.84	-0.90
ROS	20.00	19.92	19.90	20.67	20.16	0.44	2.18	0.81
Medium	**C_T**	**1**	**2**	**3**	**mean**	**std**	**% RSD**	**% RE**
CFC	18.56	20.66	17.53	18.89	19.03	1.57	8.25	2.45
CGA	57.288	63.1	53.42	59.09	58.54	4.86	8.31	2.13
SYR	41.6	46.25	41.69	45.68	44.54	2.48	5.58	6.60
PCA	128	142.5	123.84	137.05	134.46	9.60	7.14	4.81
FLC	26.24	29.23	24.97	27.57	27.26	2.15	7.88	3.73
ROS	64	71.15	61.69	69.62	67.49	5.08	7.52	5.17
High	**C_T**	**1**	**2**	**3**	**mean**	**std**	**% RSD**	**% RE**
CFC	46.4	46.9	41.86	41.48	43.41	3.03	6.97	-6.88
CGA	143.22	142.9	126.08	129.57	132.85	8.88	6.68	-7.81
SYR	104	105.2	99.95	100.99	102.05	2.78	2.72	-1.91
PCA	320	323.93	295.07	303.02	307.34	14.91	4.85	-4.12
FLC	65.6	66.47	59.51	60.69	62.22	3.72	5.99	-5.43
ROS	160	161.84	146.33	152.1	153.42	7.84	5.11	-4.29

3.5.5 Recovery

In validation of the recovery for herbal supplements of capsules and tablets, different procedures are available in the literature [85,70,72,70,59,71,86,66,67,65].

According to blank matrix is going the spiked with known concentration of analyte (theoretical concentration) and then extracted and measured (Figure 3.10.). The recovery is going the calculated as follows:

$$\% R = \frac{C_S}{C_T} * 100 \tag{3.11}$$

Where:

% R: Percent recovery

C_s: measured concentration

C_T: theoretical concentration

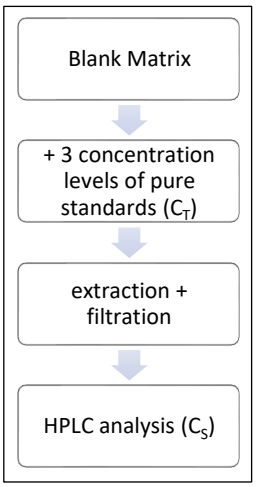

Figure 3.11: Determination Of The % R Following Blank Matrix Method.

According to sample method blank matrix is not used. Sample is divided into two portions. One portion is added with analyte (analytical concentration) and the portion is analyzed as it is (Figure 3.11). Then the % Recovery is calculated as follows:

$$\% R = \frac{C_S - C_U}{C_T} * 100 \tag{3.12}$$

C_S: sample is spiked with analyte

C_U: samples is not spiled with analyte

C_T: pure standard concentration (theoretical)

In this study, % R is going to be calculated by using both blank matrix and sample method.

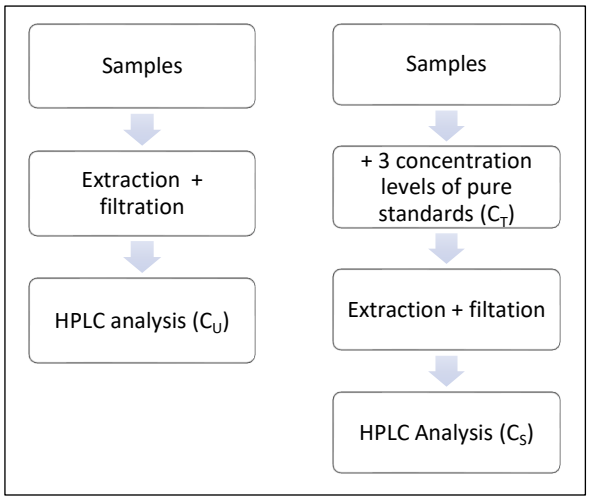

Figure 3.12: Determination Of The % Recovery By Following Sample Method.

It has been indicated that the sage capsules or tablets products may contain only pure extract or subsidiary chemicals such as sodium bicarbonate, magnesium stearate, silicon dioxide, boron, silicon, starch, zinc sulphate hepta hydrate, gum and/or chromium picolinate along with pure extracts. Blank matrix which does not contain the phenolic compounds are made by dissolving subsidiary chemicals magnesium stearate and maltodextrase in recovery validation of analysis of commercial ginseng extracts [87,88]. In some studies, dietary supplements which do not contain phenic compounds are used. For example, blank matrix in analysis of the green tea and ginkgo biloba supplement of pomegranate extracts have been used [89].

In here, recovery experiments are presented through using sample matrix since the blank matrix for salvia is not possible and difficult to obtain. Therefore, recovery experiments are done at levels of LLOQ, MLQ and HLQ by spiking appropriate number of pure standards on mg capsule and powder and magnesium stearate and magnesium dextrose mixture (MM) and obtain the recovery after the sample preparation/extraction. The recovery experiments have been designed and optimized for capsule, powder and tablet matrices at 3 levels 3 days. Extracted matrix, matrix added with standard solution are measured and compared with theoretical concentrations and the results are compared. % R has been calculated by using the equation

$$\% R = (C_S - C_U) / C_T * 100 \quad (3.13)$$

Where,

C_S: extracted matrix spiked with standard solutions at L, M, H levels

C_U: extracted matrix unspiked matrix

C_T: theoretical concentration of L, M and H levels

The acceptance criteria for the % R was 85-115% according to the regulations [5]. % R for the capsule samples were mostly within this range except a few cases (Table 3.11.). At low concentration level, FA, L7O and ROS and CA in day 2 demonstrated % R values greater or less than 85- 115%. This may be attributed to the low level of the concentration and loss of sample is significant. At medium concentration level % in FA is improved. % R for ROS in day 2 also suffers from loss of significant sample. % R of the L7O was variant and out of the desired range for the medium level which may attributed to the difficulty in desolvation

of L7O to facilitate the solvation ultrasonic bath with heat is applied. At H level % R in FA has been improved with the values around 77 – 80%. % R in L7O at high L is out of the desired range and there was not any consistency between days. % R in ROS at high conc. level has been improved. These results suggest that more appropriate sample solvation must be adopted for L7O, amount of the sample dissolved must be increased for generation of better recoveries for FA and ROS.

