T.C.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACOGNOSY

# DEVELOPMENT AND VALIDATION OF QUALITATIVE AND QUANTITATIVE HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHIC METHODS FOR ANALYSIS OF MARKER COMPOUNDS IN *MATRICARIA RECUTITA* L. AND *CYNARA SCOLYMUS* L. EXTRACTS

DOCTOR OF PHILOSOPHY THESIS

ETİL GÜZELMERİÇ, Pharm.

İstanbul-2015

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DOCTOR OF PHILOSOPHY THESIS

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SUPERVISOR
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İstanbul-2015

# THESIS APPROVAL

| | |
|---|---|
| Institute | : Yeditepe University Institute of Health Sciences |
| Programme | : Pharmacognosy |
| Title of the Thesis | : Development and Validation of Qualitative and Quantitative High Performance Thin-Layer Chromatographic Methods for Analysis of Marker Compounds in *Matricaria recutita* L. and *Cynara scolymus* L. Extracts |
| Owner of the Thesis | : Etil Güzelmeriç |
| Examination Date | : 15.06.2015 |

This study have approved as a Doctorate Thesis in regard to content and quality by the Jury.

| | | SIGNATURE |
|---|---|---|
| Chair of the Jury: (Supervisor) | Prof.Dr. Erdem Yeşilada<br>Yeditepe University, Faculty of Pharmacy, Dept. Pharmacognosy, Turkey | ..... |
| Co-supervisor: | Dr. Irena Vovk<br>National Institute of Chemistry, Laboratory for Food Chemistry, Slovenia | Irena Vovk<br>..... |
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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 18.06.2015 and numbered 2015/17-2

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

## DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

15.06.2015

Etil Güzelmeriç

**DEDICATION**

***I would like to deeply thank and dedicate this thesis to my family ...***

## ACKNOWLEDGEMENTS

Foremost, I would like to express my special appreciation to Prof.Dr. Erdem Yeşilada. His wisdom, wide knowledge and way of thinking have influenced and motivated me greatly.

I am deeply thankful to Dr. Irena Vovk for her valuable and constructive suggestions during this research and giving me the opportunity to work in National Institute of Chemistry, Laboratory for Food Chemistry in Slovenia.

I would like to acknowledge Prof.Dr. Hasan Kırmızıbekmez for his endless support and guidance all these years. I am deeply grateful to Prof.Dr. Galip Akaydın, Assist.Prof.Dr. Gizem Bulut and Dr. M. Ufuk Özbek for their contributions on plant authentication. I also thank Assist.Prof.Dr. Doğan Arslan for supplying cultivar *Matricaria recutita* L.

I am pleased to thank all my friends and colleagues who supported me in Yeditepe University, Department of Pharmacy (Turkey), National Institute of Chemistry, Laboratory for Food Chemistry (Slovenia), CAMAG Laboratory (Switzerland) and University of Belgrade, Faculty of Chemistry (Serbia) not only for sharing their knowledge and experiences but also friendship and all fun we have had during these years.

My deepest appreciation goes to my parents, grandparents and parents-in law for their unlimited love, support, encouragement and patience throughout my study. I would like to express my special thanks to my beloved husband, Barış Güzelmeriç.

Lastly, I would like to express my gratitude to The Scientific and Technological Research Council of Turkey (TUBITAK) for the scholarship provided during the Ph.D. program (2211-Yurtiçi Doktora Burs Programı).

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## ABBREVIATIONS

| | |
|---|---|
| 2D | 2-Dimensional |
| A7G | Apigenin 7-*O*-glucoside |
| ADAG | A1c-Derived Average Glucose |
| ADC | Automatic Development Chamber |
| AH | Axillary-Head |
| $Al_2O_3$ | Aluminum oxide |
| $Al(OH)_3$ | Aluminum hydroxide |
| ALP | Alkaline Phosphatase |
| ALT | Alanine Aminotransferase |
| AMD | Automated Multiple Development |
| AMPK | Adenosine Monophosphate Kinase |
| ANOVA | Analysis of Variance |
| AST | Aspartate Aminotransferase |
| cAMP | Cyclic Adenosine Monophosphate |
| CAT | Catalase |
| $CCl_4$ | Carbon Tetrachloride |
| CE | Capillary Electrophoresis |
| CEC | Capillary Electrochromatography |
| cGMP | Cyclic Guanosine Monophosphate |
| CH | Central Head |
| ChA | Chlorogenic Acid |
| COX | Cyclooxygenase |
| Crit | Critical value |
| D2 | Deuterium |
| DAD | Diodarray Detector |
| DC | Diene Conjugate |
| $DPPH^{\cdot}$ | 2,2-diphenyl-1-picrylhydrazyl radical |
| EDQM | European Directorate for the Quality of Medicines and Health Care |
| eNOS | Endothelial Nitric Oxide Synthase |
| F | Fluorescence indicator |
| FBG | Fasting Blood Glucose |
| FRAP | Ferric-Reducing Antioxidant Power |
| FU | Fluoracil |
| $GABA_a$ | γ-Aminobutyric Acid |
| GC | Gas Chromatography |
| GSH | Glutathione |
| GSH-Px | Gluthatione Peroxidase |
| GST | Glutathione Transferase |
| H | Height |
| $H_2O_2$ | Hydrogen peroxide |
| HAM-A | Hamilton Anxiety Rating |
| HDL | High Density Lipoprotein |
| HeLa | Human Cervical Carcinoma Cells |
| HETP | Height Equivalent to Theoretical Plate |
| Hg | Mercury Vapor |
| HOMA | Homeostatic Metabolic Assessment |

| | |
|---|---|
| HPLC | High-Performance Liquid Chromatography |
| HPTLC | High Performance Thin-Layer Chromatography |
| HRMS | High-Resolution Mass Spectrometry |
| HUVECs | Human Umbilical Vein Endothelial Cells |
| $IC_{50}$ | Half Maximal Inhibitory Concentration |
| ICH | International Conference on Harmonization |
| IFG | Impaired Fasting Glycaemia |
| IL | Interleukin |
| iNOS | Inducible Nitric Oxide Synthase |
| İTK | İnce-Tabaka Kromatografisi |
| IUPAC | International Union of Pure and Applied Chemistry |
| $k'$ | Capacity factor |
| L7G | Luteolin 7-*O*-glucoside |
| LDL | Low Density Lipoprotein |
| LOD | Limit of Detection |
| LOQ | Limit of Quantitation |
| LPS | Lipopolysaccharide |
| LSD | Least Significant Difference |
| MDA | Malondialdehyde |
| MEKC | Micellar Electrokinetic Capillary Chromatography |
| $MgCl_2.6H_2O$ | Magnesium chloride-6-hydrate |
| MIC | Minimum Inhibitory Concentration |
| MS | Mass Spectrometry |
| $MS^2$ | Tandem Mass Spectrometry |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MyD | Myeloid Differentiation Factor |
| N | Theoretical plate number |
| NF | Nuclear Factor |
| $NH_2$ | Amino |
| NO | Nitric Oxide |
| NP | Natural Products |
| NSAID | Nonsteroidal Anti-Inflammatory Drug |
| OPLC | Overpressured Layer Chromatography |
| PDE | Phosphodiesterase |
| PEG | Polyethylene Glycol |
| PG | Prostaglandin |
| Ph. Eur. | European Pharmacopoeia |
| $r$ | Correlation Coefficient |
| $R_F$ | Retardation Factor or Retention Factor |
| $R_s$ | Resolution |
| REM | Rapid Eye Movement |
| ROS | Reactive Oxygen Species |
| RP | Reversed Phase |
| RSD | Relative Standard Deviation |
| S/D | Signal to Noise |
| SD | Standard Deviation |
| SERS | Surface Enhanced Raman Spectroscopy |
| Si | Silica gel |

| | |
|---|---|
| $SiO_2.(H_2O)_n$ | Hydrated silicon dioxide |
| SOD | Superoxide Dismutase |
| $t_R$ | Retention Time |
| *t*-BHP | *tert*-butylhydroperoxide |
| TEA | Triethylamine |
| TFA | Trifluoroacetic Acid |
| TFEQ | Three Factor Eating Questionnaire |
| TG | Triglyceride |
| TLC | Thin-Layer Chromatography |
| TLR | Toll-Like Receptor |
| TNF | Tumor Necrosis Factor |
| TPA | 12-*O*-tetradecanoylphorbol-13-acetate |
| UPLC | Ultra-Performance Liquid Chromatography |
| UTLC | Ultra Thin-Layer Chromatography |
| UV | Ultra-Violet |
| Vis | Visible |
| W | Tungsten |
| YEF | Herbarium of the Faculty of Pharmacy in Yeditepe University |
| YPİTK | Yüksek Performanslı İnce Tabaka Kromatografisi |
| YPSK | Yüksek Performanslı Sıvı Kromatografisi |

## ABSTRACT

**Güzelmeriç, E. (2015). Development and Validation of Qualitative and Quantitative High Performance Thin-Layer Chromatographic Methods for Analysis of Marker Compounds in *Matricaria recutita* L. and *Cynara scolymus* L. Extracts. Yeditepe University, Institute of Health Sciences, Department of Pharmacognosy, Ph.D. Thesis, İstanbul.**

TLC methods have long been used in the pharmacopoeias such as European Pharmacopoeia for qualitative analysis of marker compounds in plant materials. Recent advances led to the utilization of HPTLC methods which enable both qualitative and quantitative analyses concurrently. However, there has not yet stated an HPTLC method in the European Pharmacopoeia. Therefore, HPTLC methods for qualitative and quantitative analysis of marker compounds in *Matricaria recutita* L. and *Cynara scolymus* L. (Asteraceae) extracts were developed and validated in this study. In *M. recutita* flower extract, apigenin 7-*O*-glucoside was separated from the other constituents on silica gel 60 $NH_2$ $F_{254}$s HPTLC plate using a developing solvent system of ethyl acetate-formic acid-acetic acid-water (30:1.5:1.5:3, v/v/v/v). The developed method was subsequently validated and applied both for qualitative and quantitative analyses of apigenin 7-*O*-glucoside in extracts of *M. recutita, M. recutita*-like flowers and numerous commercial *M. recutita* products. According to *C. scolymus* leaf extract, chlorogenic acid and luteolin 7-*O*-glucoside were separated on silica gel 60 $F_{254}$ HPTLC plate using a developing solvent system of ethyl acetate-formic acid-acetic acid-water (35:2:2:5, v/v/v/v). Moreover, HPTLC-bioautography experiment with $DPPH^{\cdot}$ radical was performed for *C. scolymus* leaf extract and the result revealed that chlorogenic acid and cynarin significantly contributed to antioxidant activity of *C. scolymus* leaf extract. However, due to decomposition of phenolic compounds on the plate during the chromatography, an alternative HPLC technique was used to quantify chlorogenic acid and cynarin contents. Consequently, both HPTLC and HPLC methods were used to analyze extracts of *C. scolymus* leaf, bract, receptacle, stem and *C. scolymus* food supplements sold on market.



