Use of liquid chromatography for assay of flavonoids as key constituents and antibiotics as trace elements in propolis

Investigation into the application of a range of liquid chromatography techniques for the analysis of flavonoids and antibiotics in propolis; and extraction studies of flavonoids in propolis

Ujjwala Kerba KAMBLE

Submitted for the Degree of

Doctor of Philosophy

Faculty of Life Sciences

University of Bradford

2016

Abstract

Ujjwala Kerba Kamble

Use of liquid chromatography for assay of flavonoids as key constituents and antibiotics as trace elements in propolis

Investigation into the application of a range of liquid chromatography techniques for the analysis of flavonoids and antibiotics in propolis; and extraction studies of flavonoids in propolis

Keywords- Propolis, flavonoids, HPLC, antibiotics, microemulsion, extraction, analysis.

Propolis is an approved food additive containing flavonoids as a major active constituent. Variability has been found in the composition of propolis in distinctive regions and it was noticed that there are limitations in the analysis of propolis. In this study, the identification of ten flavonoids and residual antibiotics in propolis was investigated by using several liquid chromatography techniques, including reversed-phase high-performance liquid chromatography (RP-HPLC), microemulsion LC (MELC) and ultra-performance LC (UPLC). The ten flavonoids that were selected for this research include rutin, myricetin, quercetin, apigenin, kaempferol, pinocembrin, CAPE, chrysin, galangin and acacetin while chlortetracycline, oxytetracycline and doxycycline were selected to examine the residual antibiotics in propolis. For the analysis of the selected flavonoids, routine RP-HPLC method was found to be the best method, while MELC technique was found more efficient for the analysis of the selected antibiotics. Solid phase extraction with HLB sorbent was utilised in the analysis of antibiotics for clean-up of propolis. In method development studies for flavonoids and antibiotics, one-factor-at-a-time (OFAT) approach was followed. The final optimised method for the analysis of flavonoids as well as the method

for the analysis of antibiotics was validated using the ICH guidelines, and various aspects, such as the linearity, selectivity, accuracy, recovery, robustness and stability parameters, were examined. Development of efficient conventional method for the extraction of flavonoids from propolis was studied extensively in the present research work using different extraction techniques such as maceration, hot extraction, ultrasound assisted extraction. Among all extraction experiments, ethanolic extraction using ultrasound extraction method was the best efficient approach.

This thesis shows that, in general, the performance of O/W MELC is superior to that of conventional HPLC for the determination of residual antibiotics in propolis. UPLC was not suitable for the analysis of flavonoids and antibiotics. The conventional LC was the only technique to separate the ten flavonoids but MELC was able to separate nine of the flavonoids with faster analysis time. This work also showed that MELC uses cheaper solvents. This considerable saving in both cost and time will potentially improve efficiency within quality control.

Preface

This research work has been carried out at the Centre of Pharmaceutical Engineering Science, University of Bradford, Bradford, UK between April 2012 to March 2015 under the supervision of Dr Khaled Assi and Professor Anant Paradkar. The contents are original and reference s to published work cited within this dissertation.

List of original publication

1. Kamble, U., Ragab, S., Paradkar, A. and Assi, K. (2016) Liquid chromatography methods for simultaneous determination of ten flavonoids in propolis. Submitted to: *Journal of Pharmaceutical and Biomedical Analysis.*

Poster Presentation of research work in conferences

A part of the results from this dissertation were presented at the following conferences.

1. Presented poster entitled “Development of HPLC method for analysis of flavonoids from propolis” in APS PharmSci 2014, held in University of Hertfordshire, Hatfield, UK (8^{th} - 10^{th} September, 2014).
2. Presented poster entitled “HPLC method validation for the quantification of flavonoids in propolis sample” in 30th International Symposium on Chromatography 2014, held in Salzburg, Austria (14^{th}-18^{th} September, 2014).

DEDICATED TO

Dr. B. R. AMBEDKAR

(MA., Ph.D., M.Sc., D.Sc., Barrister-at-Law, L.L.D., D.Litt)

Acknowledgement

I sincerely acknowledge the funding source Social Justice Department, Government of Maharashtra, India for providing me financial support to peruse my PhD study.

I would like to thank to my supervisors Dr Khaled Assi and Prof Anant Paradkar. I would like to express my sincere gratitude to ***Dr. Khaled Assi*** for his continuous guidance and support in my study. I am thankful to him for giving me constant motivation and suggestions throughout my work. I also appreciate his constant feedback and advices at various stages of my PhD. His cordial cooperation, enthusiasm and keen interest helped me throughout this research work. I am very grateful to my co-supervisor ***Prof. Anant Paradkar,*** who guided and supported me since my first day in University. I am very much thankful about his brilliant research ideas and knowledge about my PhD project, it helped a lot to build my confidence.

I would like to express my gratitude to Dr James from Natures laboratory, Whitby, UK for providing propolis samples to this research work. I also appreciate his motivation and suggestions. Many thanks to Analytical center, University of Bradford for providing facility for research.

I would like to appreciate the help from Dr Tristan Dew, for his valuable suggestions. I would also like to express my thanks to Dr Venugopal Vangala for his suggestions at the last phase of research.

I am also very thankful to Sue Baker, International Student Adviser for her immense support in non-academic issues. I would like to thank Anne Costigan, Subject Librarian for Pharmacy, for her help in endnote queries. I am thankful to Toheed Raza for his constant help and support in IT related problems throughout studies.

Special thank goes to my CPES colleagues, who helped me throughout my research work. I got constant help, feedback and moral support from them every time. I specially acknowledge help of Dr Chaitrali, Clive, Dr Sachin, Dr Hrushikesh, Dr Sudhir, Dr Suyog, Dr Bhanvi, Dr Amit, Dr Ratndeep, Dr Kartik, Sharad, Yousef, Nitin, Aniket, Abdul, Mukesh and Sally.

This work would not have been possible without immense support from my family members specially my mother Smt. Pramila and my lovely siblings. Thanks to late Mr. Kerba Kamble, my beloved father, who encouraged me in every stage of my life. I am thankful for blessings from Dr G. Diwan and Mrs Shalan Diwan, for their constant encouragement and unconditional love. I must acknowledge my husband Dr. Manohar Diwan; without his kind support, I would not have finished this work.

Table of Contents

List of Figures

List of Tables

Abbreviations

%	:Percentage
°C	:Degree siliceous
µg	:Microgram
µl	:Microliter
µm	:Micrometer
4,4'DDE	:Dichlorodiphenyldichloroethylene
ACN	:Acetonitrile
AD	:Anno Domini
AFB	:American Foul Brood
Ag	:Silver
Al	:Aluminium
As	:Arsenic
BC	:Before Christ
Ca	:Calcium
CAPE	:Caffeic acid phenethyl ester
CAS No.	:Chemical Abstracts Service registration number
CBA	:Carboxylic acid
CCD	:Colony Collapse Disorder
Cd	:Cadmium
CE	:Capillary Electrophoresis
Ce	:Cerium
Cu	:Copper
DDT	:Dichorodiphenyltrichloroethane
DPPH	: 2,2-diphenyl-1-picrylhydrazyl
DW	:Distilled water
ECD	:Electron capture detector
EFB	:European foul brood
ELISA	:Enzyme-linked immunosorbent assay
ETH	:Ethanol
EU	:European Union
Fe	:Iron
FPD	:Flame Photometric Detector

g	:Gram
GC	:Gas Chromatography
GC-MS	:Gas Chromatography Mass Spectrometry
GFAAS	:Graphite furnace atomic absorption spectrometry
h	:Hour
H	:Hydrodistillation
HCH	:Hexachlorocyclohexane
HCl	:Hydrochloric acid
HE	:Hot extraction
Hg	:Mercury
HIV	:Human Immunodeficiency Virus
HL-60	:Human promyelocytic leukemia cells
HLB	:Hydrophilic lipophylic balanced
HPLC	:High Pressure Liquid Chromatography
HPLC-MS-MS	:Liquid chromatography–mass spectrometry (two mass analyzers)
ICH	:International Conference on Harmonisation
IQ	:Intelligence quotient
L	:Liter
LC	:Liquid chromatography
LC-FLD	:Liquid chromatography– Fluorescence Detector
LC-MS	:Liquid chromatography–mass spectrometry
LC-UV	:Liquid chromatography–Ultraviolet Detection
LLE	:Liquid-liquid extraction
LOD	:Limit of Detection
LOQ	:Limit of Quantitation
M	:Molar
Mac	:Maceration
MELC	:Microemulaion liquid chromatography
MeOH	:Methanol
mg	:Milligram
min	:Minutes
ml	:Millilitre
mM	:Millimeter

Mn	:Manganese
MRL	:Maximum residue levels
MS	:Mass Spectrometry
Mw	:Molecular weight
n	:Number of sample/s
NaH_2PO_4	:Sodium phosphate monobasic
NaOH	:Sodium hydroxide
Ni	:Nickel
nm	:Nanometers
OFAT	:One-factor-at-a-time
PAH	:Polycyclic aromatic hydrocarbon
Pb	:Lead
PDA	:Photodiode Array detector
pH	:Hydrogen-ion concentration
ppm	:Parts per million
r^2/R^2	:Regression coefficient
RP-HPLC	:Reversed phase high pressure liquid chromatography
RSD	:Relative Standard Deviation
RT	:Retention time
Sb	:Antimony
SD	:Standard Deviation
SDS	:Sodium dodecyl sulfate
Si	:Silicon
SON	:Ultrasound sonication
SPE	:Solid phase extraction
Sr. No.	:Serial number
STDV	:Standard Deviation
THF	:Tetrahydrofuran
TLC	:Thin Layer Chromatography
UAE	:United Arab Emirates
UK	:United Kingdom
UPLC	:Ultra-performance liquid chromatography
UPLC-MS	:Ultra performance liquid chromatography-mass spectrophotometry

USA	:United States of America
UV	:Ultra-Violet
V	:Vanadium
v/v	:Volume/volume
v/v/v	:Volume/volume/volume
w/v	:Weight/volume
w/w	:Weight/weight
WCX	:Weak Cation eXchange

1. INTRODUCTION

1.1 General Introduction

From ancient period, different types of natural compounds are known from living source like plants and animals. Many of them possess very useful medicinal benefits. The emergence of new techniques in pharmaceutical and medicinal sciences facilitated the study of quality of pharmaceutical formulations. In this topic, propolis is reviewed and discussed because of its great benefit as a natural product that has a medicinal potential to be used as drug. Propolis is made by honey bees (*Apis mellifera*) and found in a honey comb. It has many health benefits. Propolis is referred as bee glue, composed of several plant chemicals. It also behaves as a natural antibiotic with strong antimicrobial properties and is used as an anti-inflammatory substance (Bogdanov 2012).

Nowadays, propolis is popularly used in many pharmaceutical preparations as food additive. It is considered in modern research because of its medicinal importance and properties such as antioxidant, antimicrobial, antifungal and antiviral. Propolis is used in skin wounds, cold sores, ulcer, gastrointestinal problems and also possess anti-cancer and anti-HIV properties (Greenaway et al. 1990; Ito et al. 2001; Bogdanov 2012; Khacha-Ananda et al. 2013). However, one of the issues of using propolis in pharmaceutical preparation is that the chemical composition of propolis varies with different geographical areas. Previous studies have reported that the main source of chemical composition of propolis is the flora of that particular region (Bankova 2000; Cuesta-Rubio et al. 2002; Cuesta-Rubio et al. 2007; Trusheva et al. 2007). This type of variation also reflects on its chemical composition and activity, which creates problems in its formulation as a drug. Some types of propolis showed lack of a particular active ingredient i.e., flavonoid (Bankova 2000). Consequently, there is a need to develop a rapid and easy method to study the

presence of active ingredients in different samples of propolis. One of the aims of the proposed study is to develop suitable analytical methods for quantification of active ingredients of propolis. This study would be useful for quality control to monitor raw propolis as well as the production of various propolis medicines and food additives.

Farmers use extensive pesticides and insecticides for more yields in field as well as in apiaries to control pests and insects; but at the same time, this pollutes the honey bee products including propolis (Pareja et al. 2011). This is another problem of propolis causing contamination and thus, it is important to determine and monitor pesticides in propolis. Similarly, the use of antibiotics in bee hives to protect bees from different diseases leads to unnecessary antibiotic traces in honey bee products including propolis (Zhou et al. 2009). Hence, there is a need to develop methods for determination of antibiotics as impurity in different types of propolis samples.

Extraction of propolis using a suitable extraction method and solvent is another area of this study. Therefore, another aim of this work is to develop a more suitable extraction method by optimising an efficient extraction technique. For this purpose, different techniques such as maceration, hot extraction, ultrasound assisted extraction and extraction using organic solvents and aqueous solutions of non-ionic surfactants of Tween 80 and Tween 20 have been examined.

There is an immense use of propolis as a natural antibiotic and its current use in pharmaceutical industries for drug production. Therefore, as discussed above, there is a need to develop efficient assay methods for determination of active ingredients and contaminants in propolis to assist the development of

pharmaceutical formulation for propolis and monitor the quality and content uniformity of these formulations.

1.2 Hypothesis

Propolis contains many active components such as flavonoids and thus the substance holds great significance because of antioxidant and antibacterial properties. However, its complex nature makes this material a challenge to analysis. In this study, ten flavonoids were chosen considering their pharmacological importance and their common appearance in different types of propolis (see section 2.2.6). These ten flavonoids had not been previously analysed in a single run. The flavonoids chosen for study were: rutin, myricetin, quercetin, apigenin, kaempferol, pinocembrin, CAPE, chrysin, galangin and acacetin. The addition of CAPE in the present study is advantageous as it shows great promise as a potent anti-cancer compound (Ozturk et al. 2012).

In the analysis of both raw and processed propolis, routine analytical techniques such as UV-spectrophotometry and high pressure liquid chromatography (HPLC) were practiced. Ultra-performance liquid chromatography (UPLC) is an advanced form of HPLC, which facilitates rapid analysis; however, UPLC was rarely utilised in the analysis of propolis. Similarly, previous researchers had not adopted the microemulsion technique in the analysis of propolis; thus, it presented a promising new approach due to the impressive ability of microemulsion liquid chromatography (MELC) to separate analytes in a complex sample (Althanyan et al. 2016). The advantages of employing microemulsion as mobile phase in Liquid Chromatography (LC) include its unique selectivity, higher speed, and green analytical technique (El-Sherbiny et al. 2003; Marsh et al. 2005; Ryan et al. 2013). The advantages of UPLC and

MELC are considered in the present research study and are utilised in the analysis of active and contaminated components of propolis. In the analysis of residual antibiotics, clean-up methods such as liquid-liquid extraction (LLE) and solid phase extraction (SPE) were used.

For the extraction of propolis, researchers utilised both conventional and new extraction techniques such as maceration, hot extraction, and ultrasound assisted extraction. In this thesis, the novel use of surfactant in the extraction of flavonoids was also studied using non-ionic surfactants such as Tween 80 and Tween 20.

From a review of relevant literature and the examination of preliminary experiment results, it is understood that there is a wide variability of product components. Once developed, robust analytical techniques will provide a better understanding of the sources of this variation.

1.3 Aim and Objectives

The aim of this work is to develop and compare different analytical methods to detect and quantify the active ingredients and impurities like antibiotics in different types of propolis preparation and raw propolis by using different analytical techniques such as HPLC, UV spectrophotometer, UPLC and MELC. Also to develop a suitable extraction method for the extraction of flavonoids from propolis is aim of current research work.

The objectives of this work are as follows-

1. To determine flavonoid contents from different types of propolis preparations using spectrophotometric technique.

2. To develop and validate a convenient analytical method for determination of ten flavonoids (active ingredients) in different types of propolis preparation utilising the following analytical techniques:

- HPLC
- UPLC
- MELC

3. To develop and validate a convenient analytical method for determination of contamination (antibiotics) in different types of propolis utilising the techniques listed in the second objective.

4. To develop a convenient and reliable extraction method for extraction of active ingredients (flavonoids) from propolis.

1.4 Outline of report

The report comprises of the following topics including the first part of brief introduction and then other topics,

Chapter 1: Introduction

This chapter explain brief introduction of this research work and provides an outline of thesis.

Chapter 2: Review and Preliminary studies of Propolis

This chapter includes knowledge about propolis. It also provides a comprehensive literature review of previous studies related to proposed research work.

Chapter 3: Material and Methods

This chapter describes details of the materials and methods adapted in this work.

Chapter 4: Analysis of flavonoids from propolis

This chapter explains overall method development and validation procedure as well as discussion of obtained results.

Chapter 5: Determination of antibiotics from propolis

This chapter discusses the method development for analysis of antibiotics, development of clean-up method and method validation procedures.

Chapter 6: Extraction studies of propolis

This chapter describes the methodology as well as discussion of results of extraction studies of propolis.

Chapter 7: General conclusion and Future work

This chapter contains an overall summary and conclusions of the work presented in this thesis. This chapter provides the scope for future work.

Topic 8: References

2. LITERATURE REVIEW

2.1 Propolis

2.1.1 History of propolis

Propolis is a honeybee product. The word ‘propolis’ originates from two Greek words ‘*pro’* and ‘*polis’* which together translates as ‘front of the city’. Records have been found about its usage from thousands of years BC by religious priests for medicines and the mummification of corpses in Egypt (Bogdanov 2012). Makashvili, (1978) has explained that folk medicinal use of propolis in Georgia was used against corns, burns, angina and respiratory tract and lung problems. Propolis has antimicrobial characteristics and was used for this purpose, using 30 % alcoholic solutions, during the Anglo-Boer war (World War II) (Makashvili 1978). In the 12th century AD, propolis was used for mouth and throat infections, dental problems, bruises and supporting wounds (Krell 1996). Renowned scientists such as Greek and Roman physicians, Aristotle, Dioscorides, Pliny and Galen have all mentioned the crude properties and possible uses of propolis (Castaldo and Capasso 2002).

2.1.2 Use of propolis

The use of propolis is very important for bees as well as for human beings.

2.1.2.1 Use for honey bees

Propolis is seepage aggregated by honey bees (*Apis melifera*) from plant’s wounds or lipophilic material on leaves, mucilage, lattices, resin, gums and is fortified with secretions from bee saliva and enzymes. Propolis is a benefit to bees because it seals holes in the hive and strengthens the thin borders of comb which is vital for avoiding droughts in the hive and makes it weather tight. It is further used for 'embalming' the dead invaders, are not transported out of the hive (Bankova 2005). Similarly, propolis is considered as a building

insulating material in a beehive and is critical for the wellbeing of hive (Greenaway et al. 1990).

2.1.2.2 Use for human beings and medicinal properties

The infamous quality of propolis is its antimicrobial property which has been known from many decades. It has antibacterial, antiviral and antifungal properties along with anti-inflammatory, anti-oxidative, antiulcer, antiseptic, antitumor, hepato-protective and local anaesthetic properties (Ghisalberti 1979; Marcucci 1995; Burdock 1998). There are considerable medicinal benefits of using propolis. It is very popular in health drinks as well as many food products and other beverages. It is available in a number of products as a powder, tablet, capsule, syrup, liquid, tincture, cream, gel etc. Most of the products are for enriching the flavonoid contents in the human body. Propolis is used as an antimicrobial treatment for tooth ache, wounds etc. It is also used to help avoiding diseases like heart troubles, inflammation, diabetes and cancer (Banskota et al. 2001). Propolis activity has been assessed against neurodermatitis, herpes simplex, genitalis, psoriasis, leg ulcers, stomatitis, influenza and colds and was found to be effective (Ghisalberti 1979; Burdock 1998). It has also been proven to have strong anti-cancerous properties (Ban et al. 1983; Scheller et al. 1989; Chiao et al. 1995).

Phenolic compounds such as CAPE, quercetin, naringenin from propolis produce an inhibitory force to the peritoneal inflammation induced by zimosin (Mirzoeva and Calder 1996). The identical anti-inflammatory outcome of propolis is due to galangin, kaempferol, kaempferide, caffeic acids and their esters were investigated by Volpert and Elstner (1996). Propolis cream has been used for the treatment of burns where a decline in microbial infection with

less inflammation was reported (Gregory et al. 2002). For eye problems like cataract and keratitis, it showed a good response (Maichuk et al. 1995; Orhan et al. 1999). The flavonoid composition of propolis is effective against *Streptococcus spp* and reduces dental microbial growth (Koo et al. 2002). It was also reported that flavonoid has an anaesthetic activity (Paintz and Metzner 1979).

CAPE is one of the active ingredients in most forms of propolis and it has an antitumor activity (Lee et al. 2000). Chen et al.(1996) reported that CAPE was able to arrest the growth of human leukaemia HL-60 cells. The antitumor property of Brazilian propolis studied by Suzuki et al (1996) using water soluble parts of propolis with other anticancer drugs, showed an inhibitory effect on Ehrlich carcinoma in rats.

The unusual role of propolis as a chemical preservative for meat products was studied earlier (Han and Park 1996). Donadieu (1979), described a 2-3 times increase in the storage life of frozen fish after the use of propolis. It has also been used in packaging because of its germicidal and insecticidal properties (Mizuno 1989b; Mizuno 1989a). To summarise, propolis has many uses in the field of preservation.

Analytical studies of propolis are an essential part of quality measurement purposes. The detection and quantification of known active compounds and contaminations from propolis are also important aspects in an analytical view.

2.1.3 Physical Characteristics

Propolis is mainly a resinous substance like a sticky gum. Its colour varies on its source and age from light green to dark brown (Burdock 1998). As reported by

Ghisalberti (1979) and Koltay (1981), the properties of propolis can change from hard and brittle in cold conditions to soft and sticky in warm conditions. There are very small amounts of wax or sometimes no wax in propolis which presents as a thin coat on the surface of comb and the content of the wax is variable according to the availability of the resins (Meyer 1956). The aroma of propolis is pleasant and it's colour differs based on source and age (Brown 1989).

2.1.4 Solubility properties of propolis

Propolis solubility is challenging because of its resinous and waxy structure. The solubility of active compounds such as flavonoids and the removal of unwanted compounds like waxes, heavy metals, portions of dead bees, organic debris and other contaminants from propolis are also important before any formulation can be made.

Ethanol or ethanol in water were used to solubilise unwanted compounds from the propolis and was followed by filtration to avoid contamination (Burdock 1998). Subsequent ethanol extraction under increasing pressure was used to extract a number of compounds from propolis (Wu and Qial 1999). In another study, You et al. (2002) used supercritical carbon dioxide for the extraction of active ingredients from propolis. Generally, ethanol is widely used to extract active constituents from propolis.

2.2 Recent studies and problems associated propolis

The presence of active ingredients distinctly varies per region. More than 300 compounds are present in the propolis. The main chemical classes are flavonoids (polyphenolic compounds), phenolic aldehydes, tannins, sesquiterpene-quinones, coumarins, amino acids, steroids, polysaccharides,

aromatic acids and inorganic compounds (Banskota et al. 2001; Castaldo and Capasso 2002). It is a challenge for manufacturers to confirm the presence of active ingredients (polyphenols) and impurities (pesticides or antibiotics). Previous studies have generated a wide range of data about the propolis composition from different regions using different analytical techniques. However, it is a challenge to analyse each and every important constituent using an available method for every propolis type. Hence, the development and standardisation of the appropriate analytical method by selecting suitable bioactive compounds for a specific propolis type is always beneficial. The analytical techniques used in these types of studies are very important. Some of the important separation techniques used in the analytical separation of bioactive compounds as well as contaminants such as antibiotics from propolis is discussed briefly in the following section. Clean up techniques are also used in the analysis of residual contaminants.

2.2.1 Analytical techniques used for analysis of propolis

2.2.1.1 UV Spectrophotometry

Spectrophotometry is rapid and simple analytical technique. The main principle of spectrophotometry is based on Beer-Lambert's Law; the absorbance of light is proportional to the intensity, and hence it is proportional to the concentration of the analyte. The main components of the spectrophotometry are white light source, monochromater, exit slit, sample cell holder and light detector. In diffraction grating, a special plate with a number of parallel grooved lines is used to separate the visible light spectrum. A monochromater is set to a desired wavelength from the diffraction grating to exit slit. The exit slit is a small hole which allows only a small amount of diffracted light pass through. The sample

cell holder holds the sample cell or cuvette. In the light detector, transmitted light hits photo amplifier in which light energy converts to electrical energy and interpreted by the computer.

Analysis of flavonoids from different plants using UV spectrophotometer

The use of UV spectrophotometer technique is very common for the analysis of flavonoids from different plant sources. The effect of different extraction procedures on the flavonoid content of the weed *Portulaca oleracea L.* was studied by UV-Vis spectrophotometry and the analytical method was found to be convenient, rapid, reliable and useful. The aluminium nitrate was used in the analytical method that reacted with flavonoid present in different extraction samples and the wavelength used 500nm (Zhu et al. 2010). A similar UV-Vis spectrophotometric methodology with aluminium nitrate and detection wavelength 500nm has been used to find out flavonoid contents from *Sedum sarmentosum* Bunge., *S. lineare* Thunb., and *S. erythrostictum* Migo., and found that this analytical technique is simple, direct, and accurate, providing a valuable reference for quality control (Chen et al. 2010). Instead of aluminium nitrate, aluminium chloride was used as a reagent for the analysis of flavonoids from monofloral honey samples from Bangladesh and the total flavonoid content from studied honey sample has been calculated using two wavelengths 450 and 720 nm (Moniruzzaman et al. 2014).

Several other studies have been reported for the analysis of total flavonoids in propolis using an UV-Vis spectrophotometric technique. Chang et al. (2002) studied two complimentary colorimetric methods for the analysis of total flavonoids from six raw and twelve market types of propolis. They suggested two different methods using aluminium chloride and 2,4-dinitrophenylhydrazine.

They were able to detect most of the flavonoids and the sum total of two sets of results provided the most accurate value for total flavonoids. Similarly, Kosalec et al. (2004) also used these techniques as two individual and complementary methods and two different wavelengths as 415nm and 495nm for flavones and flavonols with flavanones to measure the total flavonoids. By employing two methods which mentioned in earlier reference, Gülçin et al. (2010) reported the analysis of the total flavonoids of propolis from Turkey. This technique is suitable to analyse flavonoids of similar structures and to provide the preliminary information about the quantification of flavonoids in different types of propolis. At the same time, not all types of flavonoids can be detected by this technique. Thereby, new separation techniques such RP-HPLC, GC and UPLC were considered for the advanced analysis of propolis.

2.2.1.2 High performance liquid chromatography (HPLC)

HPLC is a separation technique. It has been used in a wide range of areas, mainly in pharmaceutical science, biotechnology, environmental, polymer and food industries. In this technique, eluents of interest are separated by a stationary phase which is silica based particles packed in the column and a mobile phase. The separation of components from the mixture is due to unique affinity of each component between the mobile and the stationary phase. Each of the analyte migrates at different speed in the column and emerges from it at different time. If an analyte have a high affinity towards the stationary phase, they migrate slowly and elute late as compared to analytes which have less affinity towards stationary phase. This migration time is called as a retention time which is unique for each analyte and hence, used for its identification (Pandit and Soltis 2012). There are two different approaches for the mobile

phase delivery systems, i.e. isocratic and gradient. In isocratic phase, the solvent mixture is constantly running while in the gradient phase, a time varying solvent mixture is running through column.

There are two main types of HPLC techniques: normal phase chromatography and reverse phase chromatography. In normal phase chromatography, a column with silica particles is used and in reverse phase chromatography, columns coated with C18 silica are used. The normal phase chromatography separates the analyte/s based on their affinity for a polar stationary surface (silica). This technique is based on the ability of the analyte to react with a sorbent surface through a polar interaction (hydrogen bonding, dipole-dipole interaction etc). In this technique, non-polar and non- aqueous solvents are used for example chloroform. The analytes are retained by a polar stationary phase. As the polarity of the analyte increases, the adsorption strength increases. The reversed phase chromatography has a non-polar stationary phase, and a moderately polar mobile phase. The stationary phase is made up of silica in which surface is modified with R-Me_2SiCl, where R is a straight chain alkyl group. In this type, non-polar molecules elute later while polar molecules elute first.

The HPLC system has following parts syringe compartment, column heater, solvent conditioning tray, sample compartment and detector drip tray. The schematic presentation of HPLC working pattern is shown in figure 2.1. The mobile phase from reservoir goes to solvent manager, where it is conditioned and passes through the HPLC column. Meanwhile, auto-sampler from sample manager injects samples as per the set sample method in computer and passes through HPLC column with mobile phase. In HPLC column, compounds are separated according to their affinity towards the mobile and stationary phase of

column and different peaks are eluted at different retention times which are detected by the detector. The leftover mobile phase and samples are collected in waste bottles. The resulting chromatogram containing detector response in the form of different peaks is evaluated using the software such as Empower 3.

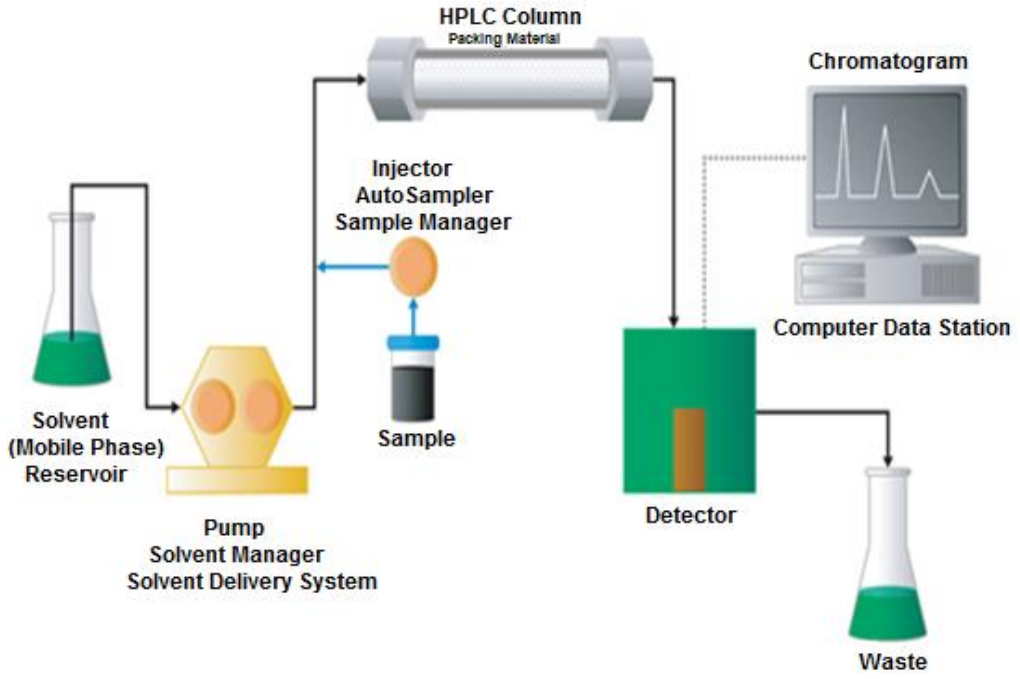


Figure 2.1: HPLC system: steps of working (Waters 2016)

The analysis of flavonoids using HPLC separation technique is much more adventitious. It covers analysis of each and every individual compound. The relationship between the structure of flavonoids and RP-HPLC are important in their retention. The hydrophobic flavonoid compounds interact with the stationary phase and elute out; depending upon the extent of hydrogen bond formed by flavonoids with the mobile phase. Hydroxylation at the positions other than at position C-3 and C-5 increases the ability of a hydrogen bond formation; hence, enhances the polarity and reduces the retention. Whereas, OH group at the position C-3 lowers retention, due to intramolecular hydrogen bonding with the carbonyl group at C-4, leading to poor separation (Stefova et al. 2004).

From the previous studies it was revealed that, C-18 stationary phase in RP-HPLC analysis technique is more superior than a normal HPLC (Svetlana et al. 2009). The analysis of flavonoids using RP-HPLC is represented in general in table 2.1.

Recently, a HPLC with mass spectroscopy technique is widely used for the analysis of flavonoids. The basic principle behind this technique is fragmentation of the analytes and determination of the masses of resulting particles. This technique is useful to identify and classify the flavonoids depending on their classification such as flavonol, flavanol and flavone (Tsimogiannis et al. 2007). This technique is successfully used for the analysis of bioactive compounds from different propolis, which is mentioned in section 2.2.2.

Table 2.1: Flavonoid analysis using RP-HPLC

Plant/source	Flavonoid and other polyphenols	Reference
Sarang semut (*Myrmecodia pendan*)	Kaempferol (13.767 mg/g), luteoline (0.005 mg/g), rutine (0.003 mg/g), quercetin (0.030 mg/g) and apigenin (4.700 mg/g) of dry extract.	(Engida et al. 2013)
Peppers	Studied effect of different extraction technique on the flavonoid content	(Bae et al. 2012)
Blumea balsamifera DC	Dihydroquercetin-7,4'-dimethyl ether, blumeatin, quercetin, 5,7,3',5'-tetrahydroxyflavanone, and dihydroquercetin-4'-methyl ether	(Nessa et al. 2005)
Plant-derived foods	17 flavonoids including catechin	(Mattila et al. 2000)
Honey	Quercetin, kaempferol, quercetin 3-methyl ether, kaempferol 3-methyl ether, quercetin 3,3-dimethyl ether, galangin, apigenin, genkwanin, chrysin, pinocembrin, pinobanksin etc	(Ferreres et al. 1991)

The RP-HPLC technique is also used for the analysis of flavonoid as well as other bioactive compounds from propolis; it is discussed in section 2.2.2.

2.2.1.3 UPLC (Ultra Performance liquid chromatography)

UPLC is an advanced version of liquid chromatography which offers greater resolution and sensitivity. The particle size of the stationary phase is reduced to less than 2mm which provides a large surface area and a high flow rate, which is utilised for high speed. The first UPLC system was developed by Waters Corporation in 2004. The operating pressure is almost doubled using this technique (15,000 psi) and achieved rapid flow rates and better resolution of compounds in a shorter time period.

The compartments of UPLC system are similar to HPLC. There are five main compartments as detector, column compartment, sample manager, binary solvent manager and sample organiser. This system is connected to computer system with Empower 3 software.

UPLC is comparatively a new technique as compared to HPLC. Therefore, there are few attempts have been made to analyse flavonoids from different samples employing this technique. The gradient method was developed for the analysis of 34 phenolic compounds which includes phenolic acids, flavonoids, catechins and coumarins by using HPLC and UPLC techniques. In order to evaluate system suitability to analyse flavonoids from the samples such as grape wine, teas etc, UPLC technique was experimented (Spáčil et al. 2008). Rapid UPLC method was developed for the analysis of 15 selected flavonoids from different species of *Epimedium*, a Chinese medicinal herb. The four marker flavonoid compounds were selected for quality control analysis of the species of *Epimedium* using UPLC technique (Chen et al. 2008). Recently, the analysis of flavonoids and polypohenols from variety of the samples were

carried out using UPLC coupled with MS detector. However, UPLC with PDA is also a very sensitive, fast and comparatively useful technique as compared to other separation techniques and can be used for successful analysis of flavonoids.

There are some evidances about the UPLC analysis of propolis. Determination of twelve active compounds including flavonoids, ferulic acid and CAPE in propolis was studied by Li et al. (2007). The method was optimised and validated by analysing 106 different propolis samples obtained from various proiduction areas in China. They found spiked recoveries in range of 90.1%-104.3%, with relative standard deviation (RSD) of 2.12%-4.9% (Li et al. (2007).

2.2.1.4 Gas chromatography

Gas chromatography involves a sample being vapourised and injected onto the head of the chromatographic column. The sample is transported through the column by the flow of inert, gaseous mobile phase. The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid (Figure 2.2).

This technique was successfully utilised for the analysis of the flavonoids from different sources using various detectors such as thermal conductivity detecor, flame ionisation detector and catalytic combustion. Nowdays, mass spectrometry detector has been widely used with GC technique. The large number of hydroxyl groups on flavonoids are responsible to choose HPLC analytical technique by many researchers, but GC technique was successfully used for the flavonoids when the HPLC was not commonly used in laboratories previously.

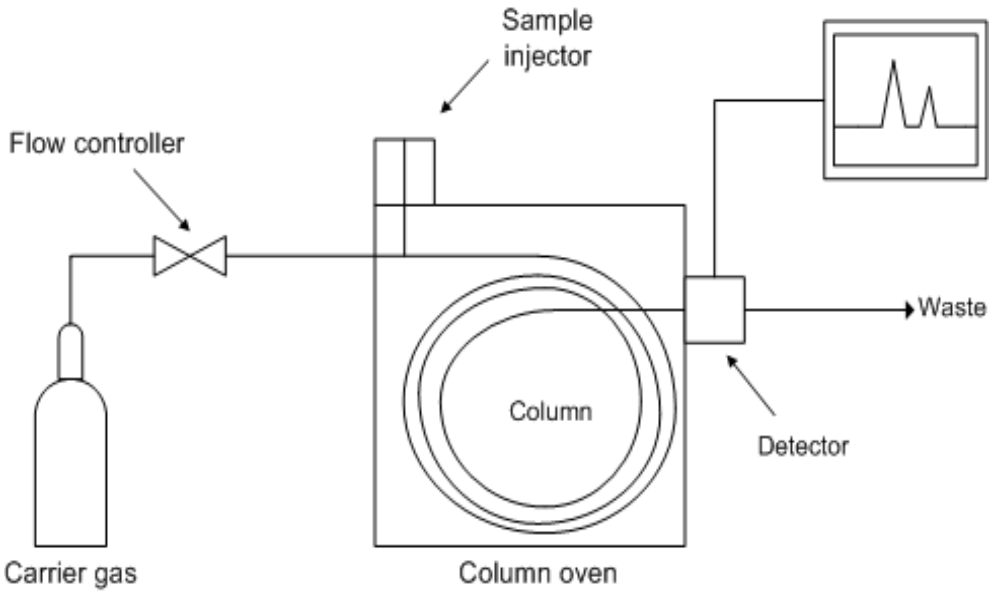


Figure 2.2: Gas chromatography (Zhao and Barron 2016)

The sample preparation methods may include liquid-liquid extraction, solid phase extraction, derivatisation etc. The early GC analysis of derivatised flavonoids employed flame ionisation detection; however, MS detection has gained popularity and is widely used today. Different subclasses of flavonoids were characterised by GC in various sample types including human plasma and urine, food, medical herbs, plants and their related products. Catechin, epicatechin and quercetin were found to be the most popular flavonoid (Nolvachai and Marriott 2013). Few of the references of bioactive compounds were found from the propolis is discussed briefly in section 2.2.2.

2.2.1.5 Microemulsion liquid chromatography (MELC)

A microemulsion is a thermodynamically balanced entity in which submicron droplets of one liquid are dispersed in another immiscible liquid (Althanyan et al. 2011). It is an isotropic liquid mixture of oil, water and surfactant. The system is mainly composed of submicron oil droplets dispersed in an aqueous, immiscible

continuous phase known as oil-in-water (o/w) microemulsion. The oil droplets are usually covered with a suitable surfactant shell and a co-surfactant. The surfactant forms an interfacial film which lowers the surface tension, thereby separating the oily phase from the aqueous phase. The co-surfactant which sets itself at the oil-water interface further reduces the surface tension, thus lowering the interfacial free energy which supports the formation of an instant and stable microemulsion (Figure 2.3).

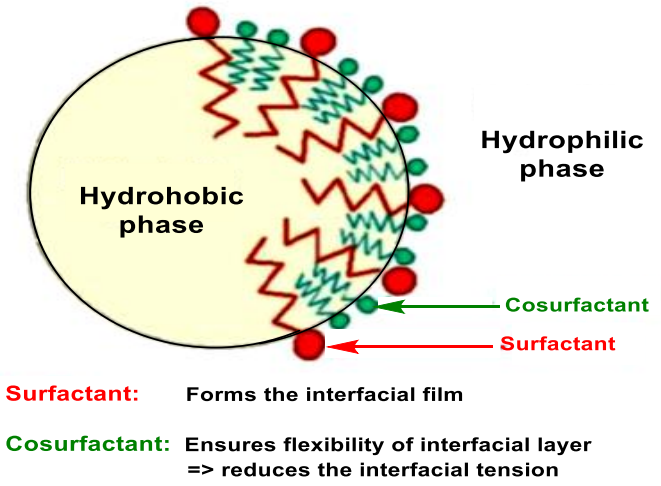


Figure 2.3: Structure of Microemulsion (Patel 2007)

The mobile phase in reversed phase HPLC is comparably polar than the stationary phase. Increased aqueous quantity of the microemulsion is more suitable with reverse phase HPLC (McEvoy et al. 2007). Microemulsion has the potential to solubilise both water soluble and non-soluble compounds; hence it can be used for the analysis of both hydrophobic and hydrophilic compounds. The advantage of MELC is development of the secondary partition mechanism, where solute partitioning is found in the aqueous phase, oil droplets and the stationary phase of column. Water insoluble compounds lie in oil droplets while water soluble compounds mainly reside in the aqueous phase and this separation is also influenced by the stationary phase. MELC can be effectively

used for the detection of compounds which detect at low wavelength (Ryan et al. 2013). This technique was crucial for the analysis of complex natural products, e.g. Polettini et al. (1995) assayed urine (complex biological fluid) sample using microemulsion HPLC technique to determine β- agonists (tarbutaline). Similarly, analysis of terbutaline in urine samples was experimented (Althanyan et al. 2016). There are no references of MELC being used for the analysis of propolis. Considering the complexity of the propolis sample, the use of this technique could be beneficial. By considering all the favourable aspects of this technique, it was used in the present study for the analysis of the complex natural product, as propolis.

MELC is a new robust and sensitive analytical technique and several reports have described application for this method for the analysis of flavonoids. *Apocynum venetum* leaf extract was studied by RP-HPLC using microemulsion mixture as a mobile phase to analyse six flavonoids such as rutin, hyperoside, quercetin-3-o-sophoroside, isoquercitrin, astragalin and quercetin. The mobile phase was consisted of 2.5% (v/v) n-butanol, 1.2% (v/v) of Genapol X-080, 0.5% (v/v) ethyl acetate and 95.8% (w/v) of aqueous 20mM phosphoric acid, pH was adjusted to 6.0 with 0.3% triethylamine. The resulting calibration curve for all six studied flavonoids were found to be linear in the range of 5-1000µg/mL and other parameters such as acuracy, recovary, etc were also studied successfully (Song and Zhou 2015). MELC technique was used for the rapid analysis of vitexin, vitexin-2″-O-rhamnoside, rutin and hyperoside in the extract of hawthorn (*Crataegus pinnatifida* Bge.) leaves. The optimised microemulsion mobile composition was composed of 1.0%(w/w) brij35, 1.1%(w/w) n-butanol, 0.1%(w/w) n-octanol and 0.3%(v/v) triethylamine, the pH was adjusted to 2.5

with phosphoric acid. The recoveries were found in the range of 98.6% to 101.6% for all the studied flavonoids (Li et al. 2009).

In this work, the analysis of flavonoid in different propolis types were carried out using various analytical techniques including MELC as this technique has not been used previously. Hence, this technique is considered in this work for flavonoid analysis from propolis.

In next section, chemical composition of propolis is discussed by reviewing various existing references.

2.2.2 Chemical composition

The crude chemical composition of propolis is, 50% resin and vegetable balsam, 10% (essential and aromatic) oil, 30% wax and 5% other substances with organic debris (Bankova et al. 2000). The main constituents in propolis are polyphenols (flavonoids, phenolic acids and their esters) which induce some hormones and neurotransmitters that reduce the activity of specific enzymes and scavenge free radicals (Havsteen 2002). Vitamins that can be found in propolis samples are vitamin B1, vitamin B2, vitamin B6, vitamin C and vitamin E; and minerals Mn, Fe, Ca, Al, V, Ag, Ce, Hg, La, Sb, Cu and Si can also be present (Deblock-Bostyn 1982; Debuyser 1983) .

The chemical composition of propolis is very complex and can vary as per location. The main variable is the regional ecology including flora (regional plant species). The renowned 'Poplar type' propolis which is mainly found in the temperate zone is well studied. The main source of this type is exudates of black poplar buds and other parts of *Populas spp;* while the main source of propolis from the northern area of Russia is considered as birch buds (*Betula*

verrucosa) and *Populus tremula* (Wollenweber and Buchmann 1997; Bankova 2000; Bankova et al. 2002). Similarly, other propolis sources are discussed briefly in table 2.2.

Table 2.2: Propolis types and its plant source

Type of propolis	Origin	Plant source	References
'Poplar type'	Temperate zone	Exudates of black poplar buds and other parts of *Populas spp;*	(Wollenweber and Buchmann 1997; Bankova 2000; Bankova et al. 2002).
Propolis from Northern area of Russia	Northern area of Russia	Birch buds (*Betula verrucosa*) and *Populus tremula*	(Wollenweber and Buchmann 1997; Bankova 2000; Bankova et al. 2002).
Brazil type	Brazil	Leaves of *Baccharis dracunculifolia*	(Bankova 2000)
Black poplar	Mediterranean type	Citrus leaves	(Martos et al. 1997)
Venezuela and Cuba type of propolis	Venezuela and Cuba	Floral resins of Clusia	(Cuesta-Rubio et al. 2007)

Minor resin content in propolis gathered from South Georgia due to scanty resins from pine forests (Johnson et al. 1994). The flavonoid pigments are the largest group of compounds of plants but these are part of the propolis tincture (Burdock 1998). It proves, there is interrelationship between the polyphenols of flora and the polyphenol content from propolis of that distinct region which is collected by honeybees (Burdock 1998). Geographical variation has an effect on the activities of propolis such as antibacterial, antioxidant and antitumor.

From table 2.3, it is clear that the polyphenolic compounds vary per region. This variation again carefully studied and main active constituents of particular type

of propolis from particular region are well explained in table 2.4. For example Poplar type of propolis (from North America, Europe, Asia) contain flavones, flavanones and cinnamic acids as active constituents and green propolis from Brazil shows active constituents such as p-coumaric and diterpenic acids. This variation demands for standardisation studies of active compounds from different types of propolis.

Some of the references of flavonoid content with regional variation are discussed here briefly. Total polyphenolic and flavonoid content from different parts of Lithuania and Czek were assayed by Savickas et al. (2005) and the quantity of total polyphenols was higher in propolis near to deciduous and mixed forests (1.64-1.53g/ 100 ml) while low quantities were found in propolis near to cultivated midows far from forests (0.18g/ 100ml). Coneac et al. (2008) determined the flavonoid contents of propolis and scrutinised its correlation with the antioxidant activity of propolis samples from the west side of Romania. They used ethanol as well as water for extraction (hot and cold extractions).

Table 2.3: Chemical composition of propolis of different types

Propolis type/ geographical region	Compounds identified	Chemical class	Analytical Technique used	Reference
Europe (South Bulgeria)	pinocembrin , galangin chrysin quercetin	Flavonoids	HPLC	(Bankova et al. 1982)
Europe (Croatia)	ferulic acid, p coumaric acid and flavonoids such as tectochrysin, galangin, pinocembrne-7 methyl ether, chrysine, apigenine, kaemferol and absence of caffeic acid and quercetin	Flavonoids	HPLC	(Barbarić et al. 2011),
Europe (Bulgeria, Italy and Switzerland)	pinocembrin, pinobanksin and its 3-O-acetate, chrysin, galangin, prenyl esters of caffeic and ferulic acids	Flavonoids	GC	(Bankova et al. 2002)
Europe (Continental and Adriatic regions of Croatia)	galangin, kaempferol, naringenin, apigenin, caffeic acid and pinocembrine	Flavonoids	TLC and HPLC	(Kosalec et al. 2003)
Europe (Eastern Anatolia)	flavonoids, aliphatic acids, aromatic acids, esters, alcohols, terpen, quinons	flavonoids, aliphatic acids, etc	GC MS	(Silici and Kutluca 2005)
Europe (Greece and Cyprus)	terpenes like totarol, sesqueterpenes, phytol, aristolone etc, some anthraquenones, flavonoids, polyphenols, aliphatic acid, aromatic acids and their esters	Terpenes, flavonoids, etc		(Kalogeropoulos et al. 2009))
Europe (Croatia)	ferulic acid, p coumaric acid and flavonoids such as tectochrysin, galangin, pinocembrne-7 methyl ether, chrysine, apigenine, kaemferol and absence of caffeic acid and quercetin	Flavonoids	HPLC	(Barbarić et al. 2011)
Zealandia (New Zealand)	pinocembrine, pinobanskin and pinobanskin-3-acetate with aromatic compounds and fatty acids	Flavonoids and fatty acids	HPLC and GC -MS	(Markham et al. 1996),
South America (Brazil)	gallic acid, diterpenes and triterpenes	Terpenes, phenolic acid	GC MS	(Velikova et al. 2000)
South America (Brazil, Red propolis)	methyl *o*-orsellinate, methyl abietate, 2,4,6 trimethylphenol, homopterocarpin, medicarpin, 4',7 -dimethoxy-2'-isoflavonol etc.	Phenols and flavonoids	GC	(Alencar et al. 2007)
Middle East Asia (Iran)	pinobanskin, pinobanskin-3-acetate, pinocembrin, pinostrobin, strobin and galangin	Flavonoids		(Mohammadzadeh et al. 2007),
Asia (Turkey, Kazan region)	isopimaric acid, androstan-1,17-dimethyl-17-hydroxy-3-one, docosa-8,14-diyn-*cis*-1,22-diol, thunbergol, steroids and long chain fatty acids	Flavonoids, steroids and fatty acids	GC	(Kartal et al. 2002)
Asia (Turkey, Marmaris region)	caffeic acid isomers, abietic acid, dehydroabietic acid, isopimaric acid	Polyphenols	GC	(Kartal et al. 2002)
Asia (Indian propolis,Tamil Nadu region)	fatty acids- 9- octadecenoic acid , decanoic acid , 9,12 hexadecanoic acid , octadecadienoic acid methyl ester and alcohols- 1-tetradecanol , octadecanol, 1-dotricontanol and 2,3 epoxy-5,8-hectadecadien-1-ol in addition with quercetin and cyclopentadiene in trace.	Phenols, flavonoids	GC	(Thirugnanasampandan et al. 2012)

The comparative study of phenolic and polyphenolic content of propolis showed the range of phenolic acid content was 18.43 - 20.13% in Romanian propolis while it was 19.79 -22.69% from Israel propolis (Croci et al. 2009). Said et al. (2006) investigated the chemical composition of propolis from Egypt and the United Arab Emirates (UAE) by using the GC-MS technique. They observed a high range of aromatic (13.7%) and aliphatic acids (14.4%) in Egyptian propolis while in the UAE samples; aliphatic acids (15.2%) were higher than the aromatic acids (4.3%). Both types showed the presence of alcohols, phenols and esters.

Geographical traceability of propolis from China was scrutinised by Zhou et al. (2008) using a HPLC technique. They developed a rapid fingerprint method to identify the type of propolis according to the region after studying 120 samples from 17 different localities of 10 provinces of China. They selected eight major flavonoids including rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrin, chrysin and galangin for this study

All of the studies concluded that the composition variation of propolis is related to the divergent plant source per region. This affects qualitative and quantitative analysis prior to any formulation. Because of the variation in propolis, the formulation is not reproducible and varies per source, weather and contaminations. The same problem is highlighted by Bankova (2005). Instead of the variety of available analytical methods, there is a need to develop a new method which includes important bioactive compound such as CAPE. From the available references (Table 2.3), it is clearly observed that many of the analytical studies have not included CAPE in their studies for ex. propolis from Europian region. In the proposed study, ten flavonoids were selected by

studying their presence and importance in Europian propolis, mainly including CAPE as a potent bioactive compound.

As noticed from the previous HPLC studies, the main disadvantage of RP-HPLC is the long run time required for analysis, the complicated elution procedure of the mobile phase, and the extensive organic solvent consumption (Kosalec et al. 2003; Kumazawa et al. 2004; Gómez-Caravaca et al. 2006; Alencar et al. 2007; Gardana et al. 2007; Coneac et al. 2008; Luo et al. 2011). Moreover, none of the reported methods have successfully identified and simultaneously separated all the 10 flavonoids (Table 2.5) in the same run. Therefore, the present work has a major advantage.

The rich polyphenolic composition of propolis is also responsible for many useful characteristics such as antioxidant and antimicrobial properties, which are briefly explained in following sub-sections.

2.2.3 Antioxidant Activity

The presence of phenolic and flavonoid content in propolis impacts on their considerable antioxidant activities, which have been studied previously. Some of the relevant references are discussed here. Antioxidant activity of propolis from different parts of Argentina was studied by Moreno et al. (2000) and they observed variation in 2,2-diphenyl-1-picrylhydrazyl (DPPH) degradation. The highest DPPH activity was found to be 67% in Banda Oeste propolis than Saenz Pena propolis (20%). Hegazi and El Hady (2002) compared the antioxidant activity of two Egyptian propolis and found that free radical scavenging activity of Al-Saff and Ismailia propolis were 88.2 and 82.2% respectively (at 100µg concentration). The antioxidant activity of water extracts

of propolis was experimented by Nagai et al. (2003) and found complete inhibition of a superoxides formation at 50 and 100mg/ ml concentration of propolis, and the similar trend was observed in hydroxyl radical scavenging activity.

The role of caffeic acid phenethyl ester (CAPE) and galangin in antioxidant activity of propolis was compared and noticed that CAPE was more anti-oxidative than galangin (Russo et al. 2002). Banskota et al. (2000) determined the antioxidant activity in propolis from different countries such as Brazil, Peru, Netherland and China and noted strong DPPH free radical scavenging activity in water extracts of six Brazilian and one Chinese propolis and methanol extracts of propolis from Netherland and Peru. Antioxidant activity of propolis from Korea region was examined by Choi et al. (2006) and stated that propolis from Yeosu (YS) and Cheorwon (CW) regions both have highest antioxidant activity (90% DPPH free radical scavenging activity) as compared to propolis from Brazil region (50% DPPH free radical scavenging activity) at 50 µg/ml concentration of each type of propolis. Mohammadzadeh et al. (2007) demonstrated regional variation in the antioxidant activity of Iranian propolis and reported that Tehran propolis has the highest antioxidant activity as compared to Khorasan propolis. Antioxidant activity of propolis from China region was explored by Ahn et al. (2007) using EEP(ethanol extracted propolis) and linoleic acid oxidation method and found regional variation in antioxidant activity which was found higher in the propolis from Hainan as compared to propolis from Yunnan region. Antioxidant activity and the total phenol content from Portugal region was studied (Moreira et al. 2008). Propolis from Bornes region showed highest DPPH radical scavenging activity i.e. 33% at 0.001mg/ml but increased

up to 94% at 0.020 mg/ml concentration of propolis extract. Comparatively, low antioxidant activity was obtained in Fundao region (as 18% at 0.020 mg/ml concentration of propolis). All these studies explained the antioxidant property of propolis. Hence, propolis has great medicinal value.

2.2.4 Antimicrobial properties of propolis

Due to the presence of aromatic/phenolic compounds, flavonoids and essential oils, propolis possesses very good antimicrobial activity, and therefore it was used as a folk medicine to cure many infectious diseases (Burdock 1998). The variation of chemical composition in propolis is also responsible and variable for its activities including antimicrobial activity (Bankova 2005). Similarly, in the previous section, the difference in anti-oxidation properties is clearly observed in different regions of propolis. Because of such variation, extensive study of antimicrobial properties of different types of propolis from different regions along with the determination of chemical composition is necessary. It will help to update and upgrade the knowledge of propolis composition and activities which have great economic value for further pharmaceutical development. The previous antimicrobial studies have been discussed in detailed in following subsections.

2.2.4.1 Antibacterial property

The propolis from Europe has antibacterial properties due to presence of phenolic compounds such as caffeic acid esters, benzyl-p-coumarate, pinobanksin, pinobanksin 3-O acetate, ferulic acid and caffeic acid and the flavonoids such as galangin and pinocembrin (Metzner et al. 1979). The derivatives of gallic acid in propolis proved to have an inhibitory effect against the Gram negative and Gram positive bacteria (Kayser and Kolodziej 1997),

which is present in propolis (Kumazawa et al. 2004; Alencar et al. 2007). Flavonoid rutin exhibited strong antimicrobial properties (Lupascu et al. 2008).

The antibacterial activity of Brazilian propolis was studied by Silva et al. (2008) using disc diffusion techniques and studied further using NMR, alkyl thiolation reaction and methylation techniques. They isolated fractions of anacardic acid derivatives and studied its effect against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Shigella spp.* Bulgarian propolis showed the antibacterial effect against most of the anaerobic bacteria like *Clostridium, Bacteroides* and *Propionibacterium* (Boyanova et al. 2006).

2.2.4.2 Antifungal activity

The well-known Poplar propolis type was found with antifungal property against different species of the yeasts *Candida* and *Trichosproron sps* (Koc et al. 2007). Caffeate esters and triterpenoids from Egyptian propolis showed antifungal property against *Candida albicans* (Hegazi and El Hady 2002). The ethanolic extracts of propolis caused effect of the different growth parameters of two verities of *Aspergillus flavus*, which was mainly due to griseofulvin compound (Ghaly et al. 1998). Antifungal activity of propolis against macrophage *Paracoccidioides brasiliensis* was studied, which is the most important pathogen causing systemic mycosis in Latin America (Murad et al. 2002).

2.2.4.3 Antiviral Activity

An antiviral activity of propolis against Type A influenza virus (*in vitro*) was studied successfully by Ioirich et al (1965). Moronic acid from Brazilian propolis showed anti-HIV activity in H9 lymphocytes (Ito et al. 2001). There are some examples for the antiviral effect of propolis such as Hegazi et al. (2012) Newcastle disease virus; small pox vaccine virus (Krivoruchko et al. 1975);

Bursal disease virus and reo virus (Hegazi et al. 2001) and (Amoros et al. 1994) found against herpes simplex virus.

2.2.5 Standardisation of propolis

The chemical composition of propolis is diverse that it is very challenging task for researchers to standardise it. Bankova (2005) suggested the need for the standardisation of propolis due to its composition, assurance and competence. The possible solution to this is to accept the typification approach according to the plant source. An active principle/marker compounds were used for the standardisation as shown by (Banskota et al. 2001) who selected CAPE (ceffeic acid phenythyl ester) as a marker compound; which is a potent ingredient of propolis. However, in some propolis types, CAPE are absent therefore this type of standardisation is not recommended. Bankova (2005) suggested a possible way for the standardisation of propolis on the basis of plant source as well as specific active ingredients. One well known Poplar type of propolis has the *Populus spp* plant source in Europe, North America and some parts of Asia (notropics), in which the main biological active substances are flavones, flavanones, cinnamic acids and their esters (Nagy et al. 1986; Greenaway et al. 1990; Bankova et al. 2000). Examples that have been studied are shown in the following table 2.4.

In this way, the above suggestion may be the most suitable solution for the standardisation of propolis; but there is a genuine need to analyse larger numbers of propolis types from different regions for their standardisation and updating.

Table 2.4: Classification of Propolis according to origin and chemical composition (Bankova 2005)

Propolis Type	Geographic Origin	Active constituents
Poplar propolis	North America, Europe and Asia	Flavones, flavanones and cinnamic acids
Birch propolis	Russia	Flavones and flavonols (different from polar)
Green propolis	Brazil	p-coumaric and diterpenic acids
Red propolis	Venezuela and Cuba	Polyprenylated benzophenones
Pacific propolis	Taiwan	C-prenylflavanones
Canarian propolis	Canary Islands	Furofuran lignans

2.2.6 Flavonoids in propolis

Flavonoids are phenolic natural substances and are abundant in natural sources such as fruits, grains, vegetables, flowers, stem, roots, bark, wine and tea (Middleton 1998; Sales et al. 2006). Flavonoids are categorised according to their chemical differences like flavon-3-ols, flavones, flavanones and flavonols as shown in figure 2.5 (Sisa et al. 2010). Flavonoids are polyphenolic compounds with a C15 ($C_6C_3C_6$) framework. The chemical structure of flavonoids contains a chroman ring (C-ring) with a second aromatic ring (B-ring) at the C-2, C-3 or C-4 position (Figure 2.4).

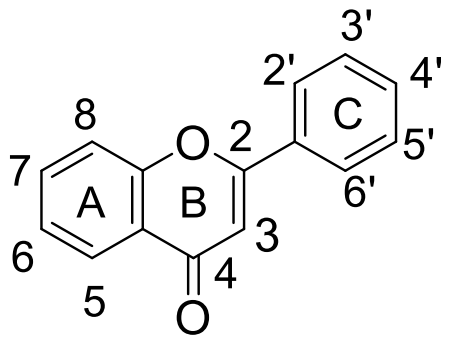
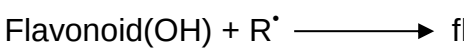


Figure 2.4: The skeleton structure of the flavones (a class of flavonoids)

In flavonoids, the variation in the structure, such as in the C-ring, and the type of the heterocyclic ring, as follows: 1) chromone derivatives (flavones, flavonols, flavanones, and flavanonols); 2) chromane derivatives (catechines and antocyanidines); and 3) flavonoids with open propane chain (chalcones) and with a furane ring (aurones) could be heterocyclic (6 member) or a five member ring (e.g. aurones) or acyclic form (chalcones). From all of these classes, flavones, flavonols and flavanones are the most abundant in plants (Stefova et al. 2004). The detailed typification is explained in figure 2.5. The flavonols have a hydroxyl at C3, where the flavones have hydrogen (Merken and Beecher 2000). The figure 2.4 showed the possible positions of substitutions at 3, 5, 6, 7, 8, 2', 3', 4', 5', and 6' responsible for hydroxylation, methoxylation, and glycosylation being the most common substitution. Thousands of flavonoids with various substitution patterns are recognised today as free flavones, flavonols, and flavanones, i.e., aglycones, and as flavonoid glycosides, which consist of flavonoid, non-sugar component aglycone, connected to the sugar moiety such as monosaccharides and disaccharides (Stefova et al. 2004).