Table 3.11: % R Of Capsule Samples.

			Day 1		**Day 2**		**Day3**	
low	**C_T**	**C_U**	**C_S**	**% R**	**C_S**	**% R**	**C_S**	**% R**
CA	5.8	11.02	17.62	113.91	17.82	117.36	17.48	111.49
CGA	17.903	7.33	24.56	96.22	24.08	93.58	24.79	97.51
SYR	13	2.82	16.06	101.82	14.43	89.26	17.12	109.95
PCA	40	1.53	40.41	97.20	37.74	90.52	41.29	99.38
FA	8.2	3.29	6.68	41.34	5.49	26.75	6.43	38.29
L7O	10.625	140.02	158.54	174.31	161.13	198.65	146.60	61.96
ROS	20	227.82	249.01	105.93	273.52	228.48	249.30	107.37
medium	**C_T**	**C_U**	**C_S**	**% R**	**C_S**	**% R**	**C_S**	**% R**
CA	18.56	11.02	29.38	98.96	26.94	85.78	29.05	97.16
CGA	57.29	7.33	62.74	96.73	55.38	83.88	60.97	93.63
SYR	41.60	2.82	43.94	98.85	40.34	90.19	45.66	102.96
PCA	128.00	1.53	127.50	98.41	114.57	88.31	127.21	98.19
FA	26.24	3.29	23.32	76.31	20.65	66.15	22.49	73.15
L7O	34.00	140.02	180.62	119.41	137.58	-7.17	152.09	35.49
ROS	64.00	227.82	293.28	102.28	262.74	54.56	292.57	101.16
high	**C_T**	**C_U**	**C_S**	**% R**	**C_S**	**% R**	**C_S**	**% R**
CA	52.73	11.02	46.40	89.91	53.48	91.52	52.46	89.31
CGA	136.72	7.33	143.22	90.34	129.69	85.43	134.69	88.93
SYR	97.77	2.82	104.00	91.29	99.72	93.17	98.91	92.39
PCA	297.71	1.53	320.00	92.56	289.48	89.98	288.85	89.78
FER	56.16	3.29	65.60	80.59	54.81	78.53	54.19	77.58
L7O	226.92	140.02	85.00	102.23	155.11	17.76	152.93	15.19
ROS	378.98	227.82	160.00	94.47	375.98	92.60	392.27	102.78

% R of the powdered sample is given in Table 3.12. The % R for the powdered sample at low level is out of the range for most of the compounds. CA, CGA and PCA have shown most rational recoveries. Indeed, % R are all improved except for L7O at M and H levels, which could be reasoned same as capsule sample.

Generally, overall recoveries are lower compared to that of capsule sample, which may be attributed to the complexity of the matrix. While the commercial capsules samples are obtained by extraction of the plant, powdered sample is cruder and contains more compounds which may interfere with analyte molecules. In analysis of powdered samples, pre-extraction stages in sample cleaning such as sample preparation could be included to eliminate interference molecules.

Table 3.12: % R For The Powdered Sample On Different Days.

			Day 1		Day 2		Day 3	
low	**C_T**	**C_U**	**C_S**	**% R**	**C_S**	**% R**	**C_S**	**% R**
CA	5.80	3.77	8.26	77.41	8.00	72.87	7.11	57.47
CGA	17.90	9.62	26.18	92.50	24.33	82.17	21.13	64.27
SYR	13.00	30.26	61.36	239.21	38.48	63.26	51.71	165.00
PCA	40.00	1.25	42.03	101.94	39.94	96.72	29.73	71.18
FER	8.20	2.87	2.96	1.10	2.39	-5.81	2.05	-10.08
L7O	10.63	153.90	160.02	57.66	161.54	71.91	177.49	222.02
ROS	20.00	266.16	270.86	23.47	301.67	177.53	325.61	297.23
medium	**C_T**	**C_U**	**C_S**	**% R**	**C_S**	**% R**	**C_S**	**% R**
CA	18.56	3.77	19.91	86.93	17.85	75.86	18.10	77.17
CGA	57.29	9.62	60.88	89.48	57.16	82.98	57.01	82.72
SYR	41.60	30.26	72.43	101.36	77.06	112.51	76.92	112.16
PCA	128.00	1.25	124.44	96.24	125.93	97.41	125.23	96.85
FER	26.24	2.87	19.36	62.83	19.55	63.57	17.60	56.12
L7O	34.00	153.90	187.40	98.54	149.75	-12.20	163.33	27.75
ROS	64.00	266.16	351.87	133.91	332.80	104.13	355.52	139.62
high	**C_T**	**C_U**	**C_S**	**% R**	**C_S**	**% R**	**C_S**	**% R**
CA	46.40	3.77	43.65	85.95	40.62	79.42	41.36	81.00
CGA	143.22	9.62	133.46	86.47	122.71	78.96	126.13	81.35
SYR	104.00	30.26	128.60	94.55	127.31	93.32	138.36	103.94
PCA	320.00	1.25	296.93	92.40	285.52	88.83	301.50	93.83
FER	65.60	2.87	55.24	79.83	51.48	74.11	53.03	76.46
L7O	85.00	153.90	217.26	74.55	135.59	-21.54	159.32	6.38
ROS	160.00	266.16	449.86	114.81	415.10	93.09	460.21	121.28

% R of the matrix containing maltodextrins and magnesium stearate represents the tablet. % R were generally in the range of 85 -11% except a few compounds. % R for FA was around 11% for all levels only at day 1, which could be attributed the non-systematic error, since the %R are 90%-100% for other days (Table 3.13). Since L7O% standard stock solution has been consumed that can not repeated for the other two days. Overall, recovery for the MM

Matrix were better compared to capsule and the plant powder indicating that the interference hinders the recovery of the analytes. Other than the extraction procedure is suitable.

Table 3.13: MM Recovery.C_U Is Zero.