**Key words:** High Performance Thin-Layer Chromatography (HPTLC), High-Performance Liquid Chromatography (HPLC), *Matricaria recutita* L., *Cynara scolymus* L., Asteraceae

# ÖZET

**Güzelmeriç, E. (2015). *Matricaria recutita* L. ve *Cynara scolymus* L. Ekstrelerinde İşaretleyici Bileşenlerin Analizi için Kalitatif ve Kantitatif Yüksek Performanslı İnce-Tabaka Kromatografi Yöntemleri Geliştirilmesi ve Validasyonu. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmakognozi ABD., Doktora Tezi, İstanbul.**

İTK yöntemleri, Avrupa Farmakopesi gibi farmakopelerde bitki materyallerindeki işaretleyici bileşenlerin kalitatif analizi için uzun yıllardır kullanılmaktadır. Son zamandaki gelişmeler, aynı anda hem kalitatif hem de kantitatif analiz sağlayan YPİTK yöntemlerinin kullanımını olanak sağlamıştır. Ancak, Avrupa Farmakopesi' nde henüz bir YPİTK yöntemi belirtilmemiştir. Dolayısıyla, bu çalışmada *Matricaria recutita* L. ve *Cynara scolymus* L. (Asteraceae) ekstrelerindeki işaretleyici bileşenlerin kalitatif ve kantitatif analizi için YPİTK yöntemleri geliştirilmiş ve valide edilmiştir. *M. recutita* çiçek ekstresindeki apigenin 7-*O*-glukozit diğer bileşenlerden, silika jel 60 $NH_2$ $F_{254}$s YPİTK plaka üzerinde yürütülen etil asetat-formik asit-asetik asit-su (30:1.5:1.5:3, v/v/v/v) solvan sistemiyle ayrıldı. Geliştirilen yöntem daha sonra valide edilerek, *M. recutita*, *M. recutita*-benzeri çiçekler ve bir çok ticari *M. recutita* ürünlerin ekstrelerinde apigenin 7-*O*-glukozitin hem kalitatif hem de kantitatif analizlerinde uygulandı. *C. scolymus* yaprak ekstresine göre, klorojenik asit ve luteolin 7-*O*-glukozit silika jel 60 $F_{254}$ YPİTK plaka üzerinde yürütülen etil asetat-formik asit-asetik asit-su (35:2:2:5, v/v/v/v) solvan sistemiyle ayrıldı. Ayrıca, *C. scolymus* yaprak ekstresi için $DPPH^{\cdot}$ radikaliyle YPİTK-biyootografi deneyi gerçekleştirildi ve sonucunda klorojenik asit ve sinarinin *C. scolymus*' un yaprak ekstresinin antioksidan etkisine önemli katkı sağladığı ortaya kondu. Ancak, kromatografi sırasında fenolik bileşenlerin plak üzerinde bozunmasından dolayı klorojenik asit ve sinarinin madde miktarı alternatif bir teknik olan YPSK ile hesaplandı. Sonuçta, hem YPİTK hem de YPSK yöntemleri *C. scolymus* yaprak, brakte, reseptakulum, sap ve piyasada satılan *C. scolymus* gıda takviyelerinin ekstrelerini analiz etmede kullanıldı.



**Anahtar kelimeler:** Yüksek Performanslı İnce-Tabaka Kromatografisi (YPİTK), Yüksek-Performanslı Sıvı Kromatografisi (YPSK), *Matricaria recutita* L., *Cynara scolymus* L., Asteraceae

## 1. INTRODUCTION and AIM

Thin-layer chromatography (TLC) is one of the mostly used analytical techniques which is performed on a solid support covered with a thin-layer of adsorbent material using a developing solvent system (1). The attractive features of TLC are its simplicity, flexibility, rapidity and cost efficiency. Also, it enables simultaneous evaluation of many samples on one plate, post-chromatographic derivatization and visual results (2). So far various innovations particularly on stationary phase led to the utilization of high performance thin-layer chromatography (HPTLC). Moreover, recent advances both in instrumentation and automation provides convenience for qualitative and quantitative conclusions in HPTLC methods (3).

TLC methods have long been stated in the pharmacopoeias such as European Pharmacopoeia (Ph. Eur.), Chinese Pharmacopoeia, United States Pharmacopoeia and Indian Herbal Pharmacopoeia mostly for analysis of marker compounds in plant materials (4). Recently, conventional TLC methods have been started to be replaced by HPTLC methods in the American Herbal Pharmacopoeia (5). However, in the Ph. Eur. which is officially approved in Turkey, no HPTLC method has referred yet. In light of the foregoing, development and validation of HPTLC methods were planned in this study for two popular medicinal plants presented in the Ph. Eur., *Matricaria recutita* L. and *Cynara scolymus* L. (Asteraceae).

Bioassay-guided processing of *M. recutita* flower extracts has revealed that many of its healing benefits such as relieving painful gastrointestinal complaints, mild sleep disorders and inflammatory diseases are highly related with its phenolic content, in particular to apigenin and apigenin 7-*O*-glucoside (A7G) (6). On the other hand, major problem is difficulty in distinguishing the genuine specimen when supplying *M. recutita* through nature-picking. Consequently flowers of other Asteraceae members resembling to *M. recutita* in appearance such as *Anthemis* spp., *Bellis* spp., *Chrysanthemum* spp. and *Tanacetum* spp. may also be gathered from nature by lay people to be used as a home remedy or for marketing in spice shops or bazaars. In the present study, we first aimed to develop and validate an HPTLC method for identification and quantification of A7G in cultivar *M. recutita* flower extract. Secondly, the validated HPTLC method was applied for analyzing A7G content in

extracts of wild *M. recutita* flowers; several morphologically similar species named as '*M. recutita*-like materials' and also commercial *M. recutita* products. The method was further interpreted for discriminating the genuine specimen, *M. recutita*, from other Asteraceae species and also adulterated products sold as *M. recutita* on market.

Many studies on *C. scolymus* have been conducted in recent years to establish its pharmacological properties such as hepatoprotective, cholagogue, antihyperlipidemic, anticarcinogenic and antioxidant effects (7). Further investigations based on activity-guided isolation revealed that especially caffeoylquinic acid derivatives such as caffeic acid, chlorogenic acid (ChA), cynarin and flavonoid fraction including mostly luteolin 7-*O*-glucoside (L7G) are responsible for most of its therapeutic activities (8). In the present study, a preliminary HPTLC-bioautography assay with 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) radical was performed for *C. scolymus* leaf extract to screen its antioxidant components. Moreover, the separation of rutin, cynarin and caffeic acid was also aimed in addition to stated marker components in the Ph. Eur. including ChA and L7G. However, due to decomposition of *C. scolymus* components on plate the during chromatography, high-performance liquid chromatography (HPLC) was used as an alternative technique to quantify ChA and cynarin contents which were significantly contribute to antioxidant activity of *C. scolymus* leaf extract. Eventually both HPTLC and HPLC were used to analyze extracts of *C. scolymus* leaf, outer, intermediate and inner bracts, receptacle and stem collected in different developmental stages and also *C. scolymus* food supplements sold on market.

## 2. GENERAL DESCRIPTION

### 2.1. Botanical Chapter

#### 2.1.1. Asteraceae (Compositae)

The order Asterales contains 11 families including Asteraceae which is one of the largest one. Asteraceae comprises over 1500 important genera and approximately 25,000 species which are distributed throughout the world. The Latin name 'Asteraceae' is derived from the type genus *Aster*, which is a Greek term that means 'star' (9).

The family Asteraceae comprises annual, biennial and perennial plants. The leaves of these herbs are mostly alternate or may be opposite, exstipulate, entire, toothed, lobed or variously dissected. Individual flowers are countless, sessile and aggregated into a capitulum covered by a protective involucre of 1-many series of phyllaries which are scarcely fused. Besides, capitula may be aggregated into a secondary capitulum-like head. Receptacle is mostly naked or having paleae with long hairs or bristles. Flowers are epigynous, either all hermaphrodite and protandrous, or female, male or neuter. Calyx is presented on the apex of ovary by a pappus of hairs, bristles, scales or awns or by a continuous corona and generally pappus may be absent. Corolla is gamopetalous, tubular, filiform, ligulate or sometimes bilabiate, particularly 3- or 5-toothed. In general, flowers have 4 or 5 stamens which are epipetalous and their filaments are commonly free and anthers are laterally fused into a cylinder round the style, rarely free or dehiscence introrse. Ovary is inferior and 1-celled with 1 basal anatropous ovule. Style is divided above into 2 branches. Additionally, disc flower styles often bear collecting hairs that lick the pollen from the anther cylinder. Moreover, the characteristic fruit type of the family is achene, commonly having a persistent or deciduous pappus (10).

The dichotomous key in the 'Flora of Turkey and the East Aegean Islands' is presented as follows (10):

*"1. Flowers all ligulate, ligules 5-toothed; plants with latex*

*Group* ***A***

*1. Flowers not all ligulate, at least central ones tubular; plants without latex (except for Gundelia in Group B)*

*2. Leaves and/or phyllaries spiny, i. e. pricking (spines rarely hooked)*

*Group **B***

*2. Neither leaves nor phyllaries spiny*

*3. Capitula radiate, marginal flowers usually with 3- (rarely 5- or more) toothed ligules obviously longer than disc flowers*

*4. Ligules bright yellow to orange*

*Group **C***

*4. Ligules white, cream, pink, red, purple or blue*

*Group **D***

*3. Capitula discoid or disciform, marginal flowers tubular sometimes elongated, widened and radiant but usually ± cylindrical, or minutely ligulate and shorter than disc flowers*

*5. Receptacle paleaceous or long-hairy (hairs at least 1/3 as long as corolla)*

*Group **E***

*5. Receptacle naked or at most with margins of alveolae (pits) ciliate or toothed*

*Group **F**"*

(Davis, 1975: 8-10)

*"Group **D***

*Capitula radiate; ligules white, pink, red or purple; plants not spiny*

*1. Receptacle paleaceous (weakly so in Uechtritzia), paleae ovate or oblong or narrower, bristle-like and present at least on inner part of receptacle*

*2. Herbs scapigerous; leaves ovate, cordate, all basal; flowers carmine, inner ones bilabiate, 1-5 cm or more long*

***94. Uechtritzia***

*2. Herbs with leafy stems; leaves variously shaped but not ovate, cordate; ray flowers white, pink or purple, disc flowers usually yellow, tubular, or, if slightly bilabiate, not more than 6 mm long*

*3. Leaves all opposite, simple, ± entire; paleae narrow, bristle-like, deciduous; annuals of marshy ground*

***3. Eclipta***

*3. Leaves alternate, usually 1-3 pinnatisect, rarely simple; paleae ovate or oblong; annuals and perennials*

*4. Achenes dorsally compressed with 2 marginal wings or distinct ribs*

*5. Outer disc achenes (at least) with scarious or hyaline wings*

*6. Base of disc corolla tube divided into 2 lobes which clasp top of achenes; leaves 2-3-pinnatisect, linear in outline, ± vermiform, segments closely conferted*

***45. Leucocyclus***

*6. Base of disc corolla tube not divided into lobes, attached to anterior side of achene; leaves 2-pinnatisect, oblong-ovate in outline, segments not conferted*

***44. Anacyclus***

*5. Outer disc or all achenes with distinct marginal ribs but not wings*

*7. Bases of disc corolla tubes pouched and enveloping tops of achenes; achenes ± compresssed, rounded, ecoronate at apex; midrib of paleae not reaching apex*

***46. Achillea***

*7. Bases of disc corolla tubes not pouched at the base; apex of achene bearing a short corona; midrib of paleae excurrent at apex into a mucro or acumen*

***42. Anthemis***

*4. Achenes terete, angular or somewhat compressed without distinct marginal ribs or wings*

*8. Disc corolla tubes prolonged at base into a spur on posterior side, partially enveloping the laterally compressed, lineolate, ecoronate achene*

***43. Chamaemelum***

*8. Disc corolla tubes not as above; achenes terete, angular or slightly compressed, usually with a rim or minute corona at apex, at least on posterior side*

***42. Anthemis***

*1. Receptacle naked*

*9. Pappus present, consisting (in par at least) of hairs*

*10. Ligules small, scarcely exserted from involucre*

*11. Pappus of 4 hairs and 4 smaller scales; leaves all radical, rosulate; annuals*

***32. Bellium***

*11. Pappus entirely of hairs; leaves not all radical; annuals and perennials*

*12. Leaves mostly cauline; capitula numerous in panicles; annuals usually more than 30 cm tall*

***33. Conyza***

*12. Leaves mostly basal; capitula solitary or few; perennials less than 20 cm tall*

***31. Psychrogeton***

*10. Ligules conspicuous, distictly exserted from involucre; biennial or perennial herbs, rarely annuals*

*13. Ray flowers neuter, without styles*

***27. Galatella***

*13. Ray flowers fertile, styles present*

*14. Indumentum strongly glandular and viscid with numerous glands; biennials; achenes abruptly contracted below pappus and almost beaked*

***30. Lachnophyllum***

*14. Indumentum sometimes glandular but not viscid; perennials; achenes not as above*

*15. Ray flowers 1-seriate, ligules commonly more than 1 mm broad; phyllaries foliaceous or membranous, relatively broad*

***26. Aster***

*15. Ray flowers 2-3-seriate, ligules usually less than 1 mm broad; phyllaries herbaceous, relatively narrow*