More than 4000 varieties of flavonoids have been identified and are responsible for the different shades of attractive colours in flowers, fruits and leaves; they

also play a protective role against coronary heart diseases (de Groot and Rauen 1998). The major aspect of flavonoids is their strong antioxidant capacity. The oxidation of flavones and catechin flevonoids by radicals results in a less reactive and more stable compound as they react with active oxygen species and become inactive (Korkina and Afanas'ev 1997). The hydroxyl group of flavonoids is crucial because it plays the main role in reactions such as the following:



Where R= Reactive species

Few flavonoids react directly with superoxides while others react with highly reactive oxygen species (Hanasaki et al. 1994).

In this proposed work, ten flavonoids were chosen for the standardisation of propolis. The analytical development studies has been carried out using following ten flavonoids such as CAPE, rutin, quercetin, acacetin, apigenin, galangin, pinocembrine, chrysin, kaempferol and myricetin. These ten flavonoids were varied in their structure (Figure 2.6) and belong to different flavonoid groups which are explained in table 2.5. The flavonoids were chosen from flavonol group in proposed study such as rutin, myricetin, quercetin, kaempferol and galangin. They are commonly found in plant polyphenols as well as in propolis (Table 2.3). The flavone type of compounds such as chrysin, apigenin and acacetin; with one flavanone pinocembrin were selected in current standardisation studies. The other chemical properties of these flavonoids are explained in table 2.5.

Caffeic acid phenethyl ester (CAPE) is one of the very important constituent of propolis and it is phenolic ester. It has anti-mitigenic, anti-carcinogenic, immunomodulatory and anti-HIV properties (Lee et al. 2003; Demestre et al. 2009; Ozturk et al. 2012). The details of the pharmacological importance of other flavonoids selected in proposed study is discussed in following table 2.5. Chemical structures are shown in figure 2.6.

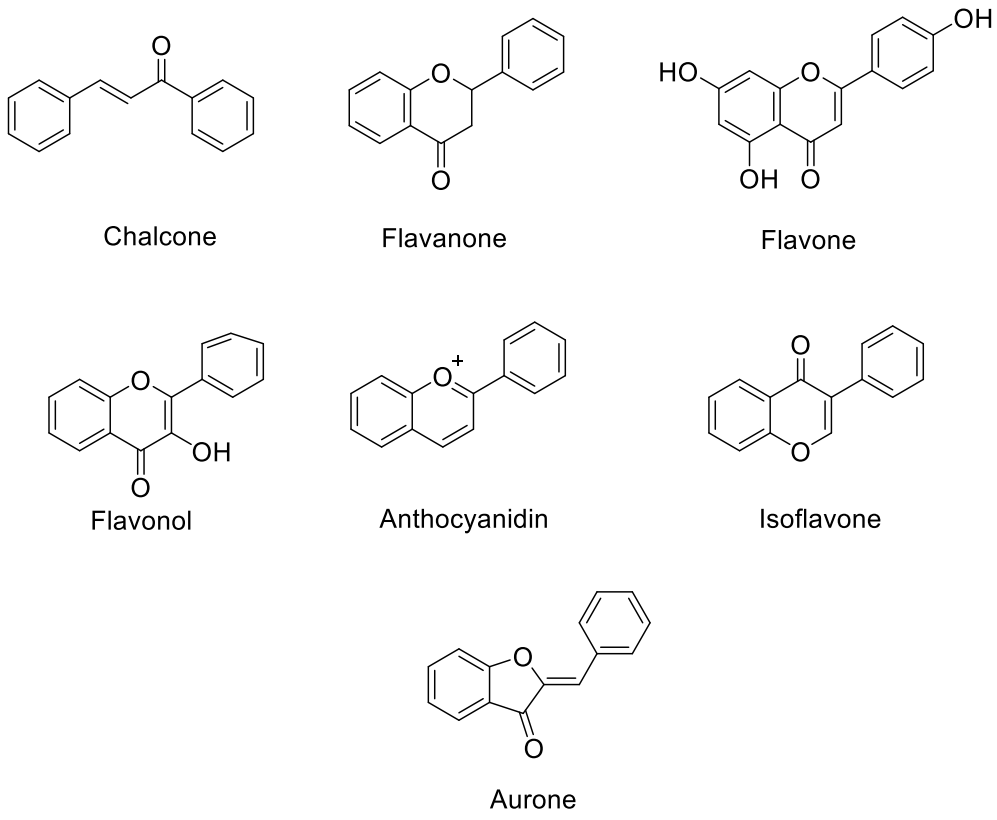


Figure 2.5: Structure of types of flavonoids

Table 2.5: Flavonoid classification and pharmacological activities of selected flavonoid compounds

(Timbola et al. 2006; He et al. 2008; Li et al. 2008; Lupascu et al. 2008; Hong et al. 2010; Shukla and Gupta 2010; Charoensin et al. 2012; Patel et al. 2012).

Compound	Flavonoid type	Pharmacological activity
Rutin	Flavonol	Antioxidant, Antimicrobial
Myricetin	Flavonol	Antimicrobial
Kaempferol	Flavonol	Antioxidant, Antiviral against HSV and coronavirus
Quercetin	Flavonol	Antihistamine, Antioxidant and Anti-ulcerogenic
Galangin	Flavonol	Antiviral against HSV and coxsackie virus, Anti-inflammatory, Antimicrobial and Antifungal
Apigenin	Flavone	Anti-inflammatory, Antimicrobial, Antioxidant and Antiviral
Chrysin	Flavone	Anti-inflammatory, Antifungal and Antiviral against coronavirus and rotavirus
Acacetin	Flavone	Anti-inflammatory and Antiviral
Pinocembrin	Flavanone	Antimicrobial, Antifungal, Local Anesthetic
CAPE	Phenolic ester	Antifungal, Anti-inflammatory, Antimicrobial, Antioxidant, cytotoxic against pancreatic and colon cancer cells

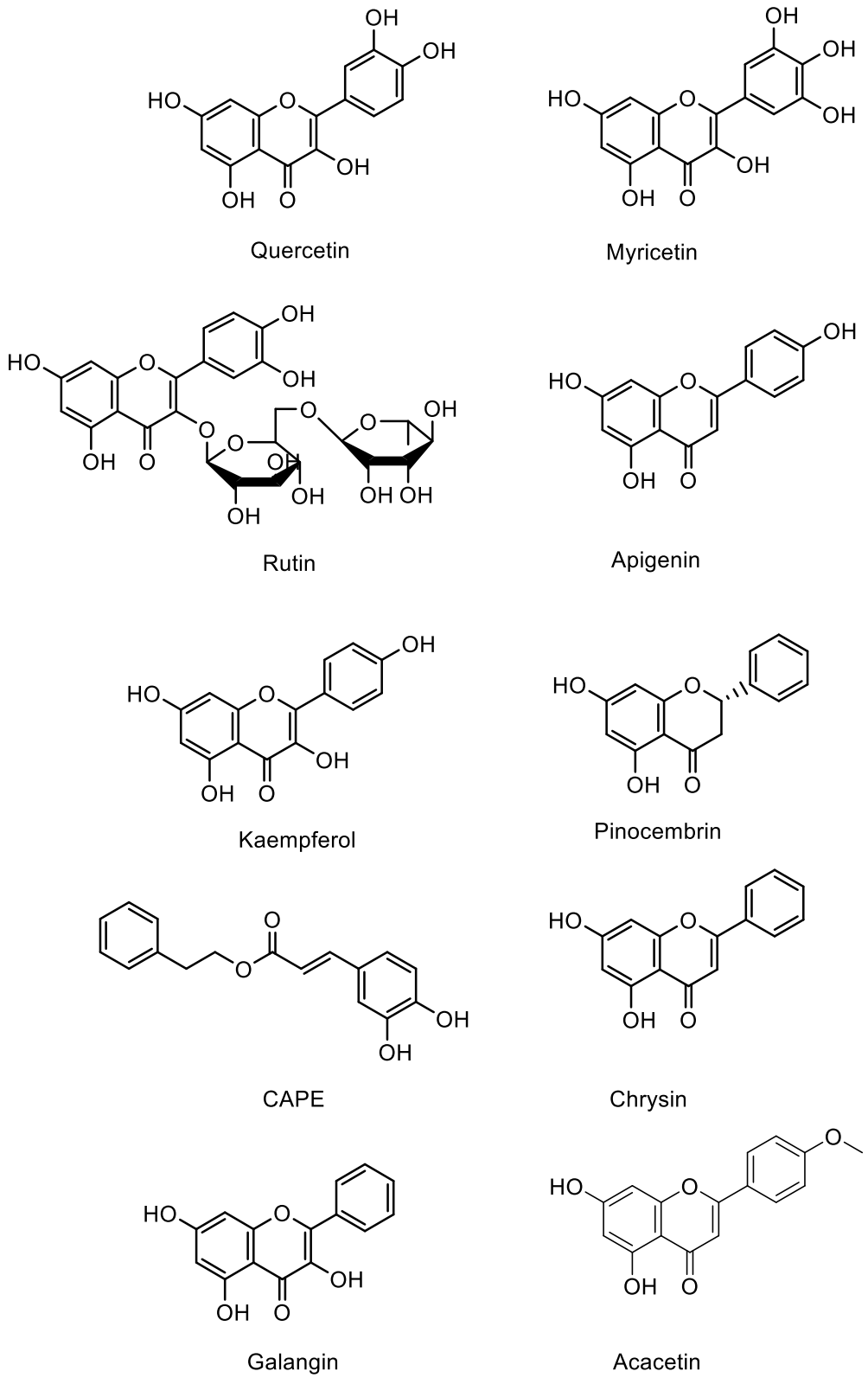


Figure 2.6: Chemical structures of different flavonoid standards

Table 2.6: Chemical properties of flavonoid standards (Wishart Research Group 2016)

Name of Flavonoid	Chemical formula	Molecular weight	pKa (Strongest Acidic)	pKa (Strongest Basic)	LogP	LogS
Rutin	$C_{27}H_{30}H_{16}$	610.51	6.45	-3.9	2.39	-3.4
Myricetin	$C_{15}H_{10}O_8$	318.24	6.43	-4.1	1.66	-3
Quecretin	$C_{15}H_{10}O_7$	302.23	6.44	-4	1.81	-3.1
Apigenin	$C_{15}H_{10}O_5$	270.23	6.63	-5.2	3.07	-3.4
Kaempferol	$C_{15}H_{10}O_6$	286.23	6.44	-3.9	1.99	-3.2
Pinocembrin	$C_{15}H_{12}O_4$	256.23	7.92	-3.92	2.85	-3.1
CAPE	$C_{17}H_{16}O_4$	283.31	9.21	-6.3	3.65	-3.7
Chrysin	$C_{15}H_{10}O_4$	254.54	6.64	-5.2	3.44	-3.4
Galangin	$C_{15}H_{10}O_5$	270.24	6.45	-3.9	2.39	-3.4
Acacetin	$C_{16}H_{12}O_5$	284.26	6.64	-4.7	3.46	-3.7

The chemical properties of all selected flavonoid compounds are mentioned in table 2.6. The pKa values for most of the compounds are more or less in the same range. In analytical separation techniques, pH of the mobile phase is one of the parameters affecting retention and separation selectivity of the flavonoids, depending mainly on the analyte pKa. Low pH such as pH 3 is favourable to maintain these analytes in unionised stable form and favours rapid separation with maintained peak shape and resolution (Esteve-Romero et al. 2005).

2.2.7 Contamination of propolis

Apart from the useful bioactive compounds, contamination of propolis is one of the major drawbacks. These contaminants are very harmful. Propolis can be contaminated by heavy metals such as lead (Pb) and cadmium (Cd), pollutants such as pesticides, antibiotics and other related chemicals. Some of the contamination studies are discussed here in brief.

2.2.7.1 Lead contamination

The heavy metal ions such as iron and copper form chelate transitions with flavonoids (Kurek-Górecka et al. 2013). The possible binding sites of heavy metals to the flavonoids are mentioned in following figure 2.7. The possibility of formation of a complex structure in case of flavonoids by chelating metal ions is due to their specific chemical structure. It is also confirmed as the antioxidant activity of flavonoids is mainly because of presence of chelating metal ions such as Fe(II), Fe(III) and Cu(I), which protect potential biological activity from oxidative stress (Malešev and Kuntić 2007).

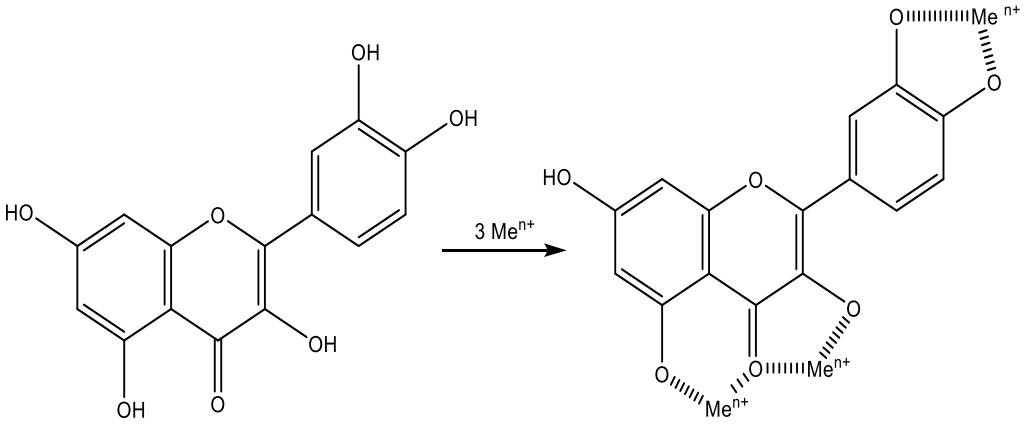


Figure 2.7: Metal-binding sites for flavonoids (Kurek-Gorecka et al. 2014)

Lead is one of the toxic heavy metal which causes harmful effects on human health at very low concentrations (Christensen and Kristiansen 1994). Neurological, behavioural and physiological effects with less IQ (Intelligence Quotient) disorders in children were observed at the low concentration of lead in blood and at higher concentrations; acute encephalopathy and memory loss may be caused (Manser et al. 1989). Therefore, the presence of lead in food and medicinal products may cause severe health issues. Government policies in different countries have been regulating and controlling a certain levels of lead in the food products by developing specific MRL (maximum residue limit) values (Davis et al. 1993). According to EU regulations, the lowest MRL of 0.02 mg/kg is set for the infant formula and milk products, while the highest MRL of 0.03 mg/kg is considered for food supplements. Lead is commonly present in surrounding atmosphere such as in air, water and soil. Due to its common presence, propolis contamination by lead is very common and hence creates problems in the further formulation process. The source of lead contamination in propolis is mainly direct from the atmosphere or through harvest, processing and extraction methods (Alcici 1997). Due to such contamination, removal of lead from propolis is become challenging, costly and laborious for manufacturers.

The detection of lead has been studied previously. The honeybees and their products are considered for monitoring the environmental pollution of a particular region. A direct connection was observed with the presence of pesticides or other contaminants in that particular region. One of such example, Conti and Botre (2001) found high levels of heavy metals such as Pb, Cr and Cd from reference sites (the area surrounding City of Rome) and compared to

the site of the centre of the city in different honey bee products including propolis. Serra Bonvehi and Orantes Bermejo (2013) observed a number of metals including heavy metals such as Pb, Cd, As and Ni in propolis samples from Spain but the concentration of lead was significantly high among other heavy metals. The GFAAS (graphite furnace atomic absorption spectrometry) method with microwave digestion technique was used for the detection of the lead from propolis with the detection limit of 0.002mg/kg and LOQ (limit of quantitation) of 0.007 mg/kg (Stanciu and Mititelu 2004). The heavy metal concentration including Pb was found to be high in propolis in comparison with honey samples.

Therefore, there exists a requirement for development of a more effective approach to facilitate the removing of lead from propolis, without changing its properties. Some of the studies reported that, the careful apiculture methods can reduce the risk of lead contamination. Zhang et al. (2012) used rush cellulose xanthogenate for lead removal. This substance illustrates good selectivity for lead (61.64% removal rate) but at the same time, it affects flavonoid content particularly rutin. Sales et al. (2006) reported the best method to harvest propolis is a use of the meshes rather than a scrap method to obtain minimum lead containing propolis. These are few effective solutions to reduce lead contamination in propolis.

2.2.7.2 Pesticide contamination in propolis

Farmers use pesticides to increase crop yield but this can affect quality of yield product. There is a big problem now of pesticide contamination in food and food products. Insecticides and pesticides are used in apiaries (over beehives) to reduce the infection by insects and pests, this also leads to a direct risk for bees

and the hive as a whole. The sap and exudates collected from pesticide contaminated fields by honey bees increases the number of incidents of pesticide pollution in their products. Due to this pollution, the depopulation of beehives has occurred in USA, Italy, France, Spain etc and also Colony Collapse Disorder (CCD) has been observed in the USA (Schmuck et al. 2001; Decourtye et al. 2003; Sbeghen-Loss et al. 2009; Bernal et al. 2010; Pettis and Delaplane 2010). Environmental pollution including pollution from pesticides was the main cause of CCD. Bees gathered pollens, nectar and resins from around 5 km area surrounded by beehive and if that region is polluted by pesticides; it changed some habits of the bees and had an effect on a behavioural phenomenon such as the communication dance, flying habits and food exchange (Colin et al. 2004).

Many studies have reported the presence of pesticide/s traces in propolis samples, which is an alarming affair for beekeepers and consumers. Pareja et al. (2011) found traces of pesticides in active and depopulated beehives from Uruguay. About 60µg/kg imidacloprid was found in propolis samples and in honey product which was detected by the HPLC method. Imidacloprid is a very hazardous chemical, it causes some lethal and sub lethal effects on bees. Other examples are presented in table 2.7.

Table 2.7: Pesticide contamination studies of propolis

Name/s of pesticides used	Propolis origin	Technique used	Results (LOD, LOQ)	Reference
Coumophos, ethion and chlorpyriphos	Uruguay	Matrix solid phase dispersion and GC with flame photometry and mass spectroscopic method	LOD 26.0 μg/kg in FPD (flame photometric detector) and 1.43 μg/kg for MS (Mass spectroscopy) detection of all studied compounds.	(Perez-Parada et al. 2011).
Organochlorine pesticide-4,4'DDE (dichlorodiphenyl dichloroethylene), endosulfan II, organochlorine	-	Gas chromatography electron capture detector technique	LOD 4,4'DDE (dichlorodiphenyldichloroethylene)- 0.8μg/kg and for enosulfan II - 11.4 μg/kg and LOQs ranged from 2.6 to 38.1 μg/kg	(Chen et al. 2009)
Chlorinated pesticides HCH (hexachlorocyclohexane) and DDT (dichoro diphenyl trichloroethane)	Bydgoszcz and Toruń (Poland)	GC	-	(Wojciech and Zommer-Urbanska 1992)
Acaricide like bromopropylate, coumaphos, fluvalinate and flumethrine	Switzerland	GC with ECD detection	Varies per different types	(Bogdanov et al. 1998)
Polycyclic aromatic hydrocarbon (PAH)	Romania	GC MS	LOD PAHs in range 0.03 to 0.12 μg/kg low up to 0.6-665.0 ng/g of some PAHs.	(Dobrinas et al. 2008)

2.2.7.3 Antibiotic contamination in propolis

Antibiotics are used for the treatment against infections in humans and animals. They are natural, semi-synthetic or synthetic compounds and are applied using oral, parenteral or topical methods (Phillips et al. 2004). The development and continuous usage of antibiotics also reflect in the development of resistant against bacterial variants, they can become more dominant and could transmit through animal host populations. Microbial resistance to antibiotics is raising concerns as bacterial strains slowly become resistant to antibiotics. Antibiotics can be found in medication, contaminated agricultural products, food products and animal industries.

In apiaries, farmers use antibiotics to control bacterial infections of bee hives as bacterial diseases such as American foulbrood (AFB) or European foulbrood (EFB), but it can also pollute bee products such as honey, pollen and propolis. There are many commercial antibiotics available for this purpose such as sulfathiazole, dihydrostreptomycin, streptomycin, terramycin, fumagillin etc (Farrar 1960). The tetracycline group of antibiotics such as tetracycline, chlortetracycline, doxycycline are also used for the same reason and are cost effective (Cherlet et al. 2003). There is strict legislation in the EU about the use of antibiotics to honeybees so the maximum residue value (MRL) is not set there and hence there is no selling of honey containing these residues. In some countries like Switzerland, UK and Belgium, MRLs have been set for each class of antibiotic in range 10-50 ppb (Hammel et al. 2008; Bernal et al. 2009). The comparative common usage of honey than propolis also impact on analytical studies; as antibiotic determination has mostly been studied in honey. All analytical studies of antibiotics from honey require specific sample preparation

techniques prior to analysis such as hydrolysis, dissolution, homogenisation, liquid-liquid extraction and solid phase extraction followed by analysis with sophisticated analytical techniques such as LC-MS, LC-UV, LC-FLD etc which are briefly mentioned by Bargańska et al. (2011). Multi residue analysis of antibiotics of the tetracycline group and pesticides were studied by Debayle et al. (2008) using HPLC MS-MS technique and they validate the method. They carried solid phase extraction by HLB cartridge to extract out contaminants. The recovery of all antibiotic compounds was found in range 64-109%. Zai et al. (2013) quantified four antibiotics tetracyclin, streptomycin, penicillin and gentamycin using TLC and HPLC methods and simple extraction techniques like centrifugation, reconstitution using nitrogen gas etc. They found tetracycline and streptomycine are common pollutants used by bee keepers to reduce against bee diseases. Along with honey, recent concern of propolis and its increasing demand points to the analysis of its contaminants such as antibiotics and only a few attempts have been made to achieve this so far. The major obstacle in the analysis of antibiotics in propolis is the intense conglomerate matrix which includes polyphenols, aromatic acids, terpenoids, wax and pollen debris with other unnecessary fragments (Zhou et al. 2009). The tetracycline group of antibiotics are commonly used in beehives for treatments so analysis of these compounds from propolis is an important exercise. Four tetracycline compounds chlortetracycline, doxycycline, tetracycline and oxytetracycline were analysed in propolis using a two-step clean-up method and a HPLC technique reported by Zhou et al. (2009). In the current analytical work, this method was followed step by step but was unable to separate out the antibiotic residue because of close elution of antibiotic and an unknown peak from the propolis sample. The main reason may be the difference between propolis

samples used in both studies. Hence, further modifications were carried out in this study.

In next part, the details of tetracyclines are explained briefly.

Tetracyclines

The tetracyclines are the broad- spectrum agents showing activity against many gram-positive, gram-negative, mycoplasmas, rickettsiae and protozoan paracites (Chopra and Roberts 2001). Tetracycline molecules has a structure with the linear fused tetracyclic nucleus (rings designated A, B, C, and D as shown in figure 2.8) to which a variety of functional groups are attached (deVries et al. 2006).

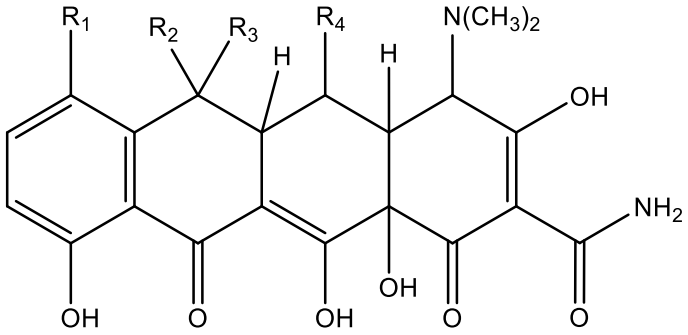


Figure 2.8: General structure of tetracycline molecule

The oxytetracycline, chlortetracycline and tetracycline are naturally occurring antibiotics whereas doxycycline is a semi-synthetic type of antibiotic. Tetracyclines are obtained from different strains of *Streptococcus* bacteria using fermentation technology. Ex. Chlortetracycline and oxytetracycline are the products of bacteria *Streptomyces aureofaciens* and *S. rimosus* respectively (Chopra and Roberts 2001). Tetracycline group of antibiotics bind with 30s ribosome (of respective microorganism) and affect the protein synthesis

process, which is the basic mechanism behind its antimicrobial activity (Brodersen et al. 2000).

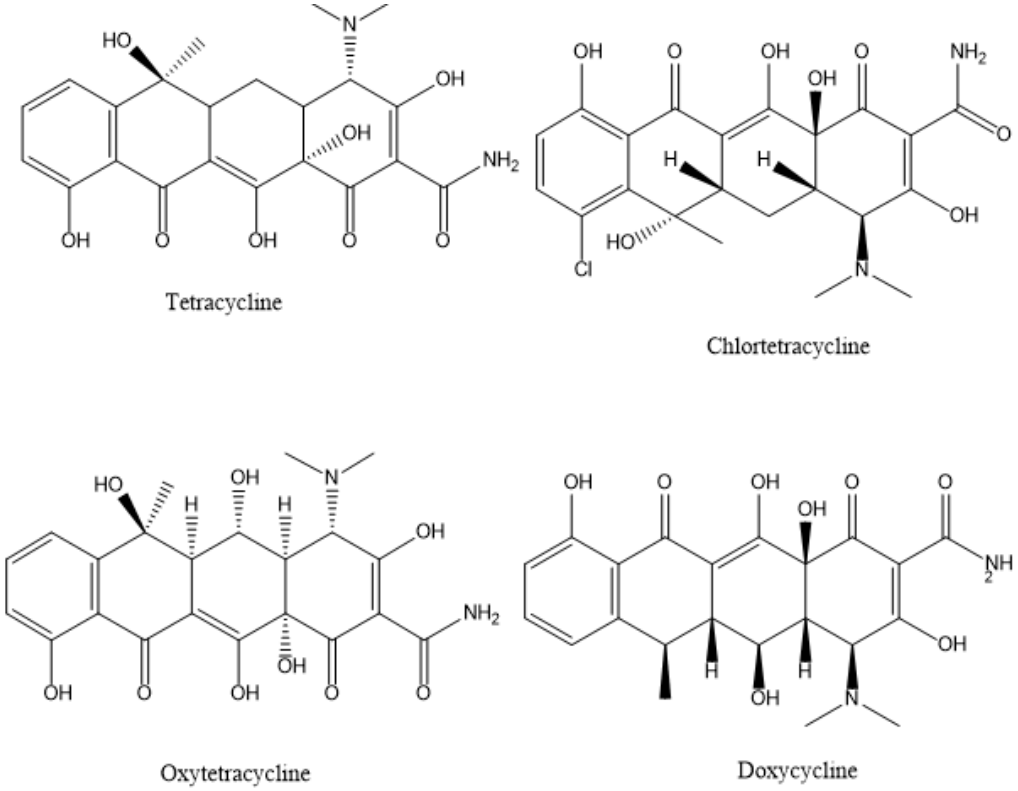


Figure 2.9: Structure of oxytetracycline, tetracycline, chlortetracycline and doxycycline

The molecular structures of four selected tetracyclines are shown in figure 2.9. Due to the presence of four hydrogen rings in all tetracyclines, they are named as tetracyclines. The physical characteristics of these four tetracycline compounds are displayed in table 2.8.

These four tetracyclines are from same group therefore, it was convenient for the analytical method developmental studies as all antibiotics behave almost in similar manner when exposed to the chromatographic conditions.

Table 2.8: Physical properties of tetracyclines (oxytetracycline, tetracycline, chlortetracycline and doxycycline) (National Center for Biotechnology Information 2016).

Name of Tetracyclines	**Molecular formula**	**Molecular weight g/mol**	**Log P**	**Log S**
Tetracycline	$C_{22}H_{24}N_2O_8$	444.43456	-0.56	-2.5
Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.43396	-0.99	-2.5
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.87962	-0.62	
Doxycycline	$C_{22}H_{24}N_2O_8$	444.43456	-0.72	-2.9

The tetracyclines are crystalline bases, yellow in colour and odourless compounds. They are amphoteric in natures and hence can produce salts with both strong acid and bases. The tetracycline exhibits three structural units and, therefore, represents three pKa values in the range of 2.8 - 3.4; 7.2-7.8 and 9.1-9.7.

3. MATERIALS AND METHODS

3.1 Material, chemicals and solvents

3.1.1 Materials

A description of the materials used for this research is provided in this section.

The four pharmaceutical preparations and raw propolis were procured from Nature's Laboratory Ltd, Whitby, North Yorkshire, UK and their details are as follows.

a. Propolis Capsules (hard gelatine capsules, Batch: 15159);

b. Purified Propolis Powder (Batch: 15159);

c. Bee Vital Propolis Tincture (Batch: 6113)

d. Bee Vital Propolis Liquid (Batch: BN0057)

e. Raw propolis (England, UK)

All propolis samples were stored in cool and dry place before and after use. The raw propolis was screened each time before carrying out any experiment. Unnecessary particles such as wood particles, nails, fibres, etc were removed from raw propolis by manual picking and by using suitable sieve. Purified propolis samples were used directly for all the experiments.

3.1.2 Standards

The details for all standard compounds that were used in this project are listed in the following table.

Table 3.1: Standards description

Name of Standard	Supplier and Grade	Purity and CAS Number
Acacetin	HPLC grade, Sigma Aldrich	(≥ 97%, CAS no.- 480-44-4)
Rutin hydrate	HPLC grade, Sigma Aldrich	(≥94%,CAS no.-207671-50-9)
Quercetin	HPLC grade, Sigma Aldrich	(≥95%, CAS no.-117-39-5)
Myricetin	HPLC grade, Sigma Aldrich	(≥96%, CAS no.- 529-44-2)
Kaempferol	HPLC grade, Sigma Aldrich	(≥90%, CAS no.- 520-18-3)
CAPE-caffeic acid phenethyl ester	HPLC grade, Sigma Aldrich	(≥97%, CAS no.-104594-70-9)
Apigenin	HPLC grade, Sigma Aldrich	(≥95%, CAS no.- 520-36-5)
Chrysin	Sigma-Aldrich	(97%)
Pinocembrin	(Fluka) Sigma-Aldrich	(95%)
Galangin	Sigma-Aldrich	(CAS no. 548-83-4)
Tetracycline	HPLC grade, Sigma Aldrich	(≥ 88%, CAS no.- 60-54-8)
Chlortetracycline hydrochloride	HPLC grade, Sigma Aldrich	(≥ 75%, CAS no.- 64-72-2)
Doxycycline	TLC grade, Sigma Aldrich	(≥ 98%, CAS no.- 24390-14-5)
Oxytetracycline hydrochloride	HPLC grade, Sigma Aldrich	(≥ 95%, CAS no.- 2058-46-0)

3.1.3 Chemicals

Specification of all chemicals are summarised below,

Table 3.2: Chemical list and details

Name of chemical	Supplier	CAS Number
Sodium phosphate (monobasic)	Sigma Aldrich	10049-21-5
Potassium phosphate dibasic	Sigma Aldrich	7758-11-4
Aluminium Chloride from	Sigma Aldrich	7446 700
Brij ®35 w/v 30 %solution in water	ACROS organics	9002-92-0
Brij ® L23 (30% w/v)	Sigma Aldrich	9002-92-0
Sodium acetate	BDH chemicals	127-09-3
Glacial acetic acid	Fischer scientific	64-19-7
Boric acid	Sigma Aldrich	10043-35-3
Sodium chloride ≥99.5%	Sigma Aldrich	7647-14-5
Orthophosporic acid	BDH chemicals	7664-38-2
Sodium hydroxide	Sigma Aldrich	1310-73-2
Formic acid (98-100%)	Merck Ltd	64-18-6
Acetic acid	Sigma Aldrich	
Oxalic acid (Unhydrous ≥ 99.0%,)	Sigma Aldrich	64-19-7
Ammonium hydroxide	Sigma Aldrich	1336-21-6
Ethylenediamine tetra-acetic acid disodium salt dihydrate (Na_2EDTA) (99.0-101.0%)	Sigma Aldrich	6381-92-6
Citric acid monohydrate (ACS reagent ≥95%)	Sigma Aldrich	5949-29-1
Sodium-n-dodecyl sulphate 99%	Alfa Aeser	151-21-3
Tween® 20	Uniqema	9005-64-5
Tween 80® (Ploysorbate 80)	Alfa Aeser	9005-65-6

3.1.4 Solvents

Specification of all the solvents is provided in the following table,

Table 3.3: Solvent list and details

Name of Solvent	Garde	Supplier	CAS Number
Acetonitrile (99.9%);	HPLC grade	Sigma Aldrich	75-05-8
Methanol (99.8%);	HPLC grade	Sigma Aldrich	67-56-1
THF- tetrahydrofuran (99.9%)	HPLC grade	Alfa Aeser	109-99-9
Ethyl alcohol	HPLC grade	Alfa Aeser	64-17-5
1 butanol (99%)	HPLC grade	Alfa Aeser	71-36-3
Ethyl acetate	HPLC grade	Fisher scientific	147-78-6

3.1.5 SPE cartridges

The following SPE cartridges were used in this work.

a. Oasis HLB (1cc vac cartridge, 30mg sorbent/cartridge, 30µm particle size) from Waters

b. Isolute CBA (25mg sorbent per cartridge, 50 µm particle size)

3.2 Instrument and apparatus

Specification of all the types of equipment used in this research are listed below,

3.2.1 UV-Visible spectrophotometer

JASCO V-630 UV-Visible spectrophotometer with intelligent remote (module iRM) and 'Spectra Manager' software was used for analysis of flavonoids. Light sources are two-deuterium lamp (190-350nm) and halogen lamp (330-1100 nm) with silicon photodiode detector. Wavelength range is from 190nm-1100nm.

Wavelength accuracy is ± 0.2nm, wavelength repeatability is ± 0.1nm with fixed spectral bandwidth 1.5nm.

3.2.2 HPLC

Water alliance e2695 separating module was used for analysis of flavonoids and antibiotics. Photodiode array of WATERS 2998 was used for advanced detection which maintains optimal spectral performance with wide, linear and constant optical band pass. It allowed to quantify both low and high level of components within single chromatographic separation and definitive identification of compounds with co elution detection. The HPLC system was consisted of a deuterium lamp with management of thermal wander for the stability of maximum baseline. A Phenomenex Sphereclone (C_{18}, 250mm x4.6mm x 5µm) HPLC analytical column was used. Analysis of data was performed using Empower 3 software.

3.2.3 UPLC

Acquity UPLC class system with PDA detector was employed in this work. The system has binary pump, which is able to work up to 15000 psi, one auto-sampler unit, fast injection cycles facility in sample injection, low injection volumes and temperature control (in a range 4–40°C). The UPLC system utilised a C18 column of Fortis Speedcore column (C18, 150mm x 2.1mm x 2.6µm).

3.2.4 Other consumables and supportive equipment

Description of other consumables and supportive equipment are provided below,

i. A vacuum filter assembly of 1 L capacity glass flask with side arm (Pyrex) and vacuum pump (Greiffenberger Antriebstechnik); nylon filter papers (0.45µl pore size, Millipore Ltd) was used for solvent filtration and propolis extract filtration.

ii. Deionised water (Millipore, 18 MΩ) was used for mobile phase preparation and degassing was carried out using a bath sonicator.

iii. The pH meter (Mettler Toledo) was used to adjust the pH of buffer and mobile phase using appropriate acid and alkali with ±0.01 variation.

iv. Vortex machine (Clifton cyclone) used for vortexing samples of LLE studies to mix two immiscible solvents with propolis.

v. Bath sonicator (Fisher brand F11013) was used to degassing the mobile phase and solvents for HPLC.

vi. Bath sonicator (3510 Branson) with heating provision is used in extraction studies at certain temperature (50°C).

vii. Centrifugation (Hettich centrifuges) was used in SPE studies to centrifuge samples at 6000rpm for 20 minutes after sonication step and before applying to SPE cartridges.

viii. Waters amber coloured screw capped 2 ml HPLC glass injection vials with pre-slit septa were used. Syringe filters (0.45 µm pore size) were used for sample and standard solution filtration and storage.

ix. SPE manifold (Varian VAC-ELUT) was used for SPE extraction.

3.3 Methods

All methods are discussed in detail in further chapters for convenience. General methods are explained in the following sections:

3.3.1 Mobile phase preparation for HPLC

In mobile phase preparation, buffer solution and solvent were used either in a mixture or separately as per requirement. For buffer preparation, appropriate amount of salt was weighed and transferred to 1L volumetric flask, and dissolved in distilled water. The pH of this solution was adjusted using orthophosphoric acid or alkali (NaOH) as appropriate and then the final volume was adjusted to 1 L with HPLC grade water. The buffer solution was filtered using 0.45µm nylon membrane filter and vacuum filter assembly. The solvent was mixed with appropriate amount of buffer. The concentration and pH of the buffer is varied in different trials. The solvent choice is also varied as per the requirement of the trial. The additional changes are explained wherever necessary in following chapters.

Similar preparation was followed in UPLC trials. The variations in different trials are explained in following chapters accordingly.

3.3.2 Mobile phase preparation for MELC

The mobile phase preparation for MELC is more complex as compared to HPLC and need to follow many steps accurately to obtain microemulsion condition. There are four main parts of microemulsion such as a surfactant, co-surfactant, oil and an aqueous phase which divided into w/w in total 100% mixture. One of the microemulsion mobile phase preparation explained here. Initially surfactant was weighed (Brij L23) of 3.5% (w/w) and mixed with the aqueous phase of 10mM sodium phosphate buffer (pH 3) of 91.5% (w/w). Then co-surfactant (1-butanol) of 3.5% (w/w) was weighed accurately and mixed with above solution and finally oil (ethyl acetate) of 1.5% (w/w) was weighed and added gradually in the prepared mixture. The resulted mixture was sonicated in

an ultrasonic bath for 15 minutes to ensure proper mixing of all compounds and to obtain microemulsion condition. Afterwards, resulting microemulsion mobile phase was filtered under vacuum through a 0.45µm filter, followed by degassing using the ultrasonic bath for another 15 minutes (Althanyan et al. 2013).

3.3.3 Solid phase extraction

In the proposed study, SPE extraction was studied for clean-up process in the analysis of antibiotics from propolis. The steps carried out during solid phase extraction were explained graphically (Figure 3.1). In the first step, sorbent of SPE column is activated using suitable solvent, which is followed by loading the sample at low flow rate. Afterward, the residue was carefully washed by selecting suitable washing solvent. Finally, elution of compounds of interest was achieved by applying a suitable elution method.

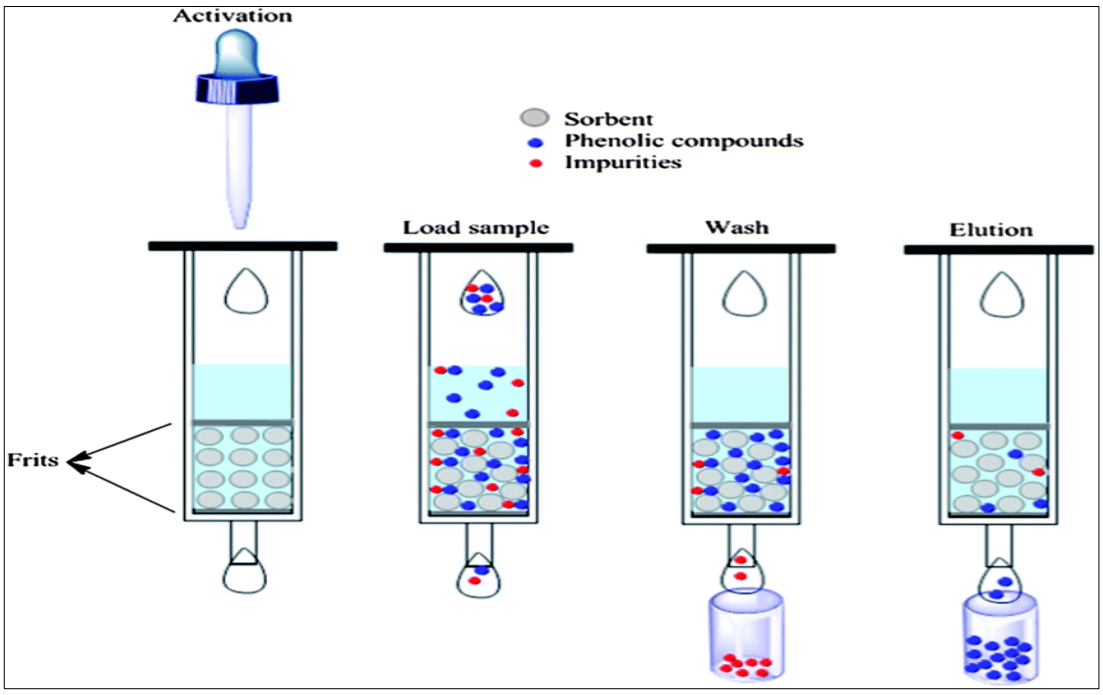


Figure 3.1: SPE steps (Su et al. 2014)

For the SPE operation, SPE manifold is used. The vacuum gauge is used to control pressure and flow of SPE cartridge.

In the proposed study, different cartridges and different solvents were used in different trials which are explained in detail in section 5.2.3.2 SPE manifold used in proposed study named as SPE manifold (Varian VAC-ELUT), with vacuum pump.

3.3.4 Liquid-liquid extraction

Liquid-liquid extraction is carried out based on difference between densities of liquids. The compounds from the liquid mixture can be separated using this principle in two immiscible liquids. The mixture is allowed to mix in both immiscible solvents by continuous shaking. For this experiment, specifically separating funnel is used which allows further collection of two different phases very easily (Figure 3.2). Liquid with higher density allowed solubilising higher density compounds while liquid with low density allow solubilisation of low density compounds. The resulting solvents with differentiated compounds were collected using funnel tap at the bottom. More than two solvents can be used in LLE extraction but the choice of two immiscible solvents is important which selected based on the type of compound which needs to be separated from the mixture, as well as the matrix of sample is also important.

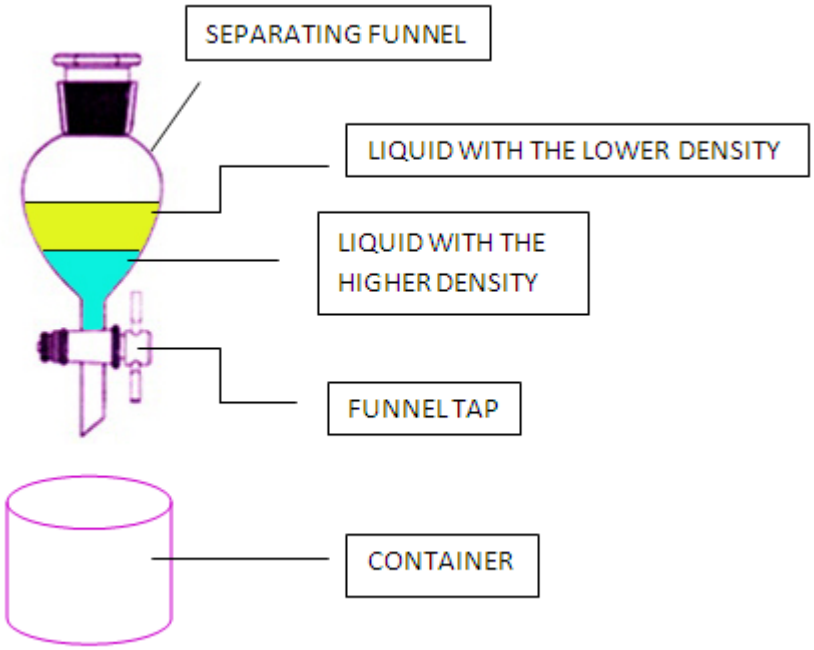


Figure 3.2: LLE separating funnel (King Saud University 2007)

In the proposed study, LLE technique is used for the extraction of tetracyclines from the propolis, which is explained in detail in section 5.2.3.1.

4. ANALYSIS OF FLAVONOIDS FROM PROPOLIS

As discussed in chapter 2 (2.2.2), flavonoids are the main constituents of propolis. They have medicinal values and great antioxidant properties (Kuropatnicki et al. 2013). The major variation among propolis types around the earth challenges possible medicinal value, as well as equality control issues. The origin of propolis is hard to define but it is crucial in the determination of propolis composition. The possible answer for this issue is to analyse different types of propolis and plant sources from different regions. It also helps to formulate “local” propolis types such as European, Brazilian etc (Bankova et al. 2000). The ongoing and future analytical studies of different regional types of propolis will help in standardisation of these types. These types of studies could produce valuable data, which will be useful to minimise critical issues around propolis. On account of all this, the development of analytical methods to analyse flavonoids in propolis, is an important aim to assist the development of pharmaceutical preparation of propolis.

Various analytical techniques were used previously for the analysis of flavonoids in propolis such as spectrophotometric, HPLC, LC-MS , GC, GC-MS etc (Greenaway et al. 1990; Bankova et al. 2000; Sforcin 2007). However, the main objective of the proposed study is to find a suitable analytical method for determination of ten flavonoids for the standardisation of the propolis. UV spectrophotometry was studied for quantitative analysis of flavonoids, while modern LC techniques were applied for quantitative analysis of flavonoids. Many reported methodologies were followed in this study to a more or less extent.

This study is clearly divided into four parts depending on the selected techniques. The UV spectrophotometric study was the first part, which was

followed by reverse phases HPLC, UPLC and microemulsion liquid chromatography. The material and common methodology is explained in chapter 3, while technique specific methods are discussed in section 4.1. Results are discussed in section 4.2.

4.1 Methods for analysis of flavonoids in propolis

Different sets of experiments were designed for the analysis of flavonoids. The details of methodologies development and method validation are explained in the following subsections.

4.1.1 Analysis of flavonoids in propolis using spectrophotometric technique

A preliminary study was performed to extract and quantify flavonoids from different propolis products using known aluminium chloride spectroscopic method (Chang et al. 2002) . The following section discusses the extraction and analysis of flavonoids in propolis by using spectrophotometric techniques.

4.1.1.1 Extraction of propolis preparations

Flavonoids were extracted from four different products of propolis. In the case of propolis capsules and propolis powder, one gram of fine and grounded powder of each was added to 25 ml of 95% ethyl alcohol separately in vials and stirred using a magnetic stirrer at 200 RPM for 24 hrs. Samples were filtered through a 0.45µm nylon membrane filter using a vacuum. The volume of filtrate was adjusted to 25 ml with 80% ethyl alcohol. Similarly, one ml of propolis tincture and one ml propolis liquid was diluted with 10 ml of 80% ethanol in separate vials. Extracted samples were prepared in triplicates (n=3). Each propolis product and flavonoid content was analysed by using the aluminium chloride spectroscopic method that was reported by (Chang et al. 2002).

4.1.1.2 Linearity curve for quercetin

Different solutions of standard quercetin were prepared by dissolving 2.5, 5, 7.5, 10 and 15 mg quercetin in100 ml of 80% ethyl alcohol to obtain 25, 50, 75, 100 and 150 µg/mL concentrations. 0.5 ml of these standard solutions were transferred into separate test tubes, followed by an addition of 1.5 ml of 95% ethyl alcohol, 0.1 ml of 10 % ethanolic aluminium chloride solution and 2.8 ml distilled water in each of the test tubes. Afterwards, the test tubes were kept for 30 mins at room temperature. A blank was prepared similarly by replacing the quercetin solution with distilled water. Solutions were prepared in triplicates of each concentration and analysed by using UV-visible spectrophotometer at 415 nm.

4.1.1.3 Analysis of extracted propolis using spectrophotometric technique

Extracted solutions of four propolis formulations were processed in triplicate (n=3) where 0.5 ml extract solution was used instead of 0.5 ml quercetin solution and the rest of the procedure was the same as explained above in section 4.1.1.2. The samples were analysed using UV-visible spectrophotometer at 415 nm.

4.1.2 Analysis of flavonoids in propolis using reverse phase RP-HPLC technique

An HPLC method was developed in this research to simultaneously determine ten different compounds present in propolis. A preliminary study was performed for selection of the appropriate solvent for different standards. Based on the literature review the medicinal value of flavonoids, 11 potential flavonoids including kaempferol, caffeic acid, galangin, acacetin, pinocembrin, myricetin,

CAPE, rutin, apigenin, chrysin and quercetin were identified and procured. Caffeic acid was eliminated after some tests because of a decomposition problem.

4.1.2.1 Preparation of flavonoid standards stock solution

A stock solution of flavonoid standards was prepared as follows. Five milligrams of each standard (kaempferol, caffeic acid, galangin, acacetin, pinocembrin, myricetin, CAPE, rutin, apigenin, chrysin and quercetin) were weighed separately and was dissolved in methanol and a final volume was made with 100 ml using methanol to get 50 µg/ml concentration of each standard. The stock solution was refrigerated at 2 to 8°C until further analysis.

In the initial HPLC method development trials, kaempferol, caffeic acid, galangin, acacetin, pinocembrin and myricetin were considered and the rest of the standards were added later on. Caffeic acid was excluded in further studies because of decomposition problems.

4.1.2.2 Preparation of mobile phase for chromatographic RP-HPLC analysis

A mobile phase was developed by performing different trials of various strength concentrations of sodium phosphate monobasic with acetonitrile, methanol or THF. Sodium phosphate monobasic solutions 30 mM, 20 mM, 10 mM and 5 mM were prepared by dissolving an appropriate amount of it in HPLC grade water. The pH of these solutions was adjusted to 3 ±0. 01 by the addition 0.01N orthophosphoric acid or 0.01N sodium hydroxide. The preparation of buffer and mobile phases is discussed in detail in section 3.4.1. The organic solvents (acetonitrile or methanol or THF) were mixed in the buffer solution by different ratios for different trials. As a result of method development, the optimum

mobile phase methanol: 5 mM sodium phosphate buffer (pH 3); 50:50 (v/v) was validated.

The variation in mobile phase composition was studied to improve chromatographic separation. The HPLC method by Pietta et al. (2002) was followed at the beginning but found that the reported method was unable to produce similar results. Hence, the mentioned gradient method was not followed in further study but only the mobile phase (5mM sodium phosphate buffer pH 3 and ACN) was chosen for the next experiments using isocratic flow. A single variation in each step including concentration of buffer, temperature and organic modifier (MeOH, THF), was employed for better chromatographic separation for the ten selected flavonoid standards. The solvent selectivity procedure was also considered in the present study (briefly discussed in results and discussion section 4.2.2.5). The pattern of variation in every trial is well described in results and discussion part (4.2.2).

4.1.3 Mobile phase preparation for UPLC

The flavonoid standards and mobile phase preparation methods followed the same procedure described in 4.1.2. The same mobile phase conditions that are used for RP-HPLC were tested initially for UPLC technique, with only the change in flow rate and injection volume using UPLC convertor (convert HPLC chromatographic condition to UPLC using calculator in Empower 3 software). Mobile phase and samples were filtered through 0.45 µm nylon membrane filter paper and syringe filter, respectively. All solvents and buffer solutions were regularly changed after alternate days and filtered regularly to avoid any blockage. The details of further variation in a mobile phase are described in the results and discussion section (4.2.3).

4.1.4 Mobile phase preparation for microemulsion LC (MELC) technique

The flavonoid standards and sample preparation methods followed the same procedure described in 4.1.2. Microemulsion mobile phase with HPLC was used in this technique to facilitate more advanced separation in the selected flavonoids. The details of microemulsion mobile phase preparation are discussed in section 3.4.2.

The method development to optimise the mobile phase and other chromatographic conditions for the separation of flavonoids are explained in sections 4.2.4.

4.1.5 Method validation for RP-HPLC method for analysis of flavonoids

The chromatographic conditions for the separation of flavonoids using RP-HPLC are as follows,

Mobile phase	:Methanol: 5mM sodium phosphate buffer (pH 3); 50:50 (v/v)
Flow rate	:1ml/min
Column temperature	:28°C
Injection volume	:20 µl
Wavelength	:265 nm
Run time	:75 min

The optimised method for separation of flavonoids using RP-HPLC was validated as per the ICH guidelines (ICH 1996). The following parameters were assessed in method validation,

Selectivity

Selectivity was demonstrated by proving non-interference of blank peak with other standard peaks as well as all peak separation.

Linearity

Linearity was performed using six solutions in a range from 10 µg/ml to 25 µg/ml. These solutions were prepared using a standard stock solution, which was explained in 4.1.2.2 and the dilutions were made as described in the following table.

Table 4.1: Preparation of standard solutions for linearity study using serial dilution

Linearity Level	Volume of standard stock solution (ml)	Final volume adjusted using methanol (ml)	Strength of solution (µg/ml)/ppm
Level-1	5	25	10
Level-2	6	25	12
Level-3	7	25	14
Level-4	8	25	16
Level-5	10	25	20
Level-6	12.5	25	25

Each linearity solution was injected in triplicate and the average area was plotted against concentration to obtain the equation of a line and correlation coefficient.

Precision

Precision was studied using five determinations at known concentration levels corresponding to low (10 µg/ml); medium (16 µg/ml) and high (25 µg/ml) levels. Standard dilutions were prepared using similar procedures in the calibration range as explained in table 4.1. Each solution was injected five times. This study was repeated for five days to determine the precision between days. The precision was evaluated by calculating RSD (Relative standard deviation) of peak area for each concentration level.

Accuracy

Three concentration levels: low (10 µg/ml); medium (16 µg/ml) and high (25 µg/ml) in the calibration range were used to study accuracy. Each solution was injected in triplicate. The accuracy of this method was assessed by standards at different concentrations then comparing with the true concentration of flavonoids.

Recovery

The recovery of the method was assessed by comparing the peak area of the extracted flavonoid with the peak area of flavonoid standards.

Robustness

The robustness of the optimised analytical method was studied by deliberately changing experimental conditions by ± 5% of the optimum condition. A standard solution of 10 µg/ml in addition to blank was selected for robustness study. The robustness was assessed by changing the following parameter of the optimum method.

Mobile Phase

Mobile phase was altered by changing methanol: buffer ratio and all other method parameters were kept unchanged. The sample was injected in triplicate for each mobile phase.

Table 4.2: Mobile phase variation in robustness studies

	Methanol	**Buffer**
Mobile phase 1	47.5	52.5
Mobile phase 2	52.5	47.5

The column was conditioned for each method for sufficient time before starting the injections. Resulted chromatogram evaluated was compared with chromatogram with the optimised analytical method.

Temperature

The temperature was altered by ± 5 % and the rest of method parameters were kept unchanged.

Table 4.3: Temperature variation in robustness studies

	ºC
Temperature 1	26.6
Temperature 2	29.4

The column was allowed to calibrate with the respective temperature. For each condition, three replicates were performed. Resulting chromatograms were compared with chromatogram of optimsed analytical method.

Flow rate (±0.1ml)

The flow rate was altered as 0.9ml/min and 1.1 ml/min and all other method parameters were kept unchanged.

Table 4.4: Flow rate variation in robustness studies

	ml/min
Flow rate 1	0.9
Flow rate 2	1.1

4.1.6 Application of validated RP-HPLC method for analysis of flavonoids in propolis

Flavonoids were extracted from four propolis products using the maceration extraction method described by (Cuesta-Rubio et al. 2007). In the case of propolis capsules and propolis powder, two gm of powder samples (granular powder inside capsule) were added separately to 15 ml methanol and stirred for three hours using a magnetic stirrer. The mixtures were filtered through Whatman filter paper and filtrates were kept in a vacuum oven at 40 $^{\circ}$C for 3-4 h

to obtain a dry extract of propolis. These extracted powder samples were stored in sealed vials.

Similarly, in the case of propolis tincture and propolis liquid products, 0.5 ml of liquid was mixed with 14-15 ml of methanol and stirred for 3 hours using a magnetic stirrer. The mixtures were filtered and stored similarly as explained earlier.

To determine flavonoids from the above four extracts using RP-HPLC, five mg of powdered extracts was dissolved in 1 ml methanol, filtered through 0.45 µm syringe filter and injected in triplicate. The quantification was done using equation of line obtained from a linearity study.

4.2 Result and discussion for analysis of flavonoids in propolis

This part of the thesis covers the method development, optimisation and validation of various analytical techniques using liquid chromatography. The overall resulting data is presented in different subsections based on the technique that was used and discussed accordingly.

4.2.1 Flavonoid quantification from propolis using spectrophotometric method

The spectrophotometric method was used during primary analysis of flavonoids from propolis. For this purpose, the aluminium chloride spectroscopic method was used. The aluminium chloride forms acid stable complex with the C4 keto group and one of the C3 and C5 hydroxyl group of flavones and flavanols (Chang et al. 2002).

Mabry et al (1970) explained that aluminium chloride forms acid labile complexes with the A or B ring flavonoids. Because of these properties, this physicochemical method was considered in the proposed study. The linearity curve obtained from an aluminium chloride spectroscopic method (Figure 4.1).

The absorbance was found to be linear in the concentration range 25 to 150 mg/ml of quercetin with coefficient of correlation (r^2) as 0.998 and equation of line as y = 0.004x +0.017.

The values of the flavonoid contents in the propolis samples were calculated using this equation and is shown in table 4.5.

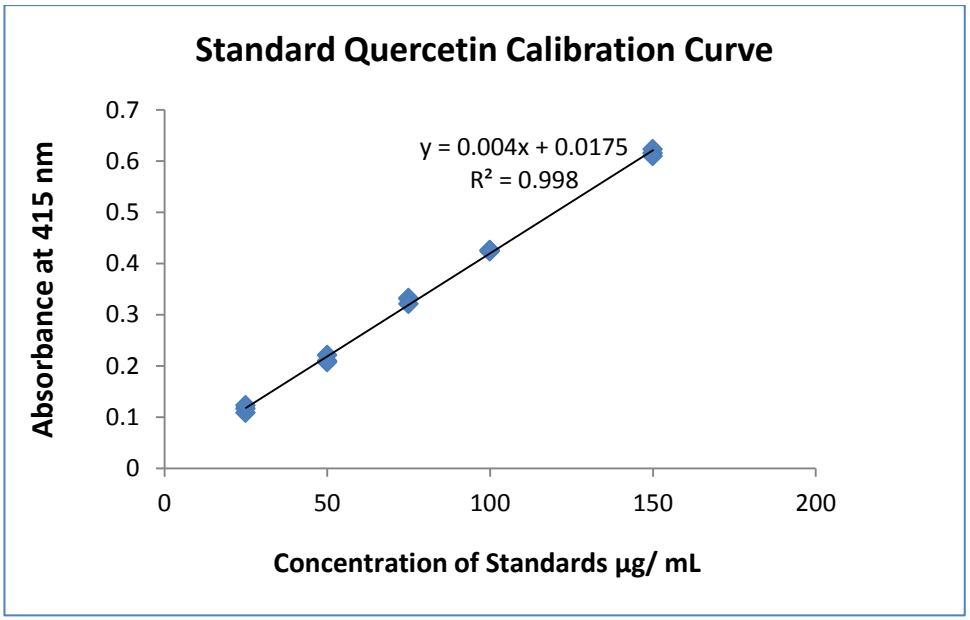


Figure 4.1: The linearity curve for quercetin by aluminium chloride spectroscopic method

Table 4.5: Result for flavonoids content in propolis products by spectrophotometric method

Propolis product	**Flavonoid content (mean) (n=3)**	**S.D.**
Propolis Capsule (A)	28.33 mg/g	1.63
Propolis Powder (B)	53.96 mg/g	3.12
Propolis Tincture (C)	20.06 mg/ml	2.79
Propolis Liquid (D)	14.24 mg/ml	2.62

The above results indicate that propolis powder contains the highest amount of flavonoid as compared to capsule, while propolis tincture contains a maximum amount of it as compared to liquid (Table 4.5). Mohammadzadeh et al. (2007) observed flavonoids in different types of propolis in a range of 1.22-7.79g/100g in similar studies. In the current study, similar flavonoid content was found as 2-5.3 g/100g propolis.

The aluminium chloride spectroscopic method for flavonoid quantification is a much faster technique to quantify total flavonoids from propolis samples. Hence, it can be used for the primary quantification or comparison between

different propolis types considering their total flavonoid value. The only and major disadvantage of this method is inadequate quantification of each flavonoid type. To overcome this problem, other techniques were taken into consideration for improved analysis such as HPLC. It is one of the modern LC techniques, widely used in analytical chemistry because of its high efficiency and usefulness. The detailed method development studies using HPLC are explained in the next section 4.2.2.