		Day 1		Day 2		Day 3	
low	**C_T**	**C_S**	**% R**	**C_S**	**% R**	**C_S**	**% R**
CA	5.80	5.17	89.05	39.49	85.11	4.98	85.92
CGA	17.90	14.63	81.69	124.43	86.88	15.36	85.78
SYR	13.00	11.67	89.77	97.74	93.98	12.11	93.18
PCA	40.00	4.47	11.18	295.11	92.22	36.57	91.43
FER	8.20	7.01	85.49	59.05	90.02	7.36	89.72
L7O	10.63	9.78	92.00				
ROS	20.00	16.96	84.78	147.31	92.07	18.52	92.60
medium	**C_T**	**C_S**	**% R**	**C_S**	**% R**	**C_S**	**% R**
CA	5.80	19.04	102.59	5.42	93.45	17.21	92.71
CGA	17.90	54.09	94.42	16.35	91.35	53.29	93.02
SYR	13.00	43.04	103.46	13.03	100.23	41.97	100.90
PCA	40.00	14.48	11.31	38.56	96.41	126.18	98.58
FER	8.20	25.72	98.00	7.82	95.37	25.34	96.56
L7O	10.63	35.88	105.53				
ROS	20.00	62.11	97.05	19.26	96.32	63.73	99.57
high	**C_T**	**C_S**	**% R**	**C_S**	**% R**	**C_S**	**% R**
CA	46.40	45.30	97.63	17.31	93.27	40.95	88.26
CGA	143.22	129.34	90.31	52.69	91.97	125.52	87.64
SYR	104.00	101.88	97.96	41.62	100.04	99.58	95.75
PCA	320.00	33.26	10.39	122.28	95.53	298.18	93.18
FER	65.60	60.87	92.79	24.88	94.82	60.01	91.47
L7O	85.00	84.73	99.68				
ROS	160.00	147.55	92.22	61.26	95.71	149.42	93.39

3.6 REAL SAMPLES

The analysis of the real samples, three brands of capsule and powder have been done and the results are given in Table 3.14. Mostly, L7O and ROS have been detected in relatively greatest amount in capsule and powder samples. There was variation in the amount of ROS among the capsule brands. Brand no 1 capsule has the highest amount of ROS. In addition, L7O has been detected higher in brand no1. While brand no 2 and 3 do not contain CA, significant amount of CA was detected in brand no1.

While there was significant amount of CGA in brand no 3, there was not CGA at all or significantly in brand no1 and 2. SYR was also not present in capsules. FA present in brand no 3 and there was low amount of FA in brand no and brand 2 does not contain FA at all. In terms of phenolic content, brand no 1 and 2 are richer. The amount of L7O and ROS were richer in powder similar to capsules. There was also appreciable amount of SYR in powder with concentration of 30.26ug/ml. CGA, CA, FA, PCA were present in powder under detection limits. As a result, the method is applicable in determination of the phenolic compounds in capsule and powder. The sample preparation with enrichment technique should be applied. These amounts could be converted in to µg/mg sample that 25 mg sample is extracted and analyzed in each case.

Table 3.14: Analysis Of Real Samples Given In Ug/Ml.

	brand no 1	**brand no 2**		**brand no 3**		**powder**		
Compound	**day 1**	**day 1**	**day 2**	**day 1**	**day 2**	**day 1**	**LOD**	**LOQ**
CA	10.43			0.59		3.77	2.03	6.77
CGA	1.5	1.64		11.66	5.5	9.62	5.81	19.38
SYR	0.08					30.26	4.40	14.67
PCA						1.25	18.03	60.10
FA	1.19			7.24	4.58	2.87	2.74	9.14
L7O	154.09	50.82	47.77	53.71	60.98	153.90	3.63	12.11
ROS	240.03	136.72	133.49	79.77	80.3	266.16	6.37	21.25

In literature there are not many examples studying the phenolic compounds in commercial capsules and powders of sage, while tablets and capsules of different plants have been analyzed [38,39,40,41,42,43,44,45,46,48,56]. Only in *Salvia miltiorrhiza* [51,52] were anlyzed by HPLC in terms of phenolic compounds other than CA, CGA, SYR, FA, PCA, L7O and ROS. Therefore, this study represents the first study for the analysis of phenolic compounds in sage capsules and powders.

4. CONCLUSION AND FUTURE WORK

In this thesis, method development and the validation of the (U)PLC/DAD method for the analysis of seven phenolic compounds CA, CGA, SYR, FA, PCA, L7O and ROS have been done by following the international validation guidelines FDA (food and drug administration), EPA (environmental protection agency), ICH (international conference on harmonization). The method validation procedures were adapted, considering the absences of the blank sample and simultaneous analysis of multiple components. System suitability has also been done. The method presented in here is particular because of the type of phenolic compounds, short run time, system suitability, method development and application to sage capsules. Different combination of phenolic compounds in different sage species have been analyzed by HPLC using UV or DAD detector.

Method development has been done by optimization of the instrumental conditions. Initially, maximum absorbances have been detected for each of the compounds and compound specific analysis has been done to improve the selectivity. Then the ideal conditions of the instrument parameters as the mobile phase composition, gradient program, injection volume, column temperature and flow rate of mobile phase. Gradient program was most difficult because of the similar polarities of the compounds, especially, CA and CGA. The total run time was 18 minutes, which is much less than the reported run time of the methods for simultaneous analysis of phenolic compounds reported in literature. 3 methods with different flow rate and gradient conditions have been developed and one is chosen for the method development.

The sample preparation and extraction of the phenolic compounds in capsule and powder has also been determined and best solvent type and composition were determined. The areas of the peaks are evaluated in a comparative manner. The data are also used to determine the calibration range. The extraction is also optimized to use the minimum amount of sample and solvent to reduce the cost and waste management. Extraction could be improved by addition of the acid.

The system suitability parameters were efficient but could be improved in terms of theoretical plate number, and resolution of the early eluting peaks that is CA and CGA. Method validation for the selectivity was adjusted for the capsule samples that there is no blank sample and the real samples may not contain all the compounds present. The method linear range was determined based on the amount of the sample to be analyzed and so the LOQ and LOD values, which could be reviewed using other approaches in different guidelines. BHT is used as an internal standard, which is eluted latest in a region crowded with impurities, therefore, direct calibration without the internal standard may be considered.