***29. Erigeron***

*9. Pappus absent, or coronate, auriculate or paleaceous*

*16. Achenes dimorphic; those of ray flowers triquetrous or 3-winged, those of disc laterally compressed*

***49. Chrysanthemum***

*16. Achenes all alike although those of rays sometimes coronate but those of disc ± ecoronate*

*17. Achenes laterally compressed with 2 distinct marginal ribs; low-growing annuals or perennials; leaves usually rosulate*

***35. Bellis***

*17. Achenes antero-poteriorly compressed or ± terete; ribs more than 2; leaves mostly cauline*

*18. Receptacle becoming hemispherical or conical at maturity; achenes unequally ribbed*

*19. Achenes strongly 3-ribbed posteriorly and bearing 1-2 reddish-brown glands near apex on anterior side; annual, biennial or perennial herbs*

***54. Tripleurospermum***

*19. Achenes weakly 3-5-ribbed on posterior surface without reddish-brown glands anteriorly; always annual*

***53. Matricaria****"*

…

(Davis, 1975: 19-20)

*"Group **E***

*Capitula discoid or disciform; receptacle paleaceous or long-hairy; plants not spiny*

*1. Capitula unisexual; male ones drooping, arranged in spike-like inflorescences; female capitula solitary, axillary*

***5. Ambrosia***

*1. Capitula bisexual*

*2. Capitula small, 5-6 mm long, sometimes aggregated into compound heads; corollas minute, 2-3 mm long, inconspicuous*

*3. Leaves entire; annuals*

*4. Achenes and female corollas ± completely enclosed within paleae and always dispersed within them*

*5. Paleae membranous and scarcely toughened at maturity, ± ovate, apex of female corolla emerging subterminally; pappus hairs present, few*

***23. Cymbolaena***

*5. Paleae obviously toughened and hardened at maturity, galeiform (hooded), apex of female corolla emerging ± laterally; pappus absent*

*6. Leaves opposite; capitula solitary, axillary; paleae felted, spinosely crested*

***24. Micropus***

*6. Leaves alternate; capitula in clusters of 2-3, terminal or axillary; paleae very densely lanate, not crested*

***22. Bombycilaena***

*4. Achenes free within concave paleae or achenes enfolded within plicate paleae and sometimes dispersed with them; female corollas free or enfolded within paleae*

*7. Pappus absent from all achenes; acaulescent or procumbent plants, leaves ± rosetted*

*7. Pappus present at least on achenes of of hermaphrodite and inner female flowers; plants caulescent, usually ascending or erect*

*8. Hairs of pappus plumose at apices; capitula in clusters of 2-5 in leaf axils along stems and branches*

***18. Ifloga***

*8. Hairs of pappus capillaceous throughout; capitula in rounded clusters of up to 60, terminal or axillary*

*9. Paleae spoon-shaped, broadened and usually somewhat saccate at base, attenuate but not strongly acuminate-aristate at apex, stellately patent in fruit*

***20. Logfia***

*9. Paleae concave or navicular (boat-shaped), not saccate at base, acute or acuminate-aristate at apex, remaining ± erect in fruit*

***19. Filago***

*3. Leaves divided; perennial herbs, subshrubs or rarely shrubs, aromatic*

***55. Artemisia***

*2. Capitula larger, commonly 10 mm or more long, not usually aggregated into compound heads; corollas usually more than 3-5 mm long, conspicuous*

*10. Flowers in axils pf paleae or stiff bristles (not more than number of flowers); pappus never hairy*

*11. Leaves opposite; pappus of 2-4 retrorsely barbed aristae; herbs of marshy ground*

***4. Bidens***

*11. Leaves alternate; pappus otherwise*

*12. Base of corolla tube downwardly produced into spongy appendages on either side of the achene and persistently enclosing it (densely white-tomentose maritime perennials)*

***48. Otanthus***

*12. Base of corolla tube otherwise*

*13. Leaves ± vermiform, pinnatisect, segments usually closely conferted; apex of achene rounded, enclosed by pouched base of corolla tube*

***47. Santolina***

*13. Leaves not vermiform entire or pinnately divided*

*14. Acaulescent herbs with entire ± rosetted leaves*

***85. Amphoricarpos***

*14. Caulescent herbs, leaves not rosetted*

*15. Style branches linear, rounded at apex; leaves entire-margined*

***10. Chrysophthalmum***

*15. Style branches flattened, truncate at apex; leaves 1-2-pinnately divided*

***42. Anthemis***

*10. Flowers surrounded at base by numerous receptacular bristles or hairs; pappus, if present, mostly hairy*

*16. Capitula heterogamous, marginal flowers neuter or rarely female, radiant or not (sometimes inconspicuous but always recognizable by absence of anther tube)*

*17. Annuals or biennials*

*18. Pappus of 5-15 lanceolate scales; leaves entire*

*19. Inner phyllaries much longer than median ones, scarious and coloured above; pappus of 5-15 short scales included within involucre*

***89. Xeranthemum***

*19. Inner phyllaries not much longer than median ones; pappus of central flowers of c. 10 whitish scales becoming exserted from involucre (and superficially appearing to be innermost phyllaries)*

***91. Chardinia***

*18. Pappus of numerous linear scales, bristles or hairs, or absent; leaves entire or divided*

*20. Phyllaries with a conspicuous appendage; achenes ± glabrous at maturity or sometimes with a few hairs around hilum; hilum lateral without any whitish lip-like protrusions at base*

*21. Innermost pappus of 3-5 bristles longer than the rest*

***76. Callicephalus***

*21. Innermost pappus shorter or not differentiated or pappus absent*

***79. Centaurea***

*20. Phyllaries without a conspicuous appendage (sometimes with a hyaline margin); achenes velutinous or pubescent at maturity, hilum basal or, if lateral, with a thick whitish lip-like protrusion at base*

*22. Pappus consisting entirely of scales, innermost not differentiated; flowers pink, marginal ones strongly radiant*

***74. Amberboa***

*22. Pappus mostly of bristles, innermost series consisting of 5-10 short blackish triangular scales; flowers purplish, marginal ones not strongly radiant*

***81. Crupina***

*17. Perennial herbs*

*23. Innermost pappus of one (rarely more) bristle(s), stouter but scarcely longer than the rest; phyllaries unappendaged, blackish-tipped*

***77. Mantisalca***

*23. Innermost pappus shorter or not differentiated; phyllaries appendaged or, if unappendaged, not blackish-tipped*

***79. Centaurea***

*16. Capitula homogamous or rarely heterogamous with functionally male marginal flowers*

*24. Pappus absent; surface of achenes somewhat wrinkled*

***83. Carthamus***

*24. Pappus present; surface of achene ± smooth*

*25. Phyllaries densely covered with yellowish tomentum*

***79. Centaurea***

*25. Phyllaries not as above*

*26. Appendages of phyllaries distinctly broader than their basal parts*

*27. Herbs up to 40 cm tall with far-creeping rootstocks; phyllaries with scarious triangular appendages*

***75. Acroptilon***

*27. Rhizomatous herbs 45-130 cm tall; phyllaries with concave spoon-shaped appendages*

***73. Rhaponticum***

*26. Appendages of phyllaries, if present, not as above*

*28. Pappus plumose*

*29. Involucre usually more than 5 cm broad; phylllaries 1-1.5 cm broad at base, obtuse (cultivated)*

***57. Cynara****"*

…

(Davis, 1975: 20-23)

### 2.1.2. *Matricaria* L.

The genus *Matricaria* contains annual herbs. Stems are erect or ascending, particularly branched and leafy. Leaves are 2-3-pinnatisect and ultimate segments are narrow. Capitula is solitary at branch ends or sometimes corymbose, radiate and heterogamous or discoid and homogamous. Phyllaries are imbricate, 2-3-seriate and scarious-margined. Receptacle is naked and conical at maturity. The ray flowers are white whereas disc flowers are yellow. Achenes are 3-10-ribbed and these ribs are more strongly developed on posterior surface. Pappus could be absent or coronate (10).

Dichotomous key for *Matricaria* species in the 'Flora of Turkey and the East Aegean Islands' is presented as follows (10):

*"1. Capitula radiate*
*2. Glabrous herbs; achenes c. 0.75 mm*

***1. chamomilla***

*2. Pubescent herbs: achenes c. 1.5-2 mm*

***2. macrotis***

*1. Capitula discoid*

***3. aurea****"*

(Davis, 1975: 293)

### 2.1.2.1. *Matricaria recutita* L.

**Synonyms:** *Matricaria chamomilla* L.; *Chamomilla recutita* (L.) Rauschert; *Matricaria suaveolens* L.; *Chamomilla vulgaris* Gray (11).

The height of glabrous stems of *M. recutita* could be 10-45 cm. The lower leaves are 5-7 cm, glabrous and oblong in outline. Besides, primary segments are 10-12-paired. In general, capitula is solitary and sometimes subcorymbose. At the beginning, involucre is 5-6 mm broad, later it reaches up to 8 mm. The phyllaries are oblanceolate, obtuse or acute, 2.5-3.5 mm. Moreover, ray flowers may be 12-15 whereas ligules 4-8 mm, patent at first and then become reflexed. Disc flowers are 1.25-1.5 mm. Achenes are brown, 0.75 mm with 5 whitish ribs on posterior surface (10).

**Flowering season:** March-April
**Habitat:** Roadsides, waste and cultivated ground
**Altitute:** -900 m (10).

**Names in different languages:** Papatya, mayıs papatyası, tıbbi papatya, adi papatya, babunç-Turkish (12); german chamomile, true chamomile, wild camomile-English; kamilica-Slovenian; echte kamille, deutsche kamille, gemeine kamille-German; camomille, matricaire, camomilla commune, camomille vulgaire-French; camomilla, camomilla comune-Italian (13).

### 2.1.3. *Cynara* L.

The genus *Cynara* is composed of perennial plants. Stems are stout, erect, ribbed, sparingly branched, spiny-winged or may be absent. Leaves are alternate, mostly pinnatisectly divided with winged rachis, spiny or unarmed. Capitula is large, solitary, homogamous and discoid, borne in 1- to few-headed corymbs. Involucre is ovoid or globose; phyllaries are pluriseriate, coriaceous and appendaged without prominent median nerve, spinose or not. Moreover, receptacle is thick, flat and densely covered with long white hairs. Flowers are violet-blue or whitish. Corolla is zygomorphic. Achenes are compressed-ovoid, angular, finely ribbed, smooth and mottled, apex mostly truncate. Pappus is pluriseriate and long stout plumose hairs are fused at base into a deciduous ring (10).

Dichotomous key for *Cynara* species in the 'Flora of Turkey and the East Aegean Islands' is stated as follows (10):

> *"1. Stems 10-20 cm, shorter than basal leaves; leaf spines short (2-6 mm); flowers dirty white*
>
> ***4. cornigera***
>
> *1. Stems much taller, longer than basal leaves; leaf spines long or absent; flowers violet-blue*
>
> *2. Leafless part of stem below capitulum long (12-35 cm); leaves with a single spine at base of each segment*
>
> ***1. syriaca***
>
> *2. Leafless part of stem below capitulum short (1-4); leaves unarmed or with a cluster of spines at base of each segment*
>
> *3. Leaves with a cluster of long spines at base of each segment; phyllaries long-spined*
>
> ***2. cardunculus***
>
> *3. Leaves unarmed, often with mucronate teeth; phyllaries ovate, obtuse or with emarginate spinose-mucronate apex*
>
> ***3. scolymus****"*
>
> (Davis, 1975: 327)

### 2.1.3.1. *Cynara scolymus* L.

**Synonyms:** *Cynara cardunculus* L. var. *sativa* Moris; *Cynara cardunculus* var. *scolymus* (L.) Fiori in Fiori & Paol (10).

The height of *C. scolymus* may reach up to 2 m. Unarmed or mucronulate leaves grow up to 40 x 15 cm. Besides, they are rarely arachnoid below; lower pinnatisect, with ovate, coarsely lobed-dentate segments; upper simple, ovate-lanceolate and irregularly dentate. Capitula is compressed-globose, 7 x 11 cm. Moreover, phyllaries are with adpressed base and cucullate ovate apical appendage 5 x 3 cm, obtuse or emarginate and spinosa-mucronate. Pappus could be 3-4 cm (10).