4.2.2 Method development of flavonoids using reverse phase HPLC (RP-HPLC) technique

The propolis comprises different types of flavonoid. In the present study, ten flavonoids were selected including rutin, quercetin, myricetin, apigenin, kaempferol, pinocembrin, CAPE, chrysin, galangin and acacetin. These are very common flavonoids found in many types of the propolis and therefore selected for this study (Pietta et al. 2002; Kosalec et al. 2003; Zhou et al. 2008; Barbarić et al. 2011; Pellati et al. 2011). Development of a suitable HPLC method for analysis of these flavonoid standards is a prime objective of the current study. HPLC method development was a complex process because of ten flavonoids selected together. A single variable at each step was followed during the HPLC method development studies, for example using acetonitrile with either 30 mM, 20 mM, 10 mM or 5 mM buffer. This experimental strategy was followed to achieve the best chromatographic condition that allows the separation of all flavonoid standards with the best resolution between flavonoid standard peaks. In the next subsections, variations of the operating parameters will be discussed.

4.2.2.1 Effect of acetonitrile and buffer concentration

In the first few trials, the effect of increasing acetonitrile (ACN) in a mobile phase composition on peak characteristics was studied. Initially, only six flavonoid standards (acacetin, kaempferol, myricetin, caffeic acid, galangin and pinocembrin) were considered. The mobile phase composition of ACN and 30mM sodium phosphate buffer solution (pH 3) was used in varying proportions (Table 4.6). Sodium phosphate 30mM buffer was previously reported in a similar kind of analysis (Pietta et al. 2002). Therefore, it was also used in the initial trials to control the pH of the mobile phase.

Table 4.6: Variation in percentage of ACN and buffer in the mobile phase for the analysis of six flavonoid standards

Sr. No.	Mobile phase		Flow rate ml/min	Temperature °C
	ACN	Buffer (30mM)		
1	20	80	1	25
2	30	70	1	25
3	40	60	1	25
4	50	50	1	25
5	60	40	1	25
6	70	30	1	25

The chromatograms of the first two trials (Table 4.6) showed close elution between the first four and the last two peaks, with a total run time of 25 minutes. While the resulting chromatograms of the last three trials showed very early peak elution (in less than 10 minutes) of all standards without separation between the peaks. Thus, from these results, it was observed that an increase in organic modifier of mobile phase composition caused a decrease in run time and increase co-elution between peaks. Increasing the amount of organic modifier increases the mobile phase elution strength; therefore, by increasing

acetonitrile ratio in mobile phase, the run time of all analytes was decreased. In a similar study of propolis, using acetonitrile and 0.1% formic acid, the mobile phase in gradient elution mode caused close elution of most of the flavonoid peaks and overlap between CAPE and pinobanksin-3-O-acetate peaks. The possible reason may be due to the concentration of the acetonitrile being set to constantly increase in gradient elution above 40% after 40 minutes (Pellati et al. 2011). In the present study, mobile phase of ACN: buffer (40:60, v/v) showed better peak resolution. Therefore, from this point onwards, ACN: buffer ratio was kept at 40:60 (v/v) and the variety in concentration of buffer was studied (Table 4.7).

4.2.2.2 Effect of buffer concentration

In the next step, trials were carried out using different concentrations of buffer in the mobile phase (Table 4.7).

Table 4.7: Variation shows the concentration of buffer in mobile phase

Sr. No.	Mobile phase		Injection volume (µl)	Temperature °C
	ACN	Buffer		
1	40	60(20mM)	50	25
2	40	60(10mM)	50	25
3	40	60 (5mM)	50	25

Peaks overlapping were observed in the first two trials (Table 4.7) with high concentration of buffers, but the resulting chromatogram of trial using low concentration of buffer (5 mM) showed better peak separation as compared to chromatograms of other trials. Therefore, 5 mM concentration of buffer was chosen and kept constant for further method optimisation.

4.2.2.3 Effect of temperature

The effect of temperature was also considered in this study (Table 4.8). Changing column temperature over a range of 20-40°C showed variation in peak characteristics. The high temperatures, 35°C and 45°C, showed reduction in run time with peaks overlapping, on the other hand 25°C and 20 °C temperature conditions showed good peak separation but 20°C increased the run time compared to 25°C column temperature. Therefore, 25°C temperature was chosen as an optimum temperature condition because it gave good separation between all peaks with reasonable analysis time. The chromatogram of best chromatographic condition is shown in figure 4.2.

Table 4.8: Effect of temperature

Sr.No.	**Mobile phase**		**Flow rate (ml/min)**	**Temperature °C**	**Analysis time (in min.)**
	ACN	Buffer			
1	40	60	1	35	22
2	40	60	1	45	20
3	40	60	1	25	28
4	40	60	1	20	25

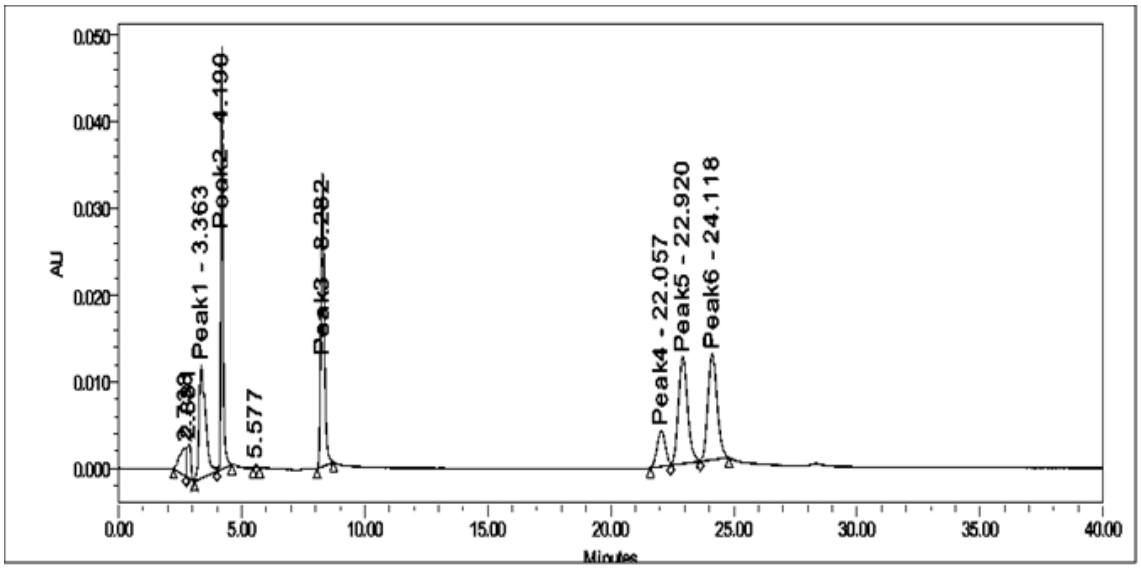


Figure 4.2: Chromatogram of six flavonoids. Chromatographic conditions: mobile phase (acetonitrile:5mM Buffer (pH 3), 40:60 v/v); temperature 25° C; injection volume 20µl; flow rate 1 ml/min.

Peak1: Caffeic acid, Peak 2: Myricetin, Peak 3: Kaempferol, Peak 4: Pinocembrin, Peak 5: Acacetin, Peak 6: Galangin.

Well-separated peaks of all standards with better resolution were observed in the above chromatogram (Figure 4.2). In reverse phase chromatography, more polar analytes such as caffeic acid and myricetin were eluted earlier as compared to less polar compounds acacetin and galangin, which were eluted later. Each peak was identified by injecting individual standards separately. The first peak was of caffeic acid eluted with retention time of 3.3 min., followed by myricetin with retention time of 4.1 min. The third peak was of kaempferol, which eluted late at RT 8.2. Fourth, fifth and sixth peaks of pinocembrin, acacetin and galangin eluted at retention time of 22.05 min., 22.9 min. and 24.1 min., respectively.

4.2.2.4 Method development for separation of ten flavonoid standards

The separation of acacetin, kaempferol, myricetin, caffeic acid, galangin and pinocembrin was achieved at acetonitrile: 5mM Buffer (pH 3), 40:60 (v/v). Five

more standards were added at this point and caffeic acid was excluded as it was showing a decomposition problem.

Accordingly, a new mixture of standards including myricetin, acacetin, galangin, pinocembrin, kaempferol, chrysin, rutin, apigenin, CAPE and quercetin was prepared. A stock solution containing all of these ten flavonoids was used for further method development studies. Further variations in mobile phase composition were conducted to optimise and develop the existing chromatographic conditions for the separation of ten flavonoid standards. In table 4.9, further studies in mobile phase are shown.

Table 4.9: Optimisation of ACN contents in the mobile phase for the separation of ten flavonoid standards

Sr. No.	Mobile phase		Flow rate ml/min	Temperature °C	Run time
	ACN	Buffer			
1	40	60	1	25	25
2	45	55	1	25	30
3	35	65	1	25	48
4	32	68	1	25	85
5	34	66	1	25	50
6	36	64	1	25	40
7	38	62	1	25	30
8	42	58	1	25	22
9	44	56	1	25	20

A wide range of run time and peak overlapping was obtained with varying the contents of ACN in mobile phase (Table 4.9). The overall outcome of these trials was not very effective in terms of chromatographic separation of ten standards but two trials comparatively showed better results. The resulting

chromatogram at mobile phase composition ACN: buffer, 40/60 (v/v) showed peak separation of nine flavonoid standards (Figure 4.3). Similar mobile phase composition showed better peak separation and peak resolution for mixture of six flavonoid standards in previous studies (Figure 4.2). But it was unsuccessful to separate the ten standards during these trials (Figure 4.3). The only advantage of this method is reasonable run time (25minutes) but peak separation and peak resolution was found to be poor.

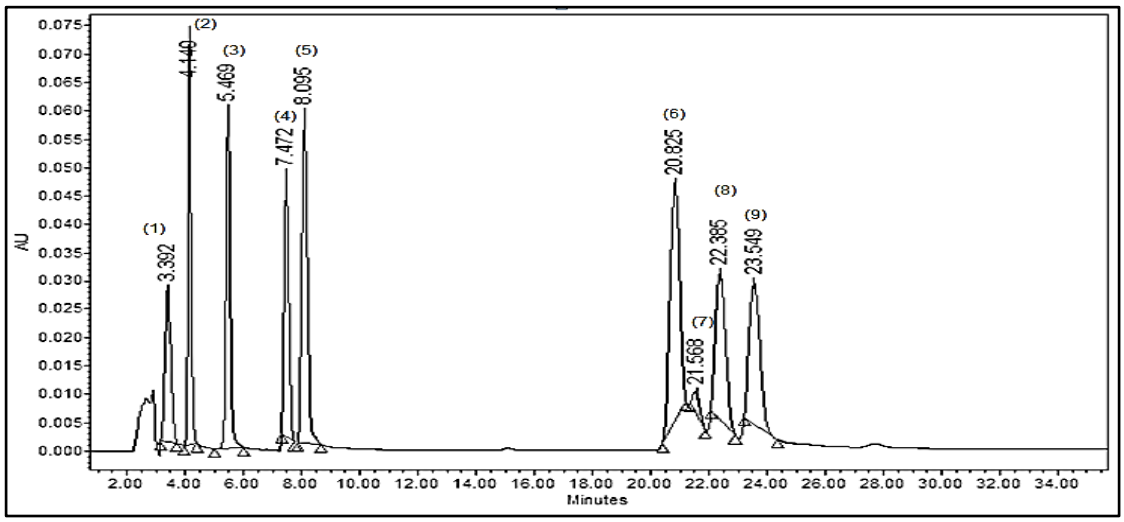


Figure 4.3: Chromatogram of 10 standard mixture of flavonoids with chromatographic conditions: Mobile phase (ACN:5mM Buffer (pH 3) (40/60 v/v); temperature 25 ° C; injection volume 20µl; flow rate 1 ml/min.

Peak 1: Rutin, Peak 2: Myricetin, Peak 3: Quercetin, Peak 4: Apigenin, Peak 5: Kaempferol, Peak 6: Chrysin and CAPE, Peak 7: Pinocembrin, Peak 8: Acacetin, Peak 9: Galangin.

The other chromatogram (Figure 4.4) of mobile phase composition ACN/buffer (35:65; v/v) showed elution of all 10 standards and all of them were identified by injecting single standards separately using the same composition. The total run time was less than 50 minutes. It can be noticed from the chromatogram that the first five standards eluted with good peak resolution in less than 12 minutes. While the last five standards eluted after 30 minutes but showed poor peak separation. The flavonoid peaks were observed in the following order: rutin (RT

3.1), myricetin (RT 4.4), quercetin (RT 6.7), apigenin (RT 10.5) and kaempferol (RT 11.7). Rutin, myricetin, quercetin, apigenin and kaempferol are more polar and having hydroxyl group at C3/C4 positions and hence elutes early (Stefova et al. 2004). Rutin was the highest polar compound amongst all of them, while galangin was the least polar of the ten standards. Chrysin and CAPE elution were the 6th and 7th with RT 36.1 and 38.4 minutes, respectively, which showed some peak overlapping. Pinocembrin eluted at RT 40.7 minutes with no overlapping. RT for acacetin and galangin was 43.8 and 45.6, respectively, which also showed some overlapping. Due to co-elution between chrysin and CAPE as well as in galangin and acacetin, this caused un-usefulness of the method for proper quantification. Hence, this chromatographic condition was not considered for further study.

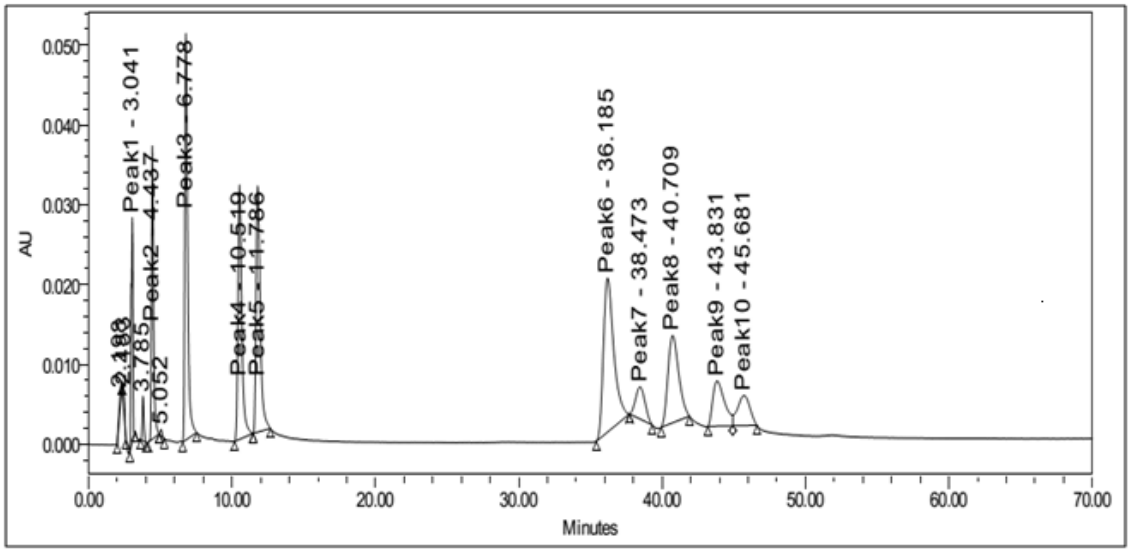


Figure 4.4: Chromatogram of standard mixture (ten standards) of flavonoids with chromatographic conditions as -Mobile phase (ACN:5mM Buffer (pH 3) (35/65; v/v)); temperature 25 °C; injection volume 20 µl; flow rate 1 ml/min.

Peak 1: Rutin, Peak 2: Myricetin, Peak 3: Quercetin, Peak 4: Apigenin, Peak 5: Kaempferol, Peak 6: Chrysin, Peak 7: CAPE, Peak 8: Pinocembrin, Peak 9: Acacetin, Peak 10: Galangin.

The chemical structures of most of the flavonoid compounds are very much similar, which was the main challenge in the method development. Apigenin and kaempferol only differs in the presence of one extra OH group in kaempferol. Similarly, the structure of chrysin and galangin are comparable, with additional OH group in galangin (Figure 2.6). Consequently, co-elution between peaks was always observed in the developed mobile phase for the separation of the ten flavonoid standards. As shown in figure 4.4, fourth and fifth peak of apigenin and kaempferol and ninth and tenth peak of acacetin and galangin showed peak overlapping.

Therefore, this method was not suitable for separation of all ten standards but can be helpful to separate rutin, myricetin, quercetin, apigenin and kaempferol standards with very short run time. Thereafter, solvent selectivity strategy was employed for further method optimisation using organic modifiers such as methanol, THF (tetrahydrofuran) and ACN.

4.2.2.5 Solvent Selectivity approach

Methanol, THF and acetonitrile were selected in solvent selectivity studies for further method optimisation. These solvents were chosen based on their solvent properties including polarity and elution strength. These solvents have acidic (methanol), basic (THF) and dipolar (ACN) nature (Figure 4.5). They have miscibility with each other and are commonly used in reverse phase chromatography techniques. The change in mobile phase properties using different solvents can affect its composition to basic or acidic or dipolar and hence helped to separate eluents, which are unable to separate by other methods (Dolan 2010).

For the demonstration of solvent selectivity, seven experiments were performed (Table 4.10). At the beginning, one of the solvents of interest was chosen and

its concentration with buffer (or water) was adjusted to obtain a desired retention pattern of eluents (Dolan 2010). In the present study, ACN: buffer, 40/60; (v/v) condition was selected and to obtain better separation in similar retention pattern, methanol: buffer 45/55; (v/v) mobile phase condition was tried based on the calculation obtained using nomograph (Dolan 2013). Similarly, THF: buffer 30/70, (v/v) mobile phase condition was also used. These proportions of different solvents were selected by studying solvent selectivity triangle (Figure 4.6) and Nomograph (Figure 4.7) as explained by (Dolan 2013). In nomograph, a possible calculation of % B (% of organic solvent) is presented in such a way that it can be used to replace solvent in RP-HPLC mobile phase without affecting the retention pattern (Figure 4.7) by considering their selectivity and elution strength. After these three trials, the next step was to carry out a mixture of two mobile phases (of all three) (Table 4.10). The final experiment conducted by using a mobile phase that combined the mobile phases in a ratio of 1:1:1 proportion.

The result from selectivity studies showed improper peak separation with poor resolution. The chromatographic conditions comprising methanol as organic modifier gave late elution of all peaks with high analysis time as compared to that of ACN. This is due to the elution strength of ACN where the aqueous phase is greater than methanol, which was demonstrated in current studies, as the sodium phosphate buffer is a diluent for all organic modified mobile phases (Shimadzu 2016). It was concluded that the mobile phase containing acetonitrile, was not suitable for the separation of flavonoid standards due to its high elution strength and the problem of co-elution of flavonoids that have similar chemical structures. On the other hand, the low elution strength of

methanol improved the separation between all flavonoid standards but with a long analysis time.

The chromatogram produced, using THF as an organic modifier, showed more than 10 small peaks with poor peak shape. The chromatograms produced using mobile phase of two organic modifiers (trial no. 5, 6 and 7 in Table 4.10) showed poor separation between the flavonoid peaks. The last trial shown in table 4.10 (1ACN 40: Buffer 60 v/v: 1 MeOH 45: Buffer 55 v/v: 1THF 30: Buffer 70 v/v) gave better separation with good baseline resolution between nine flavonoid standards but the analysis time was 150 minutes (Figure 4.8). Moreover, this method was not reproducible. The reason behind this variation was due to the complex mobile phase composition that consists of all the three organic modifiers-methanol, THF and ACN. It was found that these combinations are not suitable for the separation of the flavonoid standards. Therefore, none of these mobile phases was considered for further study.

Table 4.10: Solvent selectivity experiment in HPLC analysis of flavonoids

Trial No.	**Mobile phase**				**Injection volume (µL)**	**Flow rate ml/min**
	ACN	Methanol	THF	Buffer (5mM)		
1	-	45	-	55	20	1
2	40	-	-	60	20	1
4	-	-	30	70	20	1
5	50 (ACN:Buffer. 40:60, v/v):50 (THF:Buffer 30:70, v/v)				20	1
6	50 (MeOH:Buffer 45:55, v/v):50 (THF:Buffer 30:70, v/v)				20	1
7	50 (ACN:Buffer. 40:60, v/v): 50 (MeOH:Buffer 45:55, v/v)				20	1
8	(ACN:Buffer. 40:60, v/v): (THF:Buffer 30:70, v/v): (MeOH:Buffer 45:55, v/v), 1:1:1, v/v/v				20	1

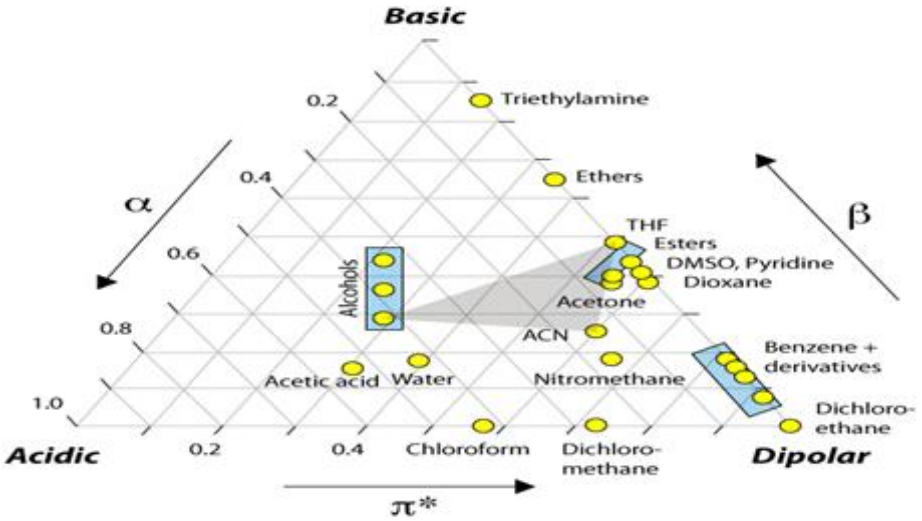


Figure 4.5: Classification of solvent properties (Dolan 2010)

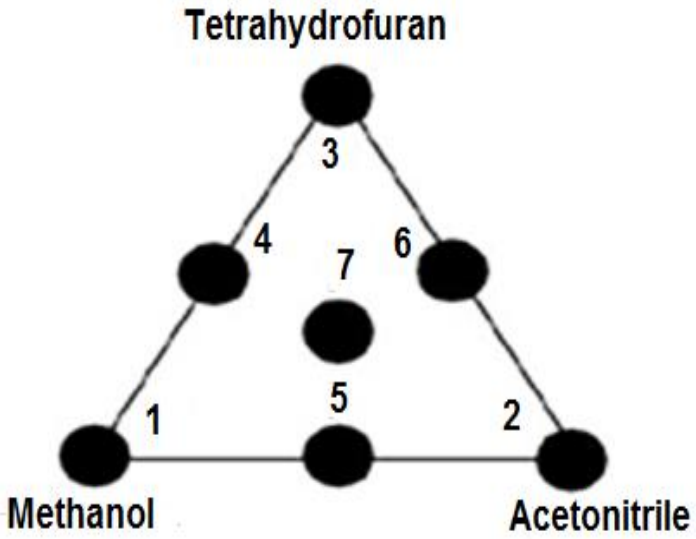


Figure 4.6: Solvent selectivity triangle (Dolan 2010)

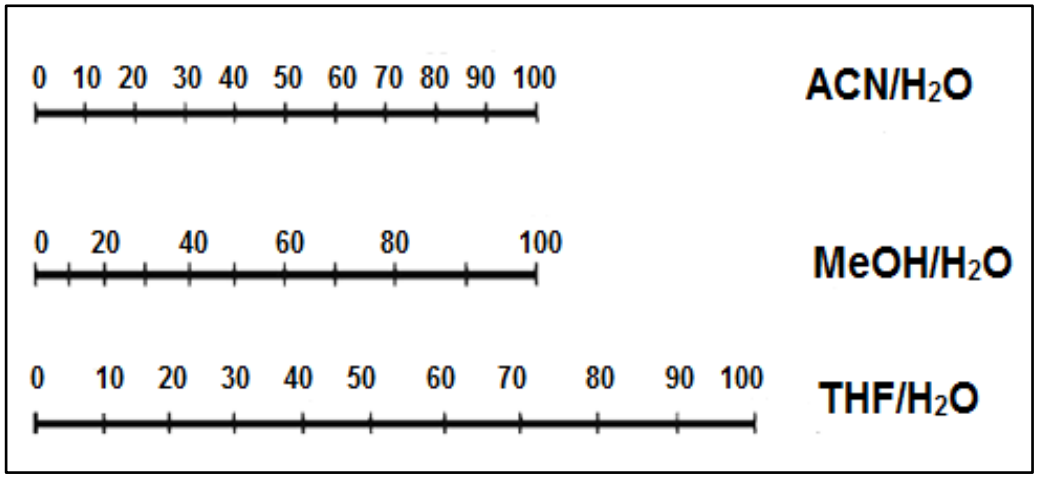


Figure 4.7: Nomograph (Dolan 2010)

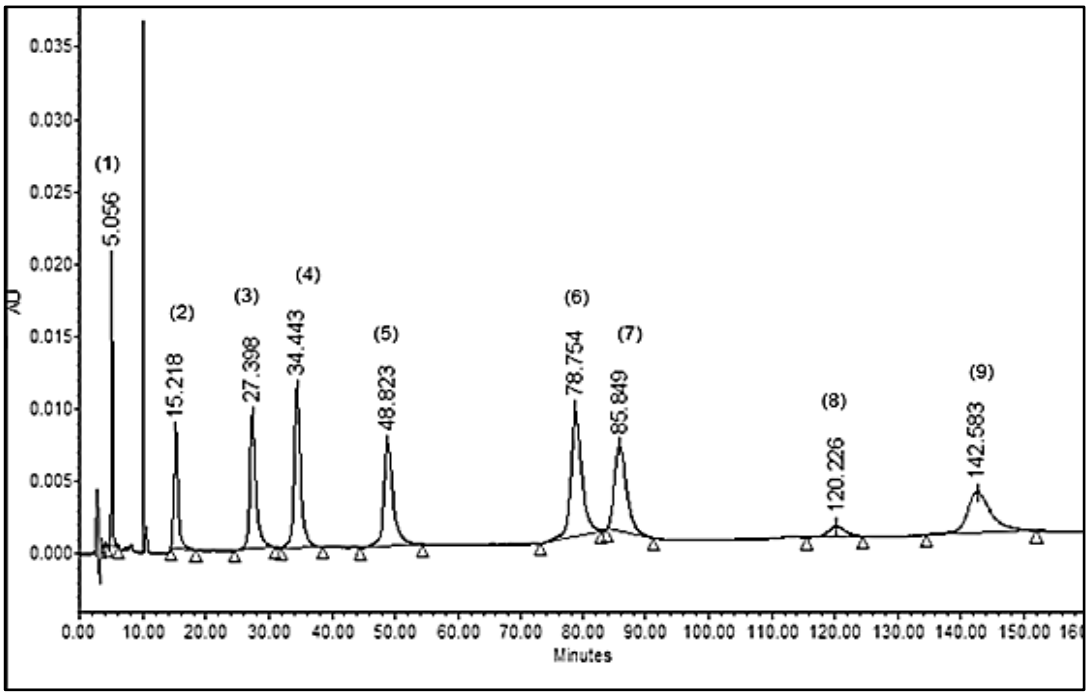


Figure 4.8: Chromatogram of a mixture of ten flavonoid standards. Chromatographic conditions: Mobile phase (ACN: Buffer (40/60 v/v) 1:1 MeOH: Buffer(45/55 v/v) :1THF :Buffer (30/70 v/v); temperature 25° C; injection volume 20 µl; flow rate 1 ml/min.

Peak 1: Rutin, Peak 2: Myricetin, Peak 3: Quercetin, Peak 4: Apigenin, Peak 5: Kaempferol, Peak 6: Chrysin, Peak 7: Pinocembrin, Peak 8: CAPE, Peak 9: Galangin.

Therefore, gradient elution mode was considered in an attempt to separate the ten flavonoid standards.

4.2.2.6 Gradient method

After demonstration of all the above isocratic experiments, gradient elution in HPLC was taken into consideration. The method reported by Pietta et al. (2002) was adapted and various solvent combinations and proportions were examined (Table 4.11).

It was found that none of the mentioned conditions (Table 4.11) was able to separate the standards, which may be due to the fact that these standards were sensitive to the continuous variation of ACN (Pellati et al. 2011). Hence, gradient elution was not suitable for the separation of flavonoids in propolis. Therefore, the isocratic method was again adapted in a proposed study for further trials.

Table 4.11: Gradient elution conditions were used for the analysis of flavonoids

Trial No.	Mobile phase		Time in minutes	Injection volume (µL)	Flow rate ml/min	Temperature ˚C
	ACN	Buffer (30mM)				
1	10-30% 40-45% 70-30%	90-70% 60-55% 70-30%	0-40 40-50 50-60	50	1	25
2	10-40% 40-70% 70-30%	90-60% 60-30% 30-70%	0-20 20-35 35-140	50	1	25
3	10-40% 40-70% 70-60% 60-30%	90-60% 60-30% 30-40% 40-70%	0-20 20-35 35-60 60-70	50	1	25
4	10-30%	90-70%	0-100	50	1	25
6	10-50%	90-60%	0-100	50	1	25
7	10-60%	90-60%	0-100	50	1	25
8	10-70%	90-60%	0-100	50	1	25
9	10-80%	90-60%	0-100	50	1	25

4.2.2.7 Method development using methanol in mobile phase preparation

From the selectivity study, it was noticed that the mobile phase prepared from methanol and buffer gave slightly improved separation compared to acetonitrile with buffer. Therefore, it was decided to optimise this condition further (Table 4.12).

The first two trials with minimum concentration of the methanol produced a chromatogram with very long run time and with noisy baseline. The gradual decrease in run time was found due to the concentration increase of methanol. This is due to an organic modifier, which modifies the elution strength of mobile phase and hence decreases the run time of all flavonoid standards. The mobile phase of (60:40 v/v) methanol/buffer showed elution of all ten standards, however, significant peak overlap was observed between the first two peaks; 3rd and 4th peaks and between 8th and 9th peaks (Figure 4.9). A similar trend was observed for mobile phases of (70:30 v/v) methanol/buffer and (80:20 v/v) methanol/buffer condition.

Table 4.12: Ratio of methanol and buffer in the mobile phase

Trial No.	**Mobile phase**		**Flow rate ml/min**	**Temperature $^{\circ}$C**
	MeOH	Buffer (5mM)		
1	30	70	1	25
2	40	60	1	25
3	50	50	1	25
4	60	40	1	25
5	70	30	1	25
6	80	20	1	25

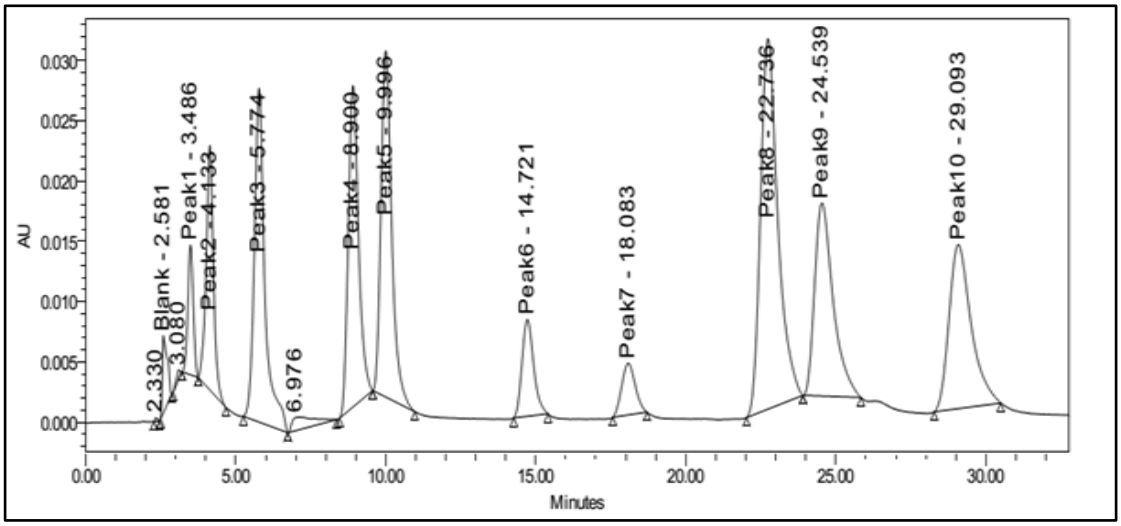


Figure 4.9: : Chromatogram of the ten flavonoids. Chromatographic conditions: mobile phase methanol/ buffer (60:40 v/v); column temperature 25°C; injection volume 20 µl; flow rate 1 ml/min.

Peak 1: Rutin, Peak 2: Myricetin, Peak 3: Quercetin, Peak 4: Apigenin, Peak 5: Kaempferol, Peak 6: Pinocembrin, Peak 7: CAPE, Peak 8: Chrysin, Peak 9: Galangin, Peak 10: Acacetin.

Mobile phase of methanol/buffer (50:50, v/v) gave the best separation with a base line resolution and appropriate peaks shape. Although run time was up to 75 mins, all flavonoid standards were well separated (Figure 4.10). The elution order was as follows, rutin (RT 4.1 minutes); myricetin (RT 5.3 minutes); quercetin (RT 9.1 minutes); kaempferol (RT 16.7 minutes); apigenin (RT 18.9 minutes); pinocembrin (RT 32.9 minutes); CAPE (RT 49.8 minutes); chrysin (RT 52.5 minutes); galangin (RT 60.1minutes) and acacetin (RT 70.6 minutes). These results were found reproducible.

To reduce total run time, some trials were carried out by changing temperature, and 28ºC temperature condition was optimised. Therefore, the optimum separation conditions are as follows, Mobile phase:(50:50, v/v) methanol:5mM sodium phosphate buffer (pH 3),Column temperature: 28ºC, Injection volume :20 µl, Flow rate:1 ml/min, Wavelength: 265nm.

Many compounds were tested for selection of internal standards using the optimised analytical condition. Some of these compounds are nicotinamide, carbamazepine, salmeterol, nifedipine, ibuprofen, isoniazid, and formoterol. Some compounds such as isoniazid, ibuprofen, carbamazepine etc eluted very early and hence showed co-elution with either solvent front or with first peak of rutin. Other compounds eluted in between flavonoid standards, but were overlapping. Hence, none of the tested compounds was selected as an internal standard for further validation process.

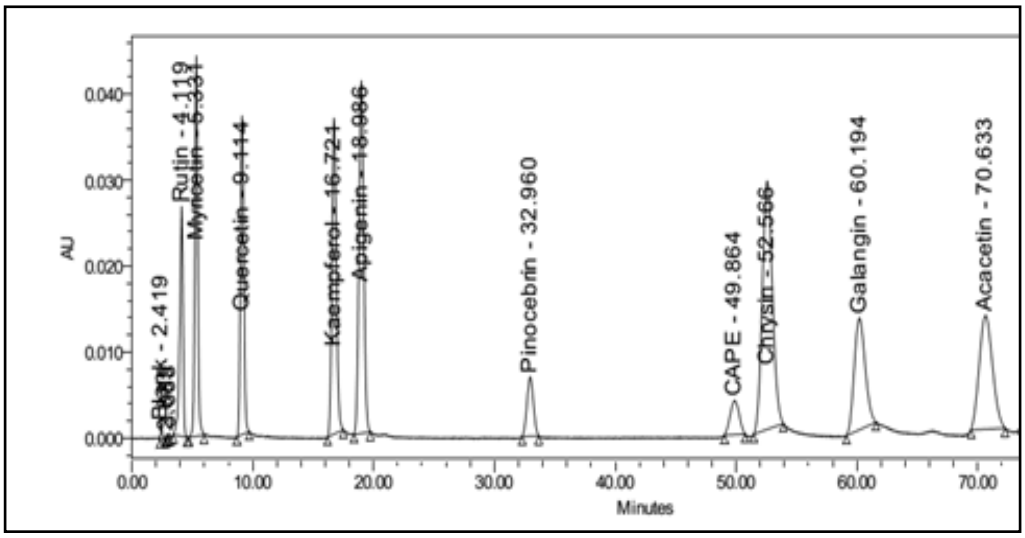


Figure 4.10: Chromatogram of the ten flavonoids rutin, myricetin, quercetin, apigenin, kaempferol, pinocembrin, CAPE, chrysin, galangin and acacetin. Chromatographic conditions: mobile phase methanol/buffer (50:50, v/v); column temperature 28°C; injection volume 20 µl; flow rate 1 ml/min.

4.2.2.8 Summary

In RP-HPLC studies, for the development of analytical method of flavonoids from propolis, showed that the main organic modifier, which can be used for such studies are methanol and acetonitrile form, from which selection may vary depending on which flavonoids are going to be studied. In our proposed study, methanol was found more suitable because it gave better peak properties and

peak separation. Besides this, use of more than one modifier in mobile phase could not improve the separation of flavonoid standards, which were assessed extensively using solvent selectivity. The addition of buffer in the mobile phase helped to maintain pH. Isocratic elution was found more suitable compared to gradient elution for the separation of ten selected flavonoid standards. The optimum temperature was found to be 28°C after examining a range of temperature 20-45°C. The wavelength was set at 265nm throughout the studies. The only disadvantage was the long run time, which induced further method development studies using different technologies and therefore most similar to HPLC, UPLC technique was selected for the next experiments.

4.2.3 Method development for the analysis of flavonoids using UPLC technique

UPLC is an advanced liquid chromatography technique, which can provide the best resolution and sensitivity, the particle size of the column is reduced. Diverse trials including isocratic and gradient flow were tried with UPLC using sodium phosphate buffer (5mM) with either methanol or acetonitrile. The first trial includes the use of methanol with buffer similar to conventional HPLC optimum method of 50:50 (v/v), the proportion was tried using a flow rate of 0.4ml/min and injection volume of 2.5 µl typical operating condition of flow rate and injection volume for UPLC. The results showed eight peaks with a very tiny peak area with very scanty area values (Figure 4.11). Improper identification and close elution of resulting peaks was observed using this isocratic method, even with changing proportion of methanol content in the mobile phase. Hence, gradient elution was considered.

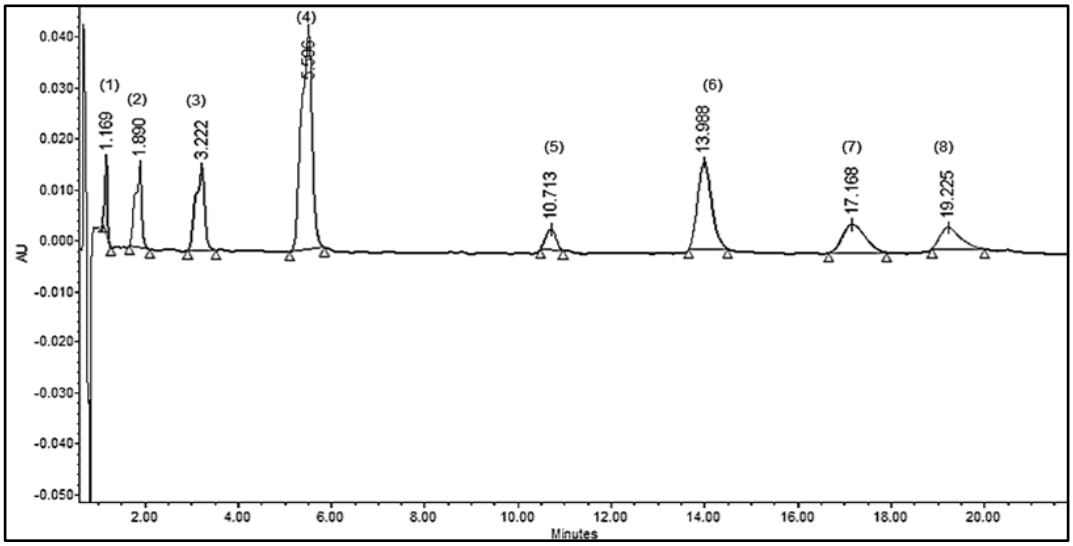


Figure 4.11: UPLC chromatogram showing elution of flavonoid standards; isocratic flow sodium phosphate buffer pH 3 and methanol (50:50, v/v); injection volume 2.5 µl; flow rate 0.4ml/min; wavelength 265nm.

Peak 1: Rutin, Peak 2: Myricetin, Peak 3: Quercetin, Peak 4: Apigenin and Kaempferol, Peak 5: Pinocembrin, Peak 6: Chrysin and CAPE, Peak 7: Acacetin, Peak 8: Galangin.

Experiments using gradient UPLC are briefly described in the following table 4.13.

Table 4.13: Gradient elution studies of UPLC for development of analytical method of flavonoids using methanol

Trial No	Mobile phase		Run time in mins	Temperature ° C
	Buffer	MeOH		
1	90-10	10-90	0-20	28
2	95-10	5-95	0-20	28
3	90-10 10-90 90	10-90 90-10 10	0-20 20-25 25-30	28
4	90-10 10-90 90	10-90 90-10 10	0-25 25-30 30-35	28
5	90-10 10-90 90	10-90 90-10 10	0-30 30-35 35-40	28
6	90-10 10-90 90	10-90 90-10 10	0-25 25-30 30-35	35

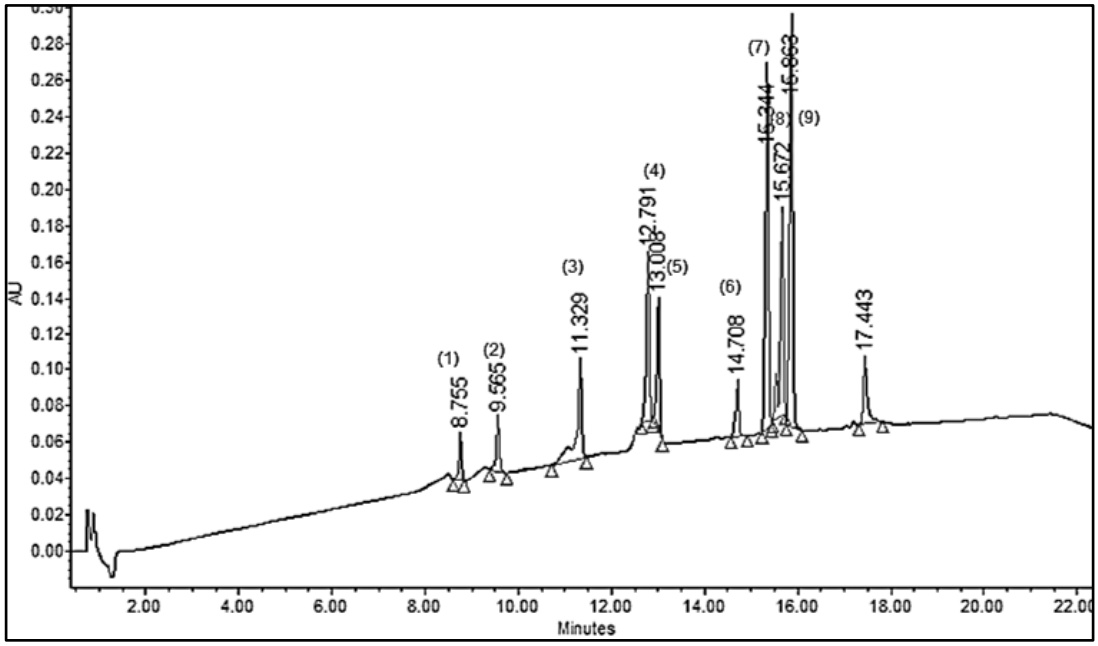


Figure 4.12: UPLC chromatogram showing elution of flavonoid standards; gradient flow from 90 -10 sodium phosphate buffer pH 3 and 10-90 methanol for first 20 minutes; injection volume 2.5 µl; flow rate 0.4ml/min; wavelength 265nm.

Peak 1: Rutin, Peak 2: Myricetin, Peak 3: Quercetin, Peak 4: Kaempferol, Peak 5: Apigenin, Peak 6: Pinocembrin, Peak 7: Chrysin, Peak 8: CAPE and Acacetin, Peak 9: Galangin.

First linear gradient showed elution of five peaks of standards with co-elution of most of the analytes. The second linear gradients failed to elute any of the peaks in total run time of 20 minutes. In the last four trials, the first three peaks were separated with good resolution but next two and last five peaks eluted very close with poor peak resolution. Even the change in run time at each step of the gradient did not result in good chromatographic separation. In the last trial, one of the previous trial (trial number 4) was repeated using high column temperature but it showed rather negative effect on peak separation. The example of one of the chromatogram from all trails of UPLC is shown if figure 4.12, gradient flow of from 90 -10 sodium phosphate buffer pH 3 and 10-90

methanol for first 20 minutes. Close elution of most of the late eluted standards is clearly seen in the chromatogram.

As compared to HPLC, the problem of overlapping between two adjacent standard peaks was found more adverse in UPLC trials. Replacing the solvent methanol with acetonitrile did not improve the resolution (Table 4.14).

Table 4.14: Gradient elution studies of UPLC for development of analytical method of flavonoids using acetonitrile

Trial No	Mobile phase		Run time in mins
	Buffer	ACN	
1	90-10 10-90 90	10-90 90-10 10	0-25 25-30 30-35
2	90-10 10-90 90	10-90 90-10 10	0-20 20-23 23-26
3	90-10 10-90 90	10-90 90-10 10	0-25 25-28 28-30

The results obtained by using acetonitrile showed similar problems of close elution as in the methanol trial. The last few peaks elute close, without any resolution and hence this work was not studied further.

Overall, this advanced liquid chromatography technique was not suitable for flavonoids. The prime element for this is analogous chemical structure and solubility properties of chosen flavonoids.

4.2.3.1 Summary

Both elution patterns either isocratic or gradient using either methanol or acetonitrile, was unable to separate peaks of flavonoid standards. Due to the failure of the UPLC studies, another technique was chosen. Microemulsion LC was not used earlier for such studies in propolis and it proves promising in

analysis of complex biological samples, therefore it was selected for further investigation.

4.2.4 Analysis and method development of flavonoid microemulsion liquid chromatography (MELC) technique

Extensive use of microemulsions in pharmaceutical applications is very common. Microemulsions' popularly is used in drug delivery systems by improving therapeutic activity because it enhances solubilisation and improves dissolution rate of poorly soluble drugs (Althanyan et al. 2011; Fanun 2012). The use of MELC in pharmaceutical analysis is affecting a growing interest. The oil in water microemulsions is mainly used in the reverse phase HPLC, which has many advantages such as unique selectivity; robustness to solvent changes and temperature; improved resolution compared to HPLC; no requirement of gradient elution hence avoids problem of irreproducibility; separation of both types of hydrophilic and lipophilic compounds and analysis of compounds without chromophores, which are detected at low UV wavelength such as 190nm (El-Sherbiny et al. 2003; Marsh et al. 2005; Ryan et al. 2013).

4.2.4.1 Microemulsion mobile phase selection

One of the recently improved microemulsion phases was selected as a starting point for this experiment. The mobile phase was prepared in the following proportions of each, 3.5% Brij-35 (surfactant); 3.5% 1-butanol (co-surfactant); 1.5% ethyl acetate (oily phase); and 91.5% sodium phosphate buffer. The preparation of this mobile phase is discussed in section 3.4.2. The resulting microemulsion was filtered under vacuum using 0.45 µm filter and degassed in an ultrasound bath for 15 minutes (Althanyan et al. 2013).

4.2.4.2 Effect of microemulsion mobile phase pH

The pH of a mobile phase affects retention and separation of the flavonoids, which depends upon the pKa value of each flavonoid. Two levels of pH low (pH 3) and high (pH 6) were studied. It was observed that the high pH did not significantly affect the flavonoid separation. The pKa values of flavonoids are weakly acidic, hence the low pH of mobile phase was found useful to maintain the analytes in unionised form and helps to get better peak separation and resolution. Buffer acidification using orthophosphoric acid was found more suitable to control pH stability of the microemulsion phase (Esteve-Romero et al. 2005).

4.2.4.3 Effect of co-surfactant concentration

The co-surfactant plays an important part in the formation of a stable microemulsion mobile phase. The co-surfactant molecules distribute themselves between the head groups of surfactant molecules and hence reduce intermolecular repulsive forces (Figure 2.3). This eventually reduces overall surface tension (Ryan et al. 2013).

Table 4.15: Variation of co-surfactant concentration in MELC analysis of flavonoids

Microemulsion mobile phase	**(%w/w)**	**(%w/w)**
Sodium phosphate buffer	91.5%	89.5%
Surfactant; Brij-35 gel	3.5%	3.5%
Co-surfactant; 1 butanol	3.5%	5.5%
Oil phase; ethyl acetate	1.5%	1.5%

The effect of variation in co-surfactant (low and high) as in the range of 3.5% and 5.5%; w/w was studied (Table 4.15). It was found that the high concentration of the co-surfactant favors a decrease in retention time of

flavonoids as compared to low concentration. Increasing the concentration of organic phase increases the solubilising capacity of microemulsions and hence retention time of analytes was reduced (Marsh et al. 2005; Althanyan et al. 2013).

4.2.4.4 Effect of surfactant concentration

Brij -35 (polyoxyethylene-23 lauryl ether) is nonionic surfactant which is used in LC analysis (Memon et al. 2012). It has the ability to interact with stationary phases and absorbs on the surface of the column, hence reducing the column's surface area and altering its efficiency (Marsh et al. 2005). The variation study in surfactant concentration was carried out (Table 4.16).

Table 4.16: Effect of surfactant concentration

Microemulsion mobile phase	**(%w/w)**	**(%w/w)**
Sodium phosphate buffer	89.5%	88.5%
Surfactant; Brij-35 gel	3.5%	4.5%
Co-surfactant; 1 butanol	5.5%	5.5%
Oil phase; ethyl acetate	1.5%	1.5%

The resulting chromatogram of two concentrations, 3.5% (Figure 4.13) and 4.5% (Figure 4.14) w/w, showed a marked difference in the separation of flavonoids. It was found that retention time of flavonoids was reduced at high surfactant concentration (4.5% w/w). A noticeable effect was observed in less polar flavonoids, which could be due to their affinity to increased volume of microemulsion droplets. The difference in RTs is shown in figure 4.15.

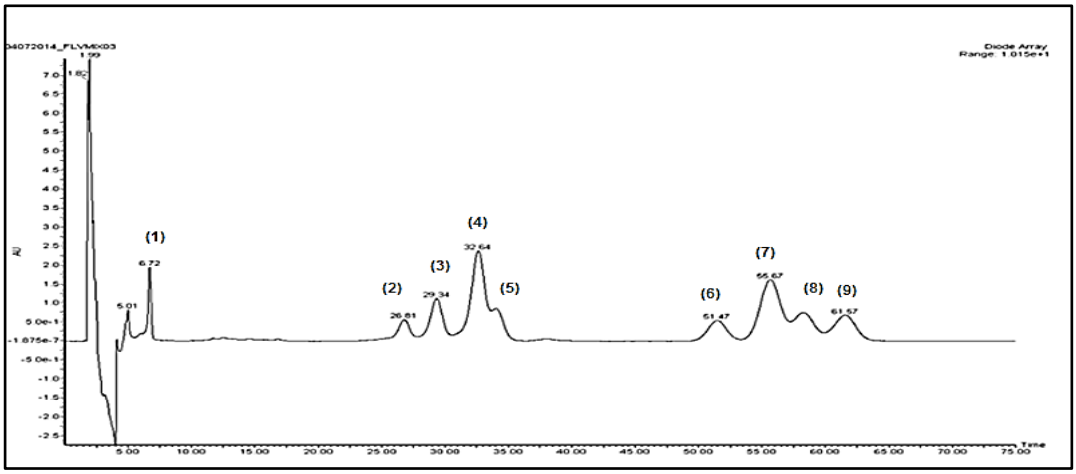


Figure 4.13: Chromatogram of test mixture of flavonoids with 3.5%w/w Brij 35. Microemulsion mobile phase composition (%w/w) 3.5% Brij-35: 5.5% 1-butanol: 1.5% ethyl acetate: 89.5% of 10mmol sodium phosphate buffer. Chromatographic conditions flow rate of mobile phase: 1ml/min, column temperature: 20 °C, injection volume: 20µl and detection wavelength 265nm.

1:Rutin, 2:Myricetin, 3:Quercetin, 4:Apigenin, 5:Kaempferol, 6:Acacetin, 7:CAPE&Chrysin, 8:Galangin, 9:Pinocembrin.

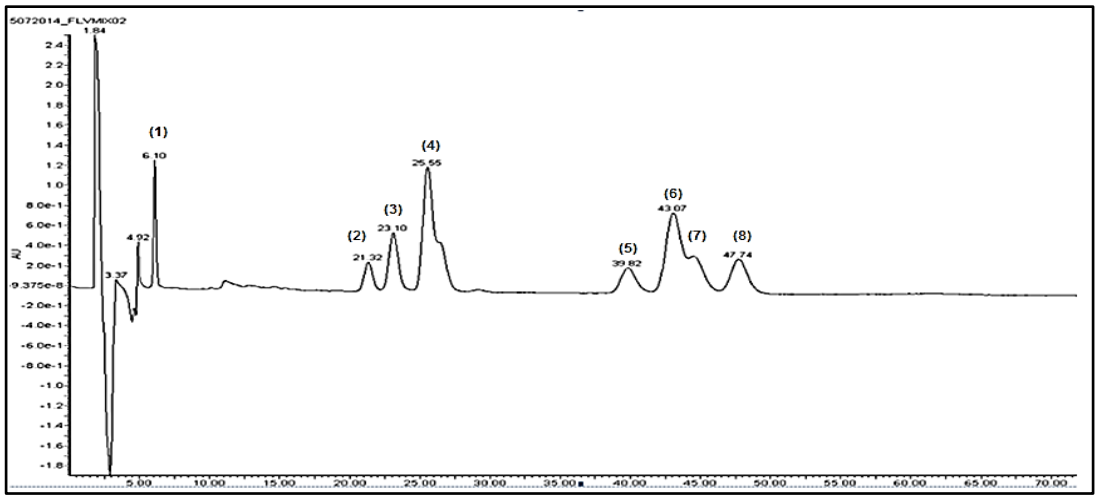


Figure 4.14: Chromatogram of test mixture of flavonoids with 4.5%w/w Brij-35. Microemulsion mobile phase composition (%w/w), 4.5% Brij-35: 5.5% 1-butanol: 1.5% ethyl acetate: 88.5% of 10mmol sodium phosphate buffer. Chromatographic conditions similar to Figure 4.13.

1:Rutin, 2:Myricetin, 3:Quercetin, 4:Apigenin and Kaempferol, 5:Acacetin, 6:CAPE&Chrysin, 7:Galangin, 8:Pinocembrin.

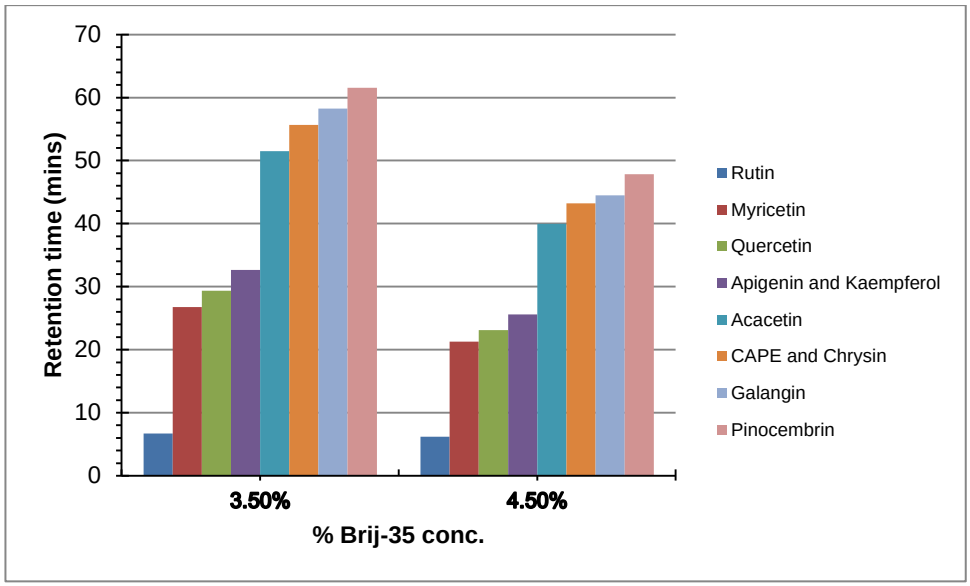


Figure 4.15: Effect of surfactant variation on separation of flavonoids.

A marked effect was observed with the less polar flavonoids with noticeable solute retention, justified by their affinity to the increased volume of microemulsion droplets (El-Sherbiny et al. 2003; Marsh et al. 2005). Better separation of flavonoid standards was achieved using surfactant concentration of 4.5% w/w compared to 3.5%w/w. Rutin, myricetin, quercetin and apigenin eluted faster in Fig.4.13 as compared to Fig.4.14 with (RT: 6.10, 21.32, 23.10, and 25.55 mins respectively). Apigenin and kaempferol peaks were merged (RT: 25.56 mins). The less polar analytes i.e. acacetin, CAPE and chrysin, pinocembrin eluted later but with significant reduction in run time RT: 39.82, 43.07 and 47.74mins respectively. Galangin was separated in Fig.4.13, whereas in Fig.4.14 it gave a merged peak with CAPE and chrysin. All flavonoids were separated with a total run time of 48 minutes due to the effect of higher surfactant concentration.

One of the drawbacks of using Brij-35 gel is that it has strong absorption on the surface of Spheroclone C-18 reverse-phase column (Ruiz-Ángel et al. 2009).

Therefore, Brij-35 gel was replaced with Brij-L23 for most of the method development.

4.2.4.5 Effect of oily phase concentration in MELC analysis of flavonoids

Oily phase is one of the influential factors in the formation of microemulsion. Increasing concentration of oil increases the number of oil droplets in microemulsion, hence favoring separation of hydrophobic compounds by reducing their interaction with stationary phase leading to less retention time. Althanyan et al. (2013) has reported the use of etheyl acetate in MELC for the determination salbutamol in metered-Dose Inhalers, therefore it was decided to use etheyl acetate in the preparation of microemulsion mobile phase in this research work. The effect of oil phase concentration was carried out as shown in the following table 4.17.

The difference in retention time of each flavonoid is shown in figure 4.16.

Table 4.17: Variation of oil concentration in MELC analysis of flavonoids

Microemulsion mobile phase	**(%w/w)**	**(%w/w)**	**(%w/w)**
Sodium phosphate buffer	90%	88.5%	88%
Surfactant; Brij-L23	4.5%	4.5%	4.5%
Co-surfactant; 1 butanol	5.5%	5.5%	5.5%
Oil phase; ethyl acetate	1%	2.5%	3%

From this figure 4.16, the decrease in the RTs of less polar flavonoids such as acacetin, CAPE and chrysin was clearly seen at higher concentration of oil phase. Galangin was not separated at low oil concentration, as well as co-elution of apigenin and kaempferol, which was also observed at the same concentration. While at high oil concentration i.e. at 3%, these peaks were well separated with better resolution between them. The hydrophilic analytes; rutin,

myricetin and quercetin, did not show much difference in their retention time at different concentrations of oil because of their affinity with aqueous phase and hence not partitioned with oily phase (Althanyan et al. 2013; Ryan et al. 2013). From these studies, it was shown that the best concentration of oil for the separation of flavonoid is 3 %.

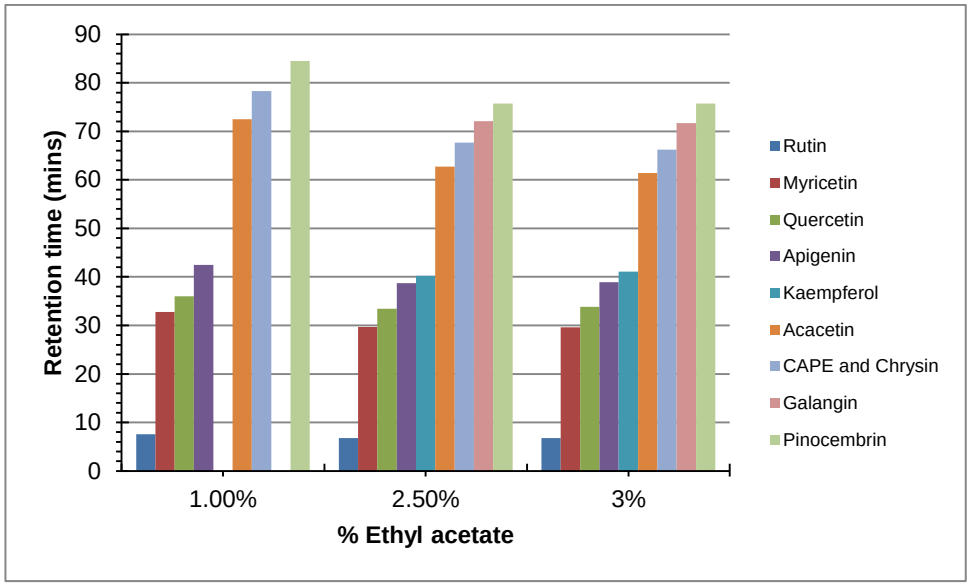


Figure 4.16: Effect of oil variation on separation of flavonoids.