The recoveries are also presented for the first time for capsule, powder and magnesium streate/maltodextrose mixture representing tablet for the first time. Recoveries have been determined by measuring the sample, since blank matrix was not available for powder and capsule.

Finally, the method has been applied for the analysis of capsule and powder. Commercial capsule samples are sold as salvia or sage which contains *Salvia officinalis* and other herbs. Powder is sold as organic sage, i.e. *Salvia officinalis* powder. Powder showed the presence ROS, L7O and SYR relatively higher compared to other compounds which were < LOQ. Capsules follow the same trends. There were significant changes among three brands in terms of the levels of CGA and CA. There was also variation in the levels of ROS and L7O.

In the future, improvements of the study in three different dimensions could be done. One of them is to improvement of the method. CA and CGA, so the other compounds could be eluted earlier by application of the higher flow rates. Suitability of different IS should also be tested, especially to evaluate the matrix effect and recovery parameters. Comparison of the UHPLC method with LC-MS/MS method could also be made in terms of selectivity, accuracy, and limit of quantitation. Extraction efficiency could be improved by using higher amounts of sample and introduction of liqiud-liqiud or solid phase extractions using polymeric sorbents or advanced molecular extractions techniques such as magnetic sorbent extractions and molecular imprinted extraction techniques.

The method could also be extended to the analysis of different commercial products containing CA, CGA, PCA, SYR, FA, L7O and ROS in one batch. Especially since a few analyses are done, different materials could be easily extended that may include the extracts of different species, compounds studied at different geographical conditions, environment and agricultural practices used in cultivation of *Salvia officinalis* species at least in terms of the analytes measured in this study.

Another important area is the application or modification of the method for the biological fluids such human serum or urine and introduction of the metabolites of the intense compounds such as ROS and L7O to the method which was found in plenty. Change of the matrix or extraction procedures requires full validation of the method.

REFERENCES

[1] M. Abbas *et al.*, "Natural polyphenols: An overview," *International Journal of Food Properties*, vol. 20, no. 8. Taylor and Francis Inc., pp. 1689–1699, Aug. 03, 2017. doi: 10.1080/10942912.2016.1220393.

[2] R. Tsao, "Chemistry and biochemistry of dietary polyphenols," *Nutrients*, vol. 2, no. 12. MDPI AG, pp. 1231–1246, 2010. doi: 10.3390/nu2121231.

[3] R. Celano *et al.*, "Oil distillation wastewaters from aromatic herbs as new natural source of antioxidant compounds," *Food Research International*, vol. 99, pp. 298–307, Sep. 2017, doi: 10.1016/j.foodres.2017.05.036.

[4] I. Cvetkovikj *et al.*, "Polyphenolic characterization and chromatographic methods for fast assessment of culinary Salvia species from South East Europe," *J Chromatogr A*, vol. 1282, pp. 38–45, Mar. 2013, doi: 10.1016/j.chroma.2012.12.068.

[5] B. Ismail and S. S. Nielsen, "Basic Principles of Chromatography," 2010, pp. 473–498. doi: 10.1007/978-1-4419-1478-1_27.

[6] D. A. Skoog, D. M. West, F. J. Holler, and S. R. Crouch, Fundamentals of Analytical Chemistry. Cengage Learning, 2013.

[7] S. Baloch and Y. Yang, "No: 858 1 Citation: Baloch S, Yang Y (2021) Review on Methods and Applications of High-Performance Liquid Chromatography J Food Process Technol," *J Food Process Technol*, vol. 12, no. 1, p. 858.

[8] M. W. Dong and K. Zhang, "Ultra-high-pressure liquid chromatography (UHPLC) in method development," *TrAC - Trends in Analytical Chemistry*, vol. 63. Elsevier B.V., pp. 21–30, Dec. 01, 2014. doi: 10.1016/j.trac.2014.06.019.

[9] T. H. Walter and R. W. Andrews, "Recent innovations in UHPLC columns and instrumentation," *TrAC - Trends in Analytical Chemistry*, vol. 63. Elsevier B.V., pp. 14–20, Dec. 01, 2014. doi: 10.1016/j.trac.2014.07.016.

[10] L. F. Shyur, C. P. Liu, and S. C. Chien, “Metabolomics in Herbal Medicine Research,” in *The Handbook of Plant Metabolomics*, Wiley-VCH, 2013, pp. 155–174. doi: 10.1002/9783527669882.ch8.

[11] S. W. Eisenman, A. O. Tucker, and L. Struwe, “Voucher Specimens are Essential for Documenting Source Material Used in Medicinal Plant Investigations,” *Journal of Medicinally Active Plants*, vol. 1, no. 1, pp. 30–43, 2012, doi: 10.7275/R50K26HC.

[12] D. Steinmann and M. Ganzera, “Recent advances on HPLC/MS in medicinal plant analysis,” *Journal of Pharmaceutical and Biomedical Analysis*, vol. 55, no. 4. pp. 744–757, Jun. 25, 2011. doi: 10.1016/j.jpba.2010.11.015.

[13] E. M. Pferschy-Wenzig and R. Bauer, “The relevance of pharmacognosy in pharmacological research on herbal medicinal products,” *Epilepsy and Behavior*, vol. 52. Academic Press Inc., pp. 344–362, Nov. 01, 2015. doi: 10.1016/j.yebeh.2015.05.037.

[14] W. L. Applequist and J. S. Miller, “Selection and authentication of botanical materials for the development of analytical methods,” *Analytical and Bioanalytical Chemistry*, vol. 405, no. 13. Springer Verlag, pp. 4419–4428, May 01, 2013. doi: 10.1007/s00216-012-6595-1.

[15] P. M. Wolsko, D. K. Solondz, R. S. Phillips, S. C. Schachter, and D. M. Eisenberg, “Lack of herbal supplement characterization in published randomized controlled trials,” *American Journal of Medicine*, vol. 118, no. 10. Elsevier Inc., pp. 1087–1093, 2005. doi: 10.1016/j.amjmed.2005.01.076.