**Flowering season:** July

**Habitat:** Widely cultivated (10).

**Names in different languages:** Enginar-Turkish (14); artichoke, globe artichoke, artichoke thistle, cardoon, cardoon artichoke-English; artičoka-Slovenian; artischocke-German; artichaut-French; articiocco, carciofo-Italian (7).

## 2.2. Theoretical Chapter

### 2.2.1. Literature Review on *Matricaria recutita*

#### 2.2.1.1. Phytochemical Studies

Several secondary metabolites including both volatile and non-volatile constituents have been identified in *M. recutita* up to now. A general chemical classification of the secondary metabolites of *M. recutita* is briefly summarized in **Figure 1** (13).

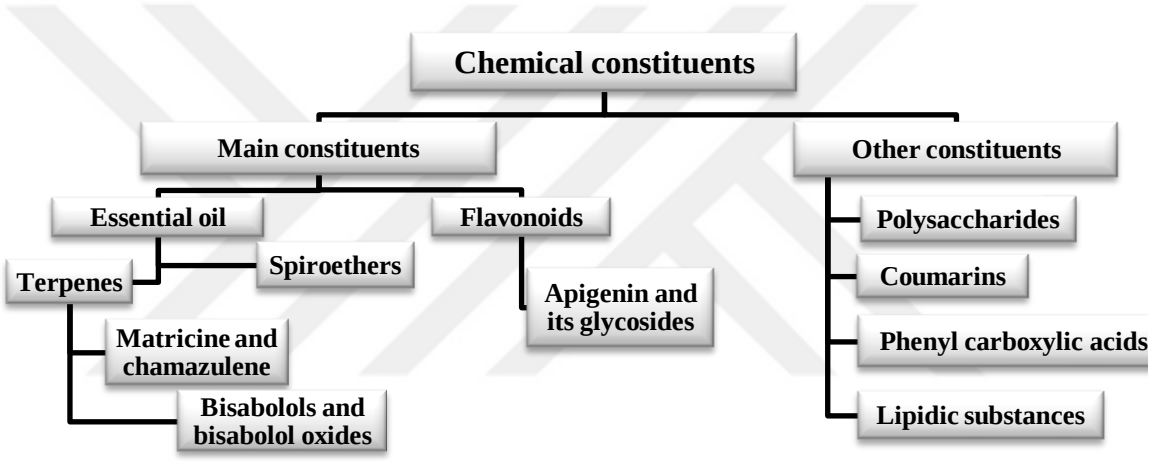



**Figure 1.** Classification of *M. recutita* secondary metabolites (13)

#### 2.2.1.1.1. Main Constituents

##### 2.2.1.1.1.1. Flavonoids

Flavonoids and their glycosides particularly found in *M. recutita* flowers may be grouped into five flavone types according to the increasing polarity of the compounds (13). These are as follows:

I. Methoxylated flavones
II. Hydroxylated flavones
III. Acetylated flavone monoglycosides
IV. Flavone monoglycosides
V. Flavone diglycosides

In **Table 1**, mostly encountered flavonoids and their glycosides in *M. recutita* are summarized.

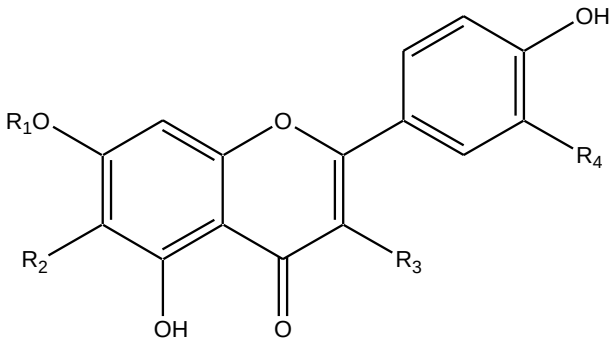



**Table 1.** Flavonoids identified in *M. recutita*

| **Compound** | **Group** | **$R_1$** | **$R_2$** | **$R_3$** | **$R_4$** | **Ref** |
|---|---|---|---|---|---|---|
| Axillarin | I | H | OMe | OMe | OH | (15) |
| Chrysoeriol | I | H | H | H | OMe | (15) |
| Chrysoplenol | I | Me | OMe | OMe | OH | (15) |
| Chrysoplenetin | I | Me | OMe | OMe | OMe | (15) |
| Eupaletin | I | Me | OMe | OH | H | (15) |
| Eupatoletin | I | Me | OMe | OH | OH | (15) |
| Isorhamnetin | I | H | H | OH | OMe | (15,16) |
| Jaceidin | I | H | OMe | OMe | OMe | (15) |
| Patuletin | I | H | OMe | OH | OH | (15) |
| Spinacetin | I | H | OMe | OH | OMe | (15) |
| Apigenin | II | H | H | H | H | (16–20) |
| Kaempferol | II | H | H | OH | H | (15) |
| Luteolin | II | H | H | H | OH | (15–17,20) |
| Quercetin | II | H | H | OH | OH | (15,16,21) |
| Isorhamnetin 7-*O*-glucoside | IV | Glc | H | OH | OMe | (20) |
| Quercetin 7-*O*-glucoside | IV | Glc | H | OH | OH | (17) |
| Quercetin 3-*O*-galactoside | IV | H | H | OGal | OH | (17) |
| Quercetin 3-*O*-rutinoside (Rutin) | V | H | H | ORut | OH | (15,16) |

Gal: Galactose; Glc: Glucose; Me: Methyl; OMe: Methoxy; Rut: Rutinose

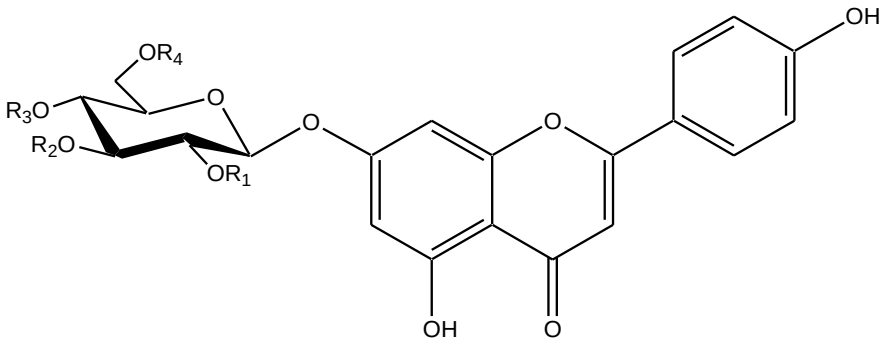



**Table 1.** Continued.

| Compound | Group | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Ref |
|---|---|---|---|---|---|---|
| Apigenin 7-(2''-*O*-acetyl)-glucoside | III | Ac | H | H | H | (22) |
| Apigenin 7-(4''-*O*-acetyl)-glucoside | III | H | H | Ac | H | (20,23) |
| Apigenin 7-(4'' -acetyl, 6''-malonyl)-glucoside | III | H | H | Ac | Mal | (23) |
| Apigenin 7-(6''-*O*-acetyl)-glucoside | III | H | H | H | Ac | (17,19,20) |
| Apigenin 7-(2'',3''-*O*-diacetyl)-glucoside | III | Ac | Ac | H | H | (23,24) |
| Apigenin 7-(3'',4''-*O*-diacetyl)-glucoside | III | H | Ac | Ac | H | (23,24) |
| Apigenin 7-(4'',6''-*O*-diacetyl)-glucoside | III | H | H | Ac | Ac | (23) |

Ac: Acetyl; Mal: Malonyl

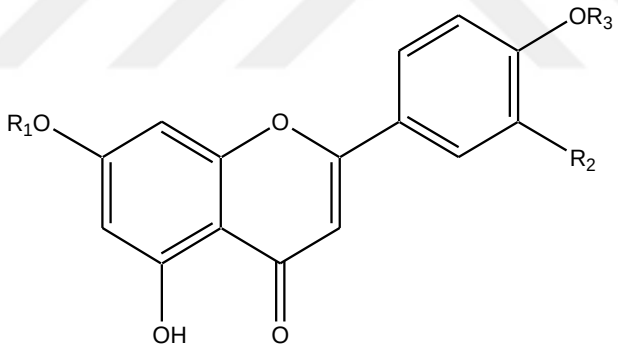



**Table 1.** Continued.

| Compound | Group | $R_1$ | $R_2$ | $R_3$ | Ref |
|---|---|---|---|---|---|
| Apigenin 7-*O*-glucoside (Apigetrin) | IV | Glc | H | H | (15,17,19,20) |
| Apigenin 7-(6''-*O*-apiosyl)-glucoside | IV | Glc-Apio | H | H | (13) |
| Apigenin 7-(6''-*O*-caffeoyl)-glucoside | IV | Glc-Caff | H | H | (23) |
| Apigenin 7-(6''-*O*-malonyl)-glucoside | IV | Glc-Mal | H | H | (23) |
| Luteolin 7-*O*-glucoside | IV | Glc | OH | H | (13) |
| Luteolin 4'-*O*-glucoside | IV | H | OH | Glc | (17) |
| Apigenin 7-*O*-rutinoside | V | Rut | H | H | (13) |
| Luteolin 7-*O*-rutinoside | V | Rut | OH | H | (17) |

Apio: Apiose; Caff: Caffeoyl; Glc: Glucose; Mal: Malonyl; Rut: Rutinose

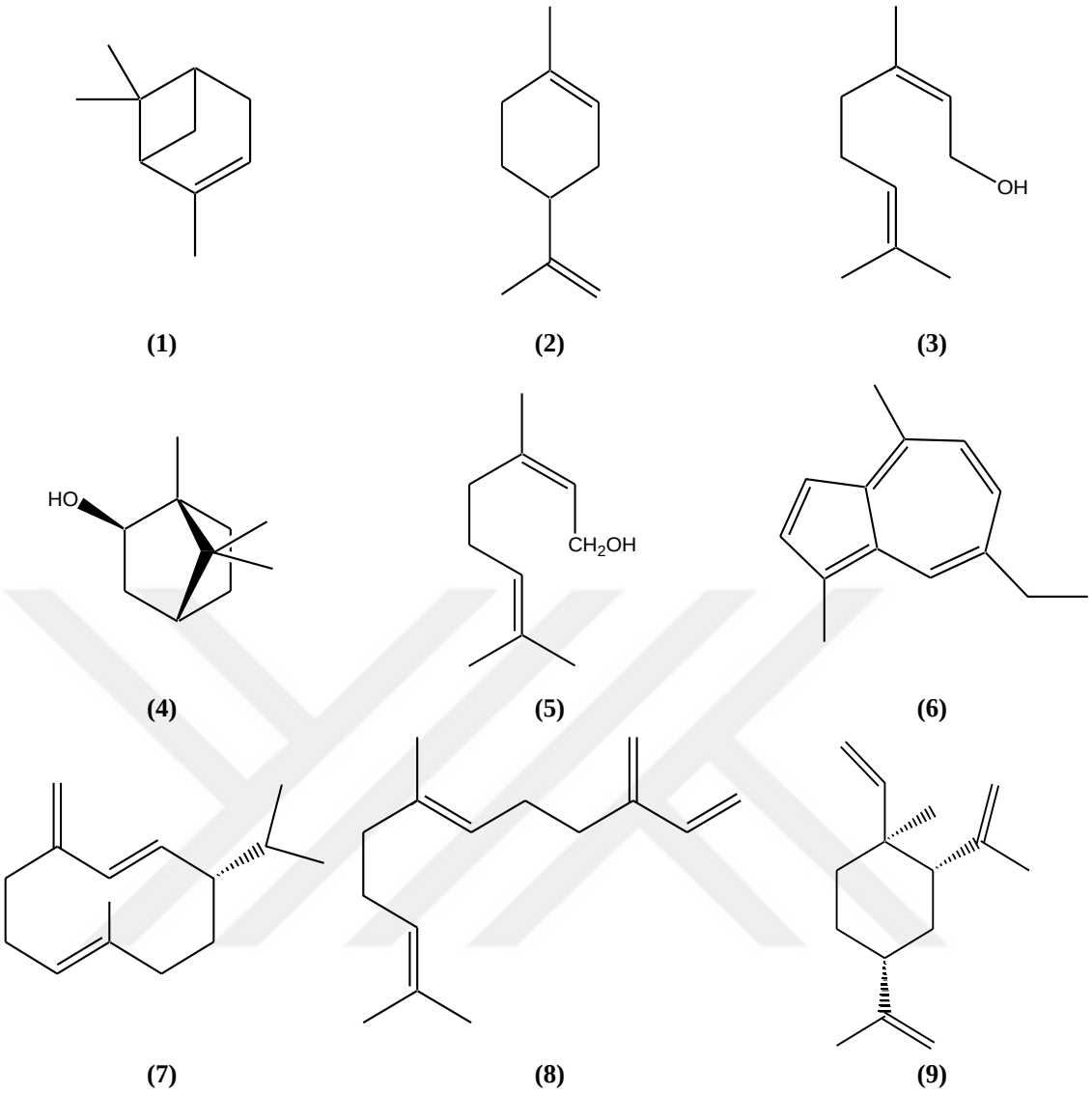
#### 2.2.1.1.1.2. Essential Oils

Blue colored *M. recutita* essential oil is commonly produced from its flower heads by steam distillation. It mainly consists of terpene derivatives (~ 75-90%, mostly sesquiterpenes) and spiroethers (~ 25%) and its amount reaches maximum value just prior to full blooming (13).