4.2.4.6 Effect of Brij-L23 surfactant on separation of flavonoids in the presence of 3% of ethyl acetate

Effect of surfactant Brij-L23 on the separation of flavonoids was examined at three different concentrations 3.5%, 4.5% and 5.5% w/w. The concentration of co-surfactant 1 butanol (5.5%) was kept constant and similarly the concentration of ethyl acetate was kept at 3% w/w (Table 4.18).

Table 4.18: Effect of the concentration of Brij-L23 on flavonoid MELC

Microemulsion mobile phase	(%w/w)	(%w/w)	(%w/w)
Sodium phosphate buffer	88%	87%	86%
Surfactant; Brij-L23	3.5%	4.5%	5.5%
Co-surfactant; 1 butanol	5.5%	5.5%	5.5%
Oil phase; ethyl acetate	3%	3%	3%

Brij L23 has the capacity to alter the surface of C18 column and causes a decrease in retention time of flavonoids (Marsh et al. 2005; Ryan et al. 2013). The resulting chromatograms of studied concentrations of surfactant (Brij L23) showed better peak separation and peak resolution at 4.5% as compared to the other two concentrations (3.5% and 5.5% w/w). Brij-L23 at concentration 4.5% was chosen for the separation of flavonoid as it was able to separate the flavonoid with good resolution between the peaks (Figure 4.17).

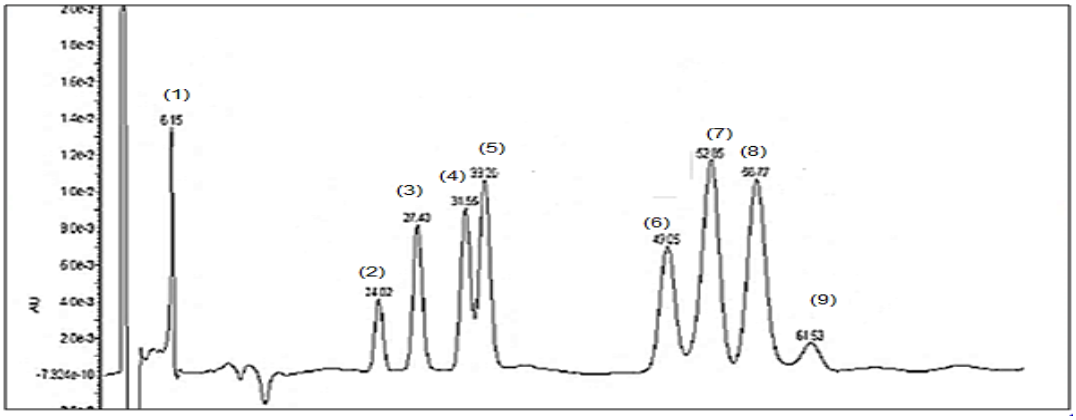


Figure 4.17: Chromatographic separation of flavonoids at: mobile phase (microemulsion of (w/w%) 4.5% Brij L23; 5.5% 1-butanol; 3% ethyl acetate; 87%10 mM phosphate buffer); flow rate 1ml/min.; column temperature 20°C; injection volume 20µl and wavelength 265nm.

Chromatographic separation: 1. Rutin; 2. Myricetin; 3. Quercetin; 4. Apigenin; 5. Kaempferol; 6. Acacetin; 7. CAPE and Chrysin; 8. Galangin and 9. Pinocembrin.

4.2.4.7 Effect of column temperature in MELC analysis of flavonoids

The effect of column temperature was assessed at four different temperatures 25°C, 30°C, 35°C and 40°C. At the optimisation of each factor in microemulsion, other factors were considered to improve chromatographic separation of flavonoids. It was reported that increasing HPLC column temperature improves separation selectivity, enhances column efficiency and hence reduces the retention time of analytes (Dolan 2002; LoBrutto and Kazakevich 2006). The retention time of flavonoids decreased with increasing column temperature. However, the temperature 40°C gave poor peak separation. Hence, 35°C temperature was chosen for the separation of flavonoids as it produced better peak resolution with total run time of 40 minutes (Figure 4.18).

4.2.4.8 Optimum microemulsion condition

Table 4.19 shows the optimum MELC condition for separation of nine flavonoid standards. However, it was not possible to separate CAPE and chrysin as both co-elute with the same retention time (Figure 4.18).

Table 4.19: Optimum MELC condition for flavonoid analysis

Mobile Phase	10Mm Sodium phosphate buffer (pH 3)-87% (w/w)
	Surfactant Brij L23 liquid- 4.5% (w/w)
	Co-surfactant 1- butanol- 5.5% (w/w)
	Oil phase ethyl acetate- 3% (w/w)
Flow rate	1ml/min
Column temperature	35°C
Injection volume	20µl

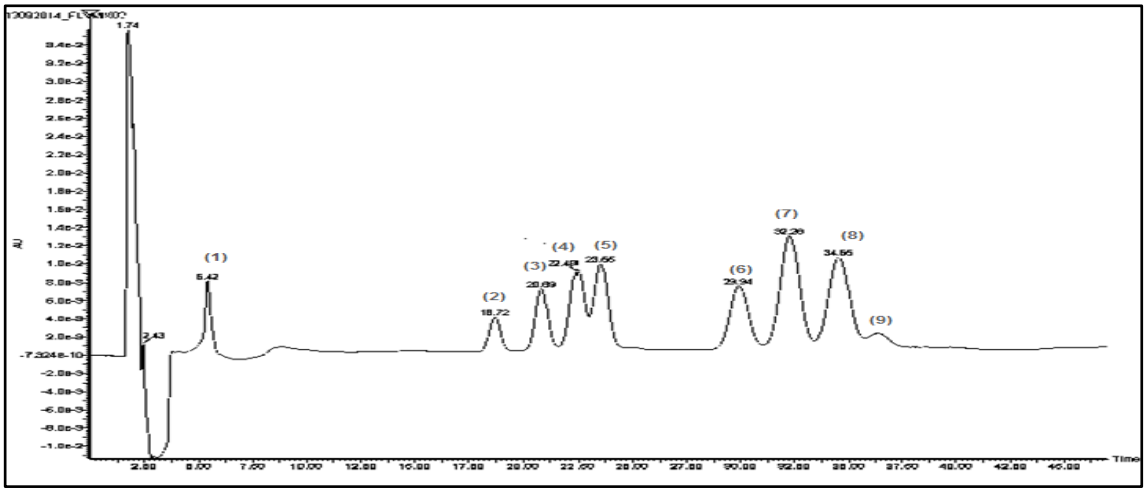


Figure 4.18: Chromatographic separation of flavonoids at: mobile phase (microemulsion of (w/w%) 4.5% Brij L23; 5.5% 1-butanol; 3% ethyl acetate; 87%10 mM phosphate buffer); flow rate 1ml/min.; column temperature 35°C; injection volume 20µl and wavelength 265nm.

Chromatographic separation: 1. Rutin; 2. Myricetin; 3. Quercetin; 4. Apigenin; 5. Kaempferol; 6. Acacetin; 7. CAPE and Chrysin; 8. Galangin and 9. Pinocembrin.

4.2.4.9 Summary

A stepwise variation for optimising MELC chromatographic conditions was carried out. The optimum condition was able to separate nine flavonoid standards with a relatively shorter run time as compared to optimised conventional HPLC method. Variation of the operating parameters; co-surfactant concentration, surfactant concentration, oil concentration and column temperature showed significant effect on the separation of flavonoids, analysis runtime and selectivity of flavonoids.

Although, MELC provided a satisfactory separation with reasonable run time of flavonoid standards, it was not considered for further studies including application to propolis pharmaceutical preparations, as it failed to separate CAPE and chrysin.

4.2.5 Selected analytical method with brief review

The optimised method using conventional HPLC technique (discussed in section 4.2.7) was considered for further studies and method development because of its ability to separate all ten flavonoid standards. Several studies were reported for extraction of flavonoids from propolis, (Coneac et al. 2008)using hot as well as cold conditions with different dilutions of ethanol and water. Methanol was used with an ultrasound assisted extraction method to extract flavonoids from propolis by Zhou et al. (2008). The decoction method with ethanol as solvent for the extraction was used by Pellati et al (2011). In the present study, for HPLC analysis, the maceration method was used as described by Cuesta-Rubio et al. (2007) using methanol as an extraction solvent before HPLC analysis. In most of the reported methods, maceration technique was used and hence it was considered in this work.

Several methods using HPLC with different mobile phases were reported for the separation of flavonoids. Several references are published regarding HPLC analysis of flavonoids with other phenolic compounds from fruits, vegetable, juices, wines, honey, propolis and plant material (Stefova et al. 2004). The most preferred solvent system for the separation of flavonoids is methanol and water followed by acetonitrile and water with the addition of acid, for example acetic acid, formic acid and phosphoric acid etc. It prevents peak tailing and improves separation of phenolic structured flavonoids (Stefova et al. 2004). Acetonitrile and water (48:52%, v/v) mobile phase was employed by Coneac et al. (2008), while methanol and 0.4% phosphoric acid (60:40%, v/v) was used by Zhou et al. (2008). Pellati et al. (2011) reported the use of a gradient elution method with 0.1% formic acid in water and acetonitrile for the separation of flavonoids from propolis. Acetonitrile with 30mM sodium phosphate buffer NaH2PO4 (pH

3) using gradient elution was used for propolis components (Pietta et al. 2002). This method was adapted in the initial trials as acetonitrile is less polar with high elution strength as compared to methanol, and hence it was thought that it would be useful for the separation of flavonoids with shorter run time. However, it failed to separate the flavonoid standards and therefore, methanol diluted with phosphate buffer was used and optimised for the separation of flavonoids (section 4.2.2.7).

RP-HPLC in combination with isocratic and gradient elution with acidic mobile phase with diode array or MS detector are most often used for flavonoid analysis. The flavonoid type varies, but in this study the following flavonoid standards rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrin, chrysin and galangin were used due to medicinal value and their common presence in propolis (Zhou et al. 2008). Two extra flavonoid standards were added CAPE and acacetin. CAPE is a potent ester that has strong anti-cancer properties (Rossi et al. 2002; Ozturk et al. 2012) while acacetin has strong antioxidant properties.

The HPLC method developed in this present study is unique and adventitious in most respects as compared to previous studies. In this method, isocratic elution was used, which is very easy to control and reproducible as compared to gradient elution. Most of the reported studies used gradient elution rather than isocratic (Pietta et al. 2002; Pellati et al. 2011), but it was difficult to reproduce any of these methods as all of them failed to separate the ten flavonoids with satisfactory resolution. In the optimised method, a mixture of methanol and 5mM NaH_2PO_4 buffer of pH 3 (50:50, v/v) was used as mobile phase with isocratic flow. Despite the long run time, the peak separation and peak

resolution is much better than the reported methods (Pietta et al. 2002; Zhou et al. 2008; Pellati et al. 2011).

4.2.6 Method validation for the reverse-phase HPLC method for analysis of flavonoids

Method validation is documentary evidence providing assurance about any developed method. As per ICH guidelines, "validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure met the requirements for the intended analytical applications" (ICH 1996). The analytical method presented in this thesis was optimised and validated in terms of selectivity, linearity, accuracy, precision, robustness and stability. All the validation procedures were carried out as per the ICH guidelines (ICH 1996).

Linearity

"Linearity is the aspect in which assay results that are directly proportional to the known concentration of the analyte" (ICH 1996). To determine linearity, at least five levels of different concentrations of standard/s or sample/s of interest with replicate measurements are required.

Six levels of concentration of standard mixture were prepared and injected in triplicate (as mentioned in section 3.3.5). The mean values for area of each peak (from 3 injections) and concentration of standards were used to plot a linearity graph. The following graphs were obtained for each standard.

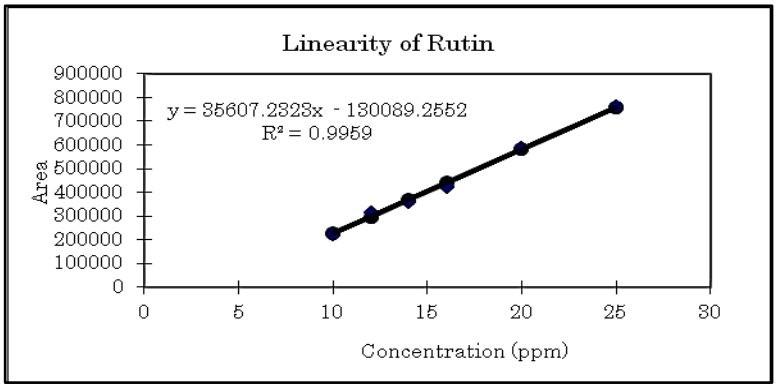


Figure 4.19: Linearity for Rutin standard in validation of flavonoids

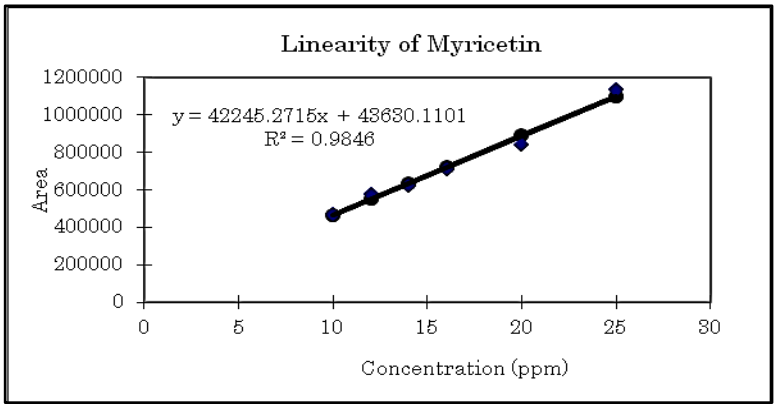


Figure 4.20: Linearity for Myricetin standard in validation of flavonoids

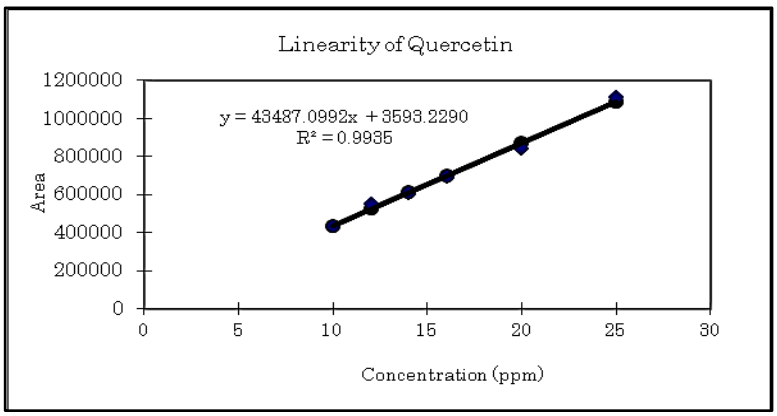


Figure 4.21: Linearity for Quercetin standard in validation of flavonoids

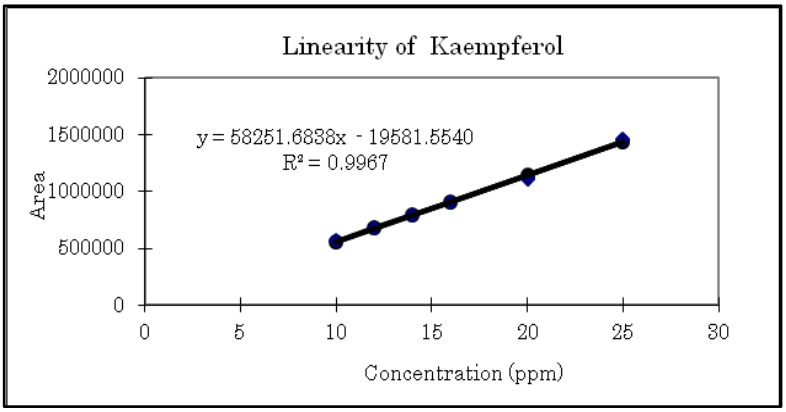


Figure 4.22: Linearity for Kaempferol standard in validation of flavonoids

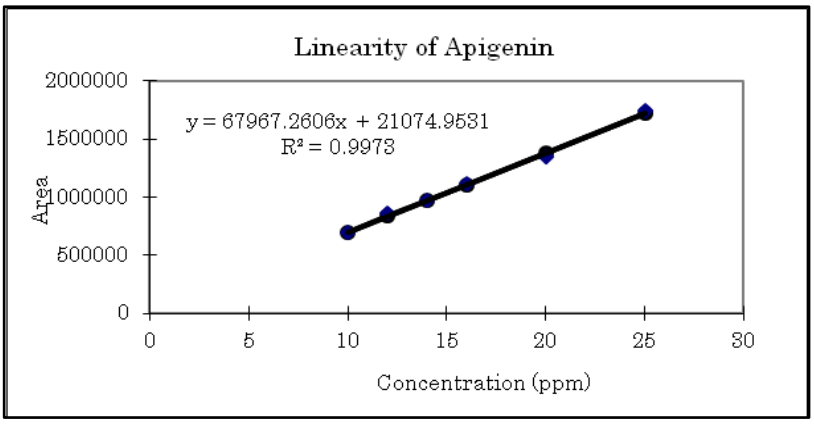


Figure 4.23: Linearity for Apigenin standard in validation of flavonoids

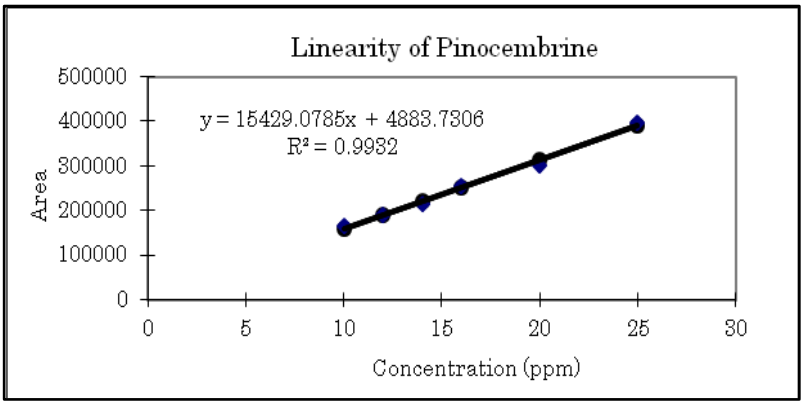


Figure 4.24: Linearity for Pinocembrin standard in validation of flavonoids

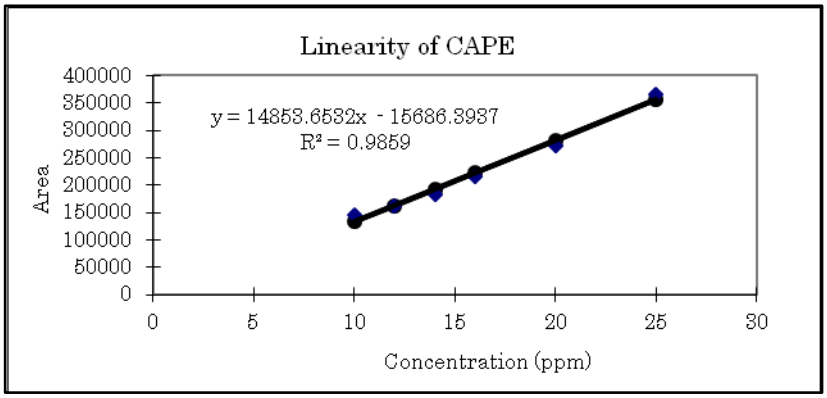


Figure 4.25: Linearity for CAPE standard in validation of flavonoids

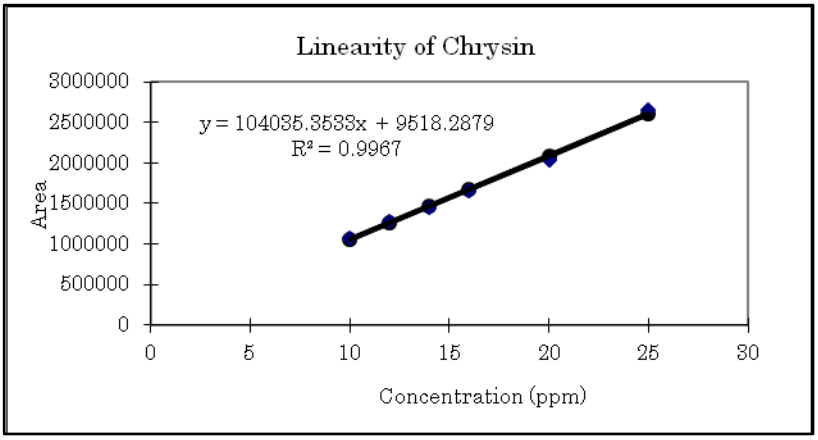


Figure 4.26: Linearity for Chrysin standard in validation of flavonoids

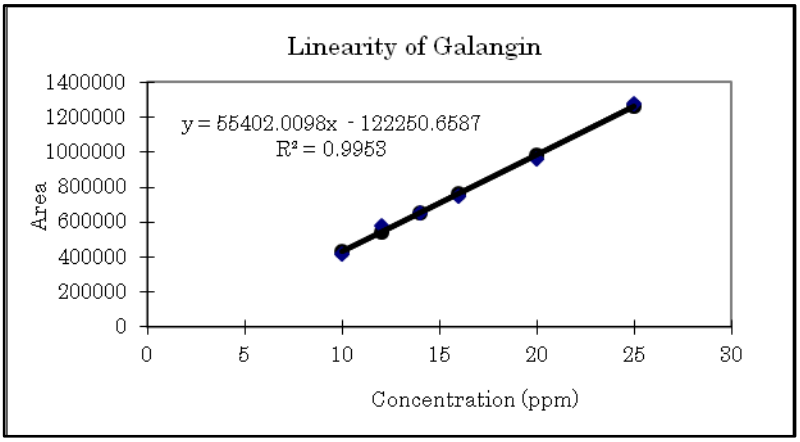


Figure 4.27: Linearity for Galangin standard in validation of flavonoids

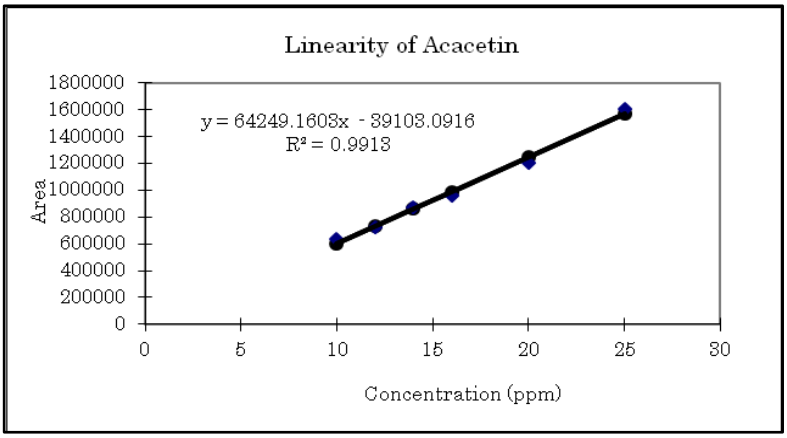


Figure 4.28: Linearity for Acacetin standard in validation of flavonoids

The regression coefficient (R^2) values are presented in table 4.20.

Table 4.20: Summary of the results for linearity experiment

Sr. No.	Name of Standard	R^2
1	Rutin	0.9959
2	Myricetin	0.9846
3	Quercetin	0.9935
4	Kaempferol	0.9967
5	Apigenin	0.9973
6	Pinocembrin	0.9932
7	CAPE	0.9859
8	Chrysin	0.9967
9	Galangin	0.9953
10	Acacetin	0.9913

HPLC method for the separation of quercetin, kaempferol, naringenin, chrysin, galangin and caffeic acid was reported by Pietta et al. (2002), the method was linear in the range of 3-80 µg/ml with R^2 0.997-0.999. In similar RP-HPLC analysis by Pellati et al.(2011) it was found that linearity (R^2) was 0.998 for tested standards such as quercetin, apigenin, kaempferol, pinocembrin, chrysin, galangin and other derivatives of CAPE. RP-HPLC fingerprints method for eight

flavonoid compounds rutin, myricetin, quercetin, apigenin, pinocembrin, chrysin and galangin was studied by Zhou et al. (2008) and used 1-500 µg/ml range for linearity studies for all standards except for galangin; where 1-1000 µg/ml range was used. The linearity R^2 was found between 0.9991-0.9999. Analysis of flavonoids using HPLC and CE techniques using linearity range of 3-200 µg/m with R^2 of 0.99 was reported (Wang et al. 2007). Similarly, flavonoid analysis of standards such as rutin, quercetin, apigenin, kaempferol, acacetin, chrysin, pinocembrin, cinnamic acid and caffeic acid was studied (Coneac et al. 2008). In this study, linearity range 10-25 µg/ml was obtained. ICH guidelines indicated that R^2 should be close to ≤1 (ICH 1996). R^2 values of the flavonoid standards in this current analytical method were between 0.984-0.997, which is very close to 1 and therefore shows a good correlation between concentrations of flavonoid standards.

Precision

According to ICH guidelines, the precision is defined as the "closeness of agreements (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed condition" (ICH 1996). RSD was calculated by multiplying SD (S) by 100 and dividing this product by average (ṡ).

$$RSD = 100 * S/\dot{s}$$

Repeatability was assessed using three different concentration levels (low, medium and high) with five injections of each concentration (preparation mentioned in 3.3.5). RSD (relative standard deviation) values for each concentration level were calculated and presented. The study was repeated over a period of five days (inter-precision). The results were expressed as % RSD and presented in table 4.21 and 4.22.

Table 4.21: Precision result for flavonoid standards: Inter-precision results

Standard	RSD		
	Concentration of mixture of standards		
	Low level	Medium level	High level
	10µg/ml	16µg/ml	25µg/ml
Rutin	1.56	1.91	0.20
Myricetin	0.55	0.46	0.22
Quercetin	0.75	0.30	0.40
Kaempferol	0.24	0.53	0.56
Apigenin	0.32	0.43	0.20
Pinocembrin	0.85	1.98	0.78
CAPE	1.84	1.98	1.26
Chrysin	1.28	0.77	0.49
Galangin	1.48	1.68	0.91
Acacetin	1.95	1.01	1.37

Table 4.22: Precision result for flavonoid standards: Intra-precision results

Standard	RSD		
	Concentration of mixture of standards		
	Low level	Medium level	High level
	10µg/ml	16µg/ml	25µg/ml
Rutin	0.82	1.58	0.18
Myricetin	0.28	0.23	0.34
Quercetin	0.78	0.06	0.53
Kaempferol	0.41	0.59	0.48
Apigenin	0.72	0.20	1.53
Pinocembrin	0.71	1.01	0.34
CAPE	1.57	0.38	1.76
Chrysin	0.92	0.70	0.35
Galangin	1.40	1.12	0.77
Acacetin	1.44	1.39	0.62

Luo et al (2011) validated an analytical method for determination of the following flavonoids in propolis quercetin, rutin, quercetrin, apigenin, kaempferol, chrysin. The RSD values for intra-day precision studies were below 2 %, while that of inter day studies was below 5%. RSD values in similar studies were found to be below 1.9 % (Pellati et al. 2011). In the current work, RSD values were found to be less than 2% and hence this method is precise according to ICH guidelines (ICH 1996).

Sensitivity

The limit of quantification and limit of detection were determined for all flavonoids and presented in tables 4.23 and 4.24.

Limit of detection

Limit of detection (LOD) defined by ICH guidelines as "lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value" (ICH 1996).

$$LOD = \quad 3.3\delta / S$$

Where- δ= standard deviation of Y intercept

S= the slope of calibration curve

LOD was calculated by injecting (n=3) six different levels of standards or samples and were repeated five times for five different groups. The results for LOD are presented in table 4.23.

The LOD values for the present method were comparable to the reported values in previous studies. Pellati et al. (2011) reported LOD or their standards between 1.6-4.6 µg/ml, which were close to values of the present study (1.96-2.16µg/ml). Zhou et al. (2008) observed values ranging between 0.1-0.2 mg/g for their studied standards while (Wang et al. 2007) obtained LOD values from 0.3-3.4 µg/ml for their standards.

Table 4.23: LOD results for flavonoid standards

Standards	LOD µg/ml
Rutin	0.96
Myricetin	2.16
Quercetin	1.27
Kaempferol	1.25
Apigenin	0.93
Pinocembrin	1.36
CAPE	1.73
Chrysin	1.14
Galangin	1.25
Acacetin	1.63

Limit of quantification

ICH guideline defines limit of quantification (LOQ) as "LOQ of individual analytical procedure as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy" (ICH 1996)

$$LOQ = 10\delta/S$$

Where- δ= standard deviation of Y intercept

S= the slope of calibration curve

Six different levels of concentrations of flavonoid standards were demonstrated for this study and were repeated five times for five different groups, results are mentioned in table 4.24.

LOQ obtained from this present study was comparable to previous studies. Pellati et al. (2011) found LOQ values ranging from 2.6-7.8 µg/ml while LOQ values ranged between 2.8-6.5 µg/ml in the present study.

Table 4.24: LOQ results for flavonoid standards

Standards	LOQ µg/ml
Rutin	2.93
Myricetin	6.57
Quercetin	3.84
Kaempferol	3.81
Apigenin	2.82
Pinocembrin	4.14
CAPE	5.25
Chrysin	3.47
Galangin	3.80
Acacetin	4.96

Accuracy

ICH defined accuracy as the 'closeness of agreement between the conventional true value and value found'. As per the guidelines, the values should be nearly 100% (ICH 1996). The accuracy of this method was assessed by adding the flavonoids into blank material at different concentrations then comparing the measured spiked concentration with the true concentration of flavonoids. To study accuracy, three experiments using three different levels of concentrations of standard mixture were demonstrated (as mentioned in section 3.3.5). The results are closer to 100% value, which indicates that this method is accurate. The results are presented below in table 4.25.

In similar flavonoids in propolis studies, Zhou et al. (2008) found an accuracy between 89.4-96.3%. Pellati et al.(2011) also studied accuracy for quercetin, kaempferol, pinocemnrin, chrysin, galangin and other flavonoids, and it was in the range of 96-105%. The accuracy results in this present study ranged from 97.14 to 102.88%, which are in the acceptable range according to ICH guidelines (ICH 1996).

Table 4.25: Accuracy Results for flavonoid

Standards		Actual concentration (µg/ml)	Observed concentration (mean ± SD., µg/ml)	% Accuracy
Rutin	Low level	10	9.91±0.14	99.17
	Medium level	16	16.02±0.12	100.14
	High Level	25	25.03±0.01	100.15
Myricetin	Low level	10	9.96±0.05	99.65
	Medium level	16	15.92±0.07	99.51
	High Level	25	25.74±0.02	102.88
Quercetin	Low level	10	10.05±0.12	100.5
	Medium level	16	15.88±0.001	99.28
	High Level	25	25.24±0.03	100.91
Kaempferol	Low level	10	10.09±0.04	100.95
	Medium level	16	15.87±0.03	99.19
	High Level	25	25.20±0.03	100.82
Apigenin	Low level	10	9.98±0.01	99.84
	Medium level	16	15.98±0.08	99.93
	High Level	25	25.13±0.07	100.54
Pinocembrin	Low level	10	10.10±0.02	101.07
	Medium level	16	15.88±0.02	99.27
	High Level	25	25.38±0.04	101.52
CAPE	Low level	10	10.05±0.05	100.54
	Medium level	16	16.01±0.28	100.05
	High Level	25	25.01±0.25	100.06
Chrysin	Low level	10	10.04±0.11	100.40
	Medium level	16	15.86±0.02	99.17
	High Level	25	25.40±0.02	101.59
Galangin	Low level	10	9.82±0.01	98.27
	Medium level	16	16.001±0.07	100.05
	High Level	25	25.54±0.65	98.16
Acacetin	Low level	10	9.72±0.01	97.15
	Medium level	16	15.81±0.17	98.84
	High Level	25	25.39±0.35	101.55

Low level-(10µg/ml), medium level- (16 µg/ml), high level (25 µg/ml).

Recovery

The recovery of the method was assessed by comparing the peak area of the extracted flavonoid with the peak area of flavonoid standards. The recovery of current validation study was found in range 97-103% (Table 4.26).

Table 4.26: Recovery results for flavonoids

Sr. No.	Standards	Low level (10µg/ml)	Medium level (16 µg/ml)	High Level (25 µg/ml)
1	Rutin	99.17	100.14	100.15
2	Myricetin	99.65	99.51	102.88
3	Quercetin	100.5	99.28	100.97
4	Kaempferol	100.95	99.19	100.82
5	Apigenin	99.84	99.93	100.54
6	Pinocembrin	101.07	99.27	101.52
7	CAPE	100.54	100.05	100.06
8	Chrysin	100.40	99.17	101.59
9	Galangin	98.27	100.05	98.16
10	Acacetin	97.15	98.84	101.55

Robustness

The definition of robustness according to ICH guidelines is “it is a measure of analytical method capacity to remain unaffected by small, but deliberate variations, in method parameters. It provides an indication of the procedures reliability during normal usage" (ICH 1996). Reliability of analytical method is studied by introducing small changes in method parameters (around ±5%) such as mobile phase proportion variation, temperature, flow rate, pH etc. The variation details and results are shown in Table 4.27.

Table 4.27: The range in robustness studies

	Variation in Chromatographic condition	Results Observed
Mobile phase variation	1) Methanol (52.5): Buffer (47.5)	Overlapping between first two peaks
	2) Methanol (47.5): Buffer (52.5)	Overlapping between 7th and 8th peak
Temperature variation	1) 29.4^{o}C	Overlapping between 7th and 8th peak
	2) 26.6^{o}C	All peaks were separated
Flow rate variation	1)1.1 ml/min	All peaks were separated
	2)0.9ml/min	All peaks were separated

The mobile phase composition, temperature and flow rate were altered to study robustness. In the mobile phase variation, buffer/ methanol (47.5:52.5, v/v) proportion gave chromatogram with all peaks well separated except the first two peaks of standard; while that of buffer /methanol (52.5:47.5, v/v) proportion, peak overlapping between 7th and 8th peaks was observed. Increasing methanol to 52.5%, led to early and co-elution of polar compounds such as rutin and myricetin, while decreases in methanol content to 47.5% led to close elution of less polar flavonoids such as CAPE and chrysin. These results show that rutin, myricetin, quercetin and kaempferol peaks were found to be more sensitive to variations in mobile phase.

Similarly, in studies with temperature variations, co-elution of peaks were observed. In the case of 29.4^{o}C column temperature, 7th (CAPE) and 8th (chrysin) peaks showed co-elution, but it was found that all peaks were separated at lower temperature i.e. at 26.6^{o}C and 28^{o}C. Therefore, CAPE and chrysin are very sensitive to high temperature practices.

In flow rate variation studies, all peaks were separated at both 0.9 ml/min. and 1.1 ml/min. Hence, this shows that the analytical method was not sensitive to changes in flow rate. At the same time, it was clearly observed that the 7th and 8th peaks of CAPE and chrysin respectively are very sensitive to mobile phase and temperature parameters. Therefore, there is a need to adhere to the optimised chromatographic conditions to achieve good resolution between all peaks, including CAPE and chrysin.

Stability

The chemical preparation of any compound can decompose at any level of laboratory practices such as during extraction, clean up and storage. Therefore, there is a need to study stability of analytes of interest. There are many different methods to check stability suggested by (ICH 1996) guidelines such as freeze and thaw stability, short-term temperature stability, long-term stability etc.

The stability of flavonoid standards was assessed using freeze and thaw procedures. Flavonoid standards and propolis samples were stored at -10^{o}C for 20 and 15 days respectively and then both standards and samples injected with freshly prepared flavonoid standards solution. Stability results of fresh and stored standards were compared. Peak areas of rutin, myricetin, keampferol and galangin distinctly decreased in stored flavonoid standards as compared to freshly prepared standards. On the other hand, the other remaining standards only show a slight change (Table 4.28).

Similarly, the stability study showed that the propolis samples are sensitive to the period of storage. For example, decreases in peak area of kaempferol in samples were found, which indicates that this compound is not stable. Other compounds such as galangin, chrysin, rutin and apigenin showed more degradation as compared to pinocembrin, CAPE and acacetin. These results

showed that the standards, as well as propolis samples, were unstable when stored for a period of 15/20 days. So, it is advisable to use these solutions within a few days after preparation.

Table 4.28: Stability results

Standards	% loss of concentration of compounds in standard solution (20 days old)	% loss of concentration of compounds in propolis sample (15 days old)
Rutin	28.99	4.18
Myricetin	19.78	2.08
Quercetin	1.34	1.89
Kaempferol	24.80	14.42
Apigenin	1.76	3.69
Pinocembrin	3.76	1.17
CAPE	18.64	0.96
Chrysin	9.01	3.26
Galangin	24.70	4.65
Acacetin	8.94	8.21

4.2.7 Application of validated RP-HPLC method to analyse propolis samples

Four types of propolis samples were used for this study including powder, capsule, liquid and tincture. The maceration technique that was described by Cuesta-Rubio et al. (2007) has been followed for the sample preparation (Section 3.4.2.4). The samples were prepared in methanol at concentration 5 µg/ml. The resulting chromatogram showed good resolution between all peaks. The peaks were identified after comparison with RTs as well as spectra of each flavonoid standard peak. The peak area of each identified peak was measured and used for further calculations. Some of the peaks were unidentified, which could indicate the presence of other types of flavonoid. The quantity of each identified flavonoid was calculated using a calibration curve. These calculations gave results in µg/ml, which are shown in table 4.29.

From the propolis sample results, it is observed that the highest amount of flavonoid is chrysin, which exists in all types of propolis. Among all flavonoids, chrysin content was found in highest amount such as 18.43 mg/ml in tincture; 15.06 mg/ml in propolis liquid; 25.55 mg/g in powder and 21.71 mg/g in capsule. Galangin was found in higher amounts next to chrysin; 27.66 mg/g in powder, 20.55 mg/g in capsule, 13.87 mg/ml in liquid and 16.73 mg/ml in tincture. Pinocembrin and rutin were found in high quantity as compared to CAPE, quercetin, apigenin, acacetin and kaempferol (Table 4.29). CAPE is a very important constituent of propolis with the highest medicinal value concern, but this study shows that CAPE is present in much less quantities in all types of propolis preparation compared to other known flavonoids. The CAPE quantity was found in powder (2.73 mg/g), 1.11 mg/g in capsule; 0.62 mg/ml in liquid and 1.04 mg/ml in tincture sample. Apigenin, quercetin, acacetin and kaempferol quantities in all types of propolis preparation were much less as compared to other known compounds and the results ranged between 0.4-3.0 mg per ml in liquid samples and 0.4-3.0 mg/g in powder samples.

Table 4.29: Recovery of flavonoids from propolis preparation

	mg/g Powder ±SD	mg/g Capsule ±SD	mg/ml Liquid ±SD	mg/ml Tincture ±SD
Rutin	17.16±0.43	15.81±0.03	2.99±0.01	3.72±0.01
Quercetin	2.29±0.33	2.03±0.09	1.20±0.01	1.32±0.03
Kaempferol	2.64±0.05	1.74±0.05	1.24±0.02	1.08±0.04
Apigenin	2.29±0.20	1.87±0.08	1.32±0.04	1.50±0.11
Pinocembrin	26.93±0.91	22.12±0.40	12.12±0.21	15.55±0.17
CAPE	2.73±0.18	1.11±0.01	0.62±0.01	1.04±0.17
Chrysin	25.55±0.09	21.71±0.05	15.06±0.02	18.43±0.04
Galangin	27.66±0.16	20.55±0.53	13.87±0.28	16.73±0.65
Acacetin	1.84±0.11	1.60±0.10	0.66±0.05	0.59±0.01

The chromatograms for the propolis samples are shown in figure 4.29-4.32. Nine of the ten flavonoid standards were identified but at the same time, a number of unidentified peaks were also found. Flavonoids from propolis samples were identified by comparing RT and spectra with standards flavonoid peaks. These chromatograms separate all unidentified as well as identified eluents with good resolution, which is to distinguish the advantage of the present analytical method compared to all reported methods. All four chromatograms of four types of propolis are similar in their peak pattern. The first identified peak was of rutin (RT 3.9 minutes), which was followed by second identified peak of quercetin (RT 11 minutes). The third and fourth identified peaks eluted consequently were of kaempferol (RT 16.5 minutes) and apigenin (RT 18 minutes) respectively. The fifth identified peak was of pinocembrin (RT 32 minutes). The sixth peak of identified compound was of CAPE (RT 49.5 minutes). The seventh peak was of chrysin (RT 51 minutes), which was followed by the eighth identified peak of galangin (RT 58.5 minutes). The last identified peak was of acacetin, which was eluted very last at RT 68 minutes.

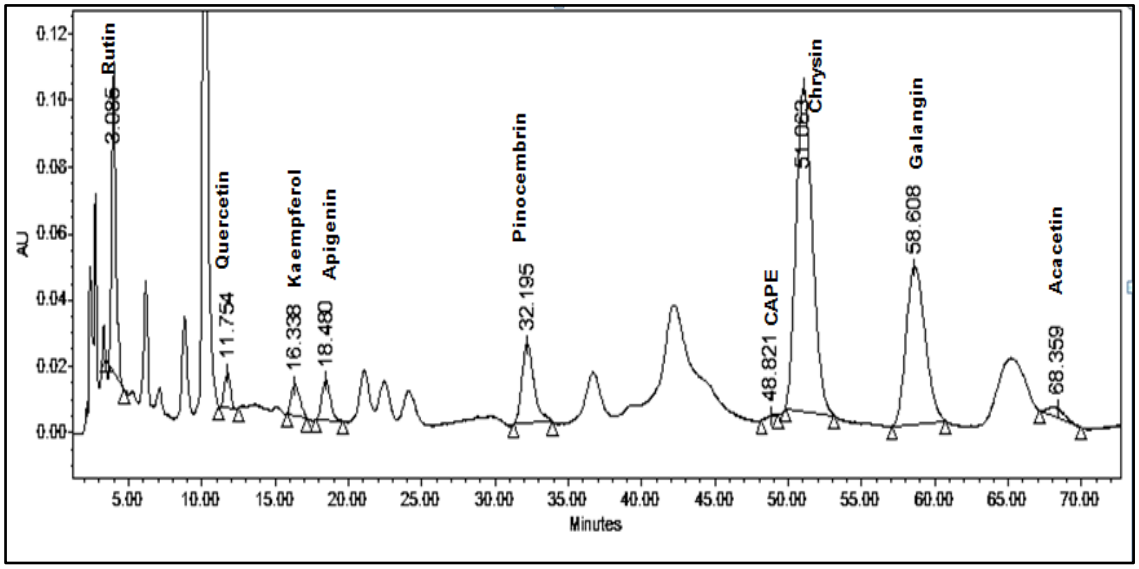


Figure 4.29: Chromatogram of propolis powder

Chromatographic conditions: mobile phase methanol/ buffer (50:50 v/v); column temperature 28°C; injection volume 20 µl; flow rate 1 ml/min

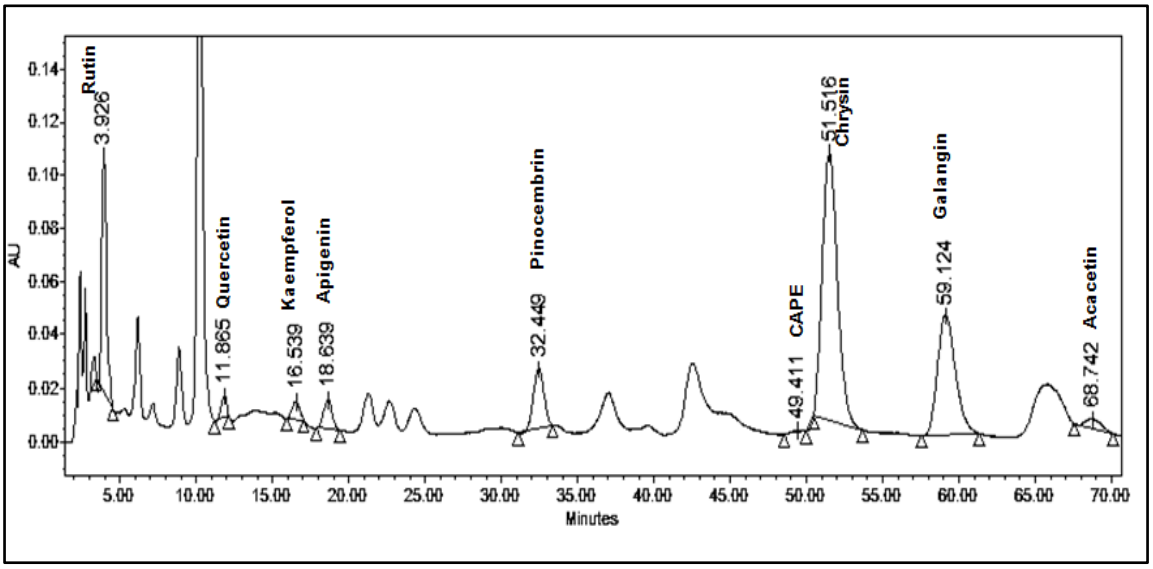


Figure 4.30: Chromatogram of propolis capsule. Chromatographic conditions: same as figure 4.29

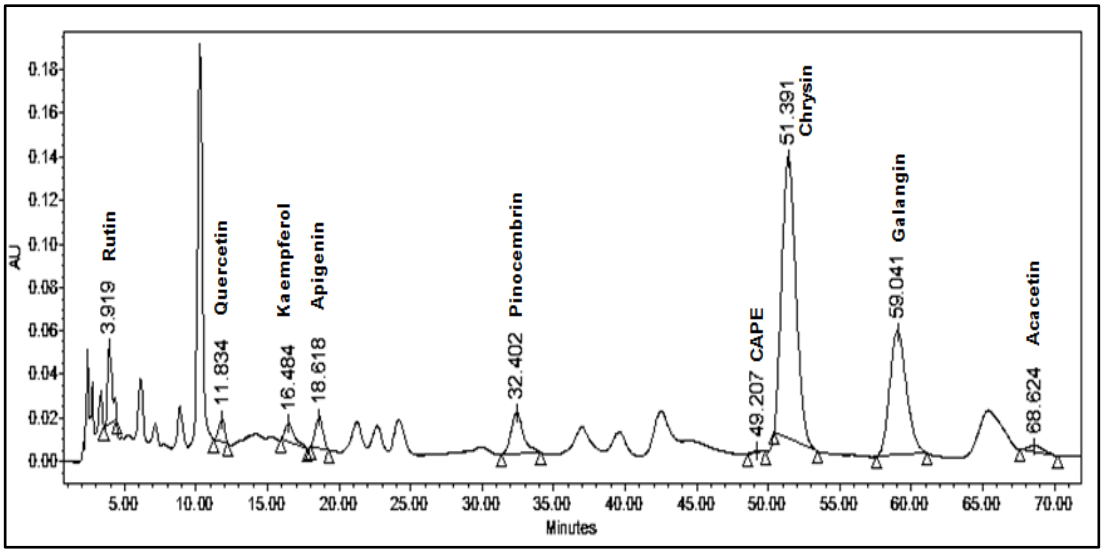


Figure 4.31: Chromatogram of propolis liquid. Chromatographic conditions: same as figure 4.29

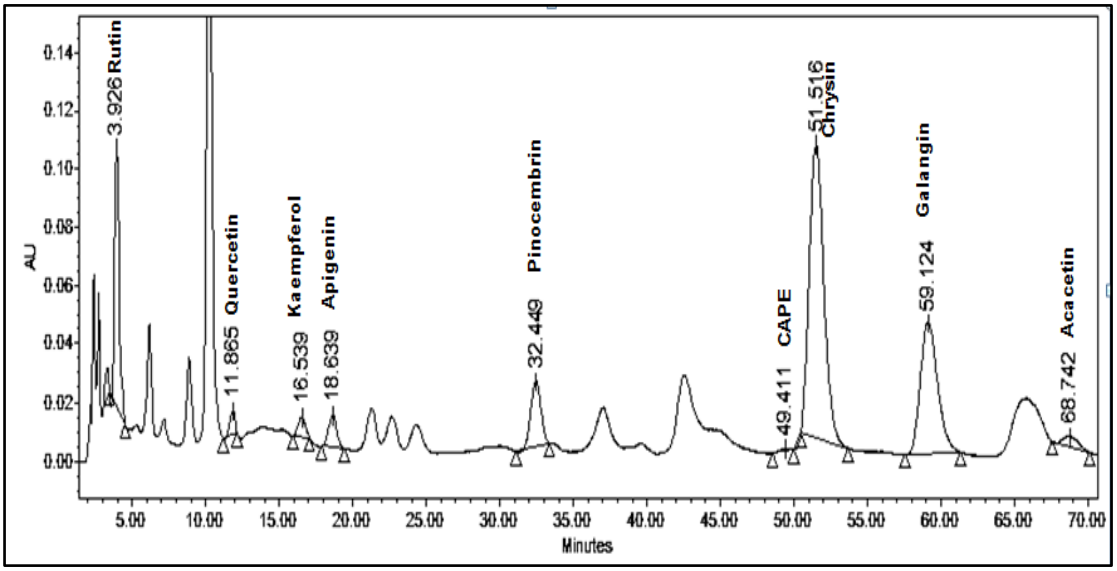


Figure 4.32: Chromatogram of propolis tincture. Chromatographic conditions: same as figure 4.29

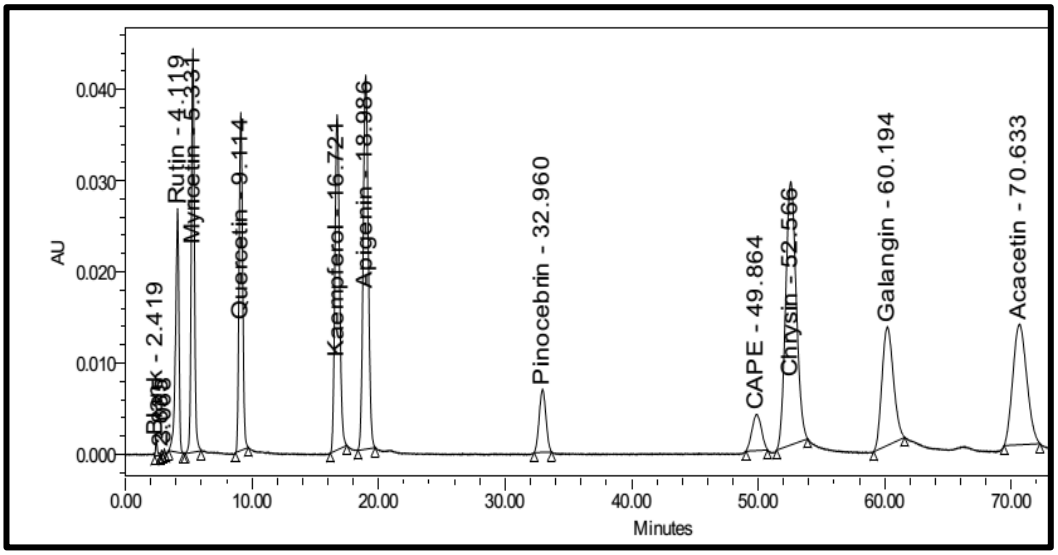


Figure 4.33: Chromatogram of Standard mixture of 25µg/ml concentration. Chromatographic conditions: same as figure 4.29

Many researchers also studied propolis samples to quantify flavonoid contents using HPLC technique. Some of their works are discussed here in brief. Repollés et al. (2006) studied different food products with propolis to check availability of flavonoids. They found the highest amount of flavonoids present in propolis as compared to other food products like green tea, red wine, orange peel and pulp and *Ginkgo biloba.* They found the highest presence of chrysin followed by galangin, naringenin and kaempferol. However, they could not detect other flavonoid compounds including quercetin in propolis samples. Their findings were very similar to this thesis, for example, they found that chrysin quantity was highest in propolis as compared to other compounds. But galangin and kaempferol quantities were much less compared to chrysin. In contrast, the quercetin content in a propolis sample of the present study was comparable to other compounds.

Coneac et al. (2008) studied different types of propolis extraction procedure using ethanol and waters (hot and cold). They found that hot ethanolic extracts

(60% and 90% ethanolic concentration) gave better results as compared to other methods. They found the chrysin content was so high (39.79 mg/g) at 60%, hot ethanolic extract and (30.22 mg/g) at 96% hot ethanolic extracts of propolis extract respectively, which followed by apigenin, but its quantity was very low (less than half of chrysin content). These results of chrysin content are similar to results of this present study where chrysin had the highest quantity found in all assessed propolis preparations. Pietta et al. (2002) studied a range of propolis types mainly market products like sprays, tablets and syrups. They obtained varied types of results for flavonoid contents in propolis. The contents of flavonoids in propolis were as follows, quercetin (0.001-1.26 mg/ml), kaempferol (0.03-0.43 mg/ml), chrysin (0.19-6.32 mg/ml), pinocembrin (0.24-7.23 mg/ml), and galangin (0.02-2.87 mg/ml). Zhou et al. (2008) studied huge number of propolis types (around 120 samples) from different provinces of China and they distinguished them by chromatogram fingerprinting. They reported that the absence or presence of any particular flavonoid standard in any sample is due to the geographical origin of that particular propolis type. However, CAPE and acacetin were not included in their study.

4.2.8 Summary

Several experiments including use of different analytical techniques end up in optimisation of RP-HPLC method. This method was found useful in separation of all ten flavonoid standards by achieving better resolution between them. This optimised chromatographic condition was validated by following ICH guidelines and found the resulting data acceptable in terms of those guidelines.

5. DETERMINATION OF ANTIBIOTICS FROM PROPOLIS

The pollution and possible contamination in food items are leading to a serious issue. This manmade issue was initially designed to treat diseases of crops, but it now affects the whole crop in terms of quality due to the contamination. The use of pesticide/ antibiotic/ insecticide treatments, treatment overdoses and other hazardous chemicals leads to harm the quality of the crop yield. Propolis is a natural honey bee product, and the chances of such contaminations are very high because of a direct and indirect exposure to the pollutants. Beekeepers practiced regular usage of these chemicals to keep away diseases from insects, bacteria and fungi (Al-Waili et al. 2012; Berry et al. 2013). A variety of these hazardous chemicals such as pesticides, antibiotics etc have been found in different honey bee products including honey (Mullin et al. 2010; Pareja et al. 2011; Al-Waili et al. 2012). The application of antibiotics is also very common in apiaries to avoid bacterial diseases (Al-Waili et al. 2012). Hence, propolis can become easily contaminated with antibiotic traces due to a direct application of the antibiotics. Contaminated food has traces of hazardous chemicals and also leads to potential health problems. Consequently, quality control and analysis of such residues is a necessary pre-requisite for obtaining pure and contamination free products. As a result, the analysis of traces of the antibiotics in raw and processed propolis is necessary.

There have been numerous cross–sectional studies of the analysis of antibiotics from honey but only a few attempts have been done in the case of propolis. The sample preparation and extraction of antibiotics from propolis is very challenging, compared to honey. The possible reason for this is the complex nature of its constituents, including wax, resin, polyphenols and aromatic compounds. The analysis of antibiotics in propolis is more difficult and tedious, especially when considering the process of clean-up methods followed by

actual analysis. Unfortunately, only one reference is published where the extraction and analysis of the tetracycline group of chemicals from propolis, following a two-step SPE method and RP-HPLC analysis, have been done (Zhou et al. 2009). The drawback of this reported method is long and tedious method. This method was initially followed in the current experimental work by selecting the same antibiotics and by using the exact same procedure but using different propolis types (raw and processed). However this method failed to produce adequate results. This failure meant that another procedure was required to secure a genuine analytical method. As mentioned earlier, it is very challenging and complex work and hence, a thorough strategy was followed to achieve this goal. The objectives were to find a suitable clean-up method as well as a suitable analytical method for the analysis of antibiotics in propolis.

In this chapter, methods are discussed in section 5.1 which is subdivided according to techniques used such as HPLC, UPLC and MELC. At the end, a validation procedure for the optimised method is explained. The analytical method development and the clean-up method development experiments were carried out simultaneously but for convenience, it was divided into different sections. The resulting chromatograms in the development of an analytical method and clean-up method along with validation results are presented and discussed in detail in 5.2 sub-sections.

5.1 Materials and methods for analysis of antibiotics from propolis

In this section, all methods for the proposed study are explained in detail. The subsections are made on the basis of the techniques used. The general methods and chemicals in detail are explained in chapter 3.

5.1.1 Analysis of antibiotics using reversed phase HPLC technique

For the analysis of the antibiotics in propolis, the study was divided into two sections. Here, analytical developments as well as sample clean-up method developments were both important. The HPLC technique was first chosen by referring to the published work of Zhou et al (2009).

5.1.1.1 Standard and sample preparation

An appropriate amount of each antibiotic was weighed accurately (one mg) and diluted with distilled water to obtain 1-100 µg/ml concentration range. Generally in most of the experiments, 2.5 µg/ml was used. The standard preparation was carried out on a regular basis after each alternate day because of the major decomposition problem of antibiotics (Osol et al. 1975).

Two types of sample preparation methods were used in the proposed study; liquid-liquid extraction (LLE) and solid phase extraction (SPE).

In LLE preparation, two immiscible polar (water) and non-polar (hexane) solvents were chosen. They were mixed in proportion of water (10 v/v) and non-polar part (10 v/v, 80%hexane and 20% ethyl acetate). Selected standards such as tetracycline, oxytetracycline, chlortetracycline and doxycycline were weighed accurately to 1mg and were added to the above solution. Afterwards, this mixture was mixed properly using a vortex machine for a limited time period and was then kept aside in a separating funnel to separate both solvents (polar and non-polar). Finally, the aqueous part was collected and analysed using HPLC.

In the propolis sample preparation, instead of standards, propolis was weighed to 1g and was added in the solvent mixture and rest of the procedure followed similar as explained above. There were so many variations studied in each and every test which are discussed in detail in the result and discussion parts.

In SPE sample preparation, the barrel type of the SPE cartridges of HLB with strongly hydrophilic, water-wettable polymer as sorbent was chosen. A specific SPE manifold supported with vacuum assembly was used to perform clean up procedures. The cartridge was firstly conditioned using an organic solvent (mostly methanol) and water. It was followed by sample loading at a very slow flow rate (1ml/min) and washing was done using the appropriate solvent or solution. The eluents were collected by using an appropriate elution medium. This preparation method has many steps. A single variable change in each experiment was carried out to help adapt and improve the method. The details of each variation are discussed in section 5.2.4.2.

For the first few trials, the published method (Zhou et al. 2009) was followed exactly. The reported procedure has many steps and preparation stages. The pre-extraction practices using an ultrasonic bath were followed. Two grams of propolis powder was placed in a 100 ml beaker and was spiked with a standard solution of known concentration, followed by an ultrasound assisted extraction (temperature 50°C) for 0.5 hours using 20 ml extraction buffer (Na_2EDTA-McIlvaine buffer 0.1mM prepared by dissolving 11.8g of citric acid monohydrate, 13.72g of Na_2HPO_4 and 33.62g of Na_2EDTA in 1L DW and the pH was adjusted to 4.0 ± 0.05 using 0.1M HCl or 0.1M NaOH). The procedure was repeated twice and the upper layer was collected in the 50ml centrifuge tube and was centrifuged for 10 min at 5000 rpm. After this pre-extraction procedure, the two-step SPE method was followed. The resulting supernatant was loaded in an

Oasis HLB cartridge equipped with a reservoir, which was pre-conditioned using 5ml methanol and 10ml 5%methanol (methanol/pure water, 5/95, v/v) and was dried completely by using the vacuum pump. A final elution was carried out using 15ml ethyl acetate. This eluate was then transferred to a CBA cartridge which was pre-conditioned by 5ml ethyl acetate. Afterwards, the cartridge was washed using 10 ml methanol followed by the drying of the cartridge. The final elution was followed using the 4ml mixture of 0.01M oxalic acid and ACN (6:4, v/v). The final eluate was evaporated using steam of nitrogen at 45°C and final 2 ml eluent was collected and filtered using 0.2µm nylon filter, this extract was used for analysis.

5.1.1.2 Mobile phase preparation

For HPLC analysis of the antibiotics, the method development was only one part of the study alongside sample preparation. The mobile phase was made up of two solvents (methanol and ACN) and one aqueous phase (diluted acid in water for example: 0.01M oxalic acid of pH 4). The mobile phase of 0.01M oxalic acid (pH 4.0), methanol and ACN (80:5:15, v/v/v) which was used in similar studies by (Zhou et al. 2009) was followed in the first few trials. The mobile phase was filtered using 0.45µ membrane filter paper and was degassed every time for 15 minutes using a sonicator bath.

Other chromatographic conditions were also followed such as a flow rate of 1ml/min, injection volume 20µl and detection wavelength 350nm. All three conditions were kept constant in each trial in the method development stage. The only solvent with an aqueous phase was also studied in some trials. Details of the variations are discussed in the results and discussion section, 5.2.1.

5.1.2 Analysis of antibiotics using UPLC technique

The UPLC studies were very similar to HPLC. The standards and sample preparation procedure followed was exactly the same in these trials. Few trials were carried out to transfer the method over UPLC. Extensive LLE trials were analysed using the UPLC technique. The trials flow is thoroughly discussed in the results and discussion section 5.2.3. The flow rate used was 0.1-0.5ml/min and the injection volume was 2.5µl. The rest of the parameters were kept similar to the RP-HPLC conditions.