[16] P. P. Fu *et al.*, “Quality assurance and safety of herbal dietary supplements,” *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*, vol. 27, no. 2, pp. 91–119, May 2009, doi: 10.1080/10590500902885676.

[17] P. Ruparel and B. Lockwood, “The quality of commercially available herbal products,” *Natural Product Communications*, vol. 6, no. 5. Natural Product Incorporation, pp. 733–744, May 01, 2011. doi: 10.1177/1934578x1100600529.

[18] M. Fotovvat, T. Radjabian, and A. Saboora, “HPLC fingerprint of important phenolic compounds in some Salvia L. Species from iran,” *Records of Natural Products*, vol. 13, no. 1, pp. 37–49, 2019, doi: 10.25135/rnp.72.18.02.228.

[19] I. Generalić, D. Skroza, I. Ljubenkov, A. Katalinić, F. Burčul, and V. Katalinić, “Influence of the phenophase on the phenolic profile and antioxidant properties of Dalmatian sage,” *Food Chem*, vol. 127, no. 2, pp. 427–433, Jul. 2011, doi: 10.1016/j.foodchem.2011.01.013.

[20] I. Generalić *et al.*, “Seasonal variations of phenolic compounds and biological properties in sage (Salvia officinalis L.),” *Chem Biodivers*, vol. 9, no. 2, pp. 441–457, Feb. 2012, doi: 10.1002/cbdv.201100219.

[21] B. Li *et al.*, “Comparison of essential oil composition and phenolic acid content of selected Salvia species measured by GC-MS and HPLC methods,” *Ind Crops Prod*, vol. 69, pp. 329–334, Jul. 2015, doi: 10.1016/j.indcrop.2015.02.047.

[22] J. B. Park, “Identification and quantification of a major anti-oxidant and anti-inflammatory phenolic compound found in basil, lemon thyme, mint, oregano, rosemary, sage, and thyme,” *Int J Food Sci Nutr*, vol. 62, no. 6, pp. 577–584, Sep. 2011, doi: 10.3109/09637486.2011.562882.

[23] T. M. Rababah, K. I. Ereifej, R. B. Esoh, M. H. Al-U’Datt, M. A. Alrababah, and W. Yang, “Antioxidant activities, total phenolics and HPLC analyses of the phenolic compounds of extracts from common Mediterranean plants,” *Nat Prod Res*, vol. 25, no. 6, pp. 596–605, Mar. 2011, doi: 10.1080/14786419.2010.488232.

[24] M. H. H. Roby, M. A. Sarhan, K. A. H. Selim, and K. I. Khalel, “Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (Thymus vulgaris L.), sage (Salvia officinalis L.), and marjoram (Origanum majorana L.) extracts,” *Ind Crops Prod*, vol. 43, no. 1, pp. 827–831, May 2013, doi: 10.1016/j.indcrop.2012.08.029.

[25] I. Fecka and S. Turek, “Determination of water-soluble polyphenolic compounds in commercial herbal teas from Lamiaceae: Peppermint, melissa, and sage,” *J Agric Food Chem*, vol. 55, no. 26, pp. 10908–10917, Dec. 2007, doi: 10.1021/jf072284d.

[26] M. B. Hossain, D. K. Rai, N. P. Brunton, A. B. Martin-Diana, and A. C. Barry-Ryan, “Characterization of phenolic composition in lamiaceae spices by LC-ESI-MS/MS,” *J Agric Food Chem*, vol. 58, no. 19, pp. 10576–10581, Oct. 2010, doi: 10.1021/jf102042g.

[27] S. Pacifico, S. Piccolella, A. Lettieri, P. Nocera, F. Bollino, and M. Catauro, “A metabolic profiling approach to an Italian sage leaf extract (SoA541) defines its antioxidant and anti-acetylcholinesterase properties,” *J Funct Foods*, vol. 29, pp. 1–9, Feb. 2017, doi: 10.1016/j.jff.2016.11.031.

[28] I. Hamrouni-Sellami, F. Z. Rahali, I. B. Rebey, S. Bourgou, F. Limam, and B. Marzouk, “Total Phenolics, Flavonoids, and Antioxidant Activity of Sage (Salvia officinalis L.) Plants as Affected by Different Drying Methods,” *Food Bioproc Tech*, vol. 6, no. 3, pp. 806–817, Mar. 2013, doi: 10.1007/s11947-012-0877-7.

[29] M. B. Hossain, N. P. Brunton, A. B. Martin-Diana, and C. Barry-Ryan, “Application of response surface methodology to optimize pressurized liquid extraction of antioxidant compounds from sage (Salvia officinalis L.), basil (Ocimum basilicum L.) and thyme (Thymus vulgaris L.),” *Food Funct*, vol. 1, no. 3, pp. 269–277, Dec. 2010, doi: 10.1039/c0fo00021c.

[30] N. Martins, L. Barros, C. Santos-Buelga, M. Henriques, S. Silva, and I. C. F. R. Ferreira, “Evaluation of bioactive properties and phenolic compounds in different extracts prepared from Salvia officinalis L.,” *Food Chem*, vol. 170, pp. 378–385, Aug. 2014, doi: 10.1016/j.foodchem.2014.08.096.

[31] I. G. Mekini} *et al.*, “In vitro Antioxidant and Antibacterial Activity of Lamiaceae Phenolic Extracts: A Correlation Study”.

[32] V. V. Milevskaya, Z. A. Temerdashev, T. S. Butyl'skaya, and N. V. Kiseleva, "Determination of phenolic compounds in medicinal plants from the Lamiaceae family," *Journal of Analytical Chemistry*, vol. 72, no. 3, pp. 342–348, Mar. 2017, doi: 10.1134/S1061934817030091.

[33] F. Sharopov, A. Valiev, M. Sobeh, E. Arnold, and M. Winka, "Bioactivity of Three Salvia Species in Relation to Their Total Phenolic and Flavonoid Contents," *Pharm Chem J*, vol. 52, no. 7, pp. 596–600, Oct. 2018, doi: 10.1007/s11094-018-1866-6.