Among its main constituents, levomenol, commonly known as (-)-α-bisabolol is a pale yellow liquid. It tends to oxidize into (-)-α-bisabolol oxide A, (-)-α-bisabolol oxide B, (-)-α-bisabolol oxide C and also (-)-α-bisabolone oxide A. Besides, pale yellow colored matricine is very unstable compound. It decomposes to chamazulene carboxylic acid by elimination of water and acetic acid during distillation procedures. Then, decarboxylation follows the process and blue colored sesquiterpene named as chamazulene occurs. It has five conjugated double bonds which are responsible for its characteristic color, ranging from blue to green. In addition, spiroethers in freshly distilled essential oil exist in two isomeric forms, *trans*- and *cis*-en-yn-dicycloether which is the major compound (25).

The approximate percentage ratios of the main components in *M. recutita* essential oil are as follows: α-bisabolol up to ~ 75%, α-bisabolol oxide A up to ~ 50% and α-bisabolol B up to ~ 55%, bisabolone oxide A up to ~ 25%, chamazulene up to ~20%, spiroethers up to ~18%, (*E*)-β-farnesene up to ~ 6% (13,25,26). On the other hand, chemical composition and relative abundance of the compounds in essential oil has been found to differ depending on the various cultivar types or growing conditions i.e. different agricultural and environmental effects (27).

The most prevalent constituents in *M. recutita* essential oil are summarized in **Table 2**.



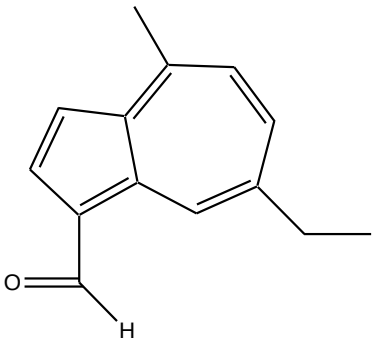

**(1)** **(2)** **(3)**

**(4)** **(5)** **(6)**

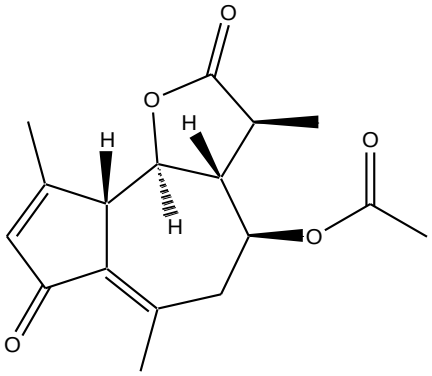
**(7)** **(8)** **(9)**

**Table 2.** Main constituents identified in *M. recutita* essential oil

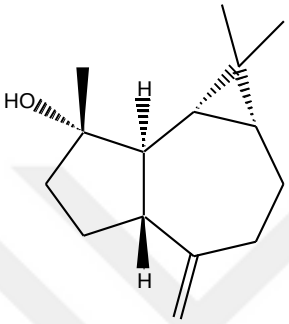
| **Compound** | **Chemical Group** | **Ref** |
|---|---|---|
| α/ β-pinene (1) | Monoterpene hydrocarbon | (28–31) |
| Limonene (2) | Monoterpene hydrocarbon | (28–32) |
| Nerol (3) | Monoterpene alcohol | (33) |
| Isoborneol (4) | Monoterpene alcohol | (33) |
| Geraniol (5) | Monoterpene alcohol | (28,29,33) |
| Chamazulene (6) | Sesquiterpene hydrocarbon | (18,28–31,33–35) |
| Germacrene D (7) | Sesquiterpene hydrocarbon | (29–31) |
| α/β-farnesene (8) | Sesquiterpene hydrocarbon | (28–31,33,34) |
| β-elemene (9) | Sesquiterpene hydrocarbon | (18,28–30,33) |



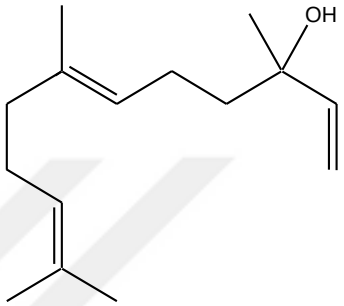

**(1)**




**(2)**




**(3)**

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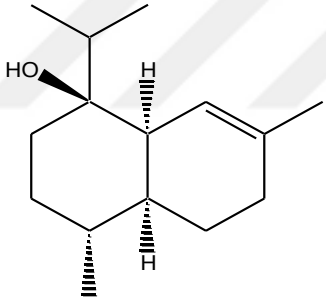


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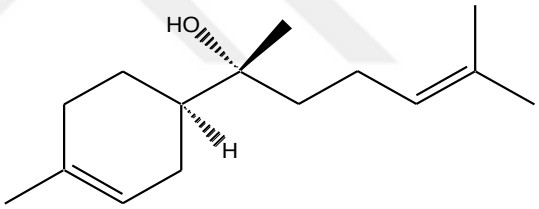
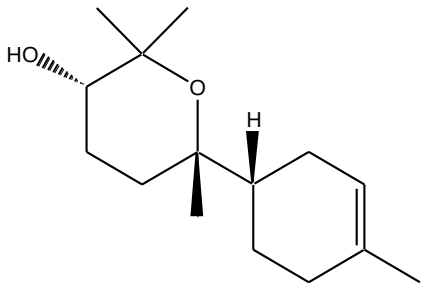


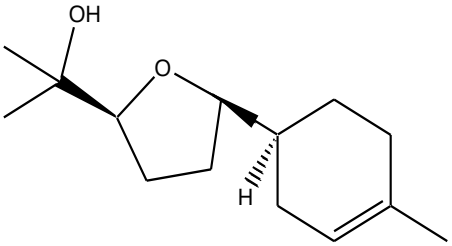

**(6)**

**Table 2.** Continued.

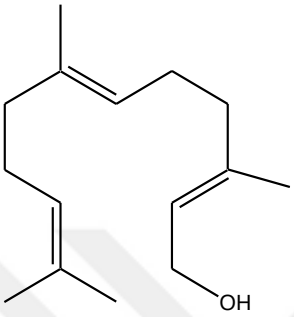
| Compound | Chemical Group | Ref |
|---|---|---|
| Chamavioline (1) | Sesquiterpene aldehyde | (36) |
| Matricarin (2) | Sesquiterpene lactone | (32,37) |
| Spathulenol (3) | Sesquiterpene alcohol | (18,28–30,32,33) |
| (*E*)-Nerolidol (4) | Sesquiterpene alcohol | (28–31,33) |
| Chamomillol (5) | Sesquiterpene alcohol | (28,29,31) |
| (-)-α-bisabolol (6) | Sesquiterpene alcohol | (18,28–35,37) |




**(1)**




**(2)**

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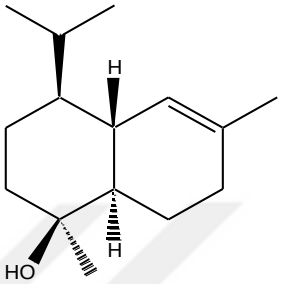


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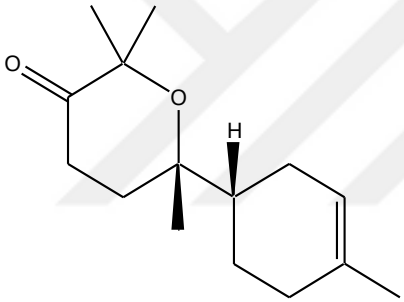
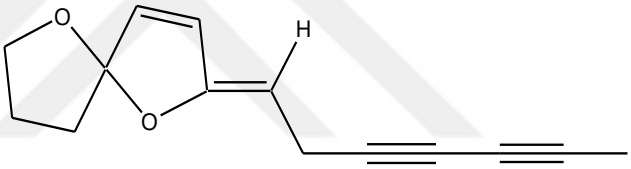



**(5)**




**(6)**

**Table 2.** Continued.

| Compound | Chemical Group | Ref |
|---|---|---|
| (-)-α-bisabolol oxide A (1) | Sesquiterpene alcohol | (18,28–35,37,38) |
| (-)-α-bisabolol oxide B (2) | Sesquiterpene alcohol | (18,28–35,37,38) |
| Farnesol (3) | Sesquiterpene alcohol | (30,33) |
| τ-Cadinol (4) | Sesquiterpene alcohol | (18,29,33) |
| (-)-α-bisabolone oxide A (5) | Sesquiterpene ketone | (18,28–33,35) |
| *cis*-en-yn-dicycloether (6) | Polyyne | (29,31–33,35,37,39) |

### 2.2.1.1.2. Other Constituents

#### 2.2.1.1.2.1. Coumarins

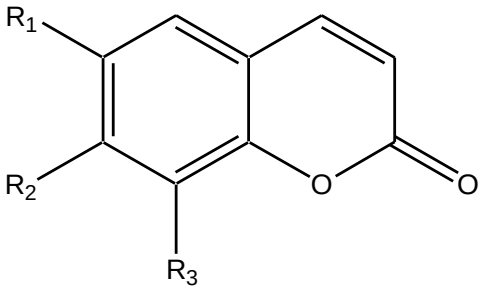



**Table 3.** Coumarins identified in *M. recutita*

| **Compound** | **$R_1$** | **$R_2$** | **$R_3$** | **Ref** |
|---|---|---|---|---|
| Coumarin | H | H | H | (40) |
| Daphnetin | H | OH | OH | (41) |
| Daphnetin 7-*O*-glucoside | H | OGlc | OH | (41) |
| Esculetin | OH | OH | H | (40) |
| Fraxidin | OMe | OMe | OH | (42) |
| Herniarin | H | OMe | H | (41) |
| Isoscopoletin | OH | OMe | H | (40) |
| Scopoletin | OMe | OH | H | (42) |
| Umbelliferone | H | OH | H | (41) |
| Umbelliferone 7-*O*-glucoside | H | OGlc | H | (41) |

Glc: Glucose; OMe: Methoxy

#### 2.2.1.2. Analytical Studies

Several qualitative and quantitative methods have been developed to analyze various phenolic components such as A7G, apigenin, quercetin, luteolin, L7G, patuletin and isorhamnetin either in aqueous or alcoholic or hydroalcoholic *M. recutita* extracts. These methods are based on overpressured layer chromatography (OPLC) (37), HPLC or ultra-performance liquid chromatography (UPLC) coupled with ultra-violet (UV) (43,44) or diodarray detectors (DAD) (16,41,45) or mass (MS) (17,46–48) or tandem mass ($MS^2$) (19,20,49) spectrometric detections, capillary electrophoresis (CE) (50,51) and capillary electrochromatography (CEC) (52). HPTLC methods have also been developed either for identification or quantification of some flavonoids in *M. recutita* flower extract (53–57).

In addition, several methods to either identify (58) or quantify *M. recutita* essential oil components calculated as percentage of total area (18,29–33) by gas chromatography (GC) coupled with MS have been developed.

Analytical studies on *M. recutita* are briefly summarized in **Table 4**.