5.1.3 Analysis of antibiotics using MELC technique

MELC technique was used in analysis of antibiotics, as it was previously studied in the analysis of flavonoids experiments.

5.1.3.1 Mobile phase preparation

As discussed earlier in 3.4.2, the mobile phase preparation in microemulsion technique is a very important task. The exact same procedure was followed here also in the preparation of the microemulsion mobile phase. In the following experiments, surfactant (Brij L23), co-surfactant (1-Butanol), oil (ethyl acetate) and various types of aqueous phases were studied for the method development studies. Each and every variation is discussed in the results and discussion section 5.2.3.The sample preparation technique followed was the same as discussed in 5.1.1.1.

5.1.4 Method validation procedures for analysis of antibiotics from propolis

At the end of all trials, the microemulsion LC method was finalised for analysis along with the SPE clean-up method using the HLB sorbent. The optimised microemulsion mobile phase is as follows:

- 10mM Sodium acetate buffer (pH 5): 91% (w/w)
- Brij L23 : 3.5 %(w/w)
- 1 Butanol: 2.5%(w/w)
- Ethyl acetate: 3 %(w/w).

The microemulsion mobile phase preparation was followed exactly the same as described in section 3.4.2. Other chromatographic conditions include the injection volume: 20µl; flow rate: 1ml/min; detection wavelength: 350nm; column temperature 30ºC.

The SPE method was optimised as follows; one gram of raw propolis sample was grounded and mixed with 10 ml distilled water and was subjected to sonication for one hour, followed by centrifugation for 20 min at 5000 rpm. Five milligrams of oxytetracycline, doxycycline and chlortetracycline were weighed separately and mixed in distilled water in a volumetric flask (250ml capacity) and the final volume was made to 250ml to obtain a 20 µg/ml concentration by DW. After carrying out appropriate dilutions, as shown in following table 5.1, these solutions were used for the clean-up procedures. Only 10ml of each antibiotic concentration was taken separately in each vial and labeled correctly.

Table 5.1: Dilution preparation for antibiotic standards

Required concentration	Volume (ml) taken from stock solution (20 µg/ml)	Final volume made using distilled water in volumetric flask	Final concentration achieved (µg/ml)
0.5 µg/ml	0.62	20	0.5
1 µg/ml	1.25	20	1
5 µg/ml	6.25	20	5
10 µg/ml	12.5	20	10
15 µg/ml	18.75	20	15
20 µg/ml	-	-	20

The resulting sample and standard solution after centrifugation were collected and mixed with 1ml 100mM phosphate buffer in each sample and standard solution. Afterwards, the resulting solutions were used for a further SPE procedure which is explained below:

- Conditioning: 1 ml distilled water followed by 1 ml methanol
- Loading: sample/ standard (slow flow 1ml/min)
- Elution: 1ml 2 % glacial acetic acid in 70 % methanol.

The following steps were studied in validation procedures followed by ICH guidelines. The parameters which were studied in this validation were selectivity, linearity, accuracy, recovery, robustness and stability.

Selectivity

Selectivity was demonstrated by proving non-interference of the blank peak with other standard peaks.

Linearity

Linearity was performed using six solutions in the range 0.5 µg/ml to 20 µg/ml. These solutions were prepared using the standard stock solution as explained in table 5.1. Each linearity solution was injected in triplicate and an average area was plotted against the concentration to obtain the equation of the line and the correlation coefficient.

Precision

Three concentration levels: low (1 µg/ml); medium (10 µg/ml) and high (20 µg/ml) were used for the precision studies. Standard dilutions were done in the same way as described in table 5.1. Each solution was injected five times. Intra-day and inter-day precision were studied by running this experiment in one day and for five successive days respectively. The precision was measured by

calculating the RSD (Relative Standard Deviation) of the peak area for each concentration level.

Accuracy

Standard solution of antibiotic (20µg/ml) was injected in triplicate and areas from the chromatogram were counted for each peak. The accuracy of this method was assessed by comparing concertation of antibiotic standards with their true concentration.

Recovery

The recovery of the method was assessed by comparing the peak area of the extracted antibiotics with the peak area of antibiotic standards (before extraction).

Robustness

The robustness of the optimised analytical method was studied by deliberately changing experimental conditions with ± 5%. One optimum concentration level of standard solution was selected and used with the blank. The changes were made as follows:

a. Temperature

The temperature was altered ± 5% and all other method parameters were kept unchanged.

Table 5.2: Temperature variation in robustness studies

	ºC
Temperature 1	28.5
Temperature 2	31.5

The column was saturated for enough time with the respective temperatures. For each condition, three replicates were performed. The resulting

chromatograms were compared with the chromatogram of the optimsed analytical method.

b. Flow rate (±0.1ml)

The flow rate was altered as 0.95 ml/min and 1.05 ml/min and all other method parameters were kept unchanged. The sample was injected in triplicate for each flow rate condition.

Table 5.3: Flow rate variation in robustness studies

	ml/min
Flow rate 1	0.95
Flow rate 2	1.05

5.2 Result and discussion for analysis of antibiotics in propolis

The analysis of antibiotics is another objective of the proposed study to compliment with the analysis of active ingredients such as flavonoids. It gives a better idea about the profile of the propolis types which were studied using these techniques. These studies have immense importance in the area of quality control, to analyse different types of raw propolis before any processing. There is very little knowledge about the analysis of residual antibiotics in propolis despite its common appearance in honey samples (Debayle et al. 2008; Bargańska et al. 2011). The analysis of residual antibiotics was extensively studied in different honey samples, which reviewed by Bargańska et al. (2011). But only one reference was found where the analysis of antibiotics in propolis was done (Zhou et al. 2009). Four antibiotics of the tetracycline group such as tetracycline, oxytetracycline, chlortetracycline and doxycycline were selected for this study because of its random use by beekeepers to control bacterial diseases (Zhou et al. 2009; Bie et al. 2012; Levy and Marshall 2013). The sample clean-up procedure is necessary in this experimental work because of the very complex nature of propolis (Zhou et al. 2009). In the case of honey, generally, extraction procedures are applied before the analysis of residual antibiotics (Bargańska et al. 2011). Hence, the analytical method development and development of a suitable clean-up method are the two main objectives of the current study.

In this section, all resulting data and chromatograms are presented and discussed. It is divided into different sub-sections depending on the techniques used. Developmental studies of each technique are supported with a detailed description of the experimental trials and chromatographic results. In the validation section, all validation results are discussed.

5.2.1 Method development of antibiotics using reverse phase HPLC technique

Along with flavonoid studies as principal constituents, the next objective was to study contaminants in propolis by developing a suitable clean-up method and analytical method.

The analysis of residual antibiotics from different sources using RP-HPLC:

The residual analysis of antibiotics from different food or animal products by using RP-HPLC have been previously studied, a few of these references are discussed here in brief. The trace analysis of three sulfonamides, sulfamethazine, sulfamonomethoxine and sulfadimethoxine from animal tissue and eggs were studied by using the HPLC technique. Tissues were extracted in acetonitrile while fat was removed by using liquid-liquid extraction. Quantification levels were found between 0.01-0.04ppm (Horii et al. 1990). Reverse phase HPLC was optimised for the analysis of tetracycline, 4-epitetracycline and oxytetracycline in milk, the samples were extracted using solid phase extraction. Oxytetracycline was found in all milk samples which were collected from a local supermarket (Fritz and Zuo 2007). The residue analysis of selected fluoroquinolones, sulfonamides and tetracyclines in 143 animal dung samples (large-scale livestock and poultry feedlots) using ultrasonic extraction and HPLC was studied and the resulting outcome showed significant statistical differences among the sampling districts and the animal species. Enrofloxacin and chlortetracycline were detected with a high occurrence in all three (cow, pig and chicken) manure samples (Zhao et al. 2010). The analytical method for the residue of four tetracyclines such as tetracycline, oxytetracycline, chlortetracycline and doxycycline from fish muscle was developed with RP-HPLC and a solid phase extraction method for sample

preparation. Excellent method reproducibility was found showing RSD values 4.22% (intra-day precision) and 5.71% (inter-day precision) and linearity in the range 100–10,000 ng/g concentration (Wen et al. 2006). The analysis of antibiotic residues of 13 antibiotics including tetracycline, macrolide, penicillin, chloramphenicol etc were studied using HPLC with UV and fluorescent detection in a large number of agricultural and fish products for example: beef (n = 148), pork (n = 78), chicken (n = 88), eel (n = 70), flatfish (n = 17) which were obtained from local markets in Korea. The variation was found in resulting data. The levels of oxytetracycline in pork and eel were found to be 0.01 and 0.05 mg/kg respectively, and In beef, the concentration of tylosin was 0.05 mg/kg (Lee et al. 2007).

The residual analysis of antibiotics from honey is well studied from many samples as compared to propolis. An investigation of the occurrence of oxytetracycline residue in 145 honey samples (collected from Ardabil provinces, Iran) using ELISA (enzyme-linked immunosorbent assay) and HPLC techniques was carried out. Both techniques confirmed a considerable presence of the oxytetracyline residue in the studied samples (Mahmoudi et al. 2014). The detailed review of the analysis of antibiotic residues from different honey types using different analytical techniques such as RP-HPLC, LC-MS and GC was carried out (Bargańska et al. 2011). From the review, it was clearly understood, sample preparation is a necessity for the analysis of antibiotics. GC is rarely used for the analysis of antibiotics in honey samples, due to the polar nature, low volatility and thermal instability of these drugs, but RP-HPLC and LC-MS were found more suitable for this purpose.

As compared to honey, propolis samples are less studied for the residue analysis of antibiotics. The development and validation of HPLC analytical

methods for the residual analysis of antibiotics were carried out for the first time by Zhou et al. (2009). Four tetracycline groups of antibiotics such as tetracycline, oxytetracycline, chlortetracycline and doxycycline were analysed by using a two-step solid phase extraction followed by an ultrasound extraction to remove water-soluble and fat-soluble flavonoids, aromatic acids, terpenoid compounds, wax, and pollen debris from propolis sample. The recoveries of all four antibiotics in this study were found in range of 61.9–88.5% and the RSDs were between 4.80% and 13.2%. The residual antibiotics were found in two propolis samples out of 30 samples. Apart from this study, no other references were found about the analysis of the antibiotic residue from propolis. Therefore, this challenging study, including the development of a suitable sample preparation method and analytical method for the analysis of tetracyclines from propolis, was performed, and RP-HPLC was selected as one of the technique for this purpose.

At first, the reported method (Zhou et al. 2009) was followed with the mobile phase of 0.01M oxalic acid (pH 4), acetonitrile and methanol (80:15:5 proportion, v/v/v). The other preparation and chromatographic condition details are explained in section 5.1.1.2. The resulting chromatogram showed relatively longer run time than expected with a broad shape of the last two peaks of doxycycline and chlortetracycline. The attempts of reproducing the reported method were not successful. Therefore, further method development was conducted using one-factor-at-a-time (OFAT) approach. All other parameters were kept constant as mentioned in 5.1.1.2. The details of further studies are shown in table 5.4.

5.2.1.1 Effect of mobile phase condition for analysis of antibiotics

Table 5.4: Effect of mobile phase composition on antibiotics

Trial No.	Mobile phase		
	0.01M oxalic acid (pH 4)	Acetonitrile	Methanol
1	80	15	5
2	70	20	10
3	70	25	5
4	70	15	15

A variation in the existing mobile phase was studied to improve chromatographic separation. The resulting chromatograms of all experiments showed a co-elution problem in adjacent peaks of first two peaks of tetracycline and oxytetracycline and last two peaks of chlortetracycline and doxycycline. However the best separation was obtained with trial 2 while third trial resulted in a co-elution of the first two peaks with a total run time 18 minutes. The total run time here is relatively long for only four compounds and the last two peaks of chlortetracycline and doxycycline showed broad peaks with uneven peak shape. The following chromatogram in figure 5.1 is for the third trial.

The long run time, peak co-elution and broad peak shapes were allowed to try further method optimisation to achieve better resolution between the peaks. In further trials, a single organic solvent was used in different proportions (Table 5.5).

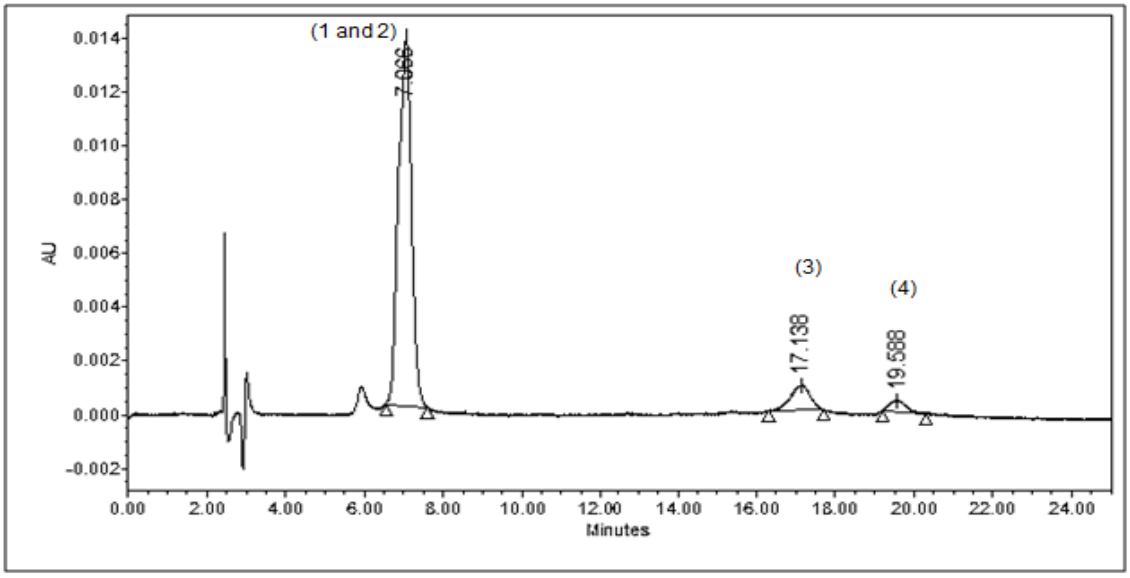


Figure 5.1: Chromatogram for antibiotic separation at chromatographic conditions: mobile phase (0.01M oxalic acid: ACN: methanol; 70:25:5; v/v/v); injection volume 2.5µl; flow rate 0.4ml/min.; column temperature 28°C and detector wavelength 350nm.

Peak 1 and 2: Tetracycline and Oxytetracycline, Peak 3: Chlortetracycline, Peak 4: Doxycycline.

5.2.1.2 Effect of solvents in mobile phase on antibiotics

The first two trials (Table5.5) with methanol showed co-elution of the first two peaks of tetracycline and oxytetracycline with peak separation and resolution between the last two peaks of chlortetracycline and doxycycline. The chromatogram for the second trial is shown in figure 5.2.

Table 5.5: Effect of solvents in mobile phase on antibiotics

Trial No.	Mobile phase	
	0.01M oxalic acid (pH 4)	Methanol
1	60	40
2	70	30
	0.01M oxalic acid (pH 4)	Acetonitrile
3	77	23
4	80	20
5	85	15

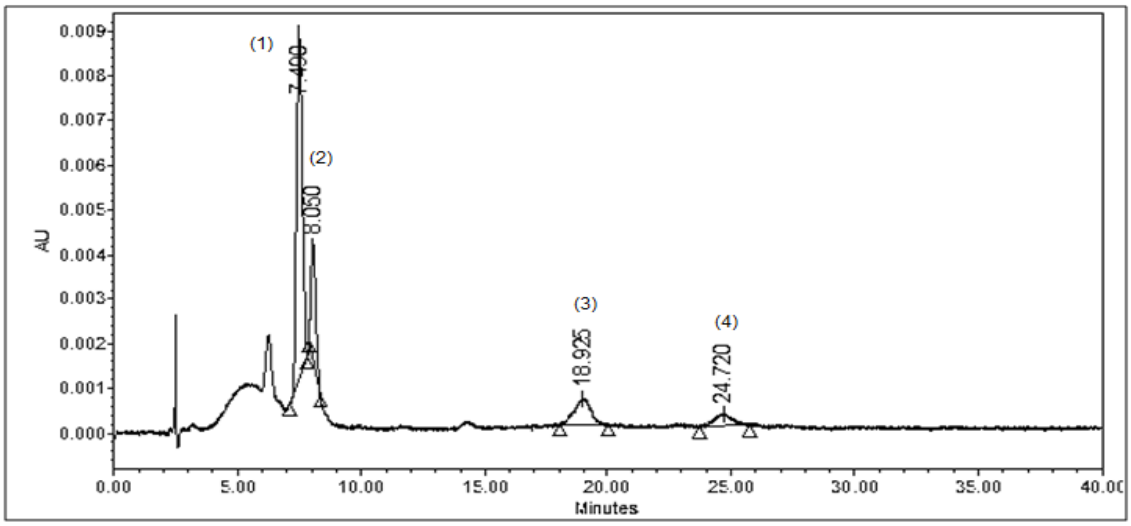


Figure 5.2: Chromatogram for antibiotic separation at chromatographic conditions: mobile phase (0.01M oxalic acid: methanol; 70:30; v/v/v); injection volume 2.5µl; flow rate 0.4ml/min.; column temperature 28°C and detector wavelength 350nm.

Peak 1 (RT 7.1 mins): Tetracycline, Peak 2 (RT 8.0 mins): Oxytetracycline, Peak 3 (RT 18.9 mins): Chlortetracycline, Peak 4 (RT 24.7 mins): Doxycycline.

In contrast, when the acetonitrile concentration decreases in the mobile phase, there is an increase in co-elution of first two peaks of tetracycline and oxytetracycline. The total retention time also increases as the acetonitrile concentration decreases and the resulting chromatograms of all trials with acetonitrile showed broad and uneven peak shapes of the last two eluted standards, chlortetracycline and doxycycline. The chromatogram of the third trial from table 5.5 is shown in figure 5.3.

Acetonitrile has more solvent strength than methanol; therefore the peaks of tetracycline and oxytetracycline were co-eluted even with a very low acetonitrile concentration. From all the above results, it was confirmed that for the separation of antibiotics, both solvents were necessary. For further optimisation of antibiotics separation, the oxalic acid was replaced by a more acidic buffer solution (10mM sodium phosphate buffer pH 3) in next trials. The trials were designed as follows in table 5.6.

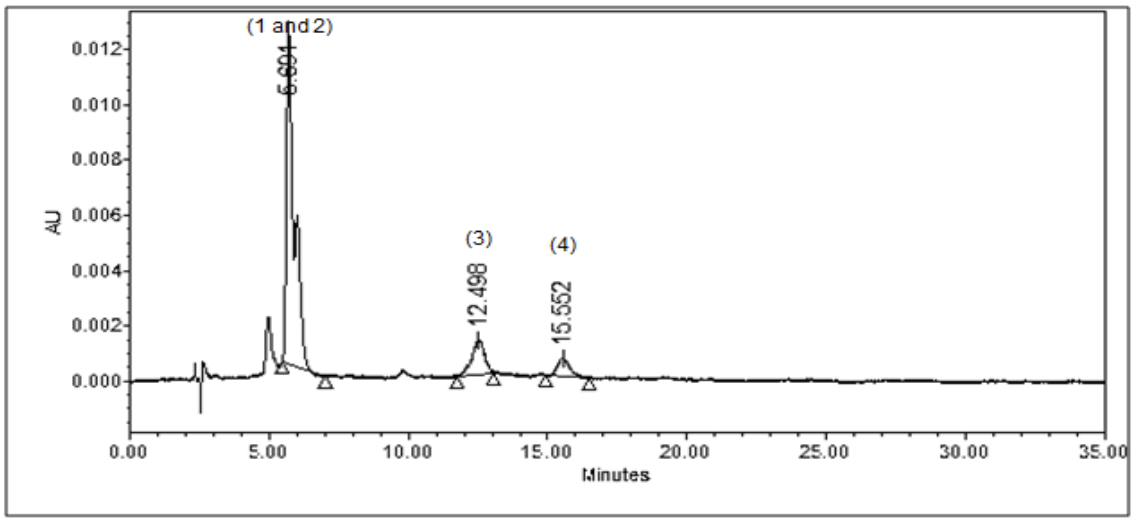


Figure 5.3: Chromatogram for antibiotic separation at chromatographic conditions: mobile phase (0.01M oxalic acid: ACN; 77:23; v/v/v); injection volume 2.5µl; flow rate 0.4ml/min.; column temperature 28°C and detector wavelength 350nm.

Peak 1 and 2: Tetracycline and Oxytetracycline, Peak 3: Chlortetracycline, Peak 4: Doxycycline.

5.2.1.3 Effect of aqueous phase in mobile phase on antibiotics

Table 5.6: Effect of aqueous phase buffer on antibiotics

Trial No.	Mobile phase		
	10mM phosphate buffer (pH 3)	Acetonitrile	Methanol
1	70	10	20
	10mM phosphate buffer (pH 3)	Acetonitrile	Methanol
2	85	15	-
3	82	18	-
4	80	20	-

The first trial was repeated here with the acidic buffer instead of using oxalic acid. This trial also gave chromatographic separation with co-elution in first two peaks and the retention time was relatively long for four eluents. The resulting chromatogram is shown in figure 5.4. The first two peaks of tetracycline and oxytetracycline were eluted closely but with peak separation with last two broad peaks of chlortetracycline and doxycycline. The reported method by Zhou et al.

(2009) had a total run time of 15 minutes and it showed a better peak separation in the first two peaks of oxytetracycline and tetracycline.

The acetonitrile trials with buffer instead of oxalic acid (Table 5.6) produced a separation with relatively long run time and improper peak shapes of eluents. Hence, another technique was considered for further experiments. UPLC technique was chosen for the development of chromatographic separation of antibiotics.

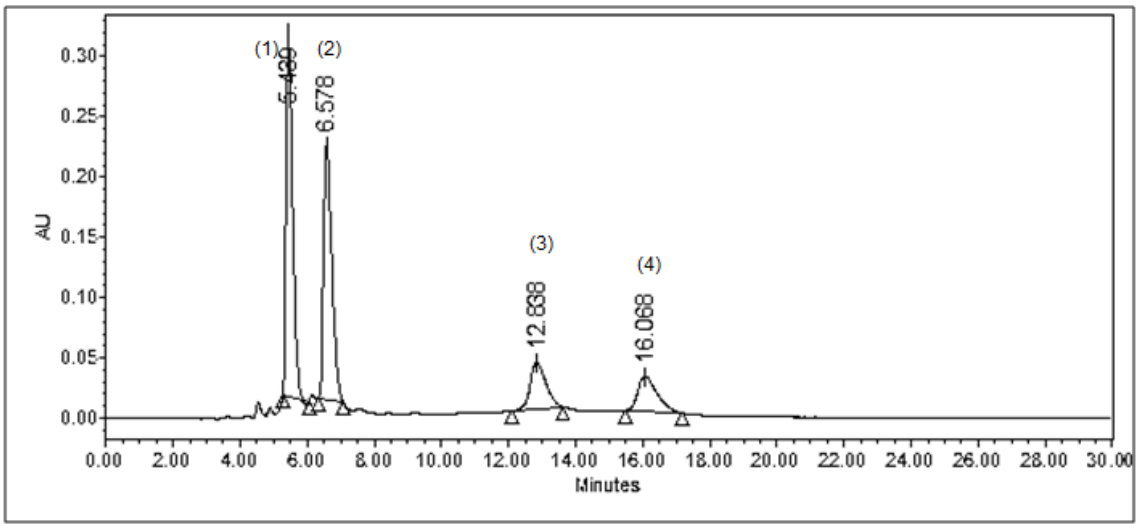


Figure 5.4:Chromatogram for antibiotic separation at chromatographic conditions: mobile phase (10mM sodium phosphate buffer (pH 3):methanol: ACN; 70:10:20; v/v/v); injection volume 2.5µl; flow rate 0.4ml/min.; column temperature 28°C and detector wavelength 350nm.

Peak 1: Tetracycline, Peak 2 : Oxytetracycline, Peak 3: Chlortetracycline, Peak 4: Doxycycline.

5.2.1.4 Summary

The RP-HPLC method development studies were not able to separate the mixture of the selected antibiotic compounds with suitable run time. In addition of that, the clean-up methods were not found to be suitable with this technique because of co-elution of the antibiotic compound with one unknown peak from

propolis sample. To overcome these limitations another techniques were studied.

5.2.2 Method development of antibiotic using UPLC technique

The analysis of antibiotic residue from different samples using the UPLC technque has not been studied much so far. For rapid screening and quantitative analysis of residues of ten antibiotics such as chloramphenicol, thiamphenicol, tetracycline, oxytetracycline, chlortetracycline, metacycline, doxycycline, cefoperazone, ceftriaxone and cefaclor were studied from milk samples using UPLC with PDA. The SPE extraction with McIlvaine buffer and methanol in ratio of 80:20, were used for clean-up procedures.This reported method has been applied satisfactorily for the analysis of antibiotcs from real milk samples (Wang and Li 2009).

For the residual analysis of antibiotics, the UPLC-MS technique was also reported. Several references were found about this analysis from different honey samples (Bargańska et al. 2011). There is no studies reported about residue analysis of antibiotics in any propolis sample so far. Hence, in this proposed study, a more promising and fast analytical technique, UPLC, was selected for the analysis of tetracyclines from propolis.

UPLC studies were initiated to obtain the appropriate chromatographic condition for the analysis of tetracycline antibiotics. The optimisation approach was similar to HPLC, experiencing variation with each solvent and with both solvents (Methanol and ACN). The trials here with UPLC were quicker because of the short retention time. In table 5.7, these variations were mentioned briefly.

Table 5.7: Effect of mobile phase composition on antibiotics

Trial No.	Mobile phase		
	10 mM sodium phosphate buffer (pH 3)	Acetonitrile	Methanol
1	70	25	5
2	60	-	40
3	70	-	30
4	80	-	20
5	90	-	10
6	60	40	-

The variations with the mobile phase were studied by maintaining other parameters constant such as the flow rate 0.4ml/min., injection volume 2.5 µl, column temperature 28°C and detector wavelength 350nm.

The resulting chromatogram of the first trial showed a very short run time with co-elution of the first two peaks. But overall peak shapes were more sharp and even as compared to the HPLC chromatograms. The following trials with methanol and buffer showed an increase in the total run time as the methanol concentration increases in the mobile phase. However, all of these trials showed co-elution of the first two peaks. The resulting chromatogram of the third trials is shown below in figure 5.5.

The chromatogram clearly showed co-elution of the first two peaks of the standards tetracycline and oxytetracycline with proper separation of the last two peaks of chlortetracycline and doxycycline (Figure 5.5). The last trial of acetonitrile failed to improve separation of the first two peaks. Hence, from these studies it was clear that both of the solvents were necessary for the mobile phase to achieve separation in all standard compounds (Zhou et al. 2009).

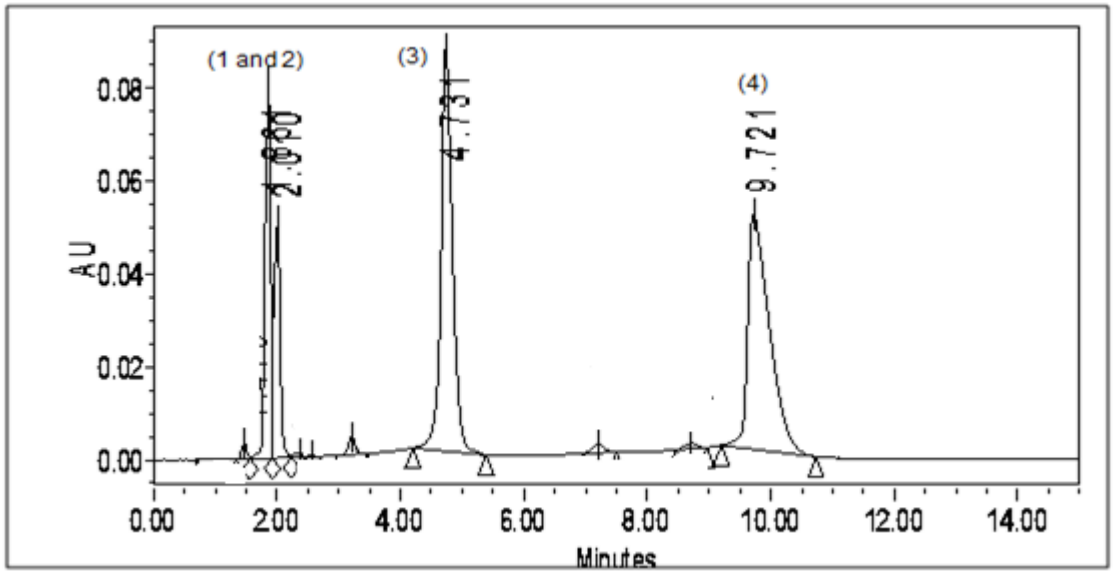


Figure 5.5:Chromatogram for antibiotic separation at chromatographic conditions: mobile phase (10mM sodium phosphate buffer (pH 3):methanol: ACN; 70:5:25; v/v/v); injection volume 2.5µl; flow rate 0.4ml/min.; column temperature off and detector wavelength 350nm.

Peak 1 and 2: Tetracycline and Oxytetracycline, Peak 3: Chlortetracycline, Peak 4: Doxycycline.

In the next step, the variation was studied in the first trial mentioned in table 5.5 with different flow rates such as 0.4ml/min; 0.3ml/min; 0.2ml/min and 0.15ml/min. The separation was found to have improved as the flow rate reduced from 0.4 to 0.15ml/min. The last flow rate of 0.15ml/min gave a good chromatographic separation and peak resolution. The resulting chromatogram is shown in figure 5.6.

In the resulting chromatogram (Figure 5.6), the first peak was of tetracycline at RT 2.2 mins, followed by peak of oxytetracycline at RT 2.4 mins, the third peak was of chlortetracycline at RT 3.6 mins and the last peak was of doxycycline at RT 3.9 mins. The chromatographic condition was finalised for further studies with the following parameters: mobile phase 10 mM sodium phosphate buffer (pH 3); methanol; acetonitrile (70:5:25, v/v/v); flow rate 0.15ml/min; column temperature off; injection volume 2.5µg/ml and detector wavelength 350nm.

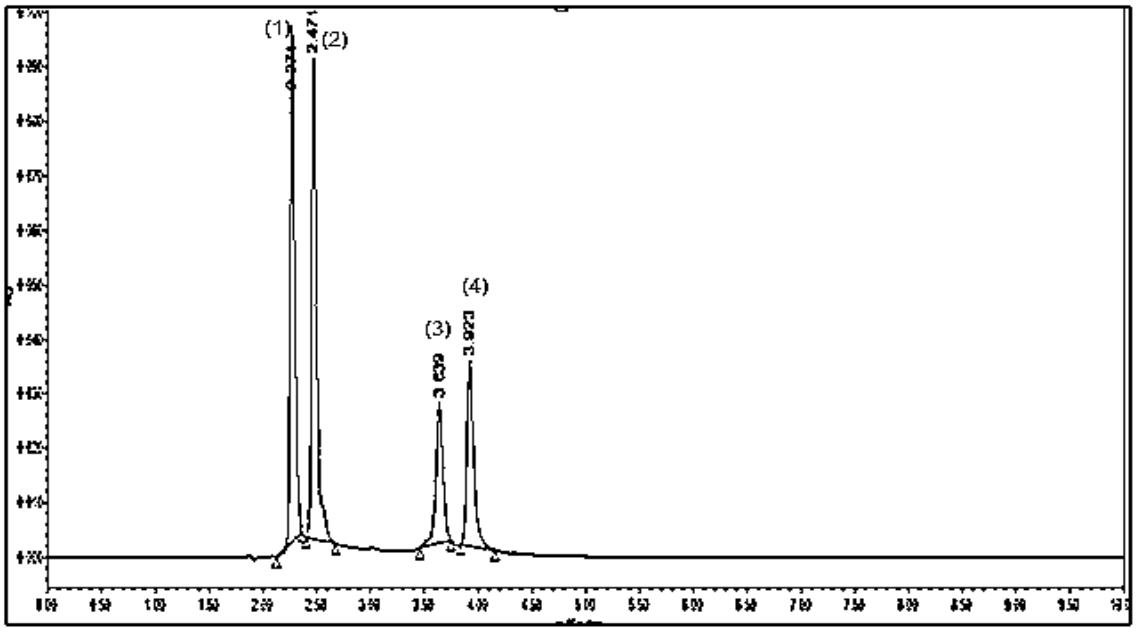


Figure 5.6: Chromatogram for antibiotic separation at chromatographic conditions: mobile phase 10 mM sodium phosphate buffer (pH 3): methanol: acetonitrile (70:5:25, v/v/v); flow rate 0.15ml/min.; column temperature off; injection volume 2.5 µl, wavelength 350nm.

Peak 1: Tetracycline, Peak 2: Oxytetracycline, Peak 3: Chlortetracycline, Peak 4: Doxycycline.

Although UPLC was able to separate the four selected antibiotics with rapid analysis time, it was decided not use this technique. This decision was due to the overlapping issue between chlortetracycline peak and unidentified compound from the sample itself, which was not possible to remove with the extraction method. This issue is discussed in section 5.2.4. Therefore, another option was selected by using microemulsion LC technique.

5.2.3 Method development for the analysis of antibiotics using microemulsion LC technique (MELC)

A microemulsion is clear and thermodynamically stable phase obtained by mixing oil, water, surfactant and co-surfactant. The use of this technique with

RP-HPLC is common nowadays and led to a new direction in the separation science.

The MELC technique using European Union Decision guidelines was reported for the analysis of antibiotics residues of danofloxacin, difloxacin, ciprofloxacin and sarafloxacin from honey samples. The samples were diluted in 1:1 0.05 M sodium dodecyl sulphate (SDS) pH 3 and the mobile phase comprised of 0.05 M SDS, 1% 1-butanol and 0.5% triethylamine buffered at pH = 3 (Tayeb Cherif et al. 2015). Rambla-Alegre et al. (2011a) reported the use of micellar liquid chromatography for the analysis of four quinolones (danofloxacin, difloxacin, flumequine and marbofloxacin) from milk and egg samples without following any extraction methods but by using procedures of homogenisation, dilution and filtration. The micellar mobile phase consisted of 0.05 M sodium dodecyl sulphate, 10% (v/v) butanol and 0.5% (v/v) triethylamine buffered at pH 3 and fluorimetric detection. A similar technique with fluorescent detection was used for the analysis of quinolenes from urine samples (Rambla-Alegre et al. 2009), and from fish samples (Rambla-Alegre et al. 2010). For the analysis of quinolones (pipemidic acid, levofloxacin, norfloxacin, and moxifloxacin) in different pharmaceutical preparations using MELC, Collado-Sánchez et al. (2010) have successfully used a mobile phase of 0.15 M sodium dodecyl sulphate, 2.5% propanol, and 0.5% triethylamine at pH 3, and a diode-array UV-Vis detection. A similar technique was used for the analysis of amoxicillin in urine samples without any extraction procedures and by using UV detection (Rambla Alegre et al. 2008). Similarly, analysis using MELC technique for four penicillin antibiotics (amoxicillin, ampicillin, cloxacillin and dicloxacillin from pharmaceutical formulations and physiological fluids (urine) were studied by Rambla-Alegre et al. (2011b).

There is no reported method was found for the analysis of antibiotics in propolis. From above referenced work, the usefulness of MELC technique for the analysis for the antibiotics is promising.

5.2.3.1 Effect of mobile phase composition on antibiotics

The MELC method that was developed for the analysis of flavonoids in propolis (Section 4.2.4) was adapted for the determination of antibiotics in propolis. The first trials were started using SDS surfactant and octanol as oil. Further development was studied as shown in following table 5.8.

Table 5.8: Effect of surfactant on antibiotics

Microemulsion mobile phase	**(%w/w)**	**(%w/w)**	**(%w/w)**
Sodium phosphate buffer	90.1%	84.2%	79.2%
Surfactant; SDS	2.5%	5%	6.6%
Co-surfactant; 1-butanol	6.6%	10%	13.4%
Oil phase; octanol	0.8%	0.8%	0.8%

The variation was studied as mentioned in table 5.8. Peak co-elution and merging was found from the first trial and increased as the SDS concentration increased in each trial. The SDS favors the microemulsion efficiency which leads to the eluting of compounds early and hence peak merging problems (El-Sherbiny et al. 2003).

The above study explained that the tetracycline group of antibiotics was not separated by the influence of the SDS surfactant. Hence, the surfactant and oil were changed in the following set of experiments.

During the MELC studies, the solubility of each antibiotic was studied in different oils and surfactants. In the following table, 5.9, the results of this experiment are shown.

Table 5.9: Solubility of antibiotic with selected surfactants and oils

	Tetracycline	Oxytetracycline	Doxycycline	Chlortetracycline
Ethyl acetate	++	++	++	++
Brij L23	++	++	++	++
Octane	++-	++-	+-	+-
SDS	-+	++	++	+-

(-+ = very poor solubility, +- = average solubility, ++- = moderate solubility, ++ = high solubility)

The results of the above study shows ethyl acetate as an oil and Brij L23 surfactant were most suitable options. These two increased the solubility for all four antibiotics as compared to octane and SDS. As a result, the oil and surfactant were changed to ethyl acetate and Brij L23, respectively.

5.2.3.2 Effect of surfactant and co-surfactant phase on antibiotics

In first few trials, the variations were studied in Brij L23 (surfactant) and butanol (co-surfactant) concentrations as shown in table 5.10.

Table 5.10: Effect of surfactant Brij L23 and co-surfactant 1-butanol on antibiotics

Microemulsion mobile phase	**(%w/w)**	**(%w/w)**	**(%w/w)**	**(%w/w)**	**(%w/w)**	**(%w/w)**
Trial No.	1	2	3	4	5	6
Sodium phosphate buffer	95.5%	94.5%	93.5%	93.5%	92.5%	91.5%
Surfactant; Brij L23	1.5%	1.5%	1.5%	**3.5%**	**4.5%**	**5.5%**
Co-surfactant; 1-butanol	***2.5%***	***3.5%***	***4.5%***	2.5%	2.5%	2.5%
Oil phase; ethyl acetate	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%

The first three trails were tried with co-surfactant variations and the last three trials with surfactant variations. The first trials resulted with the elution of only one peak while the following two trials showed co-elution of the first two peaks and separation of the last two peaks with broad peak shapes. As the concentration of the co-surfactant increases, which is directly proportional to the increment of a number of microemulsion droplets in the mobile phase, this leads to an increase in the separation of standards but peak broadening also increased with improper peak shape. By comparing all chromatograms, the lowest concentration of 1-butanol (2.5%) was selected as the optimum, considering the peak shape of last two peaks and the retention time.

In the last three trials, as the concentration of surfactant increased, it also increased the total retention time of the resulting chromatogram. All chromatograms of these trials showed co-elution of the first two peaks and separation of the last two broad peaks. By comparing all chromatograms, the lowest concentration of Brij L23 was selected for further optimisation. The chromatogram of the forth trial was shown in the figure 5.7.

To achieve better separation in antibiotic peaks, the effect of the concentration of oil was investigated.

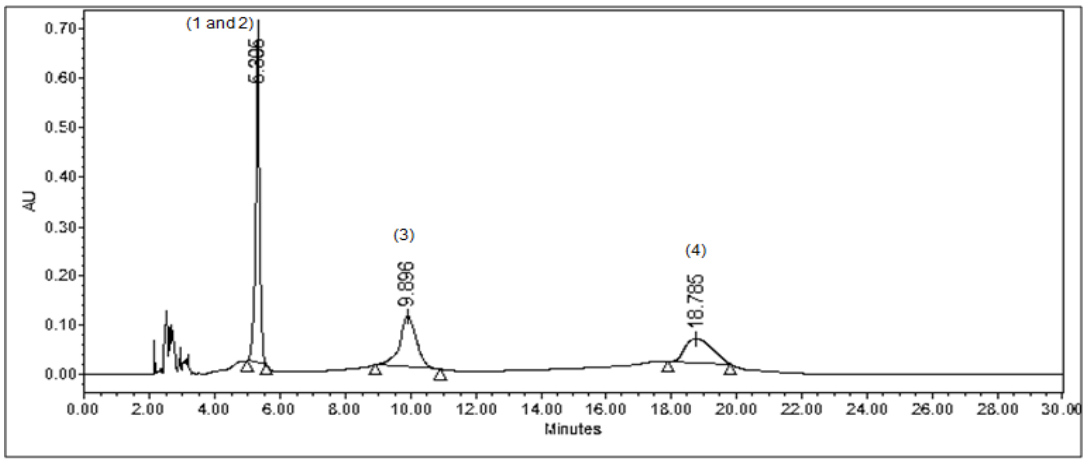


Figure 5.7: Chromatogram for antibiotic separation at: mobile phase 10 mM sodium phosphate buffer (pH 3): ethyl acetate: Brij L23: 1-butanol (93.5%: 0.5%: 3.5%: 2.5%, w/w/w/w); flow rate 1ml/min.; column temperature off; injection volume 20 µl, wavelength 350nm.

Peak 1 and 2: Tetracycline and Oxytetracycline, Peak 3: Chlortetracycline, Peak 4: Doxycycline.

5.2.3.3 Effect of oil phase on antibiotic separation

The oil concentration was studied with a high percentage of 3% instead of 0.5% (see table 5.11).

Table 5.11: Effect of oil concentrations on antibiotics

Microemulsion mobile phase	**(%w/w)**	**(%w/w)**	**(%w/w)**
Trial No.	1	2	3
Sodium phosphate buffer	93.5%	92.5	92%
Surfactant; Brij L23	3.5%	3.5	2.5%
Co-surfactant; 1-butanol	2.5%	2.5	2.5%
Oil phase; ethyl acetate	0.5%	1.5	3%

The second trial mentioned in table 5.11 showed better chromatographic separation as compared to the first trial. The first two peaks co-eluted together with the separation of the last two peaks and the total retention time was 20 minutes. The oil phase is mainly affects the retention of hydrophobic compounds but antibiotics are hydrophilic in nature. Therefore, increasing the

concentration of the oil phase did not significantly give any improvement in chromatographic separation. The highest concentration of oil was chosen and finalised. In the next part of the study, the effect of pH of the aqueous phase was examined.

5.2.3.4 Effect of pH of the aqueous phase in mobile phase on the separation of antibiotics

To achieve better peak separation and peak shape, a set of trials were organised with optimising the pH of the aqueous phase. The detailed of the modifications in the studies are explained in table 5.12.

In this study, the chromatogram of the second and third trials did not show appropriate chromatographic separation. Formic acid in the microemulsion caused very long run times for all standards and was therefore not selected for further studies. The last trial of this study gave better peak separation with sharp peak shape of each standard. The resulting chromatogram of the last trial, which included sodium acetate buffer, showed peak separation of the three standard compounds by achieving appropriate even peak shapes as compared to all previous trials. The chromatogram is shown in figure 5.8.

Table 5.12: Effect of aqueous phase on antibiotics

Microemulsion mobile phase	Trial No.1	**Sodium phosphate buffer**	**Surfactant; Brij L23**	**Co-surfactant; 1-butanol**	**Oil phase; ethyl acetate**
(%w/w)		91**%**	3.5%	2.5%	3%
	Trial No.2	**Distilled water**			
(%w/w)		91**%**	3.5%	2.5%	3%
	Trial No.3	**0.01M formic acid**			
(%w/w)		91**%**	3.5%	2.5%	3%
	Trial No.4	**10mM sodium acetate buffer pH 5**			
(%w/w)		91**%**	3.5%	2.5%	3%

Apart from the elution of only three standards instead of four, the chromatogram was showed appropriate peak separation and resolution. The weak acid, sodium acetate, played an important role in the elution and better peak separation was achieved with using a mild acidic pH 5. The main disadvantage of this method was the co-elution of the first two peaks of tetracycline and oxytetracycline. Instead, this method exhibited proper peak separation and resolution. The first peak at RT 3.3 minutes was of oxytetracycline followed by peat at RT 4.07 was of chlortetracycline and the last peak was of doxycycline at RT 6.6 (Figure 5.8). The column temperature at this experiment was optimised at 30ºC, and kept constant in further validation experiments to avoid any variation in resulting chromatographic separation.

It was then decided to select this method among the other technique UPLC and conventional HPLC for the determination of antibiotics in propolis samples. This is due to the reason that it was more suitable with extraction method. As a part of the optimisation process, we selected three antibiotics which are oxytetracycline, chlortetracycline and doxycycline. From the first two eluted standards, oxytetracycline was selected instead of tetracycline for further studies.

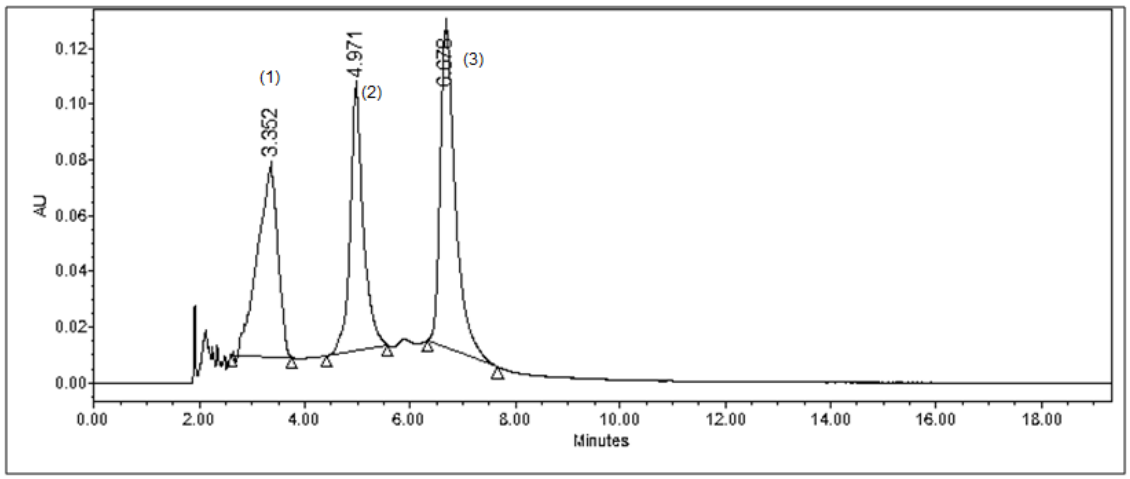


Figure 5.8: Chromatogram for antibiotic separation at: mobile phase 10 mM acetate buffer (pH 5): ethyl acetate: Brij L23: 1-butanol (91%: 3%: 3.5%: 2.5%, w/w/w/w); flow rate 1ml/min.; column temperature 30ºC; injection volume 20 µl, wavelength 350nm.

Peak 1: Oxytetracycline, Peak 2: Chlortetracycline, Peak 3: Doxycycline.

5.2.3.5 Summary

The microemulsion LC method was then validated for the determination of antibiotics in propolis samples. Three tetracyclines were selected which are oxytetracycline, chlortetracycline and doxycycline. Tetracycline was excluded because of its co-elution problem with the oxytetracycline compound.

5.2.4 Extraction studies of antibiotics from propolis-method development

The published two step solid phase extraction method (Zhou et al. 2009), for the extraction of antibiotics from propolis was followed but it did not show appropriate chromatographic results, with low recovery of antibiotics. Hence, the development of an extraction method was also carried out as a part of this study. At the beginning, simple liquid-liquid extraction (LLE) trials were carried out. For the convenience, the UPLC technique was performed using the optimised UPLC method explained in 5.2.2.

5.2.4.1 Liquid-liquid extraction

The studied antibiotics are hydrophilic in nature so therefore two immiscible solvents, including water and mixture of hexane and ethyl acetate were chosen for liquid-liquid extraction. In the initial experiments, a mixture of hexane and ethyl acetate mixture (80:20% v/v) was prepared then it was mixed with water in 1:1 proportion and was used for the first LLE trial. Water was selected because of tetracycline solubility in it, while in contrast, other non-polar immiscible solvent was selected such as hexane, to extract out unwanted compounds from propolis. The detailed experimentation of LLE is explained in the materials and method section (5.1.1). This method gave a high percentage recovery (around 80-95%) when it was examined using a standard solution. The resulting chromatogram after LLE is presented in figure 5.9. The first peak at RT 2.27 minutes was of tetracycline, followed by peak of oxytetarcycline at RT 2.44, chlortetracycline at RT 3.56 and doxycycline as the last peak at RT 3.81. However, upon extracting the antibiotics from propolis, co-elution of a large unknown peak with a chlortetracycline was observed (Figure 5.10).

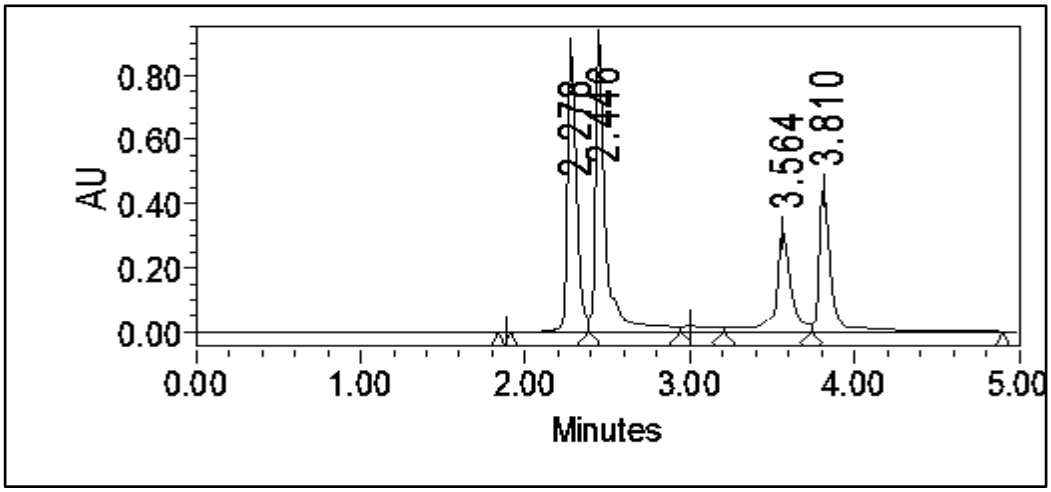


Figure 5.9:Chromatogram for antibiotic standard after LLE at: mobile phase 10 mM sodium phosphate buffer (pH 3): methanol: acetonitrile (70:5:25, v/v/v); flow rate 0.15ml/min.; column temperature off; injection volume 2.5 µl and wavelength 350nm.

Peak 1 (RT 2.2 mins): Tetracycline, Peak 2 (RT 2.4 mins): Oxytetracycline, Peak 3 (RT 3.5 mins): Chlortetracycline, Peak 4 (RT 3.8 mins): Doxycycline.

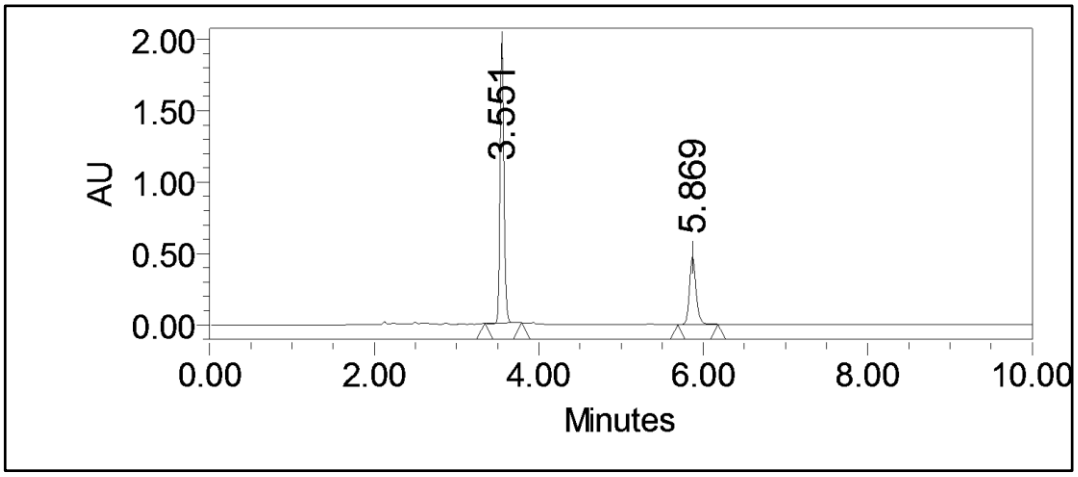


Figure 5.10: Chromatogram for propolis sample after LLE at: mobile phase 10 mM sodium phosphate buffer (pH 3): methanol: acetonitrile (70:5:25, v/v/v); flow rate 0.15ml/min.; column temperature off; injection volume 2.5 µl and detector wavelength 350nm.

Peak 1 (3.5 mins): Unknown peak, Peak 2 (5.8 mins): Unknown peak.

The elution of the unknown propolis peak and chlortetracycline has shown that this chromatographic condition is not suitable with the above LLE method.

Higher dilution of extracted antibiotics after LLE leads to no detection in propolis sample was clearly observed in resulting chromatogram (Figure 5.10). The same extracted sample was analysed using conventional HPLC and MELC methods explained in sections 5.2.1.3 and 5.2.3.4 respectively. Both of these methods were unable to separate chlortetracycline standard and unknown peak. The percentage recovery after extraction was found to be excellent, in the range of 88-100%, for antibiotic standards as well as for spiked samples of propolis with standards. At the same time, diluted concentration of antibiotic in LLE experiments caused no detection from propolis sample by the analytical method. Elution of known antibiotic peak of chlortetracycline and unknown peak from propolis is another disadvantage. The availability of different sets of analytical methods such as UPLC, HPLC and MELC were found adventitious to confirm each extraction experiment.

In further experimentation, few changes were planned to improve the chromatographic results. The variation in the ratio of polar (water) and non-polar (hexane and ethyl acetate) phases changed the percentage recovery at each experiment but the elution of the two unidentified peaks remained unchanged. The elution of two unidentified peaks and low/no detection of antibiotics from propolis samples were major drawbacks of the studies and therefore a simple LLE technique was changed to a complex solid phase extraction (SPE).

5.2.4.2 Solid phase extraction

Liquid-liquid extraction (LLE) has been used for many years for the separation of compounds of interest from liquid samples. Unlike LLE, solid phase extraction (SPE) is a reasonably new technique which has only emerged in the last two decades. SPE has more advantages than LLE, for instance it is easier to separate the liquid phase and solid phase in SPE compared to LLE; the

separation of the two immiscible liquids was more laborious in the LLE technique also. SPE was more time saving, it improved extraction efficiency with lower evaporation volumes and showed high reproducibility and produced cleaner extracts compared to LLE. It also improved the extraction recovery and clean-up due to the effect of surface area by the adsorbent (Ahadi et al. 2011). Due to the advantages of SPE over LLE, in the forthcoming extraction work, SPE was chosen for the extraction of antibiotics from propolis samples. The main purpose of solid phase extraction is to extract out semi-volatile or non-volatile analytes.

In solid phase extraction, the column containing the adsorbent (solid phase) is an important part. From it, the sample is allowed to transfer, allowing the adsorption of the analyte and washing off of any interference and ultimately the elution of the analyte using a relevant solvent. The separation of the analyte here depends on the degree of adsorption/partition of each component by the stationary phase. There are a few steps which are very important in solid phase extraction as follows,

1. Cleaning followed by activation of the sorbent.
2. Conditioning of the sorbent with the similar solvent which is in the sample.
3. Loading of the sample at a low flow rate approximately 1 ml/min.
4. Washing of the cartridge to remove the unwanted matrix components.
5. Elution of the analytes by disruption of the analyte-sorbent interactions.

There are a variety of SPE consumables available with a wide range of chemistries, adsorbent properties and sizes. Depending on the nature of the matrix of the samples and compounds of interest, the suitable SPE product can be selected (Hennion 1999). The syringe barrel types of cartridges are the most popular in SPE, followed by the disc type. The sorbents that are used in

SPE include non-endcapped C-18 silica and mono-functional C-18 silica to facilitate secondary polar interactions with solutes by additional non-functional silanol groups on bonded silica. These types of reversed phase sorbents become active after the addition of specific conditioners/wetting solvents. But for the new generation of polymers, such as Oasis, conditioning is not required and they can also elute a variety of eluents such as lipophilic, hydrophilic, acidic, basic and neutral (Hennion 1999). These polymeric columns are 'hydrophilic-lipophilic balanced' and the resin contains two monomers which allows for both hydrophilic and lipophilic retentions.

The SPE technique was considered for the extraction of antibiotics from propolis samples in the current study. Generally, reverse phase, normal phase and ion exchange columns were used for the analysis of antibiotic residues. But recently, new types of stationary phases have become available such as mixed sorbents, polymeric and graphite carbon etc.

The honey and propolis both are honey bee products and they are complex in their matrix composition. The antibiotic analysis from honey samples from different regions was well studied (Bargańska et al. 2011). Irrespective of using any analytical techniques for the analysis of antibiotics, extraction and clean-up methods were found to be necessary for honey samples. The SPE extraction method, using an Oasis HLB cartridge, was conducted for the sample clean-up for the analysis of a variety of drug residues, such as macrolides, tetracyclines, quinolones, and sulfonamides in honey (Vidal et al. 2009). SPE extraction was also employed for the analysis of pesticides (coumaphos, carbendazim, and amitraz) and antibiotics (five tetracyclines, four sulfamides) in honey (Debayle et al. 2008). Similarly, the SPE extraction method has been utilised for the analysis of antibiotics such as tetracycline, oxytetracycline, chlortetracycline,

doxycycline, minocycline and methacycline from different types of honey samples (Viñas et al. 2004). In the present study, an Oasis type of sorbent was used due to its usefulness for the extraction of antibiotics from propolis. The same type of sorbent was used by Zhou et al. (2009) in similar studies with propolis.

The published two-step SPE method (Zhou et al. 2009) was chosen first to start this experiment which includes the use of carboxylic acid (CBA) and HLB cartridges. The pre-extraction procedure also included an ultrasonic extraction with a specified buffer solution as reported by (Zhou et al. 2009). The procedure is explained in detail in the section 5.1.1.1. Considering the importance of this procedure, the final result was not perfect and antibiotic leakage was found from the first loading step. Analysis was done at each step of the SPE procedure, such as after loading, washing etc., by collecting the sample/extraction solution at each step. A gradual loss of the antibiotic standards was found step by step which caused less recovery at the end. The majority of the tetracycline was lost in the first few steps. The resulting percentage recovery for the studied antibiotics was found to be between 30-70%, which also was not reproducible in further repeat experiments. The resulting chromatograms are presented in figures 5.11-5.14. A major loss of antibiotics (peaks at RT 2.2, 2.4, 3.6 and 4.1) was observed in the chromatograms of samples collected after loading (Figure 5.11) and first washing (Figure 5.12). The sample collected after the second washing and while the last elution step showed only one peak (Figure 5.13 and 5.14). These chromatograms clearly demonstrated that this reported method was not suitable and was unable to extract tetracyclines from propolis.

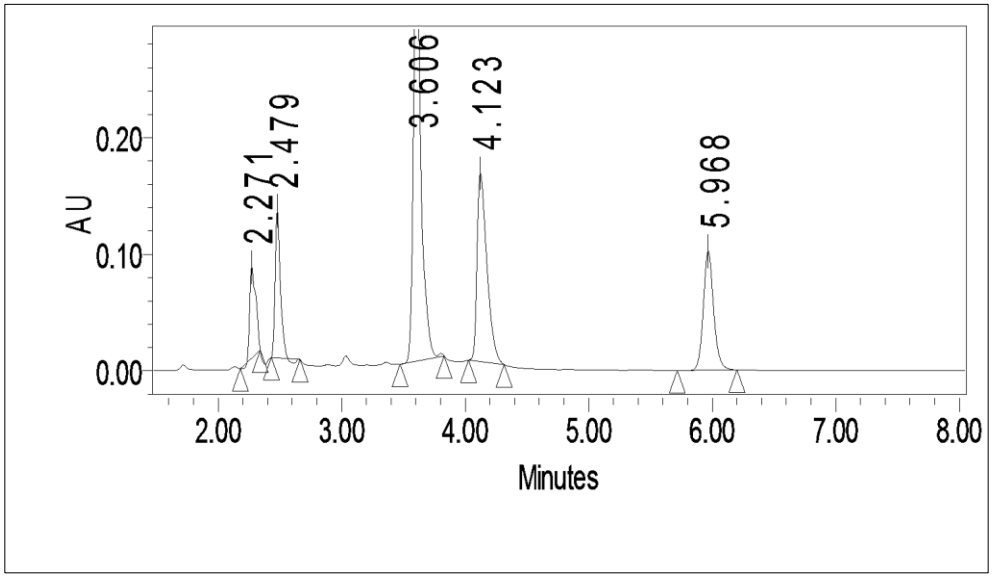


Figure 5.11: UPLC analysis of standard mixture after loading step in SPE method.

Peak 1 (RT 2.2 mins): Tetracycline, Peak 2 (RT 2.4 mins): Oxytetracycline, Peak 3 (RT 3.6 mins): Chlortetracycline, Peak 4 (RT 4.1 mins): Doxycycline.