[34] Z. Zeković *et al.*, "Utilization of sage by-products as raw material for antioxidants recovery—Ultrasound versus microwave-assisted extraction," *Ind Crops Prod*, vol. 99, pp. 49–59, May 2017, doi: 10.1016/j.indcrop.2017.01.028.

[35] B. F. Zimmermann, S. G. Walch, L. N. Tinzoh, W. Stühlinger, and D. W. Lachenmeier, "Rapid UHPLC determination of polyphenols in aqueous infusions of Salvia officinalis L. (sage tea)," *J Chromatogr B Analyt Technol Biomed Life Sci*, vol. 879, no. 24, pp. 2459–2464, Aug. 2011, doi: 10.1016/j.jchromb.2011.06.038.

[36] M. Dent, V. Dragovic-Uzelac, M. Penic, M. Brncic, T. Bosiljkov, and B. Levaj, "The Effect of Extraction Solvents, Temperature and Time on the Composition and Mass Fraction of Polyphenols in Dalmatian Wild Sage (Salvia officinalis L.) Extracts," *Food Teehnol Biotechnol*, vol. 51, no. 1, pp. 84–91, 2013.

[37] V. Dragovic-Uzelac, I. E. Garofulic, M. Jukic, M. Penic, and M. Dent, "Microwave-Assisted Extraction of Sage Polyphenols," *Food Technol Biotechnol*, vol. 50, no. 3, p. 377, 2012.

[38] Q. Chang, L. Sun, R. H. Zhao, M. S. S. Chow, and Z. Zuo, "Simultaneous determination of ten active components in traditional Chinese medicinal products containing both gegen (Pueraria iobata) and danshen (Salvia miltiorrhiza) by high-performance liquid chromatography," *Phytochemical Analysis*, vol. 19, no. 4, pp. 368–375, Jul. 2008, doi: 10.1002/pca.1063.

[39] J. Chen, J. Gao, and G. Sun, “Quantitative analysis combined with chromatographic fingerprint and antioxidant activities for the comprehensive evaluation of Compound Danshen Tablets,” *J Sep Sci*, vol. 40, no. 6, pp. 1244–1253, Mar. 2017, doi: 10.1002/jssc.201600813.

[40] M. Shi and G. Sun, “Integrated evaluation of HPLC and UV fingerprints for the quality control of Danshen tablet by systematic quantified fingerprint method combined with antioxidant activity,” *J Sep Sci*, vol. 40, no. 9, pp. 1942–1952, May 2017, doi: 10.1002/jssc.201601330.

[41] Y.-J. Wei, S.-L. Li, and P. Li, “Simultaneous determination of seven active components of Fufang Danshen 1 ORIGINAL RESEARCH ORIGINAL RESEARCH Simultaneous determination of seven active components of Fufang Danshen tablet by high performance liquid chromatography,” *Biomed. Chromatogr*, vol. 21, pp. 1–9, 2007, doi: 10.1002/bmc.

[42] D. F. Yang, Z. S. Liang, Q. M. Duan, and Y. J. Zhang, “Quality assessment of cardiotonic pills by HPLC fingerprinting,” *Chromatographia*, vol. 66, no. 7–8, pp. 509–514, Oct. 2007, doi: 10.1365/s10337-007-0359-7.

[43] L. Zhou, M. Chow, and Z. Zuo, “Improved quality control method for Danshen products-Consideration of both hydrophilic and lipophilic active components,” *J Pharm Biomed Anal*, vol. 41, no. 3, pp. 744–750, Jun. 2006, doi: 10.1016/j.jpba.2005.12.032.

[44] H. Liu *et al.*, “Core bioactive components promoting blood circulation in the traditional chinese medicine compound xueshuantong capsule (CXC) based on the relevance analysis between chemical HPLC fingerprint and in vivo biological effects,” *PLoS One*, vol. 9, no. 11, Nov. 2014, doi: 10.1371/journal.pone.0112675.

[45] Y. Lu, K. Yu, H. B. Qu, and Y. Y. Cheng, “Development of an HPLC-UV-ELSD method for quantification of multiple components of a Chinese medicine made from Radix salvia miltiorrhiza and Panax notoginseng,” *Chromatographia*, vol. 65, no. 1–2, pp. 19–24, Jan. 2007, doi: 10.1365/s10337-006-0120-7.

[46] Y. Su, Z. Fu, P. Ma, W. Wang, and J. Zhang, “Effect of ultrafine grinding on dissolving-out quantity of tanshinone IIA from Radix salvia miltiorrhiza,” *Journal Wuhan University of Technology, Materials Science Edition*, vol. 24, no. 6, pp. 973–976, Dec. 2009, doi: 10.1007/s11595-009-6973-5.

[47] M. L. Martínez *et al.*, “Oxidative stability of walnut (Juglans regia L.) and chia (Salvia hispanica L.) oils microencapsulated by spray drying,” *Powder Technol*, vol. 270, no. Part A, pp. 271–277, Jan. 2015, doi: 10.1016/j.powtec.2014.10.031.

[48] C. Högner, S. Sturm, C. Seger, and H. Stuppner, “Development and validation of a rapid ultra-high performance liquid chromatography diode array detector method for Vitex agnus-castus,” *J Chromatogr B Analyt Technol Biomed Life Sci*, vol. 927, pp. 181–190, 2013, doi: 10.1016/j.jchromb.2013.02.037.

[49] X. Shao, J. Zhao, X. Wang, and Y. Tao, “Rapid Screening and Quantitative Determination of Active Components in Qing-Hua-Yu-Re-Formula Using UHPLC-Q-TOF/MS and HPLC-UV,” *J Anal Methods Chem*, vol. 2018, 2018, doi: 10.1155/2018/8535127.