**Table 4.** Analytical studies on *M. recutita*

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **1** | Methanolic ligulate flower extract | HPLC-UV<br>λ = 335 nm | Separon SGX $C_{18}$ (7 µm, 250 mm x 4 mm, i.d) | **A:** water-acetonitrile-*o*-phosphoric acid (80:19:1)<br>**B:** 80% acetonitrile<br>**Gradient system:** 15% B (0 min), 20% B (5 min), 30% B (10 min), 35% B (15 min), 60% B (20 min), 15% B (25 min)<br>**Flow rate:** 0.7 mL/min | **Qualitative and quantitative analysis**<br>*E*/*Z*-2-β-D-glucopyranosyloxy-4-methoxy cinnamic acid, A7G, apigenin 7-*O*-(6''-malonyl)-glucoside, apigenin 7-*O*-(4''-acetyl)-glucoside, apigenin 7-*O*-(6''-caffeoyl)-glucoside, apigenin 7-*O*-(6''-acetyl)-glucoside, apigenin 7-*O*-(4''-acetyl, 6''-malonyl)-glucoside, apigenin<br>**Major component(s):** apigenin 7-*O*-(4''-acetyl, 6''-malonyl)-glucoside (15-30 mg/g); apigenin 7-*O*-(6''-malonyl)-glucoside (17-29 mg/g); A7G (14-28 mg/g) in diploid and tetraploid cultivars. | (43) |
| **2** | Aqueous fresh, freeze-dried, air-dried and oven-dried flower extracts | HPLC-UV<br>λ = 335 nm | Agilent Zorbax Eclipse XDB-$C_{18}$ (5 µm, 150 mm x 4.6 mm, i.d) | **A:** 0.025 M *o*-phosphoric acid<br>**B:** acetonitrile<br>**Gradient system:** 20-40% B (0-10 min), 40% B (10-15 min), 20% B (15-20 min)<br>**Flow rate:** 1 mL/min | **Qualitative and quantitative analysis**<br>A7G<br>**Major component(s):** A7G (3.0 mg/g) in fresh flowers.<br>Note: The effects of drying conditions on A7G content were investigated. | (44) |
| **3** | Ethanolic leaf extract and commercial tea products | HPLC-DAD<br>λ = 320 nm | Kromasil ECOM (100-7 µm, 250 mm x 4.6 mm, i.d) | **A:** water (1% trifluoroacetic acid (TFA))<br>**B:** 70% acetonitrile<br>**Gradient** system: 40% B (0-20 min); 100% B (20-30 min); 100% B (30-37 min); 0% B (37–40 min)<br>**Flow rate:** 1.1 mL/min | **Qualitative and quantitative analysis**<br>umbelliferone 7-*O*-glucoside, daphnetin 7-*O*-glucoside, 7,8-dihydroxycoumarin, *E*/*Z*-β-glucopyranosyloxy-4-methoxycinnamic acids<br>**Major component(s):** *E*- and *Z*-β-glucopyranosyloxy-4-methoxycinnamic acids 15.9 mg/g and 10.16 mg/g respectively. | (41) |

**Table 4.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **4** | Aqueous and methanolic flower and leafy flowering stem extracts | HPLC-DAD<br>λ = 280 and 370 nm | Phenomenex $C_{18}$ column (5 µm, 150 mm x 4.6 mm i.d) | **A:** water (0.1% TFA)<br>**B:** acetonitrile<br>**Gradient system:** isocratic 10% B for 3 min, from 10-15% B (12 min), isocratic 15% B for 5 min, from 15-18% B (5 min), 18-30% B (20 min) and from 30-35% (5 min)<br>**Flow rate:** 0.5 mL/min | **Qualitative and quantitative analysis**<br>3-*O*-caffeolyquinic acid, protocatechuic acid, 1,5-*O*-dicaffeolyquinic acid, 4-*O*-caffeolyquinic acid, *E*/*Z*-5-*O*-caffeolyquinic acid, *Z*-feruloyl hexoside acid, trans-feruloyl hexoside acid, 5-*O*-feruloylquinic acid, feruloyl hexoside acid dimer, 1,3,5-*O* or 1,4,5-*O*-tricaffeolyquinic, luteolin acetylhexoside hexoside, 3,4-*O*-dicaffeolyquinic acid, L7G, 3,5-*O*-dicaffeolyquinic acid, quercetin 7-O-acetylhexoside, luteolin *O*-acylhexoside, 4,5-*O*-dicaffeoylquinic acid, luteolin *O*-acylhexoside<br>**Major component(s):** luteolin *O*-acetylhexoside (2.10 g/100 g) in methanolic extract.<br>Note: A7G was not detected. | (45) |
| **5** | Methanolic ligulate and tubular flowers and receptacle extracts | HPLC-DAD<br>λ = 335 nm<br>HPLC-ESI-MS | Chrompack Intersil $C_3$ column (3 µm, 150 mm x 3.0 mm, i.d)<br>Pre-column (5 µm, 10 mm x 2.0 mm, i.d) | **A:** water (pH 2.5)<br>**B:** acetonitrile<br>**Gradient system:** 92 % A (0 min), 30 % A within 50 min<br>**Flow rate:** 0.4 mL/min | **Qualitative and quantitative analysis**<br>3-*O*-caffeoylquinic acid, 4-*O*-Caffeoylquinic acid, 5-*O*-caffeoylquinic acid, quinic acid and its derivatives, ferulic acid-l-*O*-glycoside, ferulic acid 7-*O*-glycoside, quercetin 3-*O*-glalactoside, quercetin 7-*O*-glucoside, patuletin 7-*O*-glucoside, 1,3-*O*-dicaffeoylquinic acid, A7G, quercetin derivative, luteolin 4'-*O*-glucoside, apigenin 7-*O*-glucosyl-2''-acetate, apigenin 7-*O*-glucosyl-6''-acetate, apigenin 7-*O*-glucosyl-diacetate, apigenin, luteolin 7-*O*-rutinose<br>**Major component(s):** apigenin glucoside derivatives (68.6 %) in ligulate flowers. | (17) |

**Table 4.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **6** | Methanolic and aqueous flower extracts | HPLC-ESI-MS<br>λ = 335 nm | $C_{18}$ column | **A:** water<br>**B:** acetonitrile<br>**Isocratic system:** A-B, (70:30,v/v)<br>**Flow rate:** 1 mL/min | **Qualitative and quantitative analysis**<br>A7G and apigenin derivatives<br>**Major component(s):** A7G (58.61% in aqueous extract). | (48) |
| **7** | Commercial tea products | HPLC-UV/Vis<br>λ = 306-370 nm<br>HPLC-ESI-MS-MS | Agilent Zorbax 300 SB-$C_{18}$ column (5 µm, 150 mm x 2.1 mm, i.d) | **A:** water (0.1% formic acid)<br>**B:** acetonitrile<br>**Gradient system:** 1% B (0-2 min), a linear gradient of B to 31.1% (2-80 min), a linear gradient of B to 95% (80-83 min) , 95% B (83–90 min)<br>**Flow rate:** 0.3 mL/min | **Qualitative and quantitative analysis**<br>neochlorogenic acid, ChA, cryptochlorogenic acid, ferulic acid glucosides, quercetin galactoside, quercetin glucoside, luteolin glucoside, 3,4-*O*-dicaffeoyl quinic acid 3,5-*O*-dicaffeoyl quinic acid, 4,5-*O*-dicaffeoyl quinic acid, apigenin acetylglucoside, apigenin glucoside<br>**Major component(s):** dicaffeoylquinic acids (1.7-30.4 mg/200 mL); ChA (0.6-19.2 mg/200 mL), ferulic acid glucosides (1.0-18.0 mg/200 mL); apigenin glucosides (0.6-10.2 mg/200 mL). | (49) |
| **8** | Ethanolic flower extract | HPLC-DAD<br>λ = 280 nm<br>HPLC-ESI-MS-MS<br>HPLC-NMR | AQ column $C_{18}$ column (5 µm, 150 mm x 3.0 mm, i.d) | **A:** water (0.01% formic acid)<br>**B:** acetonitrile<br>**Gradient system:** 10% B (3 min), 60% B (32 min), 90% B (15 min)<br>**Flow rate:** 0.6 mL/min | **Qualitative analysis**<br>*E/Z*-2-β-D-glucopyranosyloxy-4-methoxy cinnamic acid, apigenin 7-*O*-(6''-rhamnosyl)glucoside, apigenin 7-*O*-(6''-acetyl)glucoside, A7G, 7-methoxycoumarin, apigenin | (19) |
| **9** | Aqueous flower extract | HPLC-ESI-MS-MS | Reprosil-PUR $C_{18}$ column (3 µm, 50 mm x 2.0 mm, i.d) | **A:** water (0.1% formic acid)<br>**B:** Methanol<br>**Gradient system:** 90% A (0-3 min), 90-70% A (3-5 min), 70-50% A (5-25 min) 50-0% A (25-40 min), 0% A (40-41 min), 0-90% A (41-42 min), 90% A (42-52 min)<br>**Flow rate:** 0.3 mL/min | **Qualitative and quantitative analysis**<br>A7G, L7G, patuletin 7-*O*-glucoside, hyperoside<br>**Major component(s):** patuletin 7-*O*-glucoside (0.37-0.80%, w/v). | (59) |

**Table 4.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **10** | Aqueous and methanolic and hydroalcoholic herba extracts | UPLC-DAD<br>λ = 340 nm | Blue Orchid $C_{18}$ column (1.8 µm, 150 mm x 2.0 mm, i.d) | **A:** water (4% acetic acid)<br>**B:** acetonitrile<br>**Gradient system:** 7-21% A (18 min), hold for 3 min, 21-48% A (4 min)<br>**Flow rate:** 0.4 mL/min | **Qualitative and quantitative analysis**<br>ChA, caffeic acid, *p*-coumaric acid, salicylic acid, rutin, A7G, quercetin, luteolin, apigenin, kaempferol, isorhamnetin<br>**Major component(s):** total A7G (1.23 % (w/w) in hydrolyzed hydroalcoholic extract). | (16) |
| **11** | Methanolic flower extract and commercial products | UPLC-UV<br>λ = 200-400 nm<br>UPLC-MS | Acquity BEH $C_{18}$ column (1.7 µm, 100 mm x 2.1 mm, i.d) | **A:** water (0.05% formic acid)<br>**B:** acetonitrile (0.05% formic acid)<br>**Gradient system:** 10% B (0-10 min), 40% B (10 min), 100% B (5 min)<br>**Flow rate:** 0.25 mL/min | **Qualitative and quantitative analysis**<br>*E/Z*-2-β-D-glucopyranosyloxy-4-methoxy cinnamic acid, quercetagetin 7-*O*-glucoside, A7G, apigenin 7-*O*-(6″-acetylglucopyranoside), chamaemeloside, tonghaosu<br>**Major component(s):** *Z*-2-β-D-glucopyranosyloxy-4-methoxy cinnamic acid (0.877 mg/100g), *E*-2-β-D-glucopyranosyloxy-4-methoxy cinnamic acid (0.753 mg/100g), A7G (0.323 mg/100g) and tonghaosu (0.569 mg/100g) in methanolic extract. | (46) |
| **12** | Methanolic flower and commercial tea extracts | UPLC-ESI-MS-MS | Acquity BEH $C_{18}$ column (1.7 µm, 100 mm x 2.1 mm, i.d) | **A:** water (0.1% formic acid)<br>**B:** Methanol<br>**Gradient system:** 88.5-50% formic acid 0.1% methanol with isocratic step<br>**Flow rate:** 0.45 mL/min | **Qualitative and quantitative analysis**<br>ChA, caffeic acid, umbelliferon, quercetin 3-*O*-glucoside, rutin, A7G, quercetin, apigenin, quercitrin, luteolin, kaempferol, isorhamnetin<br>**Major component(s):** A7G (94.1-209.4 µmol/L and 7.3-127.1 µmol/L) and ChA (7.3-310.3 µmol/L and 11-116 µmol/L) in flower and tea extracts, respectively. | (47) |