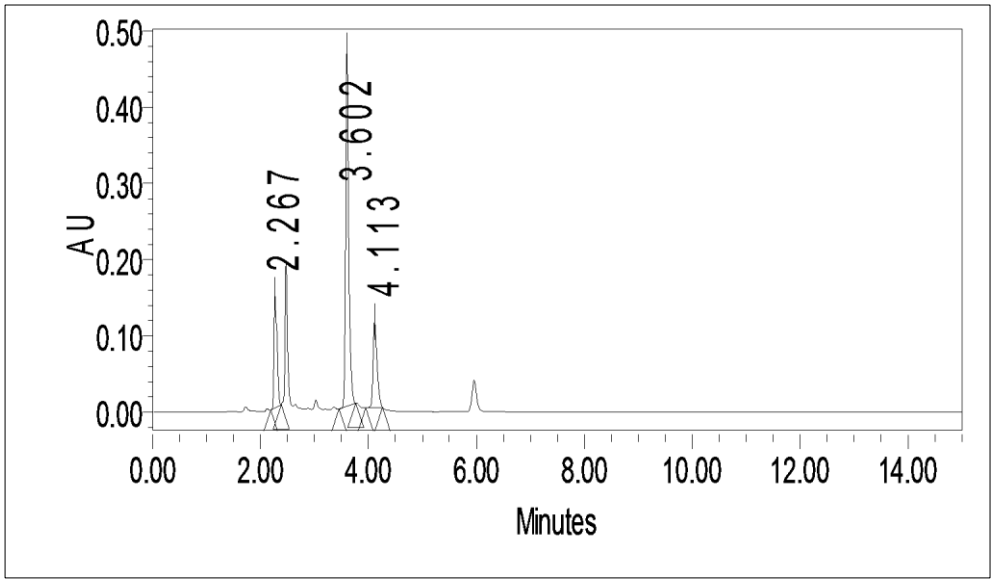


Figure 5.12: UPLC analysis of standard mixture after washing step in SPE method.

Peak 1 (RT 2.2 mins): Tetracycline, Peak 2 (RT 2.4 mins): Oxytetracycline, Peak 3 (RT 3.5 mins): Chlortetracycline, Peak 4 (RT 3.8 mins): Doxycycline, peak 5 (5.9): Unknown peak.

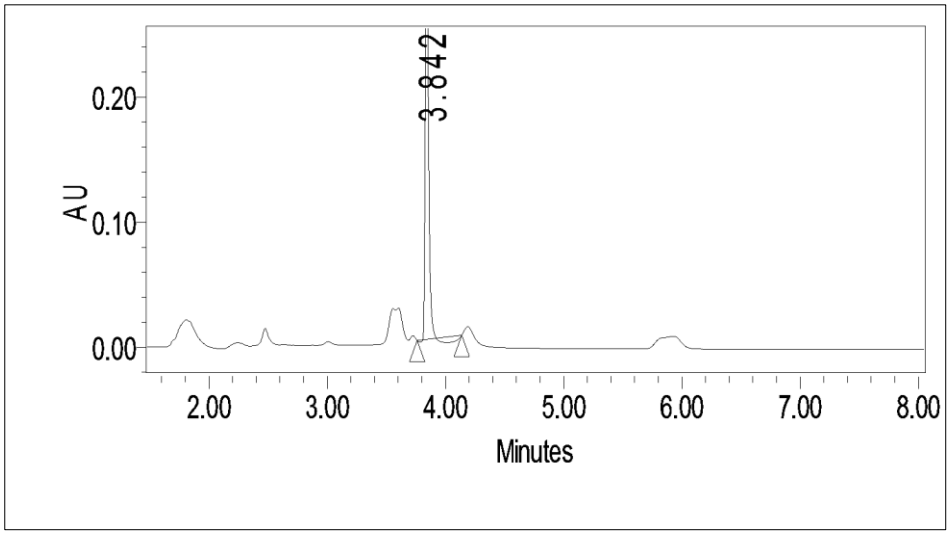


Figure 5.13: UPLC analysis of standard mixture after second washing step in SPE method.

Peak 1 (RT 3.6 mins): chlortetracycline.

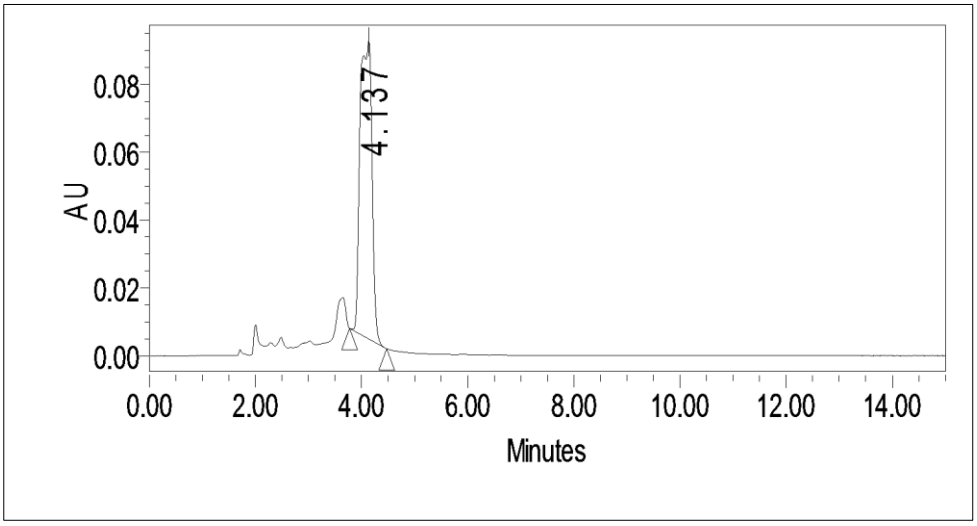


Figure 5.14: UPLC analysis of standard mixture after last elution step in SPE method.

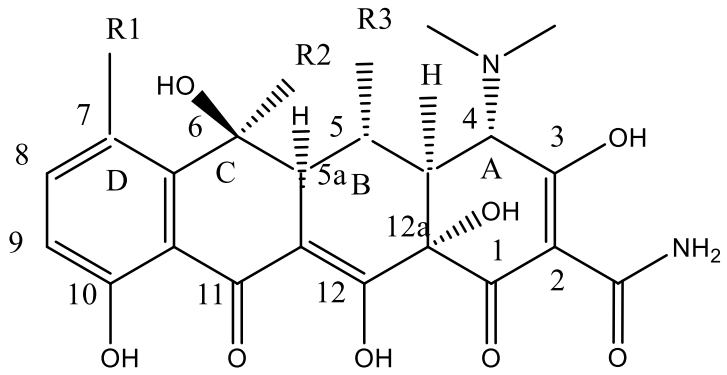
Peak 1 (4.1): Doxycycline.

The two-step SPE method was tried separately using each one of the cartridge types considering the antibiotics amphoteric nature. HLB is a hydrophilic

lipophilic balanced, water-wettable reverse phase sorbent with an equal ratio of two monomers, the hydrophilic N-vinyl pyrrolidine and lipophilic divinylbenzene. It is helpful for extracting acidic, neutral and basic compounds. Alternatively, CBA is used in WCX (weak cation exchange) to extract strong or weak cations. The pre-extraction techniques were carried out in the same way as the method reported by Zhou et al (2009). The conditioning of the sorbent (CBA and HLB separately) was attained by using 1ml methanol and 2ml McIlvaine buffer at pH 4.2, followed by loading and then elution of the same solution used by the referenced method (Zhou et al. 2009). The low pH in the WCX SPE method helped to cancel the charge on the carboxyl group of the sorbent which helped to elute the antibiotics (Stubbings et al. 2005). However, both these individual trials failed to attain a high percentage recovery and the loss of eluents was found to occur in the first two steps only. The reason for this may be the complex matrix, high impurities and irrelevant compounds (Zhou et al. 2009).

Many other related reported methods were reviewed and matched with current studies and a few of them were tried. The successful identification of formoterol from a complex urine sample using a HLB cartridge and SPE technology by (Nadarassan et al. 2007) was considered and was co-related with current studies. The SPE methodology was repeated by keeping the sonication step constant from previous method (Zhou et al. 2009) and was followed by the addition of 2 ml phospahte buffer in the propolis sample. Conditioning of the HLB cartridge was done by 2ml methanol followed by loading the sample at a low flow rate (1-1.2ml/min). Washing was carried out by using 2ml 1% NH_4OH (30% NH_4OH in 10% methanol; 1:99, v/v) and then drying of the sorbent. Elution was achieved by using 2ml glacial acetic acid in 70% methanol (2/98, v/v). The resulting analysis by using HPLC showed merged peaks for the

propolis sample while only one peak was found for the standard's mixture; this was because of loss of the antibiotics in the steps proceeded the elution. Less antibiotic wastage was found after loading and the possible reason for this may be the addition of the phosphate buffer. The resulting percentage recovery for the antibiotic standards was found to be in the range 70-100% after final elution step and a consistency in the results was found in repeat studies. Because of this successful step, pre-preparation steps of SPE were finalised which included the use of sonication at a certain temperature (50°C) followed by the addition of the phosphate buffer. After the addition of the phosphate buffer in sample, the pH of the sample (propolis/standard) was adjusted to 6.5 by adding 0.1N orthophosphoric acid solution, which allowed the hydrogen bond at C-11 and C-12 positions (Figure 5.15 and Table 5.13) with the sorbent in the HLB cartridge to form. This pH also allowed the washing of other unwanted compounds such as esters and acids from the sample itself.



Oxytetracycline: $R_{1=}H$, $R_{2=}R_{3=}OH$
Tetracycline: $R_{1=}R_{3=}H$, $R_{2=}OH$
Chloretracycline: $R_{1=}Cl$, $R_{2=}OH$, $R_{3=}H$
Doxycycline: $R_{1=}R_{2=}H$, $R_{3=}OH$

Figure 5.15: Structural presentation of tetracyclines (Anderson et al. 2005)

Table 5.13: Tetracycline charge state (Anderson et al. 2005)

	$pH<pK_{a1}$	**$pK_{a1}<pH< pK_{a2}$**	**$pK_{a2}<pH< pK_{a3}$**
O3 charge	0	-	-
O11-O12 charge	0	0	-
N4 charge	+	+	+
Overall charge	+	0	-

For further optimisation of the remaining SPE steps, another simple method (Cheng et al. 1997) was followed. It includes a HLB sorbent using SPE method, conditioning by 1ml methanol and 1ml water, followed by loading at a slow flow rate, washing by 5% methanol and final elution by 1ml methanol. As the method is mainly using methanol in each step, it adversely affected the recovery of the antibiotics and extra peaks were found in the UPLC studies. Therefore, the combination of this method and the above method was tried using the most suitable steps and a new method was developed.

In this new method, the McIlvaine buffer was replaced by water due to the un-usefulness of the buffer solution. The addition of 2ml phosphate buffer (100mM) in the propolis sample (1gm in 20ml) after sonication was applied to achieve the required pH. The conditioning of the cartridge was achieved by applying 1ml methanol and 1ml water, followed by loading at a slow flow rate. The final elution was carried out by using different solutions as follows:

a. methanol,

b. acetonitrile,

c. 2% glacial acetic acid in 95% methanol,

d. 2% glacial acetic acid in 70% methanol.

Each trial was repeated with similar steps except the elution by using these solutions separately. The washing method was found to be unnecessary because of the loss of antibiotics caused at this step in the previous trials.

A high percentage recovery was obtained using 2% glacial acetic acid in 95% methanol, compared to the other solution. Because of the acidic pH, the tetracycline compounds protonated and become more soluble with the elution solvent (see figure 5.15). A similar method was successfully used to extract out formoterol from urine using the same pH difference (Nadarassan et al. 2007).

In the next step, the loading volume was finalised as it was one of the important criterion in SPE (Hennion 1999). All other steps were kept constant except for the loading. The variation was studied by using sample volumes of 10ml, 5ml, 4ml, and 3ml. The best one was chosen by comparing the final recovery values and it was found that 5ml and 10 ml are more suitable as they offered the highest recovery values. This experiment was repeated many times to check for repeatability.

The final SPE method has following optimised parameters; it includes a HLB sorbent using SPE method, conditioning by 1ml methanol and 1ml water, followed by loading at a slow flow rate (sample after addition of phosphate buffer) and final elution by to 2% glacial acetic acid in 95% methanol.

This SPE method was tried with all analytical techniques such as UPLC,HPLC and MELC. This SPE extraction method worked well with MELC but only for three antibiotics out of four. This was because the optimised MELC method for antibiotics was only able to elute three antibiotics. Optimised MELC method includes following parameters, mobile phase 10 mM acetate buffer (pH 5): ethyl acetate: Brij L23: 1-butanol (91%: 3%: 3.5%: 2.5%, w/w/w/w); flow rate 1ml/min.; column temperature 30ºC; injection volume 20 µl, wavelength 350nm.

Tetracycline and oxytetracycline were eluted at the same RT. Chloretetracyclne is commonly used by farmers in apiculture and therefore its very common contaminant found in honey (Mascher et al. 1996; Al-Waili et al. 2012). To choose one of the antibiotics from both of them, the propolis samples were analysed using the optimised SPE method and MELC method and the presence of oxytetracycline in resulting chromatograms was found. Hence, for further validation of the process, oxytetracycline was selected with doxycyline and chlortetracycline. The optimised SPE method is described in 5.1.4 section with the optimised MELC method being validated using ICH guidelines (ICH 1996). The chromatogram of the optimised SPE extraction procedure MELC method for the selected antibiotic compounds is shown in figure 5.16. The first peak at RT 2.95 minutes was for oxytetracycline, the second peak at RT 4.4 was for chlortetracycline and the third peak at RT 5.86 was for doxycycline. All resulting peaks were eluted and were well separated from each other.

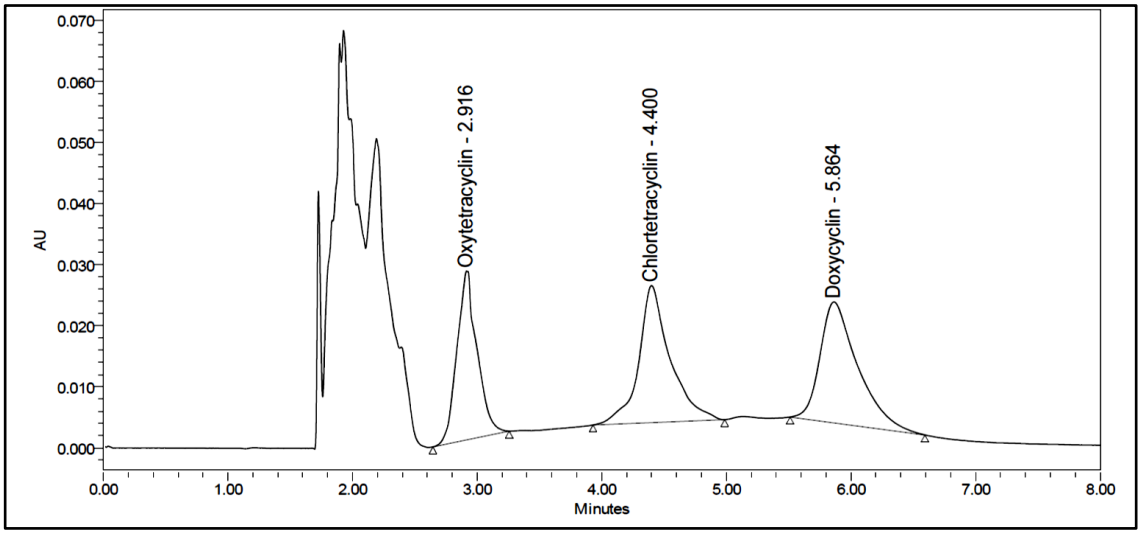


Figure 5.16: Resulting chromatogram of tetracycline antibiotics in response to optimised SPE and optimised MELC condition

5.2.5 Validation of optimum microemulsion LC method and SPE clean-up method for antibiotic analysis in propolis

The method validation was carried out by following ICH guidelines (ICH 1996). The experiment was designed into five groups A, B, C, D and E. In each group, six levels of the standard mixture were prepared. All of the experiment details are mentioned in section 5.1.4. The resulting data is discussed as follows.

Linearity

To determine the linearity, at least five levels of different concentrations of standard/s or sample/s of interest with replicate measurements were required. Five levels of concentrations were prepared for the standard mixture and were injected. The mean values for the area of each peak (from 3 injections) and the concentration of the standards were used to plot a linearity graph. The following graphs were obtained for each standard (Figures 5.17-5.19).

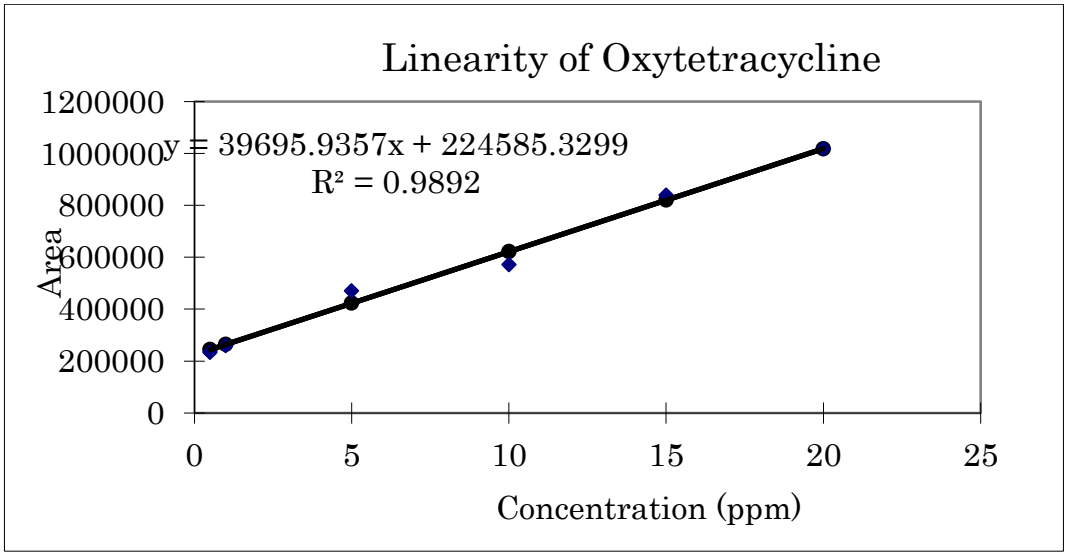


Figure 5.17: Linearity for Oxyteracycline standard in validation of antibiotics

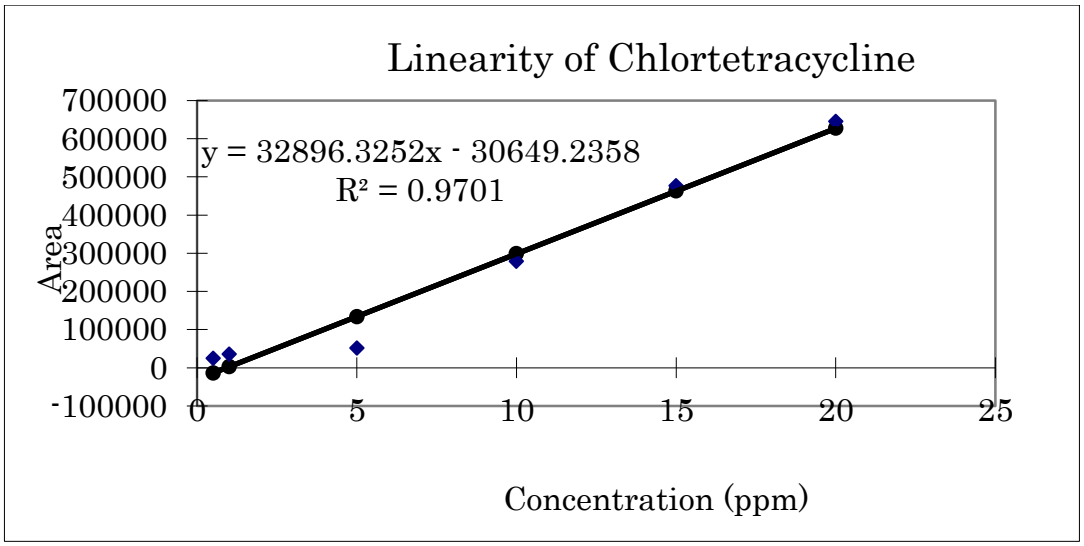


Figure 5.18: Linearity for Chlortetracycline in validation of antibiotics

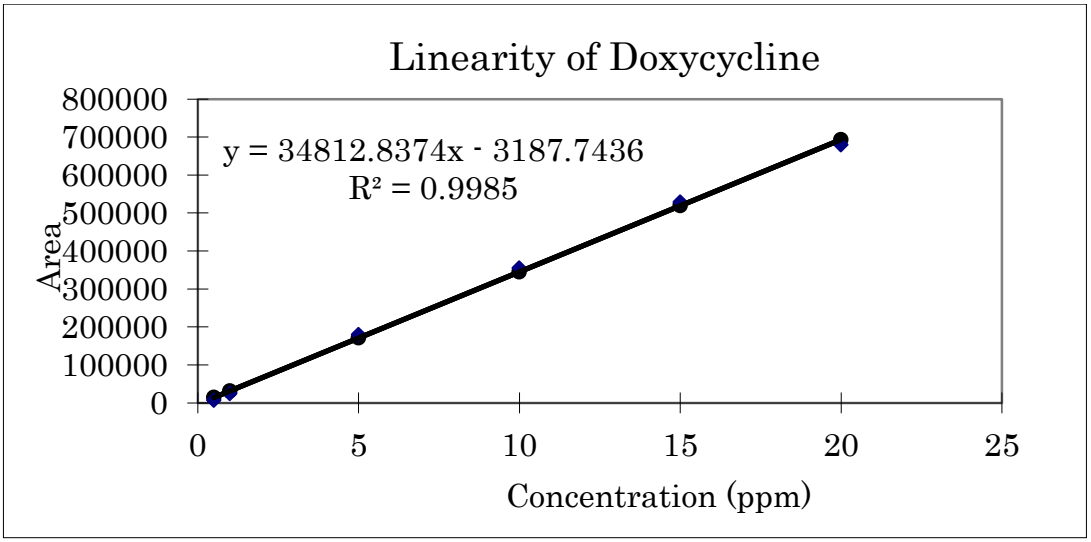


Figure 5.19: Linearity for Doxycycline in validation of antibiotics

Table 5.14: Summary of result of linearity experiment

Sr. No.	Name of Standard	R^2
1	Oxytetracycline	0.9892
2	Chlortetracycline	0.9701
3	Doxycycline	0.9985

The correlation coefficient was much closer to 1 and hence proved that there was correlation between the concentration and absorbance of the tetracycline standards after SPE.

Precision

Precision is the closeness in agreement between test results. The precision was assessed using three concentration levels (low, medium and high) and 5 injections of each concertation. RSD (relative standard deviation) values for each concentration level were calculated to correlate the data. The precision studies were carried out for inter-day and intra-day analysis. The results are as shown in tables 5.15 and 5.16.

Table 5.15: Intra-precision result for antibiotic standards

Standard	**RSD**		
	Concentration of mixture of standards		
	Low level	Medium level	High level
	1µg/ml	10µg/ml	20µg/ml
Oxytetracycline	5.89	5.29	0.75
Chlortetracycline	4.89	4.26	2.55
Doxycycline	6.65	2.66	2.12

Table 5.16:Inter-precision result for antibiotic standards

Standard	**RSD**		
	Concentration of mixture of standards		
	Low level	Medium level	High level
	1µg/ml	10µg/ml	20µg/ml
Oxytetracycline	9.18	5.34	2.47
Chlortetracycline	12.18	3.64	0.48
Doxycycline	4.87	3.50	2.78

The resulting RSD values were found to be in the acceptable range, and below 15%, according to the Food and Drug Administration (Center for Drug Evaluation and Research 1994; Zhou et al. 2009). The RSD values calculated in similar precision studies were found to be in the range 1-14% , which was also acceptable (Zhou et al. 2009).

Limit of detection (LOD)

The limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value (ICH 1996). It can be measured using the equation which is explained in section 4.4. It was calculated by injecting three injections (n=3) of five different levels of standards.

Table 5.17: LOD results for antibiotic standards

Standards	**LOD µg/ml**
Oxytetracyclin	0.49
Chlortetracycline	0.82
Doxycycline	0.18

Limit of quantitation (LOQ)

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample which can be quantitated as an exact value (ICH 1996). It can be measured using the equation which is explained in section 4.4. It was calculated by injections (n=3) of five different levels of standards.

The LOQ values here in range 0.5-2.5 µg/ml while in similar studies, these values calculated differently in range 100-150ng/g (Zhou et al. 2009).

Table 5.18: LOQ results for antibiotic standards

Standards	LOQ µg/ml
Oxytetracyclin	1.49
Chlortetracycline	2.51
Doxycycline	0.55

Accuracy

The ICH defined accuracy as the "closeness of agreement between the conventional true value and value found". The accuracy of this method was assessed by calculating concentration of the extracted antibiotic standards with the true concentration of antibiotics. The values should be close to 100% (ICH 1996).

Table 5.19: Accuracy Results for antibiotic standards

Sr. No.	Standards	Actual concentration (µg/ml)	Observed concentration (mean ± SD., µg/ml)	% Accuracy
1	Oxytetracycline	20	18.41±0.15	92.07
2	Chlortetracycline	20	21.80±0.49	109.03
3	Doxycycline	20	20.61±0.41	103.08

Recovery

The recovery of the method was assessed by comparing the peak area of the extracted antibiotic with the peak area of antibiotic standards.

Table 5.20: Recovery for antibiotic standards

Sr. No.	Standards	Recovery in % Group A	Recovery in % Group B
1	Oxytetracycline	40.64	45.07
2	Chlortetracycline	77.37	82.02
3	Doxycycline	67.86	68.45

The recovery found in range 40-83% for group A and group B antibiotic standards of 20 µg/ml concentration. The recovery values were not close to the 100% target but this was the only method which was able to recover these antibiotic compounds successfully as compared to previous studies. In similar analytical studies from propolis, a variety of percentage recovery of these compounds were found to be in the range of 60-89% (Zhou et al. 2009).

Robustness

The robustness is a measure of the analytical methods capacity to remain unaffected by small, but deliberate variations, in method parameters. It provides an indication of procedure reliability during normal usage (ICH 1996). The reliability of the analytical method was studied by introducing small changes to the method parameters (around ±5%) such as a variation in the mobile phase proportion, temperature, flow rate, pH etc. The variation details and results are shown in table 5.21.

Table 5.21: Results for Robustness studies

	Variation in Chromatographic condition	**Peak name**	**RT in min**	**Results Observed**
Optimum condition temperature 30°C and flow 1ml/min		Oxytetracycline	2.9	
		chlortetracycline	4.4	
		Doxycycline	5.8	
Temperature variation	1)28.5°C	Oxytetracycline	2.9	RT not shifted, peak separation
		chlortetracycline	4.3	
		Doxycycline	5.7	
	2) 31.5°C	Oxytetracycline	2.9	RT shifts longer, peak separation
		chlortetracycline	4.5	
		Doxycycline	6	
Flow rate variation	1)1.05 ml/min	Oxytetracycline	2.81	RT not shifted, peak separation
		chlortetracycline	4.2	
		Doxycycline	5.6	
	2)0.95 ml/min	Oxytetracycline	3.07	RT shifts longer, peak separation
		chlortetracycline	4.62	
		Doxycycline	6.18	

The optimised HPLC method was very robust and found all peaks to be similar at different variations as compared to optimum conditions. The peak resolution found between peaks was more than 1.5 in response to all variations. However, the peak area was decreased considerably at the low temperature (28.5°C) and high flow rate (1.05ml/min) as compared to optimum conditions. So, it was advisable to follow the optimum conditions to obtain accurate quantification of the resulting antibiotics.

Stability

The chemical decomposition was critical for tetracyclines as they possessed very poor stability. The freeze and thaw method was used to study the stability of the antibiotic compounds from the propolis sample and standards by

comparing them with freshly prepared samples/standards of the same concentration. Due to the awareness of sample poor stability in regular practice, samples/standards were studied only within 48 h.

Table 5.22: Stability results

Standards	**% Loss of concentration of compounds in standard solution (24h)**
Oxyteracycline	5.22
Chlortetracycline	21.51
Doxycycline	6.77

The antibiotic solutions are photosensitive and are only stable at the freeze condition, -18°C (Zhou et al. 2009). The normal freezing temperature (4°C) was not favorable for these antibiotics, even over a 24h period. Therefore throughout this study, the preparation of new standards was practiced frequently. Aluminium foil was used to cover the standards solution vials to keep them protected from light (Zhou et al. 2009).

One of the chromatograms from the validation procedure of 20µg/ml concentration is shown (Figure 5.17). Three antibiotic standards were well separated and showed sharp peak shape.

5.2.6 Validated method application for propolis sample

The validated method which included the SPE extraction method and MELC analytical method was used to check for the possibility of the presence of antibiotic contaminations in the propolis samples which were available. Four processed samples in the form of a powder, capsule, liquid and tincture were used for this study which was similarly used in flavonoid analysis. The

preparation of the experiment was repeated 3 times to identify and quantify the resulting antibiotics accurately. The preparation method that was followed was similar as the method explained in section 5.1.4. The resulting values are shown in tabular form in table 5.23.

Table 5.23: Antibiotic analysis from propolis samples

Name of sample	Name of antibiotic found	Identified compound in mg/kg±SD
Propolis powder	Oxytetracycline	1.05±0.03
Propolis capsule	Oxytetracycline	2.61±0.21
Propolis tincture	Oxytetracycline	1.95±0.13
Propolis liquid	Oxytetracycline	3.28±0.12

Only one antibiotic was found in all of the studied propolis samples, oxytetracycline. It was likely to be found in honey bee products due to its frequent use in apiaries to treat European foulbrood disease and American foulbrood diseases caused by *Paenibacilus* (Bacillus) larvae and *Streptococcus pluton* bacteria, respectively (Al-Waili et al. 2012). The traces of antibiotics found in the studied honey samples from different regions of Greece can be explained by the common practice in apiaries for disease control, especially using oxytetracycline and doxycycline (Saridaki-Papakonstadinou et al. 2006; Zhou et al. 2009). Presence of high residue levels of oxytetracycline in honey with residues of 3.7 mg/kg eight weeks after application in liquid form was observed while studying EFB treatment regime on oxytetracycline levels in honey extracted from treated honeybee colonies (Thompson et al. 2005). It was reported that the presence of oxytetracycline along with tetracycline, doxycycline, chlortetracycline and chloramphenicol was observed in honey

samples from China using the different analytical technique, high performance capillary electrophoresis; in the range of 20µg/L-40 µg/L. Very few attempts were performed to determine the concentration of the antibiotics in propolis. It appears to be more challenging than using honey samples because of the added complexity. Zhou et al. (2009) successfully developed an extraction method as well as an analytical method to analyse tetracycline antibiotics in propolis, this was initially trialed in this study. They found the residue of oxytetracycline and tetracycline in two propolis samples out of 30 samples in the range of 100-150µg/kg of raw propolis.

5.2.7 Summary

The analysis of antibiotics from propolis is challenging because of many hurdles that need to be overcome when optimising the method. The main problem was the complexity of the propolis matrix, and therefore to analyse trace amounts of antibiotics, the clean-up methods were an essential step before the analysis was carried out. As a part of this study, the development of a suitable analytical method, as well as a suitable clean-up method, was achieved by using a single variation technique at each step. Three different analytical methods were developed using HPLC, UPLC and MELC and all were practiced as convenient while studying different clean-up methods. HPLC and UPLC methods were found to be unsuitable after extraction analysis because of the interference of unknown peaks with known peaks but in MELC both the known and unknown peaks were well separated. Therefore, the MELC method was selected for further analysis.

In the clean-up method development process, LLE was used in the first few trials using immiscible solvents. This extraction method showed poor

percentage recovery of antibiotics from propolis sample and co-elution of the known antibiotic peak along with a large unknown peak. These disadvantages meant that this technique was found to be not suitable for the clean-up of unwanted compounds from the samples. Henceforth, SPE was chosen for the next few trials. The reported method of Zhou et al. (2009) was also found to be unsuitable and therefore additional trials were carried out using different SPE conditions and cartridges. The HLB cartridge was found to be more suitable for this study and was used to test further variations. The optimisation of the SPE method was carried out using relevant, existing methods (Cheng et al. 1997; Nadarassan et al. 2007). The best performing steps were chosen from both these trials and a final method was optimised. This extraction method, using HLB, was again modified by optimising a suitable loading volume and solvent for final elution. The final optimised SPE method with optimised MELC method was finally validated using ICH guidelines (ICH, 1996).

6. EXTRACTION STUDIES OF PROPOLIS

Extraction is a process of separation of medicinally active components from the plant/animal tissues using an appropriate solvent. Extraction of the bioactive components from the natural sources is a challenging task and there are different types of it, for example solvent extraction (Belova et al. 2009), high hydrostatic pressure extraction (Corrales et al. 2009) and super critical fluid extraction (Reverchon and De Marco 2006) etc. The major concern in the extraction studies is to select and identify extraction method which extracts active components with maximum yield and high purity (Shirsath et al. 2012). The main aim of this work to select most suitable extraction method for flavonoid extraction from propolis by comparing different extraction techniques. It has been studied and approved immense usefulness as antioxidant and antibacterial properties of propolis (Mohammadzadeh et al. 2007). For the availability of the active ingredients such as phenolic compounds and flavonoids from propolis, it is necessary to study different patterns of extraction methods using different solvent range. Flavonoid extraction from propolis using high hydrostatic pressure extraction was studied and found a possible effective extraction method compared to other conventional extraction methods (Shouqin et al. 2005). Maceration and sonication techniques were employed to extract phenolic and flavonoid compounds from propolis and observed that ultrasound extraction was more efficient technique to extract flavonoid (Khacha-Ananda et al. 2013). Different extraction techniques such as maceration, ultrasound extraction and microwave assisted extraction using 70% ethanolic solvents were experimented but microwave assisted extraction was found more effective than the other two extraction methods with short timeframe as an advantage (2007). Ethanolic extraction methods were studied by many researchers using different types of propolis mainly to extract phenolic and flavonoid compounds

(Nieva Moreno et al. 1999; Cunha et al. 2004; Trusheva et al. 2007; Coneac et al. 2008). A number of conventional extraction techniques have been widely used to extract the flavonoids from propolis such as maceration extraction using ethanol as a solvent of choice (Park and Ikegaki 1998; Woisky and Salatino 1998; Cunha et al. 2004; Dziedzic et al. 2013). However, various limitations are associated with these traditional methods; are time consuming and the use of solvent. Recently, new technologies including ultrasound extraction and microwave extraction have been developed for effective extraction of bioactive compounds from poplar type of propolis such as polyphenols, flavones/ flavonols and flavanones/dihydroflavonol (Popova et al. 2004; Trusheva et al. 2007). Most of these studies utilised conventional maceration extraction technique coupled with other techniques. For the extraction of active components from propolis, ethanolic extraction method is very popular and accepted technique. But there is a lack of knowledge about possible practicality of other solvents for propolis extraction. Hence, in this chapter, ranges of solvents with different extraction technique were studied for flavonoid extraction from propolis. The advantage of this work is to compare variety of possible extraction methods with combination of different solvents will increase the chances of selection of the most effective extraction method.

The purpose of the present study is to compare different conventional and new techniques and study their diverse patterns of the extraction. Additionally, the impact of various solvent systems on the extraction was studied.

Several components from propolis dissolve in different solvents and are also responsible for different activities as outlined in in the table 6.1 (Wagh 2013).

From these, three solvents such as methanol, ethanol and acetonitrile were selected in this study. Section 6.1 describes details of the materials and

methods adopted in the present work and section 6.2 contains the results and discussion of this work.

Table 6.1: Different solvents used for the extraction of propolis (Wagh 2013)

Water	Anthocyanins, starches, tannins, saponins, terpenoids, polypeptides and lectins
Methanol	Anthocyanins, terpenoids, saponins, tannins, xanthoxyline, totarol, quassinoids, lactones, flavones, phenones, polyphenols, polypeptides, and lectins
Ethanol	Tannins, polyphenol, polyacetylenes, terpenoids, sterols, and alkaloids
Chloroform	Terpenoids, flavonoids
Dichloromethane	Terpenoids, tannins, polyphenols, polyacetylenes, sterols, and alkaloids
Ether	Alkaloids, terpenoids, coumarins, and fatty acids
Acetone	Flavonols

6.1 Materials and methods for propolis extraction studies

6.1.1 Materials and chemicals

Propolis samples provided by Nature's laboratory, UK were used in this research work. All other details were mentioned in section 3.1.1. All solvents (methanol, ethanol, acetonitrile) used were of HPLC grade, other details are described in section 3.1.4.

6.1.2 Extraction methods

For this study, conventional and new extraction techniques were selected. A detailed experimentation is provided below,

6.1.2.1 Maceration

For maceration studies, finely grounded propolis powder (1g) was weighed accurately and transferred it to a clean vial. This was dissolved in 20ml of the respective solvent system and labelled it neatly. The maceration was achieved by using a magnet shaker. A magnetic bid was placed in each labelled bottle and allowed to shake for 24 h (or at different time period in other experiments) at 500rpm. The experiments were performed in duplicates and repeated if necessary to confirm the resulting performance. Different solvents such as 70% ethanol (in DW), 70% methanol (in DW), 50% ethyl acetate (in DW), 70% acetonitrile (in DW), pure acetonitrile, pure methanol, pure ethanol and some combinations of acetonitrile with non-ionic surfactant solutions of Tween 20 and Tween 80 were used (details are provided in section 6.1.2.4). The resulting mixtures were then filtered using a 0.45µm nylon membrane filter paper; vacuum dried and finally analysed using HPLC.

6.1.2.2 Hot extraction

In hot extraction, finely grounded raw propolis 1g was weighed accurately and dissolved in the respective solvents (usually 20ml). The temperature of the flask was maintained at 70°C for 1h. All the solvents/solvent mixtures were prepared in the same way as mentioned in the previous section 6.1.1.1. The resulting mixtures were allowed to cool at RT and filtered using a 0.45µm nylon membrane filter paper, vacuum dried and finally analysed using HPLC.

6.1.2.3 Ultrasound extraction

In ultrasound extraction, 1g of finely grounded propolis was weighed separately and dissolved in the respective solvents (usually 20ml) and subjected to sonication for 4h at RT using same solvents/solvent mixtures and the resulting solutions were filtered using the same procedure as mentioned in section 6.1.2.1. Final analysis was performed using HPLC.

6.1.2.4 Extraction using non-ionic surfactants

Hydro-distillation was performed using non-ionic surfactant solutions in a first stage of the experiment. The successive trails were performed using 5g of grounded propolis sample in 100ml of DW in order to optimise the temperature and pressure. Approximately 40-45°C temperature and 95mbar pressure were used as an optimised condition to achieve the desired amount of flavonoids. The solutions of non-ionic surfactants such as 0.5% solutions of Tween 20 and Tween 80 in water was used in further experiments by replacing 100ml DW in distillation. The residue of each sample was collected and dissolved in pure methanol and filtered using a 0.45µm nylon membrane filter paper in vacuum assembly. The obtained residual solution was transferred to 100ml volumetric flask and was further diluted with methanol. The prepared sample was then subjected to flavonoid analysis by HPLC.

All above extraction methods (maceration, hot extraction and ultrasound extraction) with non-ionic surfactants were also studied. Following surfactant combinations with water and mixture of water and acetonitrile were studied using maceration technique. The next part of the work is to study effect of Tween 20 aqueous solutions on different extraction methods. The details of the solvent and surfactant mixture are described in the result and discussion part 6.2. The extraction technique mirrored the procedures outlined above unless mentioned otherwise.

For analysis of all extracted samples, the house developed and validated HPLC method was used. All samples were prepared in duplicates and analysis was carried out in triplicate.

6.2 Result and discussion for extraction studies of propolis

Many attempts were made to identify conventional as well as modern extraction technologies for propolis including maceration, ultrasound, microwave assisted extraction and solvent extraction etc. In current study, some of these extraction methods were utilised and modified using different solvents/solvent mixtures. The experimental results for extraction studies are discussed in this section.

6.2.1 Maceration

Maceration is a simple yet very popular technique which is used extensively for the extraction of plant and other natural products. According to US Pharmacopeia, in this extraction technique, the crude material is grounded/cut into a suitable size/pattern depending on the type of material, mixed properly with a suitable solvent, and allowed to stand at room temperature for appropriate time in a closed container with constant agitation. After sufficient time, the resulting sample is filtered and washed if necessary with same solvent used for maceration and finally the resulting filtrates are concentrated if necessary under pressure, to obtain the desired consistency (Sagert 2008). This maceration technique is used in wine preparation and in variety of food preparations. But this technique was very much popular at ancient time for extraction of the essential oils from different plant parts (Azmir et al. 2013). This technique is also known for the extraction of the bioactive compounds such as phenolic compounds, flavonoids etc (Azmir et al. 2013). Apart from high time frame and labor, this technique is very simple and promising for extraction purpose. In propolis, the complex matrix challenges efficient extraction outcomes, and hence maceration with alcohol (ethanol) is widely used for propolis extraction from many years. The ethanolic extraction is extensively

studied further by many researchers to study antimicrobial and antioxidant activities of propolis extracts (Burdock 1998; Blonska et al. 2004; Mohammadzadeh et al. 2007). Apart from ethanol, other solvents were not studied previously with maceration technique and therefore, this technique is considered here with other solvents. This extraction work is important to find more efficient extraction techniques in terms of flavonoid yield as well as removal of unwanted compounds/ traces and to replace ethanol to more suitable solvent.

The maceration extraction was carried out using different solvents and a description of process is provided in 6.1.2.1. In the first set of experiment, the solvents (70% solvent in water) were studied for extraction of propolis. The methanol (70%) and ethanol (70%) in water showed the major difference in extraction outcomes. Five major flavonoids were selected as resulting amounts are compared (Figures 6.1-6.5).

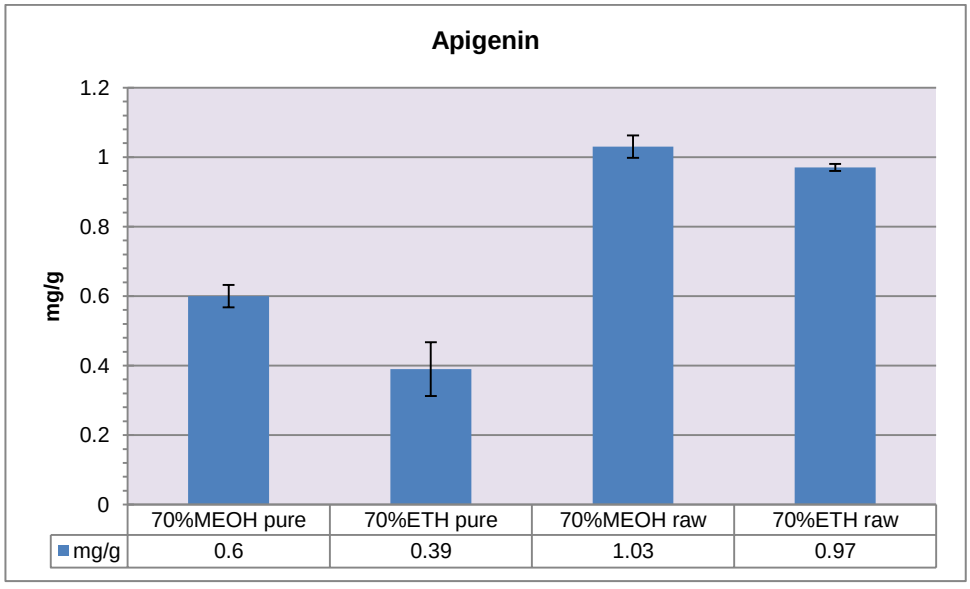


Figure 6.1: Effect of maceration extraction using aqueous solvents on Apigenin flavonoid from propolis

(70%MEOH pure: purified propolis with 70%methanol, 70%ETH pure: purified propolis with 70%ethanol, 70%MEOH raw: raw propolis with 70%methanol, 70%ETH raw: raw propolis with 70%ethanol)

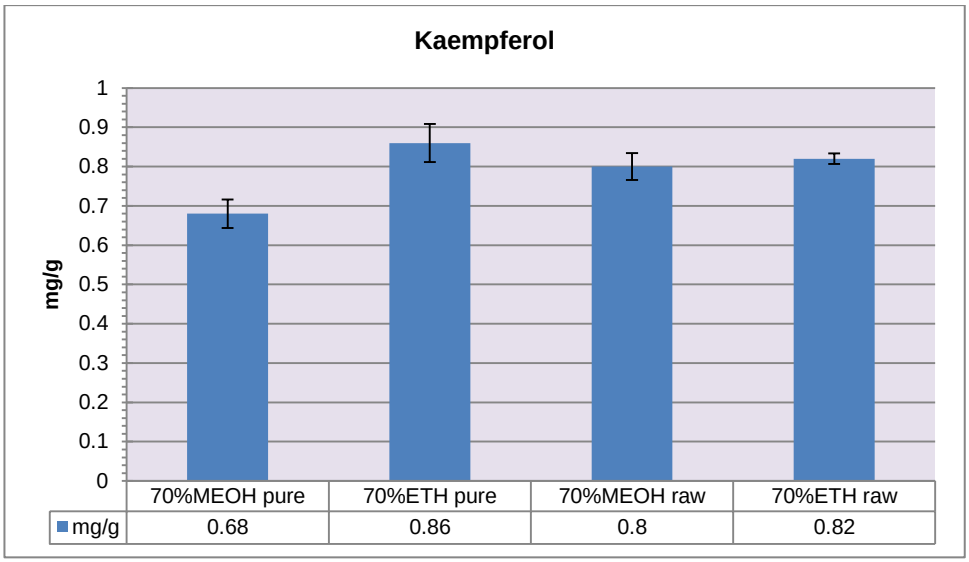


Figure 6.2: Effect of maceration extraction using aqueous solvents on Kaempferol flavonoid from propolis

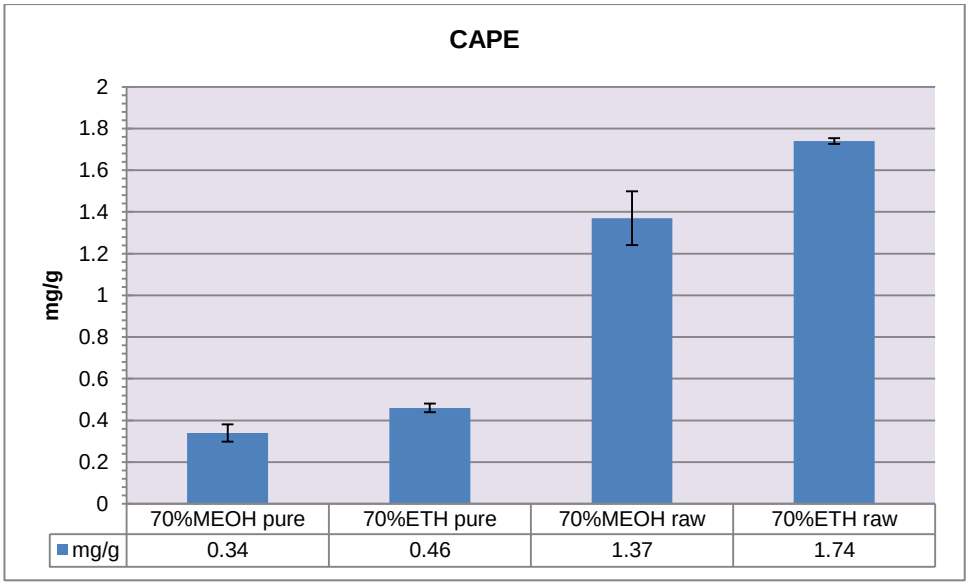


Figure 6.3: Effect of maceration extraction using aqueous solvents on CAPE from propolis

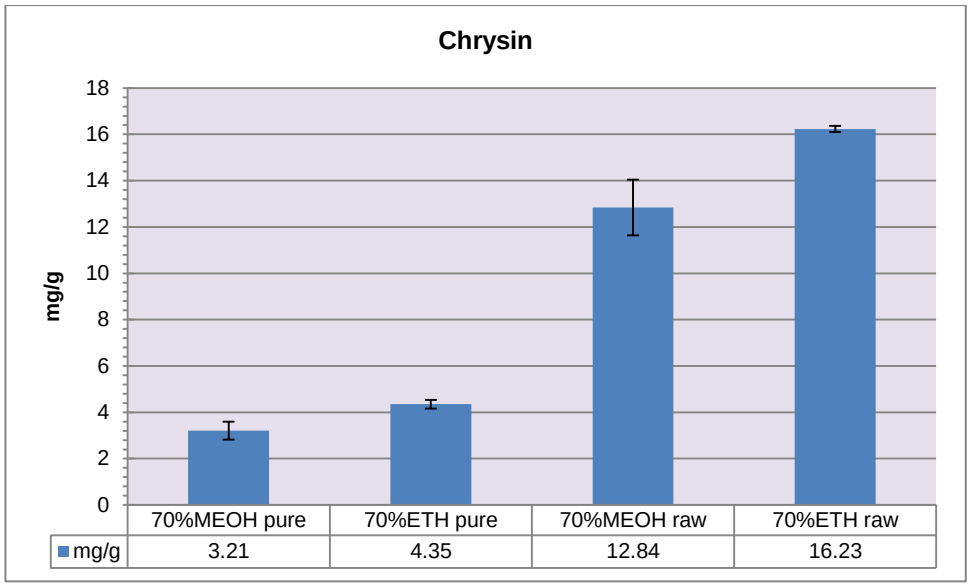


Figure 6.4: Effect of maceration extraction using aqueous solvents on Chrysin flavonoid from propolis

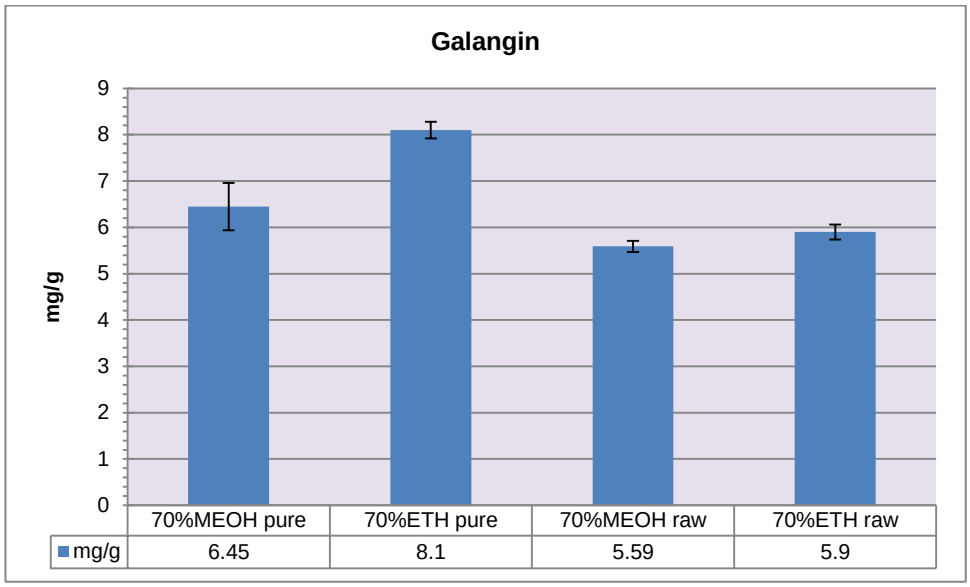


Figure 6.5: Effect of maceration extraction using aqueous solvents on Galangin flavonoid from propolis

In the figures (6.1-6.5), differentiation was observed between methanol and ethanol extraction. Propolis extraction using ethanol (70%) was resulted into the high extraction of studied flavonoids as compared to that of methanol (70%).

Difference found more acute in the two compounds such as chrysin and galangin (Figures 6.1 and 6.2) as compared to other compounds such as CAPE, apigenin and kaempferol (Figures 6.3, 6.4 and 6.5). The raw propolis showed higher amount of flavonoids compared to the purified propolis sample. Effect of different ethanol concentrations in water on propolis extraction in hot and cold conditions was studied earlier and found that higher amount of flavonoids from propolis samples can be extracted at low temperature for longer time rather than samples extracted at high temperature for shorter time (Coneac et al. 2008).

In next stage of experiment, pure organic solvents such as methanol, ethanol and acetonitrile were selected for raw propolis extraction using similar maceration technique. The major five flavonoids as mentioned above were assessed for comparative analysis.

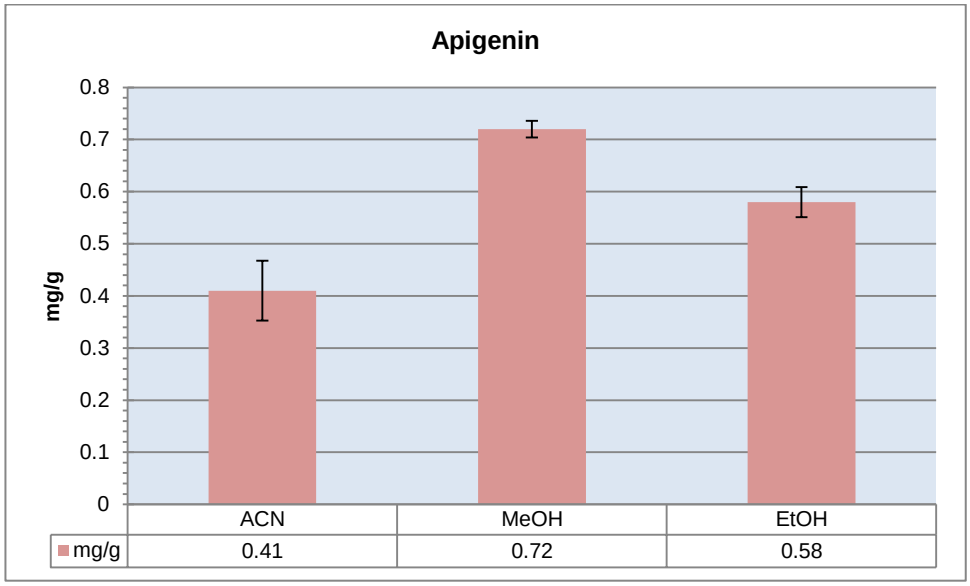


Figure 6.6: Effect of maceration extraction using pure solvents on Apigenin flavonoid from propolis

(ACN: acetonitrile, MeHO: methanol, EtOH: ethanol.)

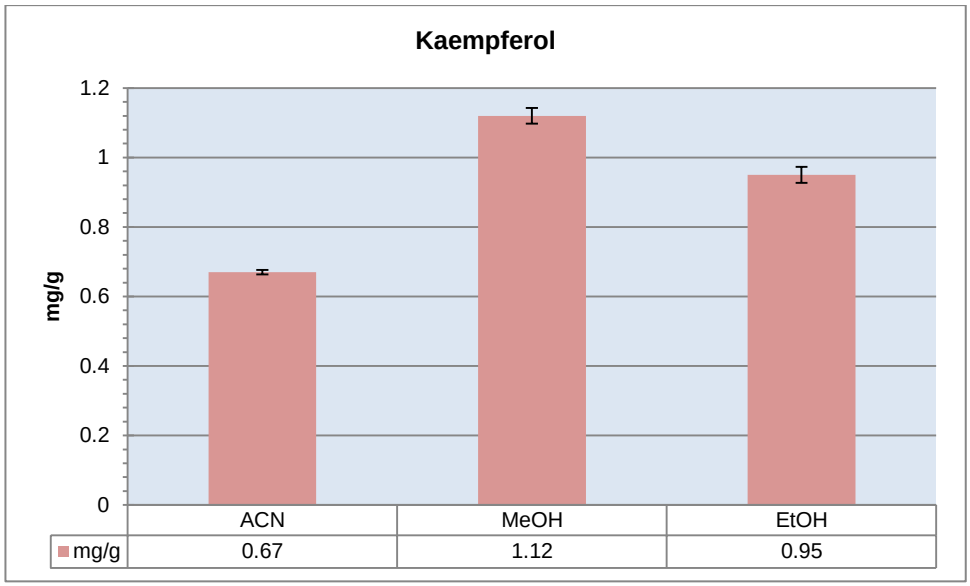


Figure 6.7: Effect of maceration extraction using pure solvents on Kaempferol flavonoid from propolis

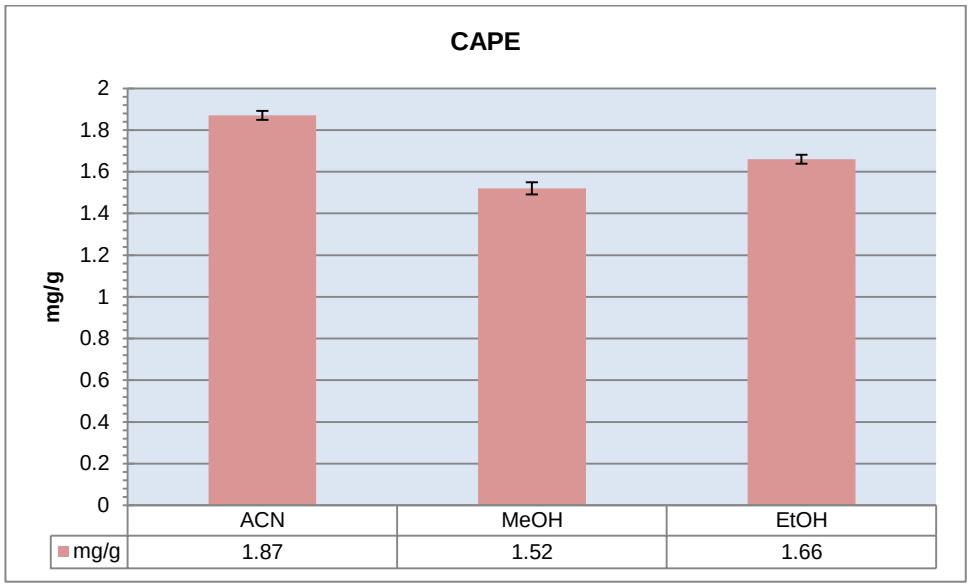


Figure 6.8: Effect of maceration extraction using pure solvents on CAPE from propolis

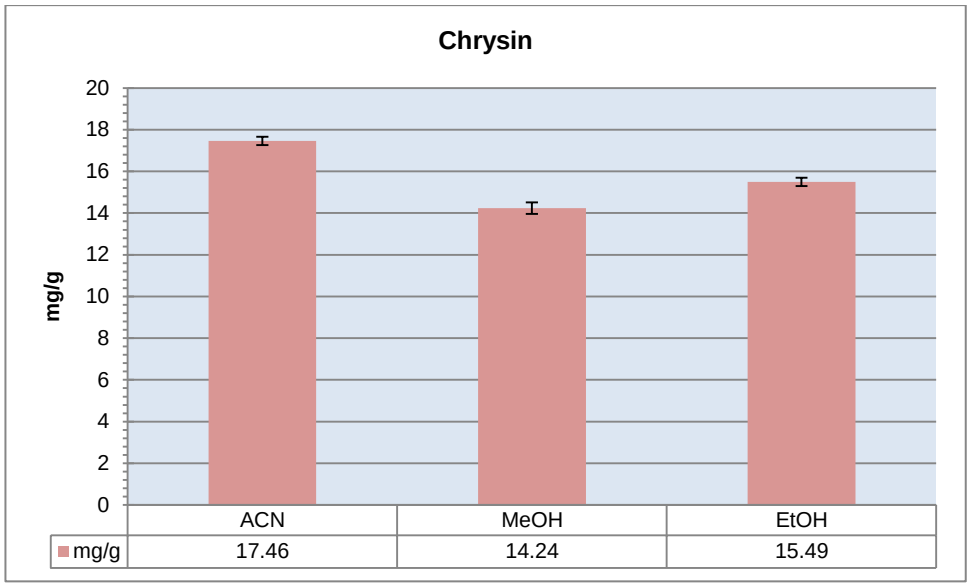


Figure 6.9: Effect of maceration extraction using pure solvents on Chrysin flavonoid from propolis

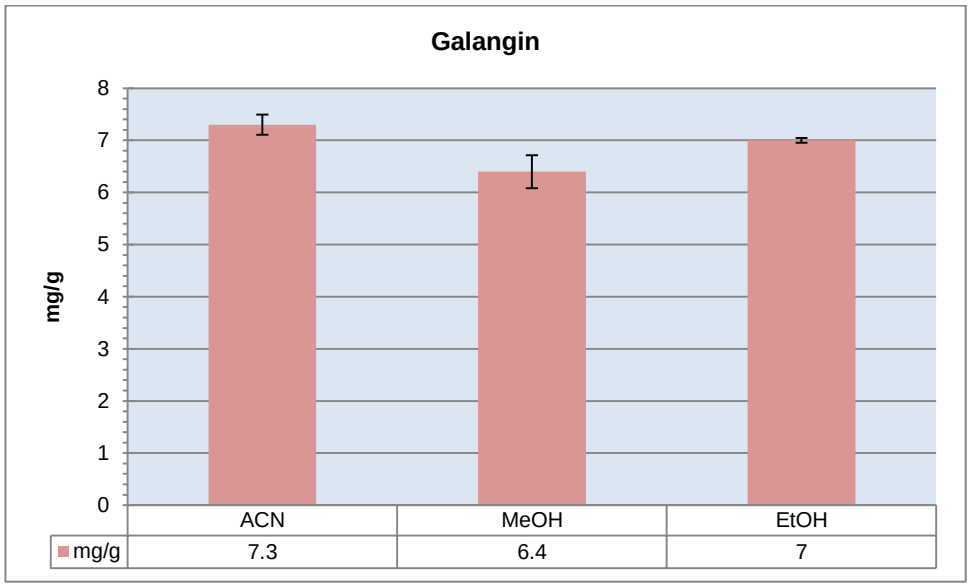


Figure 6.10: Effect of maceration extraction using pure solvents on Galangin flavonoid from propolis

The distinct variation was observed while studying pure solvents. This variation is a response of polar and non-polar compounds. Polar compounds such as apigenin and kaempferol (Figures 6.6 and 6.7) were extracted in higher amount in methanol, followed by ethanol and comparatively less extracted in

acetonitrile. Exactly opposite trend was observed in less polar flavonoids such as CAPE, chrysin and galangin (Figures 6.8, 6.9, 6.10). The highest extraction amount was observed in acetonitrile, followed by ethanol and methanol. The reason might be the presence of OH group in B ring of polar flavonoids and may cause easier structural availability for interaction with methanol to facilitate more solubility as compare to less polar flavonoids which don't have OH group in their B ring. Coneac et al. (2008) found concentrated ethanol is more suitable to extract the flavonoids as compared to ethanol with water; however, they haven't compared extraction variation using different solvents.