[50] P. Valachovic, A. Pechova, and T. J. Mason, “Towards the industrial production of medicinal tincture by ultrasound assisted extraction.” [Online]. Available: www.elsevier.nl/locate/ultsonch

[51] H. Yu, R. K. Subedi, P. R. Nepal, Y. G. Kim, and H. K. Choi, “Enhancement of solubility and dissolution rate of cryptotanshinone, tanshinone i and tanshinone IIA extracted from Salvia miltiorrhiza,” *Arch Pharm Res*, vol. 35, no. 8, pp. 1457–1464, Aug. 2012, doi: 10.1007/s12272-012-0816-1.

[52] K. Liang1, S. Zhai1, Z. Zhang1, G. Wang1, T. X. Fu, and T. Li, “Study on the small intestine absorptive kinetics characters of tanshinol and protocatechualdehyde of Salvia miltiorrhiza extracts in rats in vivo.”

[53] Qiao, Y., Wu, Z., Pei, Y., Su, Y., Wang, C., Jiao, H., & Nie, J. (n.d.). Determination of Chemical Composition of Salvia miltiorrhiza Cultivated in Jizhou. In *Medicinal Plant* (Vol. 2018, Issue 5).

[54] X. Wang and J. H. K. Yeung, “Effects of Salvia miltiorrhiza extract on the liver CYP3A activity in humans and rats,” *Phytotherapy Research*, vol. 25, no. 11, pp. 1653–1659, Nov. 2011, doi: 10.1002/ptr.3472.

[55] Y.-J. Wei, S.-L. Li, and P. Li, “Simultaneous determination of seven active components of Fufang Danshen 1 ORIGINAL RESEARCH ORIGINAL RESEARCH Simultaneous determination of seven active components of Fufang Danshen tablet by high performance liquid chromatography,” *Biomed. Chromatogr*, vol. 21, pp. 1–9, 2007, doi: 10.1002/bmc.

[56] Y. Ni, L. Zhang, J. Churchill, and S. Kokot, “Application of high performance liquid chromatography for the profiling of complex chemical mixtures with the aid of chemometrics,” *Talanta*, vol. 72, no. 4, pp. 1533–1539, Jun. 2007, doi: 10.1016/j.talanta.2007.02.003.

[57] J. Shen, K. Yang, C. Sun, and M. Zheng, “Analysis of active components in salvia miltiorrhiza injection based on vascular endothelial cell protection,” *Acta Pharmaceutica*, vol. 64, no. 3, pp. 325–334, Sep. 2014, doi: 10.2478/acph-2014-0027.

[58] J. Zhou *et al.*, “Evaluation of the antithrombotic activity of Zhi-Xiong Capsules, a Traditional Chinese Medicinal formula, via the pathway of anti-coagulation, anti-platelet activation and anti-fibrinolysis,” *Biomedicine and Pharmacotherapy*, vol. 97, pp. 1622–1631, Jan. 2018, doi: 10.1016/j.biopha.2017.11.135.

[59] P. N. Brown, L. A. Paley, M. C. Roman, and M. Chan, “Single-laboratory validation of a method for the detection and/or quantification of select alkaloids in goldenseal supplements and raw materials by reversed-phase high-performance liquid chromatography,” *Pharm Biol*, vol. 46, no. 1–2, pp. 135–144, Jan. 2008, doi: 10.1080/13880200701735171.

[60] AOAC International, “AOAC Official Methods of Analysis - Appendix K: Guidelines for Dietary Supplements and Botanicals,” *AOAC Official Methods of Analysis*, vol. 2, no. 2, 2013.

[61] USP, “Validation of Compendial Procedures, First Supplement to USP 40–NF 35, https://pdfs.semanticscholar.org/5b03/95b168eff40a79b267de263480db5020dd05.pdf,” 2017.

[62] IUPAC, “Harmonized Guidelines for Single Laboratory Validation of Methods of Analysis. http://publications.iupac.org/pac/2002/pdf/7405x0835.pdf,” 2002.

[63] EPA, “Validation and Peer Review of U.S. Environmental Protection Agency Chemical Methods of Analysis.https://www.epa.gov/sites/production/files/2016-02/documents/chemical_method_guide_revised_020316.pdf,” 2016.

[64] FDA, “Food and Drug Administration, Bioanalytical Method Validation Guidance for Industry.https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry,” 2018.

[65] E. Mudge, L. Paley, A. Schieber, and P. N. Brown, “Optimization and single-laboratory validation of a method for the determination of flavonolignans in milk thistle seeds by high-performance liquid chromatography with ultraviolet detection,” *Anal Bioanal Chem*, vol. 407, no. 25, pp. 7657–7666, Oct. 2015, doi: 10.1007/s00216-015-8925-6.

[66] E. M. Mudge and P. N. Brown, “Determination of mitragynine in Mitragyna speciosa raw materials and finished products by liquid chromatography with UV detection: Single-laboratory validation,” *J AOAC Int*, vol. 100, no. 1, pp. 18–24, Jan. 2017, doi: 10.5740/jaoacint.16-0220.

[67] E. Mudge *et al.*, “Variation of select flavonols and chlorogenic acid content of elderberry collected throughout the Eastern United States,” *Journal of Food Composition and Analysis*, vol. 47, pp. 52–59, Apr. 2016, doi: 10.1016/j.jfca.2015.12.003.

[68] P. N. Brown, E. M. Mudge, and L. Paley, “Determination of phenolic constituents in echinacea raw materials and dietary supplements by HPLC-UV: Collaborative study,” *J AOAC Int*, vol. 99, no. 5, pp. 1197–1203, Sep. 2016, doi: 10.5740/jaoacint.16-0144.

[69] J. M. Betz, P. N. Brown, and M. C. Roman, “Accuracy, precision, and reliability of chemical measurements in natural products research,” *Fitoterapia*, vol. 82, no. 1. pp. 44–52, Jan. 2011. doi: 10.1016/j.fitote.2010.09.011.

[70] P. N. Brown, “Determination of ginsenoside content in Asian and North American ginseng raw materials and finished products by high-performance liquid chromatography: Single-laboratory validation,” *J AOAC Int*, vol. 94, no. 5, pp. 1391–1399, Sep. 2011, doi: 10.5740/jaoacint.9-543.