**Table 4.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **1** | Methanolic, glycolic and hydroalcoholic flower extracts | CE-DAD<br>λ = 337 nm | Phoenix fused-silica capillary (total length 63 cm; effective length 54 cm; 75 µm i.d., 375 µm o. d.) | pressure flush (930 mbar) of 1 mol/L sodium hydroxide solution (20 min), deionized water (10 min) and electrolyte solution (30 min) followed by an electrokinetic flush of electrolyte +25 kV (15 min) | **Qualitative analysis**<br>A7G, L7G, apigenin, luteolin, naringenin, rutin, quercetin, umbelliferone, herniarin, ChA and caffeic acid | (50) |
| **2** | Methanolic, ethanolic and glycolic flower and seed extracts | CE-DAD<br>λ = 337 nm | fused-silica capillary (total length 63 cm; effective length 54 cm; 75 µm i.d., 375 µm o.d.) | pressure flush (930 mbar) of 1 mol/L sodium hydroxide solution (20 min), deionized water (10 min) and electrolyte solution (30 min) followed by an electrokinetic flush of electrolyte +25 kV (15 min) | **Qualitative and quantitative analysis**<br>apigenin<br>**Major component(s):** 903 µg/g total apigenin in methanolic extract. | (51) |
| **1** | Hydroalcoholic flower extracts | CEC<br>λ = 337 nm | Polyimide fused-silica capillaries (75 µm i. d.; 375 µm o.d.) | pH 2.8 phosphate buffer at 50 mmol/L containing 50% acetonitrile | **Qualitative analysis**<br>herniarin, umbelliferone, ChA, caffeic acid, apigenin, A7G, luteolin, L7G, quercetin, rutin, naringenin | (52) |

**Table 4.** Continued.

| No. | Extract | Method | Stationary Phase | Developing Solvent System | Derivatization | Detection | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|---|---|
| **1** | Methanolic flower extract and dietary supplements | HPTLC | HPTLC silica gel 60 $NH_2$w $F_{254}$ | dichloromethane-acetonitrile-ethyl formate-glacial acetic acid-formic acid (11:2.5:3:1.25:1.25, v/v/v/v/v) | Natural Products (NP) reagent | Scanner: reflectance/ absorption Visualizer: 366 nm | **Qualitative and quantitative analysis** rutin, L7G, chamaemeloside, A7G, luteolin, apigenin, umbelliferone **Major component(s):** A7G (2.83 mg/g, 3.91 mg/g, 2.40 mg/g in flower, capsule and tea bag extracts, respectively). | (53) |
| **2** | Commercial products | HPTLC | HPTLC silica gel 60 $F_{254}$ | toluene-methanol (10:2, v/v) | - | Scanner: reflectance/ absorption λ = 343 nm | **Qualitative and quantitative analysis** apigenin **Major component(s):** apigenin (7.5 mg/100 mL). | (54) |
| **3** | Hydroalcoholic ligulate flowers | HPTLC | HPTLC silica gel 60 $F_{254}$ | benzol-ethyl methyl ketone-methanol (5.5:3:1.5, v/v/v) | 96% sulphuric acid reagent | Visualizer: 254 nm | **Qualitative analysis** apigenin, luteolin, apigenin 7-*O*-diacetylglucoside, apigenin 7-*O*-monoacetylglucoside, A7G | (55) |
| **4** | Chloroform and ethanolic flower extracts | HPTLC | HPTLC silica gel 60 and RP-18 | hexane-ethyl acetate-acetic acid (20:10:1, v/v/v) acetone-water (90:10, v/v) | Anisaldehyde reagent | VideoScan digital image evaluation Visualizer: 366 nm, 254 nm and white light | **Qualitative and quantitative analysis** apigenin, bisabolol, chamazulene, parthenolide **Major component(s):** Chamazulene (5.35 µg/µL and 1.71 µg/µL by supercritical fluid and soxhlet extractions). | (56) |
| **5** | Steam distillated flower | HPTLC | HPTLC silica gel 60 $F_{254}$ | toluene-ethyl acetate (93:7, v/v) | Vanillin- sulphuric acid reagent | Visualizer: white light | **Qualitative analysis** bisabolol oxide A, bisabolol, azulene | (57) |

### 2.2.1.3. Traditional Usage

Infusion prepared from *M. recutita* flowers has been used for centuries either for its pleasant and calming taste or relieving painful gastrointestinal complaints i.e. diarrhea, flatulence, spasms, mild sleep disorders and inflammatory conditions such as gastritis and enteritis. In addition, the infusion is inhaled to alleviate bronchitis and nasal catarrh complains or it is applied externally as hot compress for healing wound, abscesses and hemorrhoids or the affected area is exposed to its vapors to treat acne vulgaris **(Table 5)**.

**Table 5.** Traditional usage of *M. recutita*

| Part(s) used | Administration; preparation; dosage | Usage |
|---|---|---|
| Capitulum | Internally; infusion; drink one teacup two times a day for 3-7 days (60) | Asthma, kidney stones (60), cough (60,61), flu (61), cold (60–62), spasmolytic (13,60), bronchitis (60,61,63), carminative (14,60), appetizer (14), digestive (63,64), against menstrual pains (64,65), stomachache (13,60–62,65,66), sedative (14,64,66), anti-inflammatory (13,60,66), laxative (63,66). |
| | Externally; infusion; as a gargle until recovery (60); compressed with a cloth (13,63); steam inhalation (13,66) | Acne vulgaris, bronchitis, menstrual pains (13), sore throat, to take away bad breath of mouth (60), inflamed wounds (13,60), to clean face and eyes, eye-strain (63), sinusitis (66), wounds (13,60,66). |
| | Internally; decoction (64,65) | Abdomen pains (64), sedative (65), cough (64,65). |
| | Externally; decoction; applied one a day until recovery (60) | Antiseptic (60), hemorrhoids (14), wounds (14,60). |
| Aerial parts | Internally; infusion; drink one teacup 2-3 times a day for 2-3 days (67) | Gastrointestinal diseases, headache, cancer respiratory tract diseases and flu (67). |

#### 2.2.1.4. Bioactivity Studies

Apart from traditional knowledge on therapeutic efficacy of *M. recutita*, many studies have been conducted in recent years to establish its anti-inflammatory, wound healing, antiulcer, spasmolytic, anxiolytic, antioxidant, antinociceptive, antiallergic, anticancer, antimicrobial, hepatoprotective and antidiabetic activities either through *in vitro* and *in vivo* techniques or by clinical investigations (68).

##### 2.2.1.4.1. Anti-inflammatory Activity

Safayhi et al. studied anti-inflammatory activity of chamazulene and its precursor matricin on the leukotriene production in neutrophilic granulocytes using *in vivo* assay. Chamazulene (half maximal inhibitory concentration ($IC_{50}$): 15 μM) blocked the synthesis of leukotriene B4 in intact cells dose-dependently. Additionally, it ($IC_{50}$: 2 μM) inhibited the chemical peroxidation of arachidonic acid. On the other hand, matricine did not exert any effects on the cyclooxygenase and 12-lipoxygenase activities in human platelets up to 200 μM. Consequently, chamazulene may contribute to the anti-inflammatory effect of *M. recutita* extracts via blocking the leukotriene synthesis and exerting antioxidant activity (69).

A mouse model of lipopolysaccharide (LPS)-induced proinflammatory cytokine production such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) was used by Smolinski and Pestka to investigate the anti-inflammatory effect of apigenin. Murine macrophage cell line, RAW 264.7 was also practiced to determine the molecular basis of apigenin anti-inflammatory activity. Test animals were pretreated with apigenin (50 mg/kg, p.o.) and after 1 h inflammation was triggered by LPS (1 mg/kg, i.p.) administration. The blood was collected 90 min after the administration and then the results were comparatively evaluated with control group. The percentage of the reduction caused by apigenin on LPS-induced IL-6 and TNF-α production was determined as 35% and 33%, respectively. In further analyses, RAW cells were co-administered with different concentrations of LPS and apigenin for 12 h. Co-administration with the two highest doses, 1 and 10 mg/mL of apigenin dose-dependently impaired LPS-induced IL-6 production. However, TNF-α production remarkably inhibited. Consequently, apigenin showed anti-inflammatory activity by reducing LPS-induced proinflammatory cytokine production (70).

LPS-activated RAW 264.7 macrophages were also practiced by Srivastava et al. to reveal the inhibitory activity mechanism of aqueous *M. recutita* extract on cyclooxygenase-2 (COX-2) pathway. As a result, the extract suppressed prostaglandin $E_2$ ($PGE_2$) release from LPS-activated RAW 264.7 macrophages (A7G ($IC_{50}$): 24.0 μg/mL or apigenin ($IC_{50}$): 15.0 μM). In addition, extract dose-dependently blocked COX-2 enzyme activity (A7G $IC_{50}$: 28.0 μg/mL or apigenin $IC_{50}$:17.6 μM). Moreover, it reduced COX-2 mRNA and protein expression, whereas did not affect the activity or expression of COX-1. Researchers also compared the extract efficacy with sulindac (a nonsteroidal anti-inflammatory drug, NSAID) and a specific COX-2 inhibitor, NS398. These compounds also reduced $PGE_2$ levels and blocked COX-2 activity and protein expression. However, they did not affect COX-2 mRNA expression in RAW 264.7 macrophages, challenged with LPS. Accordingly they concluded that *M. recutita* extract acts on prostaglandin synthesis by a similar mechanism to that of NSAIDs (71).

Nitric oxide (NO) plays an important role in the pathogenesis of various inflammatory diseases and inducible NO synthase (iNOS) expression. Bhaskaran et al. studied the inhibitory effects of standardized aqueous *M. recutita* extract with doses equivalent to molar concentration of A7G on NO synthesis using RAW 264.7 macrophages. Consequently, NO synthesis and iNOS expression in macrophages were blocked by extract (10-40 μg/mL). In addition, it markedly inhibited LPS-stimulated DNA binding activity of nuclear factor-κB (NF-κB) p65 subunit (RelA/p65), the upstream kinase regulating NF-κB/RelA activity and degradation of inhibitory factor-κB. These results demonstrated that *M. recutita* extract supresses NO synthesis and iNOS gene expression by inhibiting RelA/p65 activation and supported the utilization of extract as an efficient anti-inflammatory agent (72).

Skin-inflamed mouse model was used by Leite et al. to examine the anti-inflammatory effects of (-)-α-bisabolol. Moreover, its efficacy was compared with reference drugs, dexamethasone and indomethacin. The ears of mice were pretreated topically with (-)-α-bisabolol (0.7 and 1.4 mg/ear) and then dermatitis was induced by croton oil, arachidonic acid, phenol and capsaicin. The results demonstrated that (-)-α-bisabolol was topically active in the reduction of acute dermatitis induced by croton oil, arachidonic acid and phenol in a manner similar to dexamethasone (0.08 mg/ear) and

indomethacin (2 mg/ear). However, it was found to be ineffective against capsaicin-induced inflammation (73).

Hamsters induced by 5-fluoracil were analyzed by Pavesi et al. to investigate the effect of topically applied *M. recutita* ointment (Ad-Muc®) in the treatment of oral mucositis. The test animals were grouped and treated with Ad-Muc® or Celestone® (betamethasone, a corticosteroid) ointments, respectively. Further, histopathological findings revealed that Ad-Muc®-treated group exhibited the least degree of mucositis throughout the experiment in comparison with the control and Celestone®-treated groups (74). Further investigations were performed by Curra et al. to study the mechanism of action of *M. recutita* ointment. Consequently, *M. recutita* ointment exhibits anti-inflammatory effect in oral mucositis by reducing the tissue levels of IL-1β and TNF-α (75).

#### 2.2.1.4.2. Wound Healing Activity

Albino rats were practiced by Jarrahi to study wound healing potential of topically applied *M. recutita* extract which was prepared via maceration with olive oil. The specified area on rats was burned by steeping them into boiling water for 8 s. Eventually rapid wound healing was observed in topically extract-treated group when compared to the olive oil-treated group. In conclusion, *M. recutita* extract remarkably stimulates tissue regeneration and reduces wound area (76).

*In vivo* and *in vitro* methods were applied by Martins et al. to investigate *M. recutita* extract for treating ulcers comparatively with corticosteroids. They assessed the cell viability of fibroblasts by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction analysis through *in vitro* study. All experimental groups presented positive cell viability in 24 h. The extract-treated cultures showed the smallest cell viability amongst all experimental groups ($p < 0.05$). Besides, corticosteroid-treated cultures presented cell viability similar to those of control. Moreover, male rats were used for *in vivo* studies. Consequently extract-administered group showed complete wound healing 9 days before the other groups. Besides, lesions were repaired in only extract-administered group after 5 days. Moreover, the extract-administered group presented remarkably faster wound healing in comparison to those corticosteroid-

administered group. According to the obtained results, it was concluded that *M. recutita* extract promotes faster wound healing process in comparison to corticosteroids (77).