6.2.2 Hot extraction

Hot extraction method such as decoction is well known ancient Chinese method for medicinal preparation which is successfully practiced from last 3000 years (Wang et al. 2004). One of such old Chinese herbal mixture was used and studied for hydrolysis of glyosidic flavonoids after hot water extraction/decoction (Zhang et al. 2014). Hot extraction was successfully employed to extract flavonoids from calamondin using different solvents (Lou et al. 2014). Hot extraction was also studied for extraction of flavonoids from propolis using pure ethanol and different concentration of ethanol in water (Coneac et al. 2008). This is very much simpler but promising technique, requires less glassware and equipment as compared to the modified hot extraction techniques such as soxhlet extraction, hydro-distillation etc. By considering its significance in the extraction of flavonoids, this technique was considered in this work using combinations of different solvent range. The main hypothesis here is, boiling of the solvents with raw propolis sample can possibly improve the extraction of flavonoids.

In hot extraction studies, boiling of the solvents/solvent mixtures were carried out at specific temperature and for specific time as mentioned in section 6.1.2.2. In the first stage of experiments, 70% solvents in aqueous media were used for propolis extraction. In hot extraction studies, the resulting extracts were found to be opaque and contained high precipitation while studying solvent mix with water (70% ethanol, 70% methanol, 70% acetonitrile and 70% ethyl acetate). It is suspected that the reaction between wax present in propolis with water molecules and vapors which caused precipitation and cloudy extract. Due to opaque and precipitated extracts, further HPLC analysis was not carried out to avoid blockage problem of the HPLC system and the column. The clear extracts obtained from acetonitrile and methanol solvents were subjected for HPLC analysis. The flavonoid content was higher in 70% methanol as compared to 70% acetonitrile. This comparison was confirmed by calculating the total area of identified flavonoids from both the samples. Due to the precipitation problem, solvents with water using boiling conditions were not found be suitable for the extraction of flavonoids from propolis.

In the next stage of study, pure solvents were used for hot extraction and found similar result patterns as compared to maceration extraction. The results of major five flavonoid compounds such as apigenin, keampferol, CAPE, chrysin and galangin are shown in following figures (Figures 6.11-6.15) respectively.

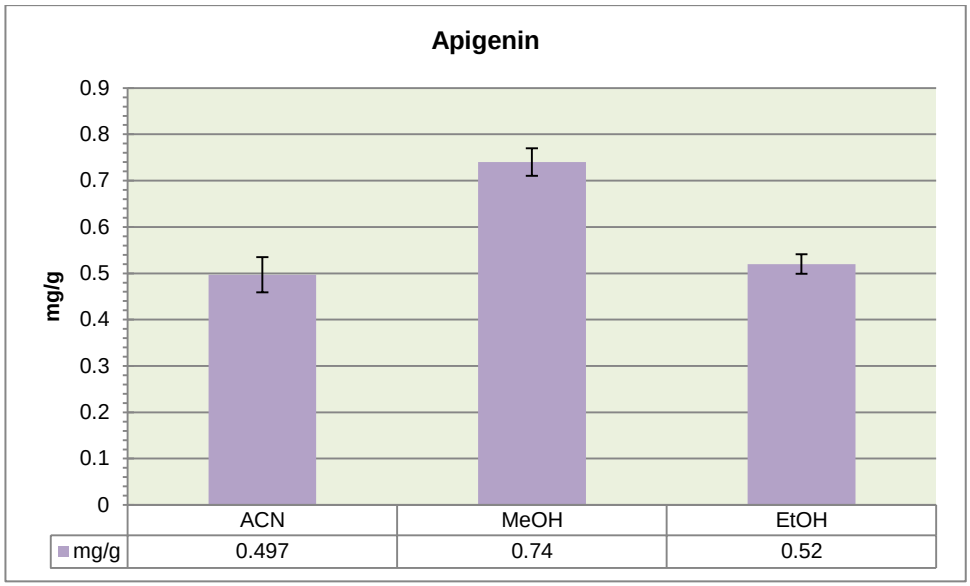


Figure 6.11: Effect of hot extraction using pure solvents on Apigenin flavonoid from propolis

(ACN: acetonitrile, MeHO: methanol, EtOH: ethanol.)

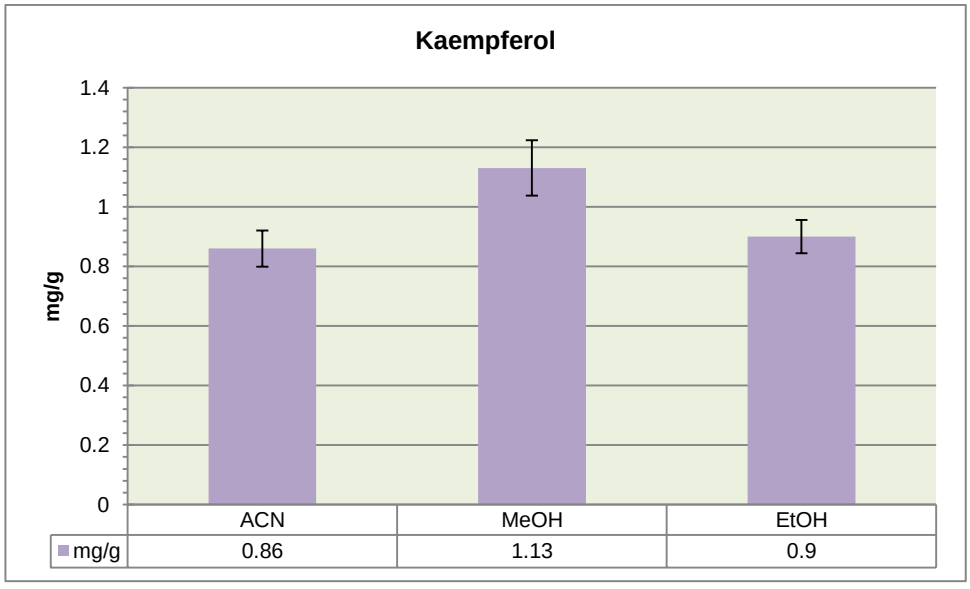


Figure 6.12: Effect of hot extraction using pure solvents on Kaempferol flavonoid from propolis

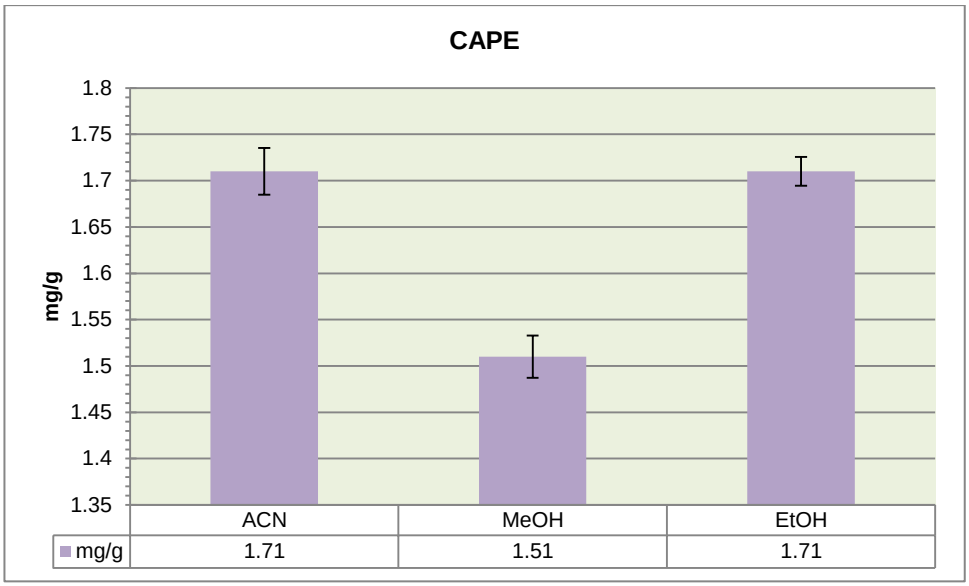


Figure 6.13: Effect of hot extraction using pure solvents on CAPE from propolis

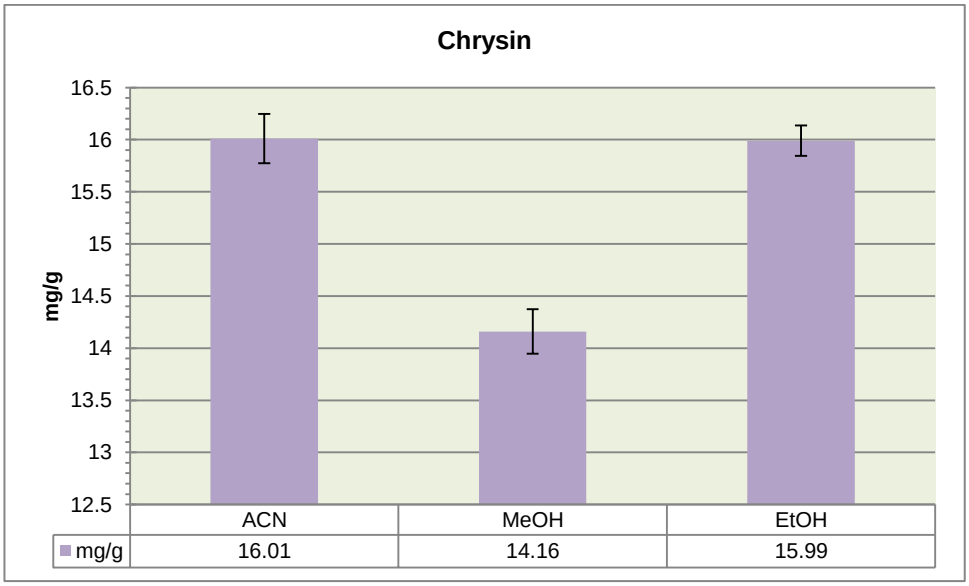


Figure 6.14: Effect of hot extraction using pure solvents on Chrysin flavonoid from propolis

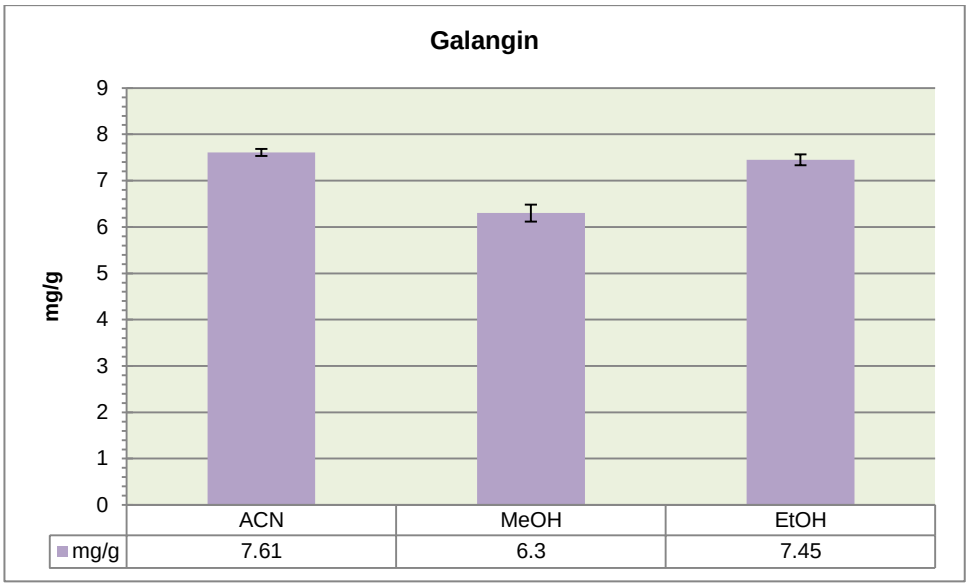


Figure 6.15: Effect of hot extraction using pure solvents on Galangin flavonoid from propolis

The extraction of flavonoids from propolis using pure solvents showed very much similar trend as compared to that of the maceration studies. The polar compounds such as apigenin and kaempferol (Figures 6.11 and 6.12) were extracted in higher amount in methanol; followed by ethanol and acetonitrile, while less polar compounds such as CAPE, chrysin and galangin (Figures 6.13,6.14 and 6.15) were extracted in high amount in acetonitrile followed by ethanol and methanol. The pattern and amount of flavonoid extraction were different after maceration and hot extraction procedures while using pure solvents. Maceration technique at low temperature found to be suitable for all flavonoids as well as for all solvents as compared to hot extraction. In contrast to these findings, it was found that extraction at a high temperature causes increase in resulting flavonoids in case of bitter melon (Tan et al. 2014). The difference between these results was mainly because of matrix difference; propolis is sticky and waxy; and dry non-living material while bitter melon is a plant material. In extraction of the phenolic compounds from *Cuphea*

aequipetala, C. aequipetala var. hispida and *C. lanceolate*, hot water bath extraction using methanol as a solvent extracted highest amount of flavonoids as compared to other techniques such as methanol stirring and hot water extraction (Cardenas-Sandoval et al. 2012).

6.2.3 Ultrasound extraction

Ultrasound extraction is a new extraction technique, employed for the extraction of natural products (Huie 2002). The principle behind ultrasound generation of extraction is bubble cavitation in the biological matrix. Cavitation is a process that includes formation of vapor bubbles, their growth and implosive collapse of the bubbles (Luque-García and Luque de Castro 2003; Vardanega et al. 2014). Ultrasound radiation facilitates and accelerates the extraction operation of organic and inorganic compounds from solid samples (Luque-García and Luque de Castro 2003). In this extraction technique, very high effective temperature increases solubility and diffusivity while effective pressure favors penetration and transport at the interference between aqueous or organic solution, subjected to ultrasonic energy and a solid matrix, combined with the oxidative energy of radicals (hydroxyl and hydrogen peroxide for water) created during sonolysis, results in a high extractive power (Luque-García and Luque de Castro 2003). Hence, this extraction method is considered in proposed study. There are two common devices used for ultrasound application, such as bath and probe units from which baths are widely used than probes. In the current extraction experiment, ultrasound bath system has been used (Figure 6.16).

In the proposed study, ultrasound extraction procedure was performed using methodology as explained in section 6.1.2.3 and 6.1.2.1. The resulting chromatograms are evaluated on the basis of calculation of amount of five

major flavonoid compounds such as apigenin, kaempferol, chrysin, CAPE and galangin. In the first set of experiment, 70% solutions of methanol and ethanol were used as solvents for flavonoid extraction from propolis using this technique. But increase in temperature during sonication process creates problem similar to hot extraction, and precipitation was observed in all resulting samples except for the extract obtained from acetonitrile solvent. Hence, it is advisable not to use aqueous solvent mixtures for extraction which includes high operational temperature.

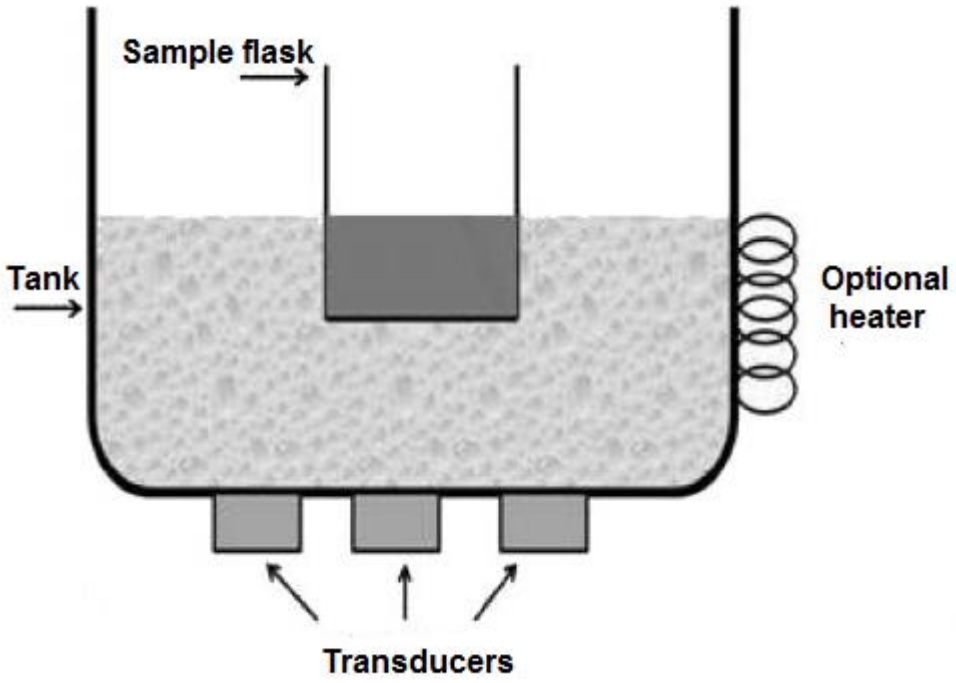
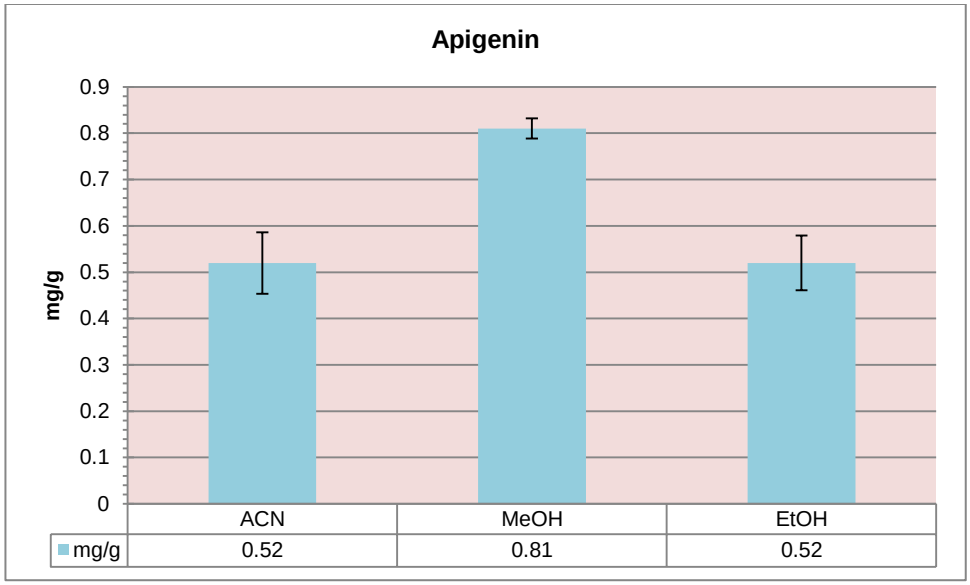


Figure 6.16: Ultrasonic bath

In the next set of experiment, pure solvents such as methanol, ethanol and acetonitrile were used to study extraction efficiency for flavonoid extraction from propolis using ultrasound extraction. The extraction pattern is different here as compared to maceration and hot extraction techniques. The methanol favors

extraction of polar compounds such as apigenin and kaempferol (Figures 6.17 and 6.18) while ethanol favors extraction of less polar flavonoids such as CAPE, chrysin and galangin (figures 6.19-6.21). The difference is a quite recognisable.



(ACN: acetonitrile, MeHO: methanol, EtOH: ethanol.)

Figure 6.17: Effect of ultrasound extraction using pure solvents on Apigenin flavonoid from propolis

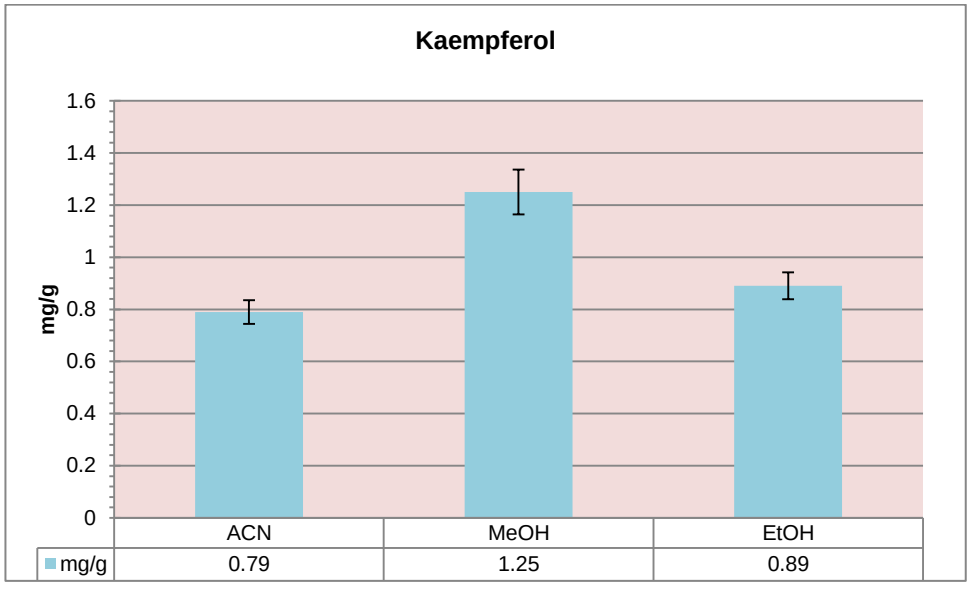


Figure 6.18: Effect of ultrasound extraction using pure solvents on Kaempferol flavonoid from propolis

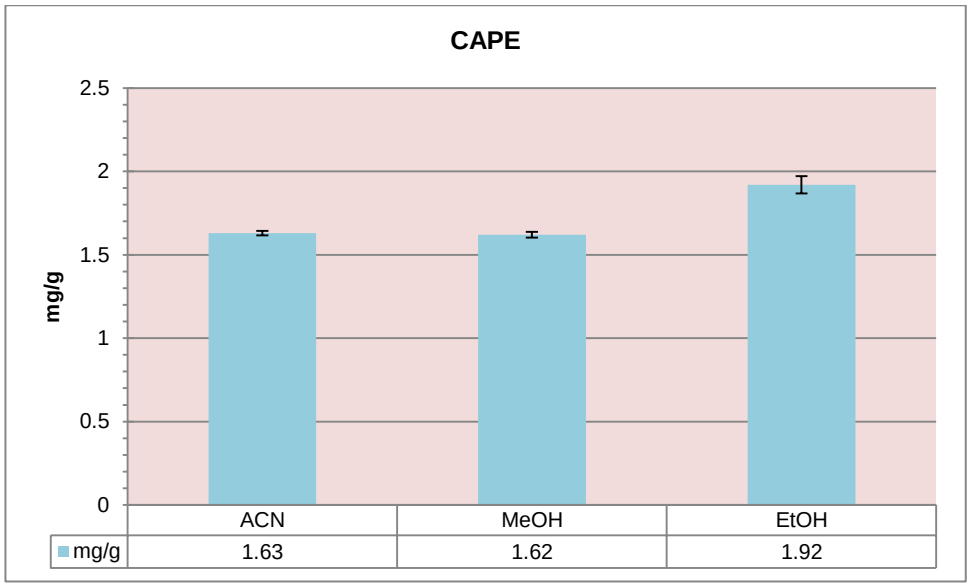


Figure 6.19: Effect of ultrasound extraction using pure solvents on CAPE from propolis

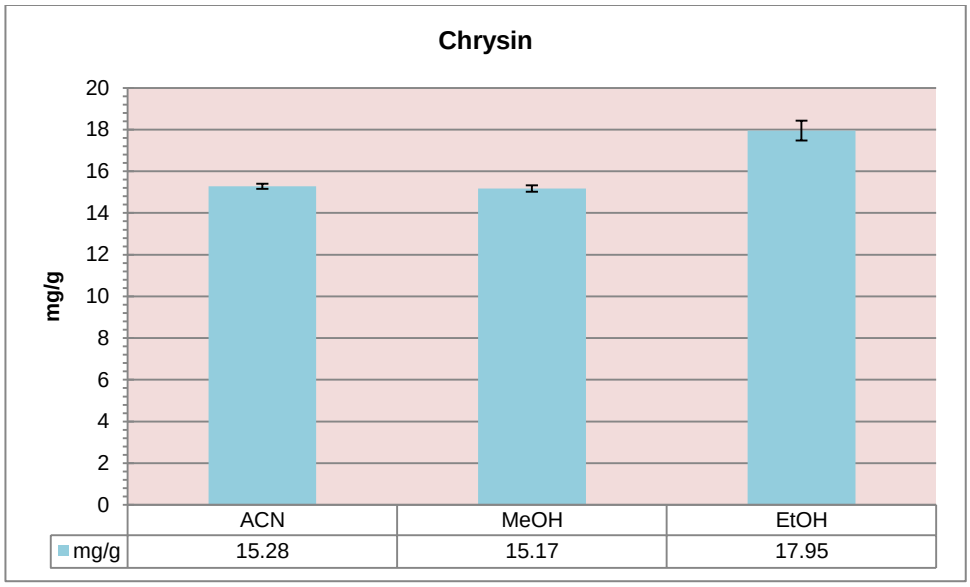


Figure 6.20: Effect of ultrasound extraction using pure solvents on Chrysin flavonoid from propolis

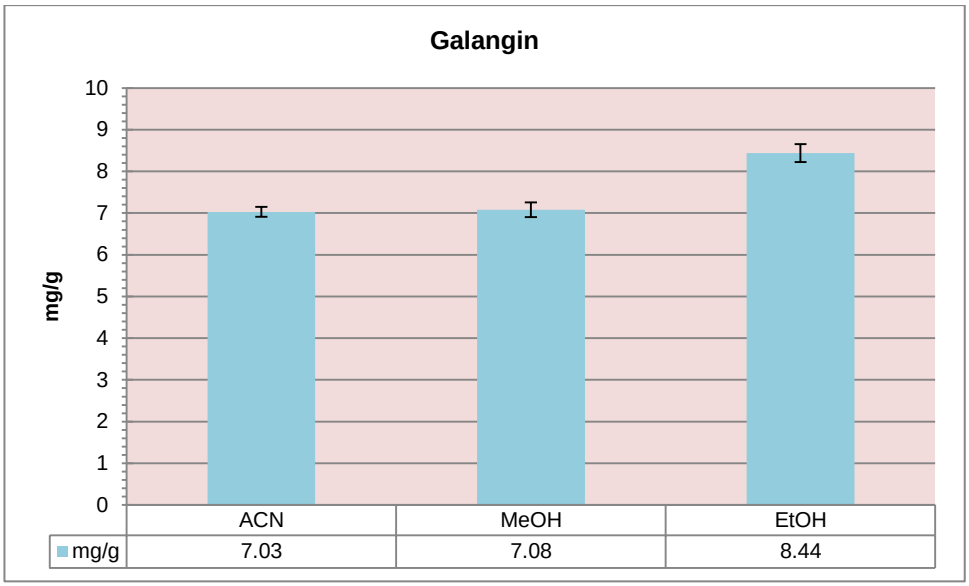


Figure 6.21: Effect of ultrasound extraction using pure solvents on Galangin flavonoid from propolis

In similar studies of extraction with propolis, Trusheva et al. (2007) found that ultrasound extraction is more efficient than other studied extraction method such as maceration. There is no evidence of similar studies which covers ultrasound extraction of flavonoids from propolis using different solvents.

6.2.4 Extraction using non-ionic surfactants

This is a novel and unique technique in which surfactant is used for extraction purpose. For the extraction of essential oil from mint, the surfactant and steam distillation process was successfully studied (McKellip et al. 1999). Effect of different non-ionic surfactant solutions on the extraction of essential oil from sage (*Salvia officinalis* L.) by using hydro-distillation technique was determined. In this study, 0.5% aqueous solutions of surfactants such as sorbitan monooleate (Span 80), sorbitan monododecanoite (Span 20), and PEG20 sorbitan monolaurate (Tween 20) were used for hydro-distillation extraction and found increase in essential oil yield in response to Tween 20 as compared to

other surfactants (Charchari and Abdelli 2014). By considering this novel use of surfactants in extraction, some of the surfactants were considered in the proposed work to study their effect on flavonoid extraction from propolis.

Two non-ionic surfactants were selected for this study named as PEG20 sorbitan monolaurate (Tween 20) and POE (20) sorbitan monooleate (Tween 80). The hydro-distillation process along with a surfactant was used in the proposed study. This is beneficial in extracting essential oil from sage (Charchari and Abdelli 2014). Here, the matrix is very different as compared to plant material sage.

In first step of experiment, hydro-distillation extraction was carried out using distilled water, 0.5% Tween 20 solution in DW and. 0.5% Tween 80 solution in DW. The detailed experimental procedure is explained in section 6.1.2.4. The pressure and temperature were optimised by using water for hydro-distillation. The residue as well as extracted water was analysed using HPLC method for flavonoids. The resulting chromatogram showed presence of number of known and unknown flavonoids in residual solution while in extracted solvents, number of early eluted compounds were observed which could be the volatile compounds from the raw propolis. Such volatile compounds were reported and identified previously using different extraction techniques ex. distillation was used for volatile compounds from Brazilian propolis samples and further analysed by using RP-HPLC, RP-HPTLC and GC-MS techniques (Maróstica Junior et al. 2008). There have been numerous cross–sectional studies of volatile compounds from different types of propolis (Melliou et al. 2007; Pellati et al. 2013; Bankova et al. 2014). In the current extraction work, the main objective was to find an efficient extraction method for flavonoids and therefore volatile compounds were not considered in further experiments.

The total areas of all identified flavonoids were considered in response to non-ionic surfactant containing extraction. In the following figure 6.22, the difference between total areas obtained after hydro-distillation using distilled water, Tween 80 and Tween 20 are shown. Tween 80 was able to extract more flavonoids as compared to other surfactant Tween 20. The difference between total area resulting to DW and Tween 20 hydro-distillation extraction was found to be negligible. The main disadvantage in the hydro-distillation technique was dissolving leftover residue in an organic solvent for flavonoid extraction at the end, which turns to un-usefulness of distillation process. Hence, this extraction method was not utilised further for extraction purpose of flavonoids from propolis.

In the next step of experiments, regular extraction methods such as maceration, hot extraction and ultrasound extraction were considered by using surfactant solutions as a medium for the extraction. The hot extraction and ultrasound extraction methods were found unsuitable with surfactants as it created precipitation in the resulting propolis samples. The potential reason was the nature of surfactant and presence of wax in raw propolis develops precipitation very easily in aqueous solution. Therefore, only the maceration technique was considered further for surfactant studies. From the previous results and current result (Figure 6.28), it was observed that acetonitrile contributes more for flavonoid extraction than other solvents such as methanol and ethanol. Hence, acetonitrile was added in the surfactant solutions to increase the extraction efficiency. The detailed experimental plan is showed in table 6.2.

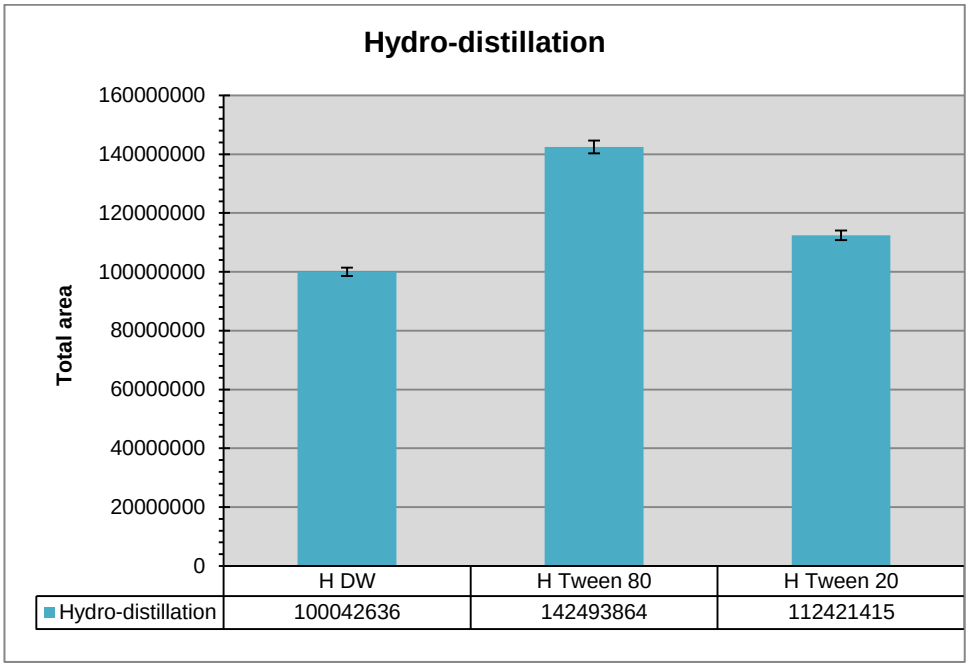


Figure 6.22: Effect of surfactant mediated hydro-distillation on extraction of flavonoids

(H DW: hydro-distillation using DW, H Tween 80: hydro-distillation using Tween 80 solution, Tween 20: hydro-distillation using Tween 20 solution)

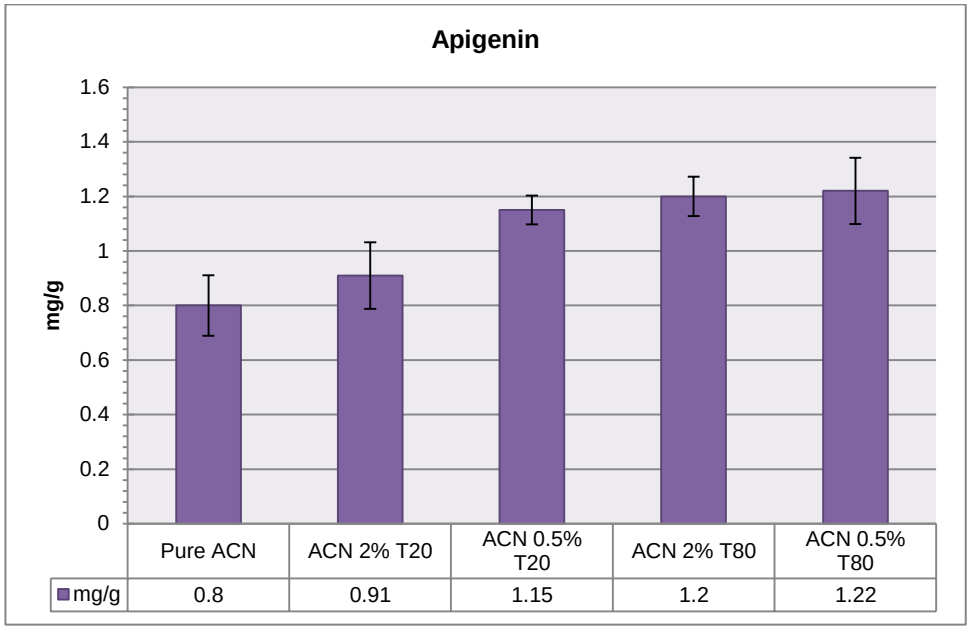
Table 6.2: Effect of surfactant on extraction of flavonoids using maceration technique

Acetonitrile(v/v)	**T20 (v/v)**	**T80 (v/v)**
80(16ml)	20 (2% in DW) (4ml)	-
80(16ml)	20 (0.5% in DW)(4ml)	-
80(16ml)	-	20 (2%in DW) (4ml)
80(16ml)	-	20 (0.5% in DW)(4ml)

(T20: Tween 20, T80: Tween 80, DW: distilled water)

In the above table 6.2, the combination of surfactant and acetonitrile solvent is explained. Different percentage of surfactant solutions were prepared to understand their effect on the extraction outcome of flavonoids. Five major

flavonoids such as apigenin, kaempferol, CAPE, chrysin and galangin were calculated and compared for each variation.



(ACN: acetonitrile, ACN 2% T20: 2% Tween 20 solution in acetonitrile, ACN 0.5% T20: : 0.5% Tween 20 solution in acetonitrile, ACN 2% T20: 2% Tween 80 solution in acetonitrile, ACN 0.5% T20: : 0.5% Tween 80 solution in acetonitrile)

Figure 6.23: Effect of surfactant on extraction of Apigenin flavonoid from propolis

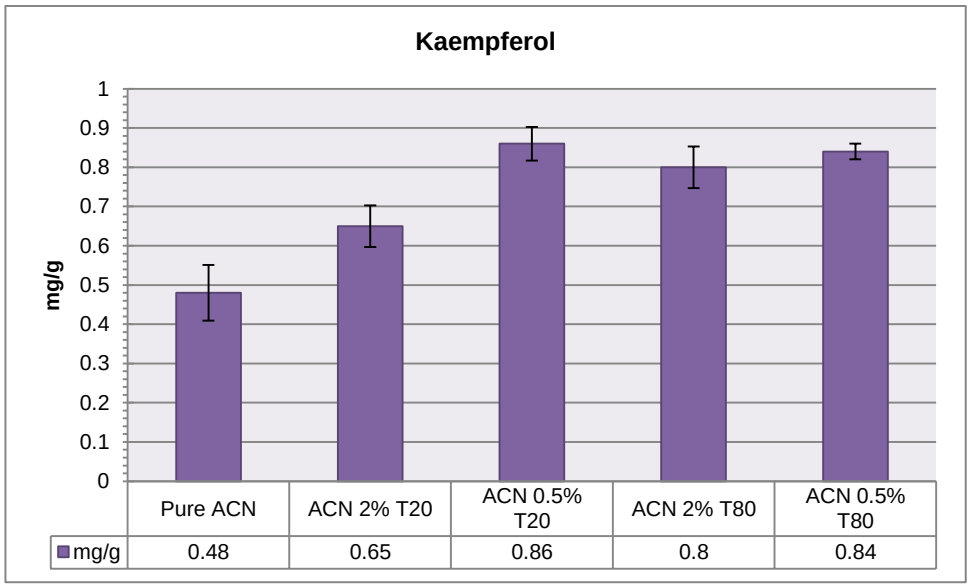


Figure 6.24: Effect of surfactant on extraction of Kaempferol flavonoid from propolis

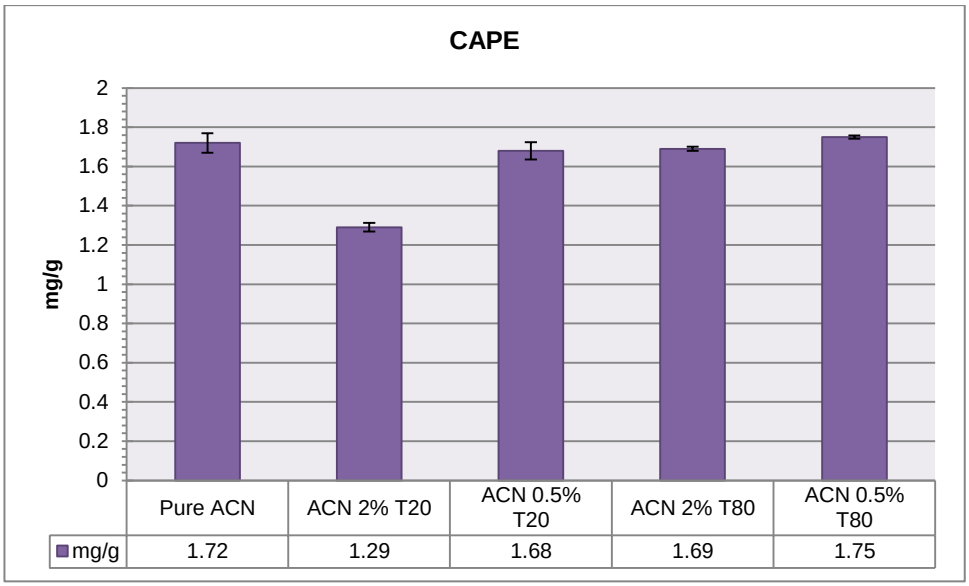


Figure 6.25: Effect of surfactant on extraction of CAPE f from propolis

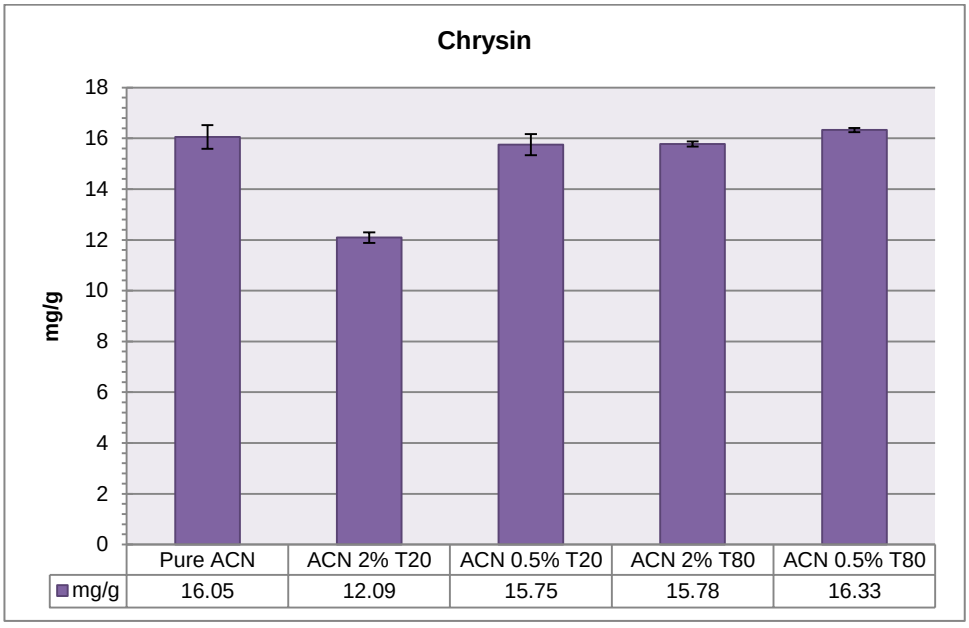


Figure 6.26: Effect of surfactant on extraction of Chrysin flavonoid from propolis

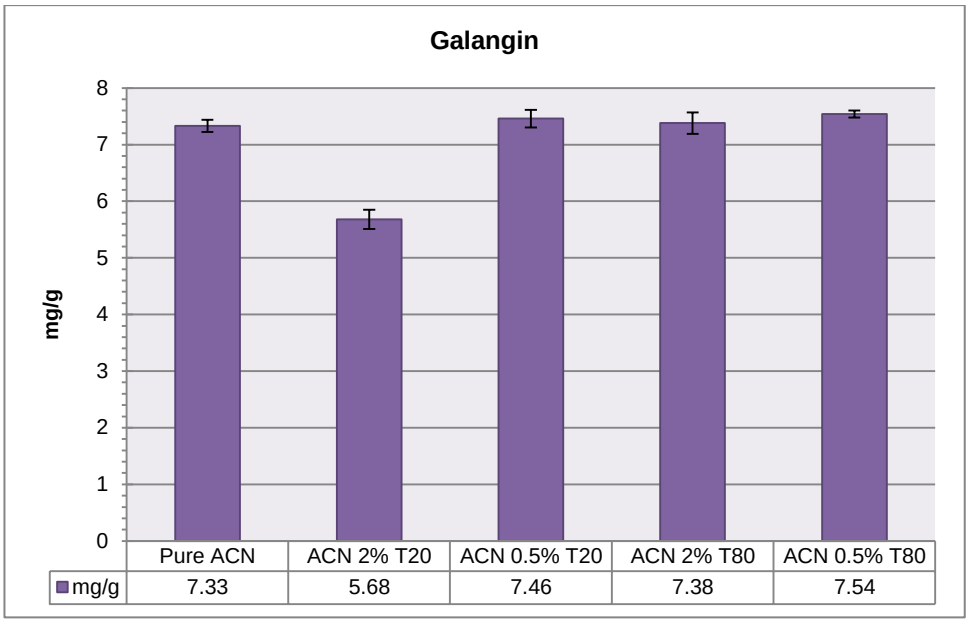


Figure 6.27: Effect of surfactant on extraction of Galangin flavonoid from propolis

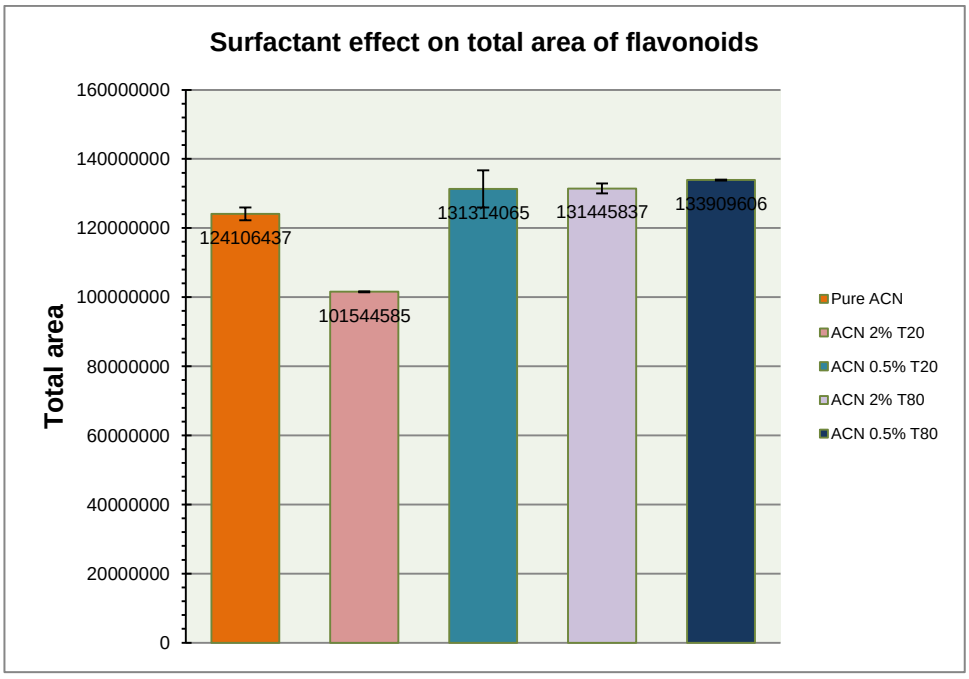


Figure 6.28: Effect of surfactant on the total area of flavonoids from propolis

In these studies, it was observed that at the low concentration levels of surfactants were positively influence high extractions of each flavonoid (Figure 6.23-6.27). The solution of 2% Tween 20 caused low extraction of flavonoids as

compared to other variations (Figure 6.28). But, interestingly it was found that acetonitrile was as a good solvent as compared to other surfactant solutions for the extraction of flavonoids (Figures 6.23-6.28). The polar compounds like kaempferol and apigenin showed higher extraction in response to surfactants (Figures 6.23-6.24), but the less polar comparatively major eluted compound such as chrysin, CAPE and galangin, not showed significant difference to surfactant solutions as compared to pure acetonitrile. Hence, the organic solvent acetonitrile was found as a good solvent as surfactant solutions for extraction of flavonoids and finally this solvent is recommended for the extraction of maximum amount of flavonoids from propolis.

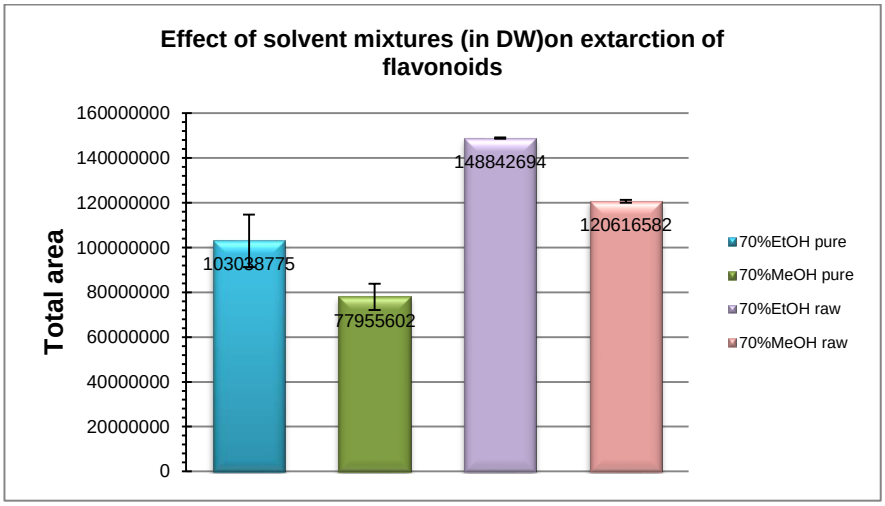
6.2.5 Comparative results of all extraction types

The extraction work includes different extraction techniques as well as different organic solvents. Hence, there is necessity to correlate and compare all results to identify the most efficient extraction method. The total areas or the total flavonoid contents of known flavonoids were considered for further comparison (Figure 6.29 and 6.30).

The extraction techniques used in the present study with pure solvents is well explained graphically in figure 6.30. Ethanol solvent showed much better results as compared to other solvents in all the extraction methods. While, methanol was found to be less effective solvent. Ethanol is commonly used for the extraction of polyphenolic compounds such as flavonoids from propolis (Burdock 1998; Blonska et al. 2004; Mohammadzadeh et al. 2007).

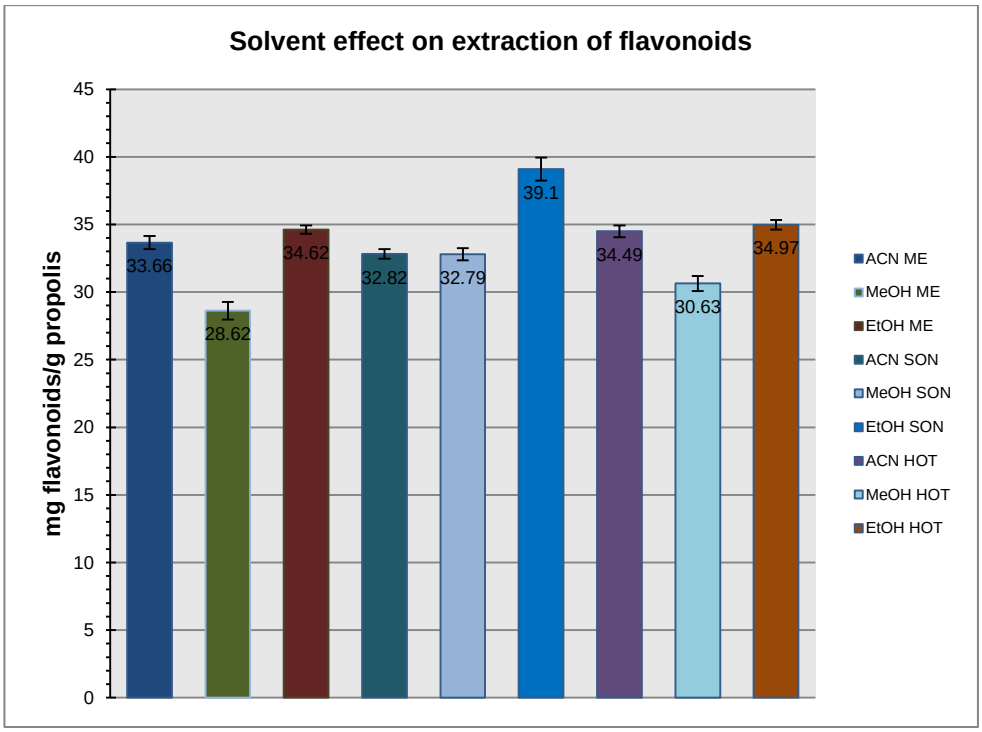
In the previous results, little differentiation was observed between extraction of polar and less polar flavonoids in response to acetonitrile and methanol in maceration and hot extraction. Therefore, it is advisable to check the polarity

and then select a suitable solvent such as for more polar flavonoids methanol is more suitable while for less polar flavonoids, acetonitrile is suitable for extraction (section 6.2.1, 6.2.2).



(70%MEOH pure: purified propolis with 70%methanol, 70%ETH pure: purified propolis with 70%ethanol, 70%MEOH raw: raw propolis with 70%methanol, 70%ETH raw: raw propolis with 70%ethanol)

Figure 6.29: Effect of solvent mixture (in DW) on extraction of flavonoids



(ACN: acetonitrile, MeOH: methanol, EtOH: ethanol, ME: maceration extraction, SON: ultrasound extraction, HOT: hot extraction)

Figure 6.30: Effect of solvents on extraction of flavonoids using different extraction procedures

Hot extraction causes reduction in less polar flavonoids such as chrysin and galangin. The high temperature obstructs the phenolic structures and causes damage reported from honey sample (Biesaga and Pyrzyńska 2013). But, in current extraction experiments, the solvent solutions was heated at 70°C temperature for 1h in a hot water bath is not found to be destructive for flavonoids as compared to maceration extraction which was carried out at RT. The variation of ethanolic extraction techniques such as maceration, ultrasound extraction and microwave assisted extraction for bioactive compounds from propolis was studied (Trusheva et al. 2007), and found that microwave assisted extraction is very efficient and time saving extraction technique as compared to other methods. The ethanolic extraction at different temperatures using pure as well as different % solutions of ethanol with water were observed (Coneac et al.

2008). They found propolis extracted at lower temperature for long time have higher concentrations of flavonoids compared with those obtained at higher temperature in a short time. But in the current extraction work, 70°C temperature for 1h was not showed decrease in flavonoid content, as compared to maceration technique (Figure 6.30).

6.2.6 Summary

The comparative study of extraction of flavonoids from propolis showed very interesting results. Ethanol and acetonitrile are the best solvents for flavonoid extraction however; there is no much variation in flavonoid content using other methods (Figure 6.30). Among all extraction experiments, ethanolic extraction using ultrasound extraction method is more efficient approach as compared to all other methods. Hence, it is concluded that any extraction method can be used for flavonoid extraction as per convenience.

7. CONCLUSION AND FUTURE WORK

7.1 Conclusion

The present study aimed to investigate the analytical methods used to determine the presence of active ingredients (flavonoids) and contaminants (antibiotics) in propolis samples. This study utilised various analytical techniques such as UV spectrophotometer, HPLC, UPLC, and microemulsion HPLC. Another aim of the present study is the development of efficient methods for the extraction of flavonoids from propolis. The majority of research objectives were achieved via the completion of optimisation strategies and the validation of methods used in the analysis of flavonoids and antibiotics. Significant results of this study are as follows:

7.1.1 Optimisation of liquid chromatography methods in the analysis of flavonoids

Propolis possesses a number of active compounds, including those that are classified as polyphenolic. To determine the presence of flavonoids in propolis, the basic quantification of total flavonoids in four different propolis formulations was studied using a UV-Vis spectrophotometric technique. This method was beneficial for the primary quantification of total flavonoids in raw or purified propolis samples.

During the next stage of experiments, modern separation techniques were conducted to make an advanced determination regarding the presence of flavonoids in propolis samples using ten common flavonoids. Selected flavonoids were used for method standardisation, including rutin, quercetin, myricetin, apigenin, kaempferol, pinocembrin, CAPE, chrysin, galangin, and acacetin. Originally, HPLC was to be utilised for such purposes. Various parameters were tested during method development experiments, including

isocratic elution, gradient elution, solvent selectivity, the use of different solvents, the use of different concentrations of buffer solutions, the use of different column temperatures, and the use of different proportions of aqueous buffer phases and organic solvents. Continuous peak overlapping of examined flavonoids in the resulting chromatogram allowed to study a greater variation of ongoing experiments. Of all the variation studies, a methanol and 5mM sodium phosphate buffer (pH 3), 50:50, a v/v with isocratic elution, a flow rate of 1ml/min, a column temperature of 28ºC, an injection volume of 20 µl, and a detection wavelength of 265nm were shown to be optimal parameters. This optimised method was used to successfully separate all ten selected flavonoid compounds.

The time necessary to complete a total analysis of the optimised HPLC method was high, at approximately 75 minutes. Hence, other analytical techniques were considered for further optimisation. The UPLC technique was selected and variation studies similar to those conducted for HPLC were carried out. However, the resulting peak overlapping figure was found to be peculiar. UPLC allowed for the early elution of peaks due to its high speed separation while simultaneously affecting the separation and resolution of resulting peaks. Neither isocratic nor gradient flow trials were able to resolve the peak overlapping problem, and thus the UPLC studies were terminated.

A microemulsion HPLC technique was considered for next stage of the experiment, as it was shown to be useful in the analytical separation of complex samples. The unique preparation required during the microemulsion mobile phase, to include the aqueous, oil, and surfactant phases, was found to be promising in the separation of high mixture samples such as propolis. Single variations in each experiment were carried out using various concentrations of

oil phase (ethyl acetate), surfactant (Brij L23), co-surfactant (1-butanol), and aqueous phase (buffer solutions such as phosphate buffer, acetate buffer). Variations in the pH of aqueous phase and column temperature were also conducted. The overlapping of studied flavonoid peaks was again an issue during MELC experimentation, similar to those witnessed in HPLC and UPLC studies. The optimised MELC method was able to separate only nine out of ten flavonoid compounds, with peak overlapping occurring between CAPE and chrysin.

Due to the unsuitableness of UPLC and MELC methods for the separation of ten flavonoid compounds, an optimised HPLC method was considered for further validation.

7.1.2 Validation of optimised HPLC method for the analysis of flavonoids

For the validation experimentation, ICH guidelines were followed and following parameters were studied such as selectivity, linearity, accuracy, recovery, precision, stability and robustness. All parameters were successfully studied and resulting data is discussed accordingly. The correlation coefficients of all of the flavonoids ranged from 0.984-0.997, while the limit of detection ranged from 0.93-2.16 µg/ml. This method had an accuracy ranging from 97.15%-102.88%, and the intra and inter-precision % relative standard deviation (RSD) values of the peak areas were less than 2. The flavonoids identified from the propolis samples were further quantified by using the linearity equation of standard solutions. Overall, this proposed method is efficient and can be used for the analysis of flavonoids, including caffeic acid phenethyl ester (CAPE).

7.1.3 Optimisation of method for the analysis of antibiotics

Four common antibiotic contaminates were selected in this study including tetracycline, oxytetracycline, chlortetracycline and doxycycline. For the method development of antibiotics, HPLC was considered first and published method of (Zhou et al. 2009) was initially followed. The resulting chromatogram for this trial was not similar as reported; improper peak shapes were observed with relatively long run time. Due to this issue, this method was developed further by optimising parameters such as mobile phase including the use of different organic solvents such as methanol and acetonitrile, use of different aqueous solutions in mobile phase such as oxalic acid and phosphate buffer. A relatively long run time as well as poor peak shape of broad peaks was obtained in resulting chromatogram. This has allowed to study other analytical technique for method optimisation.

The next technique used for this purpose was UPLC, the method development was performed using single variation step at each experiment. The analysis time for UPLC was relatively short as compared to HPLC and the quality of peak shape was also improved. The optimised UPLC method was as follows mobile phase 10 mM sodium phosphate buffer (pH 3): methanol: acetonitrile (70:5:25, v/v/v); flow rate 0.15ml/min.; column temperature off; injection volume 2.5 µl, wavelength 350nm. Most of the clean-up experiments including LLE and SPE were studied using UPLC technique. But due to one un-avoidable problem of diluted concentration of antibiotic and could not be detected by the analytical method, causes un-usefulness of this technique and hence allowed to switch another analytical technique to achieve analysis of antibiotics.

In next experimentation, MELC technique was utilised for separation of antibiotics. The variation studies were carried out using single variation at each

step. The parameters studied in this work includes concentration of surfactant (Brij L23), concentration of oil (ethyl acetate), concertation of co-surfactant (1-butanol), different aqueous phases such as formic acid, acetate buffer, phosphate buffer etc. The final optimised method has the following optimum condition; mobile phase 10 mM acetate buffer (pH 5): ethyl acetate: Brij L23: 1-butanol (91%: 3%: 3.5%: 2.5%, w/w/w/w); flow rate 1ml/min.; column temperature 30ºC; injection volume 20 µl, wavelength 350nm. This optimised condition able to separate three selected antibiotics from four such as oxytetracycline, chlortetracycline and doxycycline. These three antibiotics considered for further validation process by considering common presence of oxytetracycline in propolis samples.