[71] P. N. Brown, R. Yu, C. H. Kuan, J. Finley, E. M. Mudge, and S. Dentali, “Determination of aloin A and aloin B in Aloe vera raw materials and finished products by high-performance liquid chromatography: Single-laboratory validation,” *J AOAC Int*, vol. 97, no. 5, pp. 1323–1328, Sep. 2014, doi: 10.5740/jaoacint.13-028.

[72] P. N. Brown, M. Chan, and J. M. Betz, “Optimization and single-laboratory validation study of a high-performance liquid chromatography (HPLC) method for the determination of phenolic Echinacea constituents,” *Anal Bioanal Chem*, vol. 397, no. 5, pp. 1883–1892, Jul. 2010, doi: 10.1007/s00216-010-3763-z.

[73] L. G. da S. Solon, I. P. de B. Lima, F. H. A. Nogueira, J. P. de Araújo, C. A. Vivacqua, and C. F. S. Aragão, “Development and validation of an UHPLC method for the determination of betamethasone valerate in cream, gel, ointment and lotion,” *Steroids*, vol. 106, pp. 70–77, Feb. 2016, doi: 10.1016/j.steroids.2015.12.011.

[74] F. Jirjees *et al.*, “A validated size exclusion chromatography method coupled with fluorescence detection for rapid quantification of bevacizumab in ophthalmic formulations,” *J Pharm Biomed Anal*, vol. 174, pp. 145–150, Sep. 2019, doi: 10.1016/j.jpba.2019.05.038.

[75] K. M. Kalili and A. De Villiers, “Recent developments in the HPLC separation of phenolic compounds,” *Journal of Separation Science*, vol. 34, no. 8. pp. 854–876, Apr. 2011. doi: 10.1002/jssc.201000811.

[76] AOAC, “Official Methods of Analysis. Appendix K: Guidelines for Dietary Supplements and Botanicals.http://www.eoma.aoac.org/app_k.pdf,” 2013.

[77] J. M. Betz, P. N. Brown, and M. C. Roman, “Accuracy, precision, and reliability of chemical measurements in natural products research,” *Fitoterapia*, vol. 82, no. 1. pp. 44–52, Jan. 2011. doi: 10.1016/j.fitote.2010.09.011.

[78] L. Nováková, Z. Spáčil, M. Seifrtová, L. Opletal, and P. Solich, “Rapid qualitative and quantitative ultra high performance liquid chromatography method for simultaneous analysis of twenty nine common phenolic compounds of various structures,” *Talanta*, vol. 80, no. 5, pp. 1970–1979, Mar. 2010, doi: 10.1016/j.talanta.2009.10.056.

[79] M. Schwarz, M. C. Rodríguez, D. A. Guillén, and C. G. Barroso, “Development and validation of UPLC for the determination of phenolic compounds and furanic derivatives in Brandy de Jerez,” *J Sep Sci*, vol. 32, no. 11, pp. 1782–1790, 2009, doi: 10.1002/jssc.200800706.

[80] K. J. Yan *et al.*, “Qualitative and quantitative analyses of Compound Danshen extract based on 1H NMR method and its application for quality control,” *J Pharm Biomed Anal*, vol. 131, pp. 183–187, Nov. 2016, doi: 10.1016/j.jpba.2016.08.017.

[81] European Medicines Agency, “International Conference on Harmonisation - ICH Q2 (R1) Validation of analytical procedures: text and methodology,” *Encyclopedia of Toxicology: Third Edition*, 1995.

[82] O. Martínez-Cruz and O. Paredes-López, “Phytochemical profile and nutraceutical potential of chia seeds (Salvia hispanica L.) by ultra high performance liquid chromatography,” *J Chromatogr A*, vol. 1346, pp. 43–48, Jun. 2014, doi: 10.1016/j.chroma.2014.04.007.

[83] J. Y. Jia *et al.*, “Pharmacokinetics of depside salts from Salvia miltiorrhiza in healthy Chinese volunteers: A randomized, open-label, single-dose study,” *Curr Ther Res Clin Exp*, vol. 71, no. 4, pp. 260–271, Aug. 2010, doi: 10.1016/j.curtheres.2010.08.004.

[84] S. Magiera, I. Baranowska, and A. Lautenszleger, “UHPLC-UV method for the determination of flavonoids in dietary supplements and for evaluation of their antioxidant activities,” *J Pharm Biomed Anal*, vol. 102, pp. 468–475, Jan. 2015, doi: 10.1016/j.jpba.2014.10.004.

[85] P. N. Brown, E. M. Mudge, and L. Paley, “Determination of phenolic constituents in echinacea raw materials and dietary supplements by HPLC-UV: Collaborative study,” *J AOAC Int*, vol. 99, no. 5, pp. 1197–1203, Sep. 2016, doi: 10.5740/jaoacint.16-0144.

[86] E. M. Mudge and P. N. Brown, “Determination of alkaloids in mitragyna speciosa (kratom) raw materials and dietary supplements by HPLC-UV: Single-laboratory validation, first action 2017.14,” *J AOAC Int*, vol. 101, no. 4, pp. 964–965, Jul. 2018, doi: 10.5740/jaoacint.18-0026.

[87] E. Mudge, L. Paley, A. Schieber, and P. N. Brown, “Optimization and single-laboratory validation of a method for the determination of flavonolignans in milk thistle seeds by high-performance liquid chromatography with ultraviolet detection,” *Anal Bioanal Chem*, vol. 407, no. 25, pp. 7657–7666, 2015, doi: 10.1007/s00216-015-8925-6.

[88] P. N. Brown and R. Yu, “Determination of ginsenoside content in panax ginseng C.A. meyer and panax quinquefolius L. root materials and finished products by high-performance liquid chromatography with ultraviolet absorbance detection: Interlaboratory study,” *J AOAC Int*, vol. 96, no. 1, pp. 12–19, 2013, doi: 10.5740/jaoacint.12-153.

[89] S. Magiera, I. Baranowska, and A. Lautenszleger, “UHPLC-UV method for the determination of flavonoids in dietary supplements and for evaluation of their antioxidant activities,” *J Pharm Biomed Anal*, vol. 102, pp. 468–475, 2015, doi: 10.1016/j.jpba.2014.10.004.