Another area of the analysis of antibiotic study was to develop clean up method for the cleaning of propolis sample and to extract antibiotics from it. For this purpose, LLE as well as SPE methods were studied. SPE was considered further due to unsuitableness of LLE technique for this particular study. The reported SPE method of (Zhou et al. 2009) was initially followed but further optimisation was necessary. The final optimised SPE method include a HLB sorbent using SPE method, conditioning by 1ml methanol and 1ml water, followed by loading at a slow flow rate sample after addition of phosphate buffer and final elution by to 2% glacial acetic acid in 95% methanol.

7.1.4 Validation of optimised MELC method for the analysis of antibiotics

ICH guidelines were followed here for validation. The parameters were studied in validation such as selectivity, linearity, accuracy, recovery, precision, stability and robustness. All parameters were successfully studied and resulting data was discussed accordingly. The correlation coefficient value for three antibiotics

oxytetracycline, chlortetracycline and doxycyline ranged between 0.97-0.998, limit of quantitation ranged in 0.5-2.5 µg/ml and limit of detection was in range 0.18-0.82 µg/ml. This method had accuracy in range 92-104%, the intra and inter-precision % relative standard deviation (RSD) was found to be less than 15%, and recovery values were found in the range of 45-83%. This validated method employed for the identification of residual antibiotics from propolis samples. The compounds were determined by MELC technique by applying calibration curve calculation for the standards of those antibiotics.

7.1.5 Extraction studies of propolis for extraction of flavonoids

Another aim of this thesis was to develop a more efficient extraction method particularly to extract flavonoids from propolis. For this purpose, extraction techniques such as maceration, hot extraction and ultrasound assisted extraction were considered using a range of solvents including methanol, ethanol and acetonitrile. The use of non-ionic surfactants such as Tween 20 and Tween 80 were also utilised as an aqueous solution to facilitate more extraction of flavonoids from propolis. At the end, a variety of resulting outcome suggested that ultrasound extraction including pure ethanol is the most efficient method.

7.2 Future work

Propolis standardisation is an important research area considering its immense power of flavonoid source and antimicrobial properties. The development of new analytical techniques is frequently required due to the variability found in various propolis types. The chemical profiles also differ considerably in all propolis types. Hence, the findings from this research are valuable but there is still potential for future work on the analysis of propolis.

In the analysis of flavonoids and antibiotics, method development practices were studied extensively but there is still a need for further work, especially in MELC technique. The separation of flavonoids in propolis could be improved by studying different type of surfactants, co-surfactants and oils. The unknown compounds found while studying flavonoids, can be identified using other separation techniques such as LC-MS or LC-MS MS.

In the analysis of antibiotics, the application of polymer based reversed phase columns with pH range 0-14, may improve analysis of antibiotics at high pH, where they exist in neutral form.

The unknown compounds found while studying flavonoids as well as antibiotics, can be identified using other separation techniques such as LC-MS or LC-MS MS.

In extraction studies, it could be beneficial to examine an advanced extraction techniques such as microwave extraction.

8. REFERENCES

Ahadi, A., Partoazar, A., Abedi-Khorasgani, M.-H. and Shetab-Boushehri, S. V. (2011) Comparison of liquid-liquid extraction-thin layer chromatography with solid-phase extraction-high-performance thin layer chromatography in detection of urinary morphine. *Journal of Biomedical Research* 25 (5), 362-367.

Ahn, M.-R., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F. and Nakayama, T. (2007) Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chemistry* 101 (4), 1383-1392.

Al-Waili, N., Salom, K., Al-Ghamdi, A. and Ansari, M. J. (2012) Antibiotic, Pesticide, and Microbial Contaminants of Honey: Human Health Hazards. *The Scientific World Journal* 2012, 9.

Alcici, N. M. F. (1997) *Heavy metals in propolis - Practical and simple procedures to reduce the lead level in the Brazilian propolis.* Bee Products: Properties, Applications, and Apitherapy. Springer US.

Alencar, S. M., Oldoni, T. L. C., Castro, M. L., Cabral, I. S. R., Costa-Neto, C. M., Cury, J. A., Rosalen, P. L. and Ikegaki, M. (2007) Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis. *Journal of Ethnopharmacology* 113 (2), 278-283.

Althanyan, M., Clark, B., Hanaee, J. and Assi, K. (2013) Development of a Microemulsion High Performance Liquid Chromatography (MELC) Method for Determination of Salbutamol in Metered-Dose Inhalers (MDIS). *BioImpacts : BI* 3 (1), 37-42.

Althanyan, M. S., Assi, A.-A. N. H., Clark, B. J. and Assi, K. H. (2016) Microemulsion High Performance Liquid Chromatography (MELC) for Determination of Terbutaline in Urine Samples. *Int. J. Pharm. Sci. Rev. Res.* 36 (1), 298-292.

Althanyan, M. S., Assi, K. H., Clark, B. J. and Hanaee, J. (2011) Microemulsion high performance liquid chromatography (MELC) method for the determination of terbutaline in pharmaceutical preparation. *J Pharm Biomed Anal* 55 (3), 397-402.

Amoros, M., Lurton, E., Boustie, J., Girre, L., Sauvager, F. and Cormier, M. (1994) Comparison of the anti-Herpes simplex virus activities of propolis and 3-methyl-but-2-enyl caffeate. *Journal of Natural Products* 57 (5), 644-647.

Anderson, C. R., Rupp, H. S. and Wu, W. H. (2005) Complexities in tetracycline analysis-chemistry, matrix extraction, cleanup, and liquid chromatography. *J Chromatogr A* 1075 (1-2), 23-32.

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N. and Omar, A. K. M. (2013) Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering* 117 (4), 426-436.

Bae, H., Jayaprakasha, G. K., Jifon, J. and Patil, B. S. (2012) Extraction efficiency and validation of an HPLC method for flavonoid analysis in peppers. *Food Chemistry* 130 (3), 751-758.

Ban, J., Popovic, S. and Maysinger, D. (1983) Cytostatic Effects of Propolis Invitro. *Acta Pharmaceutica Jugoslavica* 33 (3-4), 245-255.

Bankova, V. (2000) Determining Quality in Propolis Samples. *J Am Apither Soc* 7 (2).

Bankova, V. (2005) Chemical diversity of propolis and the problem of standardization. *Journal of Ethnopharmacology* 100 (1–2), 114-117.

Bankova, V., Popova, M., Bogdanov, S. and Sabatini, A. G. (2002) Chemical composition of European propolis: Expected and unexpected results. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences* 57 (5-6), 530-533.

Bankova, V., Popova, M. and Trusheva, B. (2014) Propolis volatile compounds: chemical diversity and biological activity: a review. *Chemistry Central Journal* 8 (1), 28.

Bankova, V. S., de Castro, S. L. and Marcucci, M. C. (2000) Propolis: recent advances in chemistry and plant origin. *Apidologie* 31 (1), 3-15.

Bankova, V. S., Popov, S. S. and Marekov, N. L. (1982) High-performance liquid chromatographic analysis of flavonoids from propolis. *Journal of Chromatography A* 242 (1), 135-143.

Banskota, A. H., Tezuka, Y., Adnyana, I. K., Midorikawa, K., Matsushige, K., Message, D., Huertas, A. A. G. and Kadota, S. (2000) Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and China. *Journal of Ethnopharmacology* 72 (1-2), 239-246.

Banskota, A. H., Tezuka, Y. and Kadota, S. (2001) Recent progress in pharmacological research of propolis. *Phytotherapy Research* 15 (7), 561-571.

Barbarić, M., Mišković, K., Bojić, M., Lončar, M. B., Smolčić-Bubalo, A., Debeljak, Ž. and Medić-Šarić, M. (2011) Chemical composition of the ethanolic propolis extracts and its effect on HeLa cells. *Journal of Ethnopharmacology* 135 (3), 772-778.

Bargańska, Ż., Namieśnik, J. and Ślebioda, M. (2011) Determination of antibiotic residues in honey. *TrAC Trends in Analytical Chemistry* 30 (7), 1035-1041.

Belova, V. V., Voshkin, A. A., Kholkin, A. I. and Payrtman, A. K. (2009) Solvent extraction of some lanthanides from chloride and nitrate solutions by binary extractants. *Hydrometallurgy* 97 (3-4), 198-203.

Bernal, J., Garrido-Bailon, E., del Nozal, M. J., Gonzalez-Porto, A. V., Martin-Hernandez, R., Diego, J. C., Jimenez, J. J., Bernal, J. L. and Higes, M. (2010) Overview of Pesticide Residues in Stored Pollen and Their Potential Effect on Bee Colony (Apis mellifera) Losses in Spain. *Journal of Economic Entomology* 103 (6), 1964-1971.

Bernal, J., Jesus Nozal, M., Jose Jimenez, J., Teresa Martin, M. and Sanz, E. (2009) A new and simple method to determine trace levels of sulfonamides in honey by high performance liquid chromatography with fluorescence detection. *Journal of Chromatography A* 1216 (43), 7275-7280.

Berry, J. A., Hood, W. M., Pietravalle, S. and Delaplane, K. S. (2013) Field-Level Sublethal Effects of Approved Bee Hive Chemicals on Honey Bees (Apis mellifera L). *PLoS ONE* 8 (10), e76536.

Bie, M., Li, R., Chai, T., Dai, S., Zhao, H., Yang, S. and Qiu, J. (2012) Simultaneous determination of tetracycline antibiotics in beehives by liquid chromatography--triple quadrupole mass spectrometry. *Adv. Appl. Sci. Res* 3, 462-468.

Biesaga, M. and Pyrzyńska, K. (2013) Stability of bioactive polyphenols from honey during different extraction methods. *Food Chemistry* 136 (1), 46-54.

Blonska, M., Bronikowska, J., Pietsz, G., Czuba, Z. P., Scheller, S. and Krol, W. (2004) Effects of ethanol extract of propolis (EEP) and its flavones on inducible gene expression in J774A.1 macrophages. *Journal of Ethnopharmacology* 91 (1), 25-30.

Bogdanov, S. (2012) *Propolis: Composition, Health, Medicine: A Review.* *www.bee-hexagon.net/files/file/fileE/Health/PropolisBookReview.pdf* Accessed on 05/08/2013.

Bogdanov, S., Kilchenmann, V. and Imdorf, A. (1998) Acaricide residues in some bee products. *Journal of Apicultural Research* 37 (2), 57-67.

Boyanova, L., Kolarov, R., Gergova, G. and Mitova, I. (2006) In vitro activity of Bulgarian propolis against 94 clinical isolates of anaerobic bacteria. *Anaerobe* 12 (4), 173-177.

Brodersen, D. E., Clemons Jr, W. M., Carter, A. P., Morgan-Warren, R. J., Wimberly, B. T. and Ramakrishnan, V. (2000) The Structural Basis for the Action of the Antibiotics Tetracycline, Pactamycin, and Hygromycin B on the 30S Ribosomal Subunit. *Cell* 103 (7), 1143-1154.

Brown, R. (1989) Hive Products - Pollen, Propolis and RoyalJelly. *Bee World* 70 (3), 109-117.

Burdock, G. A. (1998) Review of the biological properties and toxicity of bee propolis (propolis). *Food and Chemical Toxicology* 36 (4), 347-363.

Cardenas-Sandoval, B. A., López-Laredo, A. R., Martínez-Bonfil, B. P., Bermúdez-Torres, K. and Trejo-Tapia, G. (2012) Advances in the phytochemistry of Cuphea aequipetala, C. aequipetala var. hispida and C. lanceolata: Extraction and quantification of phenolic compounds and antioxidant activity. *Revista mexicana de ingeniería química* 11, 401-413.

Castaldo, S. and Capasso, F. (2002) Propolis, an old remedy used in modem medicine. *Fitoterapia* 73, S1-S6.

Center for Drug Evaluation and Research. (1994) *Reviewer Guidance' Validation of Chromatographic methods.* US Food and Drug Administration. *http://webcache.googleusercontent.com/search?q=cache:WFnNkNAJ9hgJ:www.fda.gov/downloads/Drugs/.../Guidances/UCM134409.pdf+&cd=1&hl=en&ct=clnk&gl=uk* Accessed on 05/08/2013

Chang, C. C., Yang, M. H., Wen, H. M. and Chern, J. C. (2002) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis* 10 (3), 178-182.

Charchari, S. and Abdelli, M. (2014) Enhanced Extraction by Hydrodistillation of Sage (Salvia officinalis L.) Essential Oil Using Water Solutions of Non-ionic Surfactants. *Journal of Essential Oil Bearing Plants* 17 (6), 1094-1099.

Charoensin, S., Punvittayagul, C., Pompimon, W., Mevatee, U. and Wongpoomchai, R. (2012) Toxicological and clastogenic evaluation of pinocembrin and pinostrobin isolated from Boesenbergia pandurata in Wistar rats. *Thai Journal Toxicology* 25 (1), 29-40.

Chen, F., Chen, L., Wang, Q., Zhou, J., Xue, X. and Zhao, J. (2009) Determination of organochlorine pesticides in propolis by gas chromatography-electron capture detection using double column series solid-phase extraction. *Analytical and Bioanalytical Chemistry* 393 (3), 1073-1079.

Chen, J. H., Shao, Y., Huang, M. T., Chin, C. K. and Ho, C. T. (1996) Inhibitory effect of caffeic acid phenethyl ester on human leukemia HL-60 cells. *Cancer Letters* 108 (2), 211-214.

Chen, X. J., Ji, H., Zhang, Q. W., Tu, P. F., Wang, Y. T., Guo, B. L. and Li, S. P. (2008) A rapid method for simultaneous determination of 15 flavonoids in Epimedium using pressurized liquid extraction and ultra-performance liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis* 46 (2), 226-235.

Chen, Y., Wang, J. and Wan, D. (2010) Determination of total flavonoids in three Sedum crude drugs by UV–Vis spectrophotometry. *Pharmacognosy Magazine* 6 (24), 259-263.

Cheng, Y. F., Phillips, D. J. and Neue, U. (1997) Simple and rugged SPE method for the determination of tetracycline antibiotics in serum by HPLC using a volatile mobile phase. *Chromatographia* 44 (3-4), 187-190.

Cherlet, M., Schelkens, M., Croubels, S. and De Backer, P. (2003) Quantitative multi-residue analysis of tetracyclines and their 4-epimers in pig tissues by high-performance liquid chromatography combined with positive-ion electrospray ionization mass spectrometry. *Analytica Chimica Acta* 492 (1-2), 199-213.

Chiao, C., Carothers, A. M., Grunberger, D., Solomon, G., Preston, G. A. and Barrett, J. C. (1995) Apoptosis and Altered Redox State induced by Caffeic Acid Phenethyl Ester (CAPE) in Transformed Rat Fibroblast Cells. *Cancer Research* 55 (16), 3576-3583.

Choi, Y. M., Noh, D. O., Cho, S. Y., Suh, H. J., Kim, K. M. and Kim, J. M. (2006) Antioxidant and antimicrobial activities of propolis from several regions of Korea. *Lwt-Food Science and Technology* 39 (7), 756-761.

Chopra, I. and Roberts, M. (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and molecular biology reviews* 65 (2), 232-260.

Christensen, J. and Kristiansen, J. (1994) *Lead Handbook on Metals in Clinical and Analytical Chemistry.* New York: Sigel and Sigel.

Colin, M. E., Bonmatin, J. M., Moineau, I., Gaimon, C., Brun, S. and Vermandere, J. P. (2004) A method to quantify and analyze the foraging activity of honey bees: Relevance to the sublethal effects induced by systemic insecticides. *Archives of Environmental Contamination and Toxicology* 47 (3), 387-395.

Collado-Sánchez, M. A., Rambla-Alegre, M., Carda-Broch, S. and Esteve-Romero, J. (2010) Simultaneous Separation and Determination of Quinolones in Pharmaceuticals by Micellar Liquid Chromatography. *Journal of Liquid Chromatography & Related Technologies* 33 (4), 513-525.

Coneac, G., Gafiţanu, E., Hădărugă, D., Hădărugă, N., Pînzaru, I., Bandur, G., Urşica, L., Păunescu, V. and Gruia, A. (2008) Flavonoid contents of propolis from the west side of Romania and correlation with the antioxidant activity. *Chemical Bulletin "Politehnica" University Timisoara* 53 (67), 56-60.

Conti, M. E. and Botre, F. (2001) Honeybees and their products as potential bioindicators of heavy metals contamination. *Environmental Monitoring and Assessment* 69 (3), 267-282.

Corrales, M., García, A. F., Butz, P. and Tauscher, B. (2009) Extraction of anthocyanins from grape skins assisted by high hydrostatic pressure. *Journal of Food Engineering* 90 (4), 415-421.

Croci, A. N., Cioroiu, B., Lazar, D., Corciova, A., Ivanescu, B. and Lazar, M. I. (2009) HPLC Evaluation of Phenolic and Polyphenolic Acids from Propolis. *Farmacia* 57 (1), 52-57.

Cuesta-Rubio, O., Frontana-Uribe Bernardo, A., Ramírez-Apan, T. and Cárdenas, J. (2002) *Polyisoprenylated Benzophenones In Cuban Propolis; Biological Activity Of Nemorosone* .

Cuesta-Rubio, O., Lisa Piccinelli, A., Campo Fernandez, M., Marquez Hernandez, I., Rosado, A. and Rastrelli, L. (2007) Chemical characterization of Cuban propolis by HPLC-PDA, HPLC-MS, and NMR: the Brown, Red, and Yellow Cuban varieties of propolis. *Journal of Agricultural and Food Chemistry* 55 (18), 7502-7509.

Cunha, I., Sawaya, A. C., Caetano, F. M., Shimizu, M. T., Marcucci, M. C., Drezza, F. T., Povia, G. S. and Carvalho, P. d. O. (2004) Factors that influence the yield and composition of Brazilian propolis extracts. *Journal of the Brazilian Chemical Society* 15 (6), 964-970.

Cushnie, T. P. T. and Lamb, A. J. (2005) Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents* 26 (5), 343-356.

Davis, J. M., Elias, R. W. and Grant, L. D. (1993) Current issues in human lead exposure and regulation of lead. *Neurotoxicology* 14 (2-3), 15-27.

de Groot, H. and Rauen, U. (1998) Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundamental & Clinical Pharmacology* 12 (3), 249-255.

Debayle, D., Dessalces, G. and Grenier-Loustalot, M. (2008) Multi-residue analysis of traces of pesticides and antibiotics in honey by HPLC-MS-MS. *Analytical and Bioanalytical Chemistry* 391 (3), 1011-1020.

Deblock-Bostyn, G. (1982) The bee and its products. *Bulletin de la Societe de Pharmacie de Lille* 38 (3/4), 181-203.

Debuyser, E. (1983) *La propolis.* Docteur en Pharmacie Thesis. France: Université de Nantes, France.

Decourtye, A., Lacassie, E. and Pham-Delegue, M. H. (2003) Learning performances of honeybees (Apis mellifera L) are differentially affected by imidacloprid according to the season. *Pest Management Science* 59 (3), 269-278.

Demestre, M., Messerli, S. M., Celli, N., Shahhossini, M., Kluwe, L., Mautner, V. and Maruta, H. (2009) CAPE (Caffeic Acid Phenethyl Ester)-based Propolis Extract (Bio 30) Suppresses the Growth of Human

Neurofibromatosis (NF) Tumor Xenografts in Mice. *Phytotherapy Research* 23 (2), 226-230.

deVries, T., Boissonneault, R. and Muldoon, B. (2006) *Tetracycline metal complex in a solid dosage form*. Google Patents.

Dobrinas, S., Birghila, S. and Coatu, V. (2008) Assessment of polycyclic aromatic hydrocarbons in honey and propolis produced from various flowering trees and plants in Romania. *Journal of Food Composition and Analysis* 21 (1), 71-77.

Dolan, J. (2013) HPLC Solutions #48: Solvent Nomograph.

Dolan, J. W. (2002) Temperature selectivity in reversed-phase high performance liquid chromatography. *Journal of Chromatography A* 965 (1–2), 195-205.

Dolan, J. W. (2010) Selectivity in Reversed-Phase LC Separations, Part I: Solvent-Type Selectivity. *LC GC North America* 28 (12).

Donadieu, Y. (1979) *La Propolis.* Natural Therapeutic. Paris:

Dziedzic, A., Kubina, R., Wojtyczka, R. D., Kabala-Dzik, A., Tanasiewicz, M. and Morawiec, T. (2013) The Antibacterial Effect of Ethanol Extract of Polish Propolis on Mutans Streptococci and Lactobacilli Isolated from Saliva. *Evidence-Based Complementary and Alternative Medicine*.

El-Sherbiny, D. T. M., El-Ashry, S. M., Mustafa, M. A., Abd-El-Rahman El-Emam, A. and Hansen, S. H. (2003) Evaluation of the use of microemulsions as eluents in high-performance liquid chromatography. *Journal of Separation Science* 26 (6-7), 503-509.

Engida, A. M., Kasim, N. S., Tsigie, Y. A., Ismadji, S., Huynh, L. H. and Ju, Y.-H. (2013) Extraction, identification and quantitative HPLC analysis of flavonoids from sarang semut (Myrmecodia pendan). *Industrial Crops and Products* 41, 392-396.

Esteve-Romero, J., Carda-Broch, S., Gil-Agustí, M., Capella-Peiró, M.-E. and Bose, D. (2005) Micellar liquid chromatography for the determination of

drug materials in pharmaceutical preparations and biological samples. *TrAC Trends in Analytical Chemistry* 24 (2), 75-91.

Fanun, M. (2012) Microemulsions as delivery systems. *Current Opinion in Colloid & Interface Science* 17 (5), 306-313.

Farrar, C. L. (1960) Caution In The Use of Chemicals, Drugs, and Antibiotics. *American Bee Journal* 100 (5), 192-193.

Ferreres, F., Tomáas-Barberáan, F. A., Gil, M. I. and Tomáas-Lorente, F. (1991) An HPLc technique for flavonoid analysis in honey. *Journal of the Science of Food and Agriculture* 56 (1), 49-56.

Fritz, J. W. and Zuo, Y. (2007) Simultaneous determination of tetracycline, oxytetracycline, and 4-epitetracycline in milk by high-performance liquid chromatography. *Food Chemistry* 105 (3), 1297-1301.

Gardana, C., Scaglianti, M., Pietta, P. and Simonetti, P. (2007) Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal* 45 (3), 390-9.

Ghaly, M. F., Ezzat, S. M. and Sarhan, M. M. (1998) Use of propolis and ultragriseofulvin to inhibit aflatoxigenic fungi. *Folia Microbiologica* 43 (2), 156-160.

Ghisalberti, E. L. (1979) Propolis: A Review. *Bee World* 60 (2), 59-84.

Greenaway, W., Scaysbrook, T. and Whatley, F. R. (1990) The Composition and Plant Origins of Propolis: A Report of Work at Oxford. *Bee World* 71 (3), 107-118.

Gregory, S. R., Piccolo, N., Piccolo, M. T., Piccolo, M. S. and Heggers, J. P. (2002) Comparison of propolis skin cream to silver sulfadiazine: A naturopathic alternative to antibiotics in treatment of minor burns. *Journal of Alternative and Complementary Medicine* 8 (1), 77-83.

Gómez-Caravaca, A. M., Gómez-Romero, M., Arráez-Román, D., Segura-Carretero, A. and Fernández-Gutiérrez, A. (2006) Advances in the

analysis of phenolic compounds in products derived from bees. *Journal of Pharmaceutical and Biomedical Analysis* 41 (4), 1220-1234.

Gülçin, İ., Bursal, E., Şehitoğlu, M. H., Bilsel, M. and Gören, A. C. (2010) Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey. *Food and Chemical Toxicology* 48 (8–9), 2227-2238.

Hammel, Y.-A., Mohamed, R., Gremaud, E., LeBreton, M.-H. and Guy, P. A. (2008) Multi-screening approach to monitor and quantify 42 antibiotic residues in honey by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* 1177 (1), 58-76.

Han, S. K. and Park, H. K. (1996) A study on the preservation of meat products with water extracted propolis (WEP). *Korean Journal of Animal Science* 38 (6), 605-612.

Hanasaki, Y., Ogawa, S. and Fukui, S. (1994) The Correlation between active oxygens scavenging and Antioxidative effects of flavonoids. *Free Radical Biology and Medicine* 16 (6), 845-850.

Havsteen, B. H. (2002) The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics* 96 (2-3), 67-202.

He, S., Sun, C. and Pan, Y. (2008) Red wine polyphenols for cancer prevention. *International Journal of Molecular Sciences* 9 (5), 842-853.

Hegazi, A., Abdou, A. M. and Allah, F. A. (2012) Egyptian Propolis 9- Its Effect on Chicken Productivity and Immune Response Against Newcastle Disease Vaccine. *British Journal of Poultry Sciences* 1 (3), 25-30.

Hegazi, A. G. and El Hady, F. K. A. (2002) Egyptian propolis: 3. Antioxidant, antimicrobial activities and chemical composition of propolis from reclaimed lands. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences* 57 (3-4), 395-402.

Hegazi, A. G., Farghaly, A. A. and Abd El Hady, F. K. (2001) Antiviral activity and Chemical compositions of Europian and Egyptian Propolis. *APIMONDIA.* Durban, South Africa. International Apicultural Congress.

Hennion, M.-C. (1999) Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography. *Journal of Chromatography A* 856 (1–2), 3-54.

Hong, T. B., Rahumatullah, A., Yogarajah, T., Ahmad, M. and Yin, K. B. (2010) Potential Effects of Chrysin on MDA-MB-231 Cells. *International Journal of Molecular Sciences* 11 (3), 1057-1069.

Horii, S., Momma, C., Miyahara, K., Maruyama, T. and Matsumoto, M. (1990) Liquid chromatographic determination of three sulfonamides in animal tissue and egg. *J Assoc Off Anal Chem* 73 (6), 990-2.

Huie, C. W. (2002) A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Anal Bioanal Chem* 373 (1-2), 23-30.

ICH. (1996) *Validation of Analytical Procedures: Text and Methodology.* Geneva: ICH.

Ioirìch, N., Derevici, A. and Petrescu, A. (1965) Virulicidal action in vitro of alcoholic extracts of drone larvae and propolis. *lucr. stünt stat. Cent. Seri. Apic.* 5 (107-110).

Ito, J., Chang, F. R., Wang, H. K., Park, Y. K., Ikegaki, M., Kilgore, N. and Lee, K. H. (2001) Anti-AIDS agents. 48. Anti-HIV activity of moronic acid derivatives and the new melliferone-related triterpenoid isolated from Brazilian propolis. *Journal of Natural Products* 64 (10), 1278-1281.

Johnson, K. S., Eischen, F. A. and Giannasi, D. E. (1994) Chemical-Composition of North-American Bee propolis and biological activity towards Larvae of Greater Wax moth (Lepidoptera: Pyralidae) *Journal of Chemical Ecology* 20 (7), 1783-1791.

Kalogeropoulos, N., Konteles, S. J., Troullidou, E., Mourtzinos, I. and Karathanos, V. T. (2009) Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. *Food Chemistry* 116 (2), 452-461.

Kartal, M., Kaya, S. and Kurucu, S. (2002) GC-MS analysis of propolis samples from two different regions of Turkey. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences* 57 (9-10), 905-909.

Kayser, O. and Kolodziej, H. (1997) Antibacterial activity of extracts and constituents of Pelargonium sidoides and Pelargonium reniforme. *Planta Medica* 63 (6), 508-510.

Khacha-Ananda, S., Tragoolpua, K., Chantawannakul, P. and Tragoolpua, Y. (2013) Antioxidant and anti-cancer cell proliferation activity of propolis extracts from two extraction methods. *Asian Pacific journal of cancer prevention* 14 (11), 6991-6995.

King Saud University (2007) *Liquid liquid extraction.* King Saud University. http://faculty.ksu.edu.sa/18856/Extraction/Forms/DispForm.aspx?ID=6 Accessed on 22/02/2016.

Koc, A. N., Silici, S., Mutlu-Sariguzel, F. and Sagdic, O. (2007) Antifungal activity of propolis in four different fruit juices. *Food Technology and Biotechnology* 45 (1), 57-61.

Koltay, M. (1981) *Isolation and Identification of Constituents of Propolis.* PhD. Concordia University. http://clues.concordia.ca/search/c?SEARCH=SF%20539%20K68+

Koo, H., Rosalen, P. L., Cury, J. A., Park, Y. K. and Bowen, W. H. (2002) Effects of compounds found in propolis on Streptococcus mutans growth and on glucosyltransferase activity. *Antimicrobial Agents and Chemotherapy* 46 (5), 1302-1309.

Korkina, L. G. and Afanas'ev, I. B. (1997) Antioxidant and chelating properties of flavonoids. *Advances in Pharmacology; Antioxidants in disease mechanisms and therapy.*Advances in Pharmacology. Vol. 38. 151-163.

Kosalec, I., Bakmaz, M. and Pepeljnjak, S. (2003) Analysis of propolis from the continental and Adriatic regions of Croatia. *Acta Pharm* 53 (4), 275-85.

Kosalec, I., Bakmaz, M., Pepeljnjak, S. and Vladimir-Knezevic, S. (2004) Quantitative analysis of the flavonoids in raw propolis from northern Croatia. *Acta Pharmaceutica (Zagreb)* 54 (1), 65-72.

Krell, R. (1996) *Value- Added Products from Beekeeping.* FAO Agricultural Services Bulletin. Milan: FAO Publications.

Krivoruchko, V. F., Degtyarenko, V. I., Zaitseva, T. I. and Ganchenko, M. G. (1975) Study of anti-viral effect of propolis in an experiment. *Virusy i Virusnye Zabolevaniya (Kiev)* 3, 61-64.

Kumazawa, S., Hamasaka, T. and Nakayama, T. (2004) Antioxidant activity of propolis of various geographic origins. *Food Chemistry* 84 (3), 329-339.

Kurek-Gorecka, A., Rzepecka-Stojko, A., Gorecki, M., Stojko, J., Sosada, M. and Swierczek-Zieba, G. (2014) Structure and Antioxidant Activity of Polyphenols Derived from Propolis. *Molecules* 19 (1), 78-101.

Kurek-Górecka, A., Rzepecka-Stojko, A., Górecki, M., Stojko, J., Sosada, M. and Świerczek-Zięba, G. (2013) Structure and Antioxidant Activity of Polyphenols Derived from Propolis. *Molecules* 19 (1), 78.

Kuropatnicki, A. K., Szliszka, E. and Krol, W. (2013) Historical Aspects of Propolis Research in Modern Times. *Evidence-based Complementary and Alternative Medicine : eCAM* 2013, 964149.

Lee, J.-B., Chung, H.-H., Chung, Y.-H. and Lee, K.-G. (2007) Development of an analytical protocol for detecting antibiotic residues in various foods. *Food Chemistry* 105 (4), 1726-1731.

Lee, Y. J., Kuo, H. C., Chu, C. Y., Wang, C. J., Lin, W. C. and Tseng, T. H. (2003) Involvement of tumor suppressor protein p53 and p38 MAPK in caffeic acid phenethyl ester-induced apoptosis of C6 glioma cells. *Biochemical Pharmacology* 66 (12), 2281-2289.

Lee, Y. J., Liao, P. H., Chen, W. K. and Yang, C. C. (2000) Preferential cytotoxicity of caffeic acid phenethyl ester analogues on oral cancer cells. *Cancer Letters* 153 (1-2), 51-56.

Levy, S. B. and Marshall, B. M. (2013) Honeybees and Tetracycline Resistance. *mBio* 4 (1), e00045-13.

Li, G.-R., Wang, H.-B., Qin, G.-W., Jin, M.-W., Tang, Q., Sun, H.-Y., Du, X.-L., Deng, X.-L., Zhang, X.-H., Chen, J.-B., Chen, L., Xu, X.-H., Cheng, L.-C., Chiu, S.-W., Tse, H.-F., Vanhoutte, P. M. and Lau, C.-P. (2008) Acacetin, a natural flavone, selectively inhibits human atrial repolarization potassium currents and prevents atrial fibrillation in dogs. *Circulation* 117 (19), 2449-2457.

Li, N., Huang, G.-L., Li, Y.-L., Wang, T.-J. and Gao, C.-K. (2009) Simultaneous Determination of Four Flavonoids in Extract of Hawthorn Leaves by Microemulsion Liquid Chromatography. *Chinese Journal of Analytical Chemistry* 37 (12), 1791-1794.

Li, Y., Zhao, J., Xue, X. and Zhou, J. (2007) [Determination of twelve active compounds in propolis using ultra-performance liquid chromatography]. *Se Pu* 25 (6), 857-60.

LoBrutto, R. and Kazakevich, Y. (2006) Reversed-Phase HPLC. *HPLC for Pharmaceutical Scientists.* John Wiley & Sons, Inc. 139-239. http://dx.doi.org/10.1002/9780470087954.ch4 Accessod on 07/08/2015

Lou, S.-N., Hsu, Y.-S. and Ho, C.-T. (2014) Flavonoid compositions and antioxidant activity of calamondin extracts prepared using different solvents. *Journal of Food and Drug Analysis* 22 (3), 290-295.

Luo, C., Zou, X., Li, Y., Sun, C., Jiang, Y. and Wu, Z. (2011) Determination of flavonoids in propolis-rich functional foods by reversed phase high performance liquid chromatography with diode array detection. *Food Chemistry* 127 (1), 314-320.

Lupascu, D., Tuchilus, C. and Profire, L. (2008) Physico - Chemical And Antimicrobial Properties Of Novel Rutin Derivatives With 6-Aminopenicillanic Acid. *Farmacia Lvi* 5, 501-506.

Luque-García, J. L. and Luque de Castro, M. D. (2003) Ultrasound: a powerful tool for leaching. *TrAC Trends in Analytical Chemistry* 22 (1), 41-47.

Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *THE SYSTEMATIC IDENTIFICATION OF FLAVONOIDS.* The Systematic Identification of Flavonoids.

Mahmoudi, R., Moosavy, M., Norian, R., Kazemi, S., Nadari, M. R. A. and Mardani, K. (2014) Detection of oxytetracycline residues in honey samples using ELISA and HPLC methods. *Pharmaceutical Sciences* 19 (4), 145-50.

Maichuk, I. F., Orlovskaia, L. E. and Andreev, V. P. (1995) The use of ocular drug films of propolis in the sequelae of ophthalmic herpes. *Voenno-meditsinskii zhurnal* (12), 36-80.

Makashvili, Z. A. (1978) *From the history of propolis. In Remarkable hive product :Propolis. Scientific data and suggessions concerning its composition, properties and possible use in therapeutics.* Bucharest: Apimondia.

Malešev, D. and Kuntić, V. (2007) Investigation of metal-flavonoid chelates and the determination of flavonoids via metal-flavonoid complexing reactions. *Journal of the Serbian Chemical Society* 72 (10), 921-939.

Manser, W. W., Khan, M. A. and Hasan, Z. (1989) Trace element studies in Karachi populations. Part III: Blood copper, zinc, magnesium and lead levels in psychiatric patients with disturbed behaviour. *JPMA. The Journal of the Pakistan Medical Association* 39 (9), 235-8.

Marcucci, M. C. (1995) Propolis: chemical composition, biological properties and therapeutic activity. *Apidologie* 26 (2), 83-99.

Markham, K. R., Mitchell, K. A., Wilkins, A. L., Daldy, J. A. and Lu, Y. (1996) HPLC and GC-MS identification of the major organic constituents in New Zeland propolis. *Phytochemistry* 42 (1), 205-211.

Marsh, A., Clark, B. J. and Altria, K. D. (2005) A review of the background, operating parameters and applications of microemulsion liquid chromatography (MELC). *Journal of Separation Science* 28 (15), 2023-2032.

Martos, I., Cossentini, M., Ferreres, F. and TomasBarberan, F. A. (1997) Flavonoid composition of Tunisian honeys and propolis. *Journal of Agricultural and Food Chemistry* 45 (8), 2824-2829.

Maróstica Junior, M. R., Daugsch, A., Moraes, C. S., Queiroga, C. L., Pastore, G. M. and Parki, Y. K. (2008) Comparison of volatile and polyphenolic compounds in Brazilian green propolis and its botanical origin Baccharis dracunculifolia. *Food Science and Technology (Campinas)* 28, 178-181.

Mascher, A., Lavagnoli, S. and Curatolo, M. (1996) Determination of residual oxytetracycline in honey with an immunoassay kit. *Apidologie* 27 (4), 229-233.

Mattila, P., Astola, J. and Kumpulainen, J. (2000) Determination of Flavonoids in Plant Material by HPLC with Diode-Array and Electro-Array Detections. *Journal of Agricultural and Food Chemistry* 48 (12), 5834-5841.

McEvoy, E., Donegan, S., Power, J. and Altria, K. (2007) Optimisation and validation of a rapid and efficient microemulsion liquid chromatographic (MELC) method for the determination of paracetamol (acetaminophen) content in a suppository formulation. *Journal of Pharmaceutical and Biomedical Analysis* 44 (1), 137-143.

McKellip, L. L., Bing, P. and Trost, B. M. (1999) *Method for extraction of essential oils from plant material*. Google Patents.

Melliou, E., Stratis, E. and Chinou, I. (2007) Volatile constituents of propolis from various regions of Greece – Antimicrobial activity. *Food Chemistry* 103 (2), 375-380.

Memon, N., Shaikh, H. I. and Solangi, A. R. (2012) Selectivity of Brij-35 in Micellar Liquid Chromatographic Separation of Positional Isomers. *2012* 2012.

Merken, H. M. and Beecher, G. R. (2000) Measurement of Food Flavonoids by High-Performance Liquid Chromatography: A Review. *Journal of Agricultural and Food Chemistry* 48 (3), 577-599.

Metzner, J., Bekemeier, H., Paintz, M. and Schneidewind, E. (1979) Anti microbial acivity of Propolis and propolis constituents. *Pharmazie* 34 (2), 97-102.

Meyer, W. (1956) Propolis bees and their activities. *Bee World* 37, 25-36.

Middleton, E. (1998) Effect of plant flavonoids on immune and inflammatory cell function. *Flavonoids in the Living System.*Advances in Experimental Medicine and Biology. Vol. 439. 175-182. *<Go to ISI>://WOS:000076150000013*

Mirzoeva, O. K. and Calder, P. C. (1996) The effect of propolis and its components on eicosanoid production during the inflammatory response. *Prostaglandins Leukot Essent Fatty Acids* 55 (6), 441-9.

Mizuno, M. (1989a) *Food packaging materials containing propolis as a preservative.*

Mizuno, M. (1989b) *Propolis or its extract containing resin compositions.*

Mohammadzadeh, S., Sharriatpanahi, M., Hamedi, M., Amanzadeh, Y., Sadat Ebrahimi, S. E. and Ostad, S. N. (2007) Antioxidant power of Iranian propolis extract. *Food Chemistry* 103 (3), 729-733.

Moniruzzaman, M., Yung An, C., Rao, P. V., Hawlader, M. N. I., Azlan, S. A. B. M., Sulaiman, S. A. and Gan, S. H. (2014) Identification of Phenolic Acids and Flavonoids in Monofloral Honey from Bangladesh by High Performance Liquid Chromatography: Determination of Antioxidant Capacity. *BioMed Research International* 2014, 11.

Moreira, L., Dias, L. G., Pereira, J. A. and Estevinho, L. (2008) Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal. *Food and Chemical Toxicology* 46 (11), 3482-3485.

Moreno, M. I. N., Isla, M. I., Sampietro, A. R. and Vattuone, M. A. (2000) Comparison of the free radical-scavenging activity of propolis from several regions of Argentina. *Journal of Ethnopharmacology* 71 (1-2), 109-114.

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., vanEngelsdorp, D. and Pettis, J. S. (2010) High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLoS ONE* 5 (3), e9754.

Murad, J. M., Calvi, S. A., Soares, A., Bankova, V. and Sforcin, J. M. (2002) Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against Paracoccidioides brasiliensis. *Journal of Ethnopharmacology* 79 (3), 331-334.

Nadarassan, D. K., Chrystyn, H., Clark, B. J. and Assi, K. H. (2007) Validation of high-performance liquid chromatography assay for quantification of formoterol in urine samples after inhalation using UV detection technique. *Journal of Chromatography B* 850 (1–2), 31-37.

Nagai, T., Inoue, R., Inoue, H. and Suzuki, N. (2003) Preparation and antioxidant properties of water extract of propolis. *Food Chemistry* 80 (1), 29-33.

Nagy, E., Papay, V., Litkei, G. and Dinya, Z. (1986) Investigation of the chemical constituents, particularly the flavonoid components, of propolis and *Populi gemma* by the GC/MS method. *Studies in Organic Chemistry* 23, 223-232.

National Center for Biotechnology Information (2016) *PubChem Compound.* National Center for Biotechnology Information,. *http://www.ncbi.nlm.nih.gov/pccompound* Accessed on 03/03/2016.

Nessa, F., Ismail, Z., Karupiah, S. and Mohamed, N. (2005) RP-HPLC method for the quantitative analysis of naturally occurring flavonoids in leaves of Blumea balsamifera DC. *J Chromatogr Sci* 43 (8), 416-20.

Nieva Moreno, M. I., Isla, M. I., Cudmani, N. G., Vattuone, M. A. and Sampietro, A. R. (1999) Screening of antibacterial activity of Amaicha del Valle (Tucumán, Argentina) propolis. *Journal of Ethnopharmacology* 68 (1–3), 97-102.

Nolvachai, Y. and Marriott, P. J. (2013) GC for flavonoids analysis: Past, current, and prospective trends. *Journal of Separation Science* 36 (1), 20-36.

Orhan, H., Marol, S., Hepsen, I. F. and Sahin, G. (1999) Effects of some probable antioxidants on selenite-induced cataract formation and oxidative stress-related parameters in rats. *Toxicology* 139 (3), 219-232.

Osol, A., Hoover, J. E. and al., e. (1975) *Remington's Pharmaceutical Sciences.* 15th ed. edition. Easton, Pennsylvania: Mack Publishing Co.

Ozturk, G., Ginis, Z., Akyol, S., Erden, G., Gurel, A. and Akyol, O. (2012) The anticancer mechanism of caffeic acid phenethyl ester (CAPE): review of melanomas, lung and prostate cancers. *Eur Rev Med Pharmacol Sci* 16 (15), 2064-2068.

Paintz, M. and Metzner, J. (1979) Local-Anesthetic action of Propolis and some of its constituents. *Pharmazie* 34 (12), 839-841.

Pandit, N. K. and Soltis, R. P. (2012) *Introduction to the pharmaceutical sciences : an integrated approach.* Baltimore, MD: Lippincott Williams & Wilkins.

Pareja, L., Colazzo, M., Pérez-Parada, A., Niell, S., Carrasco-Letelier, L., Besil, N., Cesio, M. V. and Heinzen, H. (2011) Detection of Pesticides in Active and Depopulated Beehives in Uruguay. *International Journal of Environmental Research and Public Health* 8 (10), 3844-3858.

Park, Y. K. and Ikegaki, M. (1998) Preparation of Water and Ethanolic Extracts of Propolis and Evaluation of the Preparations. *Bioscience, Biotechnology, and Biochemistry* 62 (11), 2230-2232.

Patel, K., Gadewar, M., Tripathi, R., Prasad, S. K. and Patel, D. K. (2012) A review on medicinal importance, pharmacological activity and bioanalytical aspects of beta-carboline alkaloid "Harmine". *Asian Pacific Journal of Tropical Biomedicine* 2 (8), 660-664.

Patel, M. (2007) Microemulsions : As Novel Drug Delivery Vehicle. *Pharmainfo.net,* .

Pellati, F., Orlandini, G., Pinetti, D. and Benvenuti, S. (2011) HPLC-DAD and HPLC-ESI-MS/MS methods for metabolite profiling of propolis extracts. *J Pharm Biomed Anal* 55 (5), 934-48.

Pellati, F., Prencipe, F. P. and Benvenuti, S. (2013) Headspace solid-phase microextraction-gas chromatography–mass spectrometry characterization of propolis volatile compounds. *Journal of Pharmaceutical and Biomedical Analysis* 84, 103-111.

Perez-Parada, A., Colazzo, M., Besil, N., Geis-Asteggiante, L., Rey, F. and Heinzen, H. (2011) Determination of coumaphos, chlorpyrifos and ethion residues in propolis tinctures by matrix solid-phase dispersion and gas chromatography coupled to flame photometric and mass spectrometric detection. *Journal of Chromatography A* 1218 (34), 5852-5857.

Pettis, J. S. and Delaplane, K. S. (2010) Coordinated responses to honey bee decline in the USA. *Apidologie* 41 (3), 256-263.

Phillips, I., Casewell, M., Cox, T., de Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R. and Waddell, J. (2004) Does the use of antibiotics in food animals pose a risk to human health? A reply to critics. *Journal of Antimicrobial Chemotherapy* 54 (1), 276-278.

Pietta, P. G., Gardana, C. and Pietta, A. M. (2002) Analytical methods for quality control of propolis. *Fitoterapia* 73, Supplement 1 (0), S7-S20.

Polettini, A., Montagna, M., Hogendoorn, E. A., Dijkman, E. and Vanzoonen, P. a. V. L. A. (1995) Applicability of coupled-column liquid-chromatography to the analysisof Beta-agonists in urine by direct sample injection .1. Development of a single-residue reversed-phase liquid-chromatography UV method for clenbuterol and selection of chromatographic conditions suitable for multi-residue analysis. *Journal of chromatography* 695 (1), 19 - 31.

Popova, M., Bankova, V., Butovska, D., Petkov, V., Nikolova-Damyanova, B., Sabatini, A. G., Marcazzan, G. L. and Bogdanov, S. (2004) Validated methods for the quantification of biologically active constituents of poplar-type propolis. *Phytochem Anal* 15 (4), 235-40.

Rambla Alegre, M., Carda Broch, S. and Esteve Romero, J. (2008) Development and validation of a method to determine amoxicillin in physiological fluids using micellar liquid chromatography. *J Sep Sci* 31 (15), 2813-9.

Rambla-Alegre, M., Collado-Sanchez, M. A., Esteve-Romero, J. and Carda-Broch, S. (2011a) Quinolones control in milk and eggs samples by liquid chromatography using a surfactant-mediated mobile phase. *Anal Bioanal Chem* 400 (5), 1303-13.

Rambla-Alegre, M., Esteve-Romero, J. and Carda-Broch, S. (2009) Validation of a MLC method with fluorescence detection for the determination of quinolones in urine samples by direct injection. *J Chromatogr B Analyt Technol Biomed Life Sci* 877 (31), 3975-81.

Rambla-Alegre, M., Marti-Centelles, R., Esteve-Romero, J. and Carda-Broch, S. (2011b) Application of a liquid chromatographic procedure for the analysis of penicillin antibiotics in biological fluids and pharmaceutical formulations using sodium dodecyl sulphate/propanol mobile phases and direct injection. *J Chromatogr A* 1218 (30), 4972-81.

Rambla-Alegre, M., Peris-Vicente, J., Esteve-Romero, J. and Carda-Broch, S. (2010) Analysis of selected veterinary antibiotics in fish by micellar liquid chromatography with fluorescence detection and validation in accordance with regulation 2002/657/EC. *Food Chemistry* 123 (4), 1294-1302.

Repollés, C., Herrero-Martínez, J. M. and Ràfols, C. (2006) Analysis of prominent flavonoid aglycones by high-performance liquid chromatography using a monolithic type column. *Journal of Chromatography A* 1131 (1–2), 51-57.

Reverchon, E. and De Marco, I. (2006) Supercritical fluid extraction and fractionation of natural matter. *Journal of Supercritical Fluids* 38 (2), 146-166.

Rossi, A., Longo, R., Russo, A., Borrelli, F. and Sautebin, L. (2002) The role of the phenethyl ester of caffeic acid (CAPE) in the inhibition of rat lung cyclooxygenase activity by propolis. *Fitoterapia* 73 Suppl 1, S30-7.

Ruiz-Ángel, M. J., García-Álvarez-Coque, M. C. and Berthod, A. (2009) New Insights and Recent Developments in Micellar Liquid Chromatography. *Separation & Purification Reviews* 38 (1), 45-96.

Russo, A., Longo, R. and Vanella, A. (2002) Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. *Fitoterapia* 73, S21-S29.

Ryan, R., Altria, K., McEvoy, E., Donegan, S. and Power, J. (2013) A review of developments in the methodology and application of microemulsion electrokinetic chromatography. *Electrophoresis* 34 (1), 159-77.

Sagert, K. B. (2008) *United States Pharmacopeia and National Formulary (USP-NF). Encyclopedia of Global Health. SAGE Publications, Inc.* Thousand Oaks, CA: SAGE Publications, Inc.

Said, S. A., Khan, S. A., Ahmad, I. and Ali, H. S. (2006) Chemical composition of Egyptian and UAE propolis. *Pakistan journal of pharmaceutical sciences* 19 (1), 58-61.

Sales, A., Alvarez, A., Areal, M. R., Maldonado, L., Marchisio, P., Rodriguez, M. and Bedascarrasbure, E. (2006) The effect of different propolis harvest methods on its lead contents determined by ET AAS and UV-visS. *Journal of Hazardous Materials* 137 (3), 1352-1356.

Saridaki-Papakonstadinou, M., Andredakis, S., Burriel, A. and Tsachev, I. (2006) Determination of tetracycline residues in Greek honey. *Trakia Journal of Sciences* 4 (1), 33-36.

Savickas, A., Majiene, D., Ramanauskiene, K., Pavilonis, A., Muselik, J., Masteikova, R. and Chalupova, Z. (2005) Chemical composition and antimicrobial activity of Lithuanian and Czech propolis. *BIOLOGIJA* 4, 59-63.

Sbeghen-Loss, A., Barboza, E. G. G., Matto, M., Cesio, M. V., Frizzo, C., Monteiro de Barros, N. and Heinzen, H. (2009) Lime wax influences

feeding behaviour of cryptothermes brevis and the viability of its foaina sp. symbionts. 54, 1-14.

Scheller, S., Krol, W., Swiacik, J., Owczarek, S., Gabrys, J. and Shani, J. (1989) Antitumoral Property of Ethanolic Extract of Propolis in Mice-Bearing Ehrlich Carcinoma, as compared to Bleomycin. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences* 44 (11-12), 1063-1065.

Schmuck, R., Schoning, R., Stork, A. and Schramel, O. (2001) Risk posed to honeybees (Apis mellifera L. Hymenoptera) by an imidacloprid seed dressing of sunflowers. *Pest Management Science* 57 (3), 225-238.

Serra Bonvehi, J. and Orantes Bermejo, F. J. (2013) Element content of propolis collected from different areas of South Spain. *Environmental Monitoring and Assessment* 185 (7), 6035-6047.

Sforcin, J. M. (2007) Propolis and the immune system: a review. *Journal of Ethnopharmacology* 113 (1), 1-14.

Shimadzu (2016) *Differences between using Acetonotrile and Methanol for reverse phase chromatography.* Shimadzu. http://www.shimadzu.com/an/hplc/support/lib/lctalk/35/35lab.html Accessed 08/03/2016.

Shirsath, S. R., Sonawane, S. H. and Gogate, P. R. (2012) Intensification of extraction of natural products using ultrasonic irradiations—A review of current status. *Chemical Engineering and Processing: Process Intensification* 53, 10-23.

Shouqin, Z., Jun, X. and Changzheng, W. (2005) High hydrostatic pressure extraction of flavonoids from propolis. *Journal of Chemical Technology and Biotechnology* 80 (1), 50-54.

Shukla, S. and Gupta, S. (2010) Apigenin: A Promising Molecule for Cancer Prevention. *Pharmaceutical Research* 27 (6), 962-978.

Silici, S. and Kutluca, S. (2005) Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. *Journal of Ethnopharmacology* 99 (1), 69-73.

Silva, M. S. S., De Lima, S. G., Oliveira, E. H., Lopes, J. A. D., Chaves, M. H., Reis, F. A. M. and Citó, A. M. G. L. (2008) Anacardic acid derivatives from Brazilian propolis and their antibacterial activity. *Eclética Química* 33, 53-58.

Sisa, M., Bonnet, S. L., Ferreira, D. and Van der Westhuizen, J. H. (2010) Photochemistry of Flavonoids. *Molecules* 15 (8), 5196-5245.

Song, R. and Zhou, J. (2015) Microemulsion liquid chromatographic method for simultaneous separation and determination of six flavonoids of Apocynum venetum leaf extract. *J Chromatogr B Analyt Technol Biomed Life Sci* 995-996, 8-14.

Spáčil, Z., Nováková, L. and Solich, P. (2008) Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography. *Talanta* 76 (1), 189-199.

Stanciu, G. and Mititelu, M. (2004) Study of some heavy metals from propolis and honey. *4th AACD Congress.* Turkey.

Stefova, M., Stafilov, T. and Kulevanova, S. (2004) HPLC analysis of flavonoids. *Encyclopedia of Chromatography 2004 Update Supplement*, 113.

Stubbings, G., Tarbin, J., Cooper, A., Sharman, M., Bigwood, T. and Robb, P. (2005) A multi-residue cation-exchange clean up procedure for basic drugs in produce of animal origin. *Analytica Chimica Acta* 547 (2), 262-268.

Su, P., Wang, R., Yu, Y. and Yang, Y. (2014) Microwave-assisted synthesis of ionic liquid-modified silica as a sorbent for the solid-phase extraction of phenolic compounds from water. *Analytical Methods* 6 (3), 704-709.

Suzuki, I., Takai, H., Koide, M. and Yamamoto, H. (1996) Antitumor effect of immunologically active fractions obtained from Brazilian Propolis, given with combination in anticancer drug Ehrlich carcinoma bearing mice. *Mitsubachi Kagaku* 17, 1-6.

Svetlana, K., Marina, S. and Trajce, S. (2009) Flavonoids: HPLC Analysis. *Encyclopedia of Chromatography, Third Edition.* Vol. null. Taylor & Francis. 882-889. http://dx.doi.org/10.1081/E-ECHR3-120016287

Tan, S. P., Parks, S. E., Stathopoulos, C. E. and Roach, P. D. (2014) Extraction of Flavonoids from Bitter Melon. *Food and Nutrition Sciences* Vol.05No.05, 8.

Tayeb Cherif, K., Peris-Vicente, J., Carda-Broch, S. and Esteve-Romero, J. (2015) Analysis of danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey using micellar liquid chromatography and validation according to the 2002/657/EC decision. *Analytical Methods* 7 (15), 6165-6172.

Thirugnanasampandan, R., Raveendran, S. B. and Jayakumar, R. (2012) Analysis of chemical composition and bioactive property evaluation of Indian propolis. *Asian Pacific Journal of Tropical Biomedicine* 2 (8), 651-654.

Thompson, H. M., Waite, R. J., Wilkins, S., Brown, M. A., Bigwood, T., Shaw, M., Ridgway, C. and Sharman, M. (2005) Effects of European foulbrood treatment regime on oxytetracycline levels in honey extracted from treated honeybee (Apis mellifera) colonies and toxicity to brood. *Food Addit Contam* 22 (6), 573-8.

Timbola, A. K., de Souza, C. D., Giacomelli, C. and Spinelli, A. (2006) Electrochemical oxidation of quercetin in hydro-alcoholic solution. *Journal of the Brazilian Chemical Society* 17 (1), 139-148..

Trusheva, B., Trunkova, D. and Bankova, V. (2007) Different extraction methods of biologically active components from propolis: a preliminary study. *Chemistry Central Journal* 1, 13-13.

Tsimogiannis, D., Samiotaki, M., Panayotou, G. and Oreopoulou, V. (2007) Characterization of Flavonoid Subgroups and Hydroxy Substitution by HPLC-MS/MS. *Molecules* 12 (3), 593.

Vardanega, R., Santos, D. T. and Meireles, M. A. A. (2014) Intensification of bioactive compounds extraction from medicinal plants using ultrasonic irradiation. *Pharmacognosy Reviews* 8 (16), 88-95.

Velikova, M., Bankova, V., Marcucci, M. C., Tsvetkova, I. and Kujumgiev, A. (2000) Chemical composition and biological activity of propolis from Brazilian Meliponinae. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences* 55 (9-10), 785-789.

Vidal, J. L. M., Aguilera-Luiz, M. d. M., Romero-González, R. and Frenich, A. G. (2009) Multiclass Analysis of Antibiotic Residues in Honey by Ultraperformance Liquid Chromatography−Tandem Mass Spectrometry. *Journal of Agricultural and Food Chemistry* 57 (5), 1760-1767.

Viñas, P., Balsalobre, N., López-Erroz, C. and Hernández-Córdoba, M. (2004) Liquid chromatography with ultraviolet absorbance detection for the analysis of tetracycline residues in honey. *Journal of Chromatography A* 1022 (1–2), 125-129.

Volpert, R. and Elstner, E. F. (1996) Interactions of different extracts of propolis with leukocytes and leukocytic enzymes. *Arzneimittel-Forschung/Drug Research* 46 (1), 47-51.

Wagh, V. D. (2013) Propolis: A Wonder Bees Product and Its Pharmacological Potentials. *Advances in Pharmacological Sciences* 2013, 11.

Wang, L. and Li, Y.-Q. (2009) Simultaneous determination of ten antibiotic residues in milk by UPLC. *Chromatographia* 70 (1-2), 253-258.

Wang, S.-P., Fu, M.-D. and Wang, M.-H. (2007) Separation mechanism and determination of flavanones with capillary electrophoresis and high-performance liquid chromatography. *Journal of Chromatography A* 1164 (1–2), 306-312.

Wang, Y.-H., Zhang, R.-F. and Wang, Y.-Z. (2004) Factors influencing pharmacodynamics of the Chinese materia medica decoction and the prospect of its development. *Pharmaceutical Care and Research* 4 (1), 77-79.

Waters (2016) *How Does High Performance Liquid Chromatography Work?* Waters. http://www.waters.com/waters/en_GB/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=en_GB Accessed on 22/02/2016.

Wen, Y., Wang, Y. and Feng, Y.-Q. (2006) Simultaneous residue monitoring of four tetracycline antibiotics in fish muscle by in-tube solid-phase microextraction coupled with high-performance liquid chromatography. *Talanta* 70 (1), 153-159.

Wishart Research Group (2016) *FooDB.* University of Alberta. http://foodb.ca/ Accessed on 22/02/2016.

Woisky, R. G. and Salatino, A. (1998) Analysis of propolis: some parameters and procedures for chemical quality control. *Journal of Apicultural Research* 37 (2), 99-105.

Wojciech, P. and Zommer-Urbanska, S. (1992) Determination of a chlorinated pesticides residue in propolis extract. *Acta poloniae pharmaceutica* 49 (5-6), 19-21.

Wollenweber, E. and Buchmann, S. L. (1997) Feral honey bees in the Sonoran Desert: Propolis sources other than poplars (Populus spp.). *Zeitschrift Fur Naturforschung C-a Journal of Biosciences* 52 (7-8), 530-535.

Wu, J. and Qial, L. (1999) *Supercritical multiple bee glue extracting process.*

You, G. S., Lin, S. C., Chen, C. R., Tsai, W. C., Chang, C. M. J. and Huang, W. W. (2002) Supercritical carbon dioxide extraction enhances flavonoids in water-soluble propolis. *Journal of the Chinese Institute of Chemical Engineers* 33 (3), 233-241.

Zai, I. U. M., Rehman, K., Hussain, A. and Shafqatullah (2013) Detection and Quantification of Antibiotics Residues in Honey Samples by Chromatographic Techniques. *Middle-East Journal of Scientific Research* 14 (5), 638-687.

Zhang, L., Li, Y. and Tong, P. (2012) Study on the application of common rush cellulose xanthogenate for removal of lead from propolis. *Science & Technology of Food Industry* 33 (1), 226-229.

Zhang, W. L., Chen, J.-P., Lam, K. Y.-C., Zhan, J. Y.-X., Yao, P., Dong, T. T.-X. and Tsim, K. W.-K. (2014) Hydrolysis of Glycosidic Flavonoids during the Preparation of Danggui Buxue Tang: An Outcome of Moderate Boiling of Chinese Herbal Mixture. *Evidence-Based Complementary and Alternative Medicine* 2014, 11.

Zhao, L., Dong, Y. H. and Wang, H. (2010) Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Science of The Total Environment* 408 (5), 1069-1075.

Zhao, Z. and Barron, A. R. (2016) *Dynamic Headspace Gas Chromatography Analysis.* CNX. https://cnx.org/contents/HwglYzq6@2/Dynamic-Headspace-Gas-Chromato Accessed on 01/03/2016.

Zhou, J., Li, Y., Zhao, J., Xue, X., Wu, L. and Chen, F. (2008) Geographical traceability of propolis by high-performance liquid-chromatography fingerprints. *Food Chemistry* 108 (2), 749-759.

Zhou, J., Xue, X., Li, Y., Zhang, J., Chen, F., Wu, L., Chen, L. and Zhao, J. (2009) Multiresidue determination of tetracycline antibiotics in propolis by using HPLC-UV detection with ultrasonic-assisted extraction and two-step solid phase extraction. *Food Chemistry* 115 (3), 1074-1080.

Zhu, H., Wang, Y., Liu, Y., Xia, Y. and Tang, T. (2010) Analysis of Flavonoids in Portulaca oleracea L. by UV–Vis Spectrophotometry with Comparative Study on Different Extraction Technologies. *Food Analytical Methods* 3 (2), 90-97.