Charousaei et al. compared the effects of *M. recutita* solution and 1% hydrocortisone ointment for management of peristomal skin lesions on colostomy patients. Lesions healed markedly faster in *M. recutita* solution than hydrocortisone ointment, mean healing time was 8.89 ± 4.89 and 14.53 ± 7.6 days ($p$ = 0.001), respectively. Moreover, comparing the long term topical corticosteroid use, *M. recutita* solution may be considered to be much safer choice (78).

Rats having wound on their tongues were examined by Duarte et al. to evaluate the effect of *M. recutita* ointment (0.04 mL/day) on wound healing. Eventually rapid epithelialization, lessened wound size, increased number of fibroblasts and hydroxyproline content and also reduced inflammation were observed in *M. recutita* ointment-treated animals (79).

**2.2.1.4.3. Antiulcer Activity**

Balb/c mice were used by Karbalay-Doust and Noorafshan to investigate the antiulcerogenic activity of *M. recutita* extract. Test animals were grouped and received either 400 mg/kg sucralfate, a cytoprotective agent, or 400 mg/kg extract respectively through intragastric route. After 30 min, gastric ulceration was stimulated by oral administration of 1.0 mL of a 0.3 M solution of hydrochloric acid in 60% ethanol in all animals. After 1 h, stereological method was used to determine the area of the gastric lesions and hemorrhage. The authors have further studied possible toxicity when administered at a single dose of 5000 mg/kg extract. Histopathological evaluation of liver, kidneys, lung and heart tissues 14 days after the administration has revealed that the extract was safe up to 5000 mg/kg. Then they concluded that *M. recutita* extract at 400 mg/kg can be effective in prevention of gastric ulceration in mice and does not produce toxic effects in doses up to 5000 mg/kg (80).

Rats having gastric mucosal injury were analyzed by Cemek et al. to investigate antiulcerogenic activities of hydroalcoholic *M. recutita* extract. The test animals were fed on 25 to 400 mg/kg of extracts or 20 mg/kg of famotidine by gavage, respectively. After 1 h, gastric mucosal injury was stimulated by oral administration of ethanol and

then all groups were sacrificed. The gastric ulcer index was calculated, besides malondialdehyde (MDA), reduced glutathione (GSH) in whole blood and gastric tissue, serum ascorbic acid, retinol and β-carotene levels were determined in all rats. It was concluded that gastric lesions significantly reduced in *M. recutita* extract-pretreated group. In addition, MDA levels significantly lessened whereas GSH increased in gastric tissue or whole blood. Serum β-carotene and retinol levels were found to be higher in the 200 mg/kg extract-treated group than the control. As a result, *M. recutita* extract showed protective activity against ethanol-induced gastric mucosal lesions and this effect might possibly due to the reduction in lipid peroxidation and enhancement in antioxidant activity (81).

#### 2.2.1.4.4. Spasmolytic Activity

Foster et al. compared the antispasmodic effects of ethanolic *M. recutita* extract with spasmogenic agents, acetylcholine and histamine in guinea pig ileum. The extract was found to possess significant inhibitory activity on the contractility stimulated by acetylcholine as well as histamine (82).

*In vitro* tests were used by Maschi et al. to study the spasmolytic activity of aqueous *M. recutita* extract on cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate phosphodiesterases (cGMP-PDE). Human platelet cAMP-PDE and recombinant PDE-5A1 were analyzed during the study. As a result, the extract blocked cAMP-PDE activity ($IC_{50}$= 17.9-40.5 μg/mL), whereas cGMP-PDE5 was found to be less effected (-15% at 50 μg/mL). These data support that smooth muscle relaxation caused by *M. recutita* extract is mediated by decreasing of cAMP degradation rather than that of cGMP (59).

#### 2.2.1.4.5. Anxiolytic Activity

*In vitro* and *in vivo* methods were applied by Viola et al. to evaluate pharmacological effect of apigenin. *In vitro* tests were resulted that apigenin acts as a ligand for binding to central benzodiazepine receptors (γ-aminobutyric acid-$GABA_a$) and competitively blocked the binding of flunitrazepam, an anxiolytic drug, to $GABA_a$ receptors. Besides, *in vivo* test revealed that it shows a clear anxiolytic activity in mice in the elevated plus-maze assay without having any significant side effects such as

convulsion or muscle relaxation (83). Avallone et al. also reported pharmacological effects of apigenin. Cultured cerebellar granule cells were used to perform *in vitro* tests. They reported that apigenin dose-dependently reduced GABA-activated $Cl^-$ currents and the effect was blocked by the co-application of Ro 15-1788, a specific benzodiazepine receptor antagonist. Eventually, apigenin reduced the latency in the onset of picrotoxin, a non-competitive channel blocker for the $GABA_a$ receptor chloride channels, stimulated convulsions. Moreover, apigenin reduced locomotor activity when injected intraperitoneally to rats whereas did not show anxiolytic, myorelaxant, or anticonvulsant activities. Eventually, contrary to the hypothesis of Viola et al., they concluded that the inhibitory activity of apigenin cannot be attributed to an interaction with $GABA_a$-benzodiazepine receptor because it is not blocked by Ro 15-1788 (84).

Sleep-deprived rat model was evaluated by Shinomiya et al. to study the hypnotic activities of aqueous *M. recutita* extract. Wistar rats were fed on 30 to 300 mg/kg extract for 7 days. Then, test animals were anesthetized by pentobarbital sodium application (35 mg/kg) and exposed to electroencephalogram and electromyogram measurements. The sleep-wake states and sleep latency were measured. 300 mg/kg extract remarkably lessened the sleep latency. Besides, total times of wakefulness, non-rapid eye movement (REM) sleep and REM sleep were not influenced. Eventually hypnotic activity of *M. recutita* extract possibly because of binding of apigenin to the benzodiazepine receptors (85).

Ross studied the efficacy and tolerability of a standardized *M. recutita* extract including 1.2% apigenin in patients experiencing moderate generalized anxiety disorder. In randomized, double-blinded, placebo-controlled, parallel-group trial, 61 patients were treated with either extract or a placebo for 5 weeks. Hamilton Anxiety Rating (HAM-A) scores were used to evaluate the results. Consequently, oral administration of the extract significantly reduced the mean total HAM-A score ($p$ = 0.047) when compared to the placebo group. *M. recutita* extract may have a potential of anxiolytic activity (86).

#### 2.2.1.4.6. Antioxidant Activity

$DPPH^·$ and ferric thiocyanate assays were tested by Hernández-Ceruelos to determine *M. recutita* essential oil antioxidant activity. Eventually they found that *M.*

*recutita* showed dose-dependently antioxidant capacity (87). In another study, *M. recutita* essential oil antioxidant activity was determined by β-carotene-bleaching method with the $IC_{50}$ value of 45.93 ± 6.05 μg/mL. Among the major components in essential oil, α-bisabolol exerted the highest antioxidant potential the $IC_{50}$ value 95.18 ± 12.14 (88).

Macrophage RAW 264.7 cells were practiced by Bhaskaran et al. to demonstrate the cytoprotective effects of *M. recutita* extract. Hydrogen peroxide ($H_2O_2$) was used to induce the cellular damage. Extract-pretreated cells dose-dependently reduced $H_2O_2$-induced cell viability loss. Protection mechanism against oxidative stress was through the induction of several antioxidant enzymes including NAD(P)H:quinone oxidoreductase, superoxide dismutase (SOD), and catalase (CAT) as well as increase nuclear accumulation of the transcription factor Nrf2 and its binding to antioxidant response agents. Furthermore, *M. recutita* dose-dependently reduced $H_2O_2$-mediated increase in the intracellular levels of reactive oxygen species (ROS). Consequently they analyzed two major peaks in extract could be associated with antioxidant activity were A7G (63.3%) and apigenin 7-*O*-neohespridoside (27.7%), respectively (89). In addition to these findings, Sebai et al. investigated antioxidant properties of aqueous *M. recutita* extract on male Wistar rats against castor oil-induced oxidative damage. Castor oil administration stimulated MDA formation in stomach and intestine mucosa indicating an increase in lipid peroxidation and depletion of antioxidant activities of SOD, CAT and glutathione peroxidase (GSH-Px). Castor oil also increased gastric and intestinal mucosa $H_2O_2$ and free iron levels. Oral application of aqueous *M. recutita* extract (25, 50 and 100 mg/kg) dose-dependently prevented all these alterations and the highest dose repaired their levels (90).

#### 2.2.1.4.7. Antinociceptive Activity

Cisplatin is an anticancer drug and one of the basic treatments of solid tumors such as colerectal, ovarian, lung cancer. However, it is characterized by a painful sensory neuropathy dose-dependently. Namvaran Abbas Abad et al. studied the effects of hydroalcoholic *M. recutita* extracts on cisplatin-induced peripheral neuropathy using albino rats and compared with an analgesic drug morphine. Morphine decreased cisplatin-induced pain in the first (0-5 min) and second phases (15-40 min) of formalin

administration. Moreover, first and second phases of the pain response remarkably decreased in extract-pretreated groups. On the other hand, co-administration of extract and cisplatin has markedly decreased only the second of cisplatin-induced pain (91).

Leite et al. investigated the effect of (-)-α-bisabolol in test animals of visceral nociception induced by acetic acid, capsaicin, formalin, and the contribution of the NO system, $\alpha_2$, $K_{ATP}^{+}$, 5-$HT_3$ and TRPV1 receptors on mustard oil-evoked nociceptive behaviors. Mice were pretreated orally with (-)-α-bisabolol (50, 100 and 200 mg/kg) or vehicle, and the pain-related behavioral responses to intraperitoneal acetic acid or intracolonic mustard oil administration were analyzed. Consequently, (-)-α-bisabolol significantly suppressed the nociceptive behaviors independently to the administered dose (92). Leite et al. further tested the effects of (-)-α-bisabolol on two acute models of visceral pain in mice. Visceral pain was triggered by intraperitoneal administration of cyclophosphamide, a chemotherapeutic agent, or by intracolonic mustard oil loading. In groups pretreated with (-)-α-bisabolol (100 or 200 mg/kg, p.o.) the nociceptive behaviors induced by cyclophosphamide (400 mg/kg, p.o.) and mustard oil inhibited dose-independently (73).

#### 2.2.1.4.8. Antiallergic Activity

Anaphylaxis and pruritis models induced by 40/80 compound were analyzed by Chandrashekhar et al. to study the protective effect of methanolic *M. recutita* extract against acute phase of hypersensitivity reactions. The extract showed inhibitory effect on compound 48/80-stimulated anaphylaxis in mast cells. It also showed antipruritic effect dose-dependently by suppressing mast cell degranulation. The extract also reduced histamine release and lowered NO levels in serum and bronchoalveolar lavage fluid as well as stabilized mast cell membrane (93).

Since itching is one of the basic symptoms of skin allergy, skin itching in test animals is experimentally triggered by dextran, 4-aminopyridine or histamine. Wu et al. demonstrated that administration of *M. recutita* essential oil and its aqueous phase obtained during steam distillation markedly inhibited itching via suppressing the histamine released from local mast cell (94).

#### 2.2.1.4.9. Anticancer Activity

Zheng et al. studied the inhibitory activity of apigenin on the growth of human cervical carcinoma cells (HeLa) through apoptotic pathway. They concluded that apigenin remarkably decreased the viability of HeLa cells at 37-74 μM concentrations and the $IC_{50}$ value was found as 35.89 μM (95).

Hernández-Ceruelos et al. showed the inhibitory effect of *M. recutita* essential oil on the sister chromatid exchanges produced by daunorubicin and methyl methanesulfonate in mouse bone marrow cells (96).

Human cancer cell lines namely, A-549 (human lung cancer), PC-3 (human prostate cancer), and MCF-7 (human breast cancer) were examined by Zu et al. to study *M. recutita* essential oil anticancer activity. MTT assay was used to determine the cell viability. Eventually essential oil (0.002%, v/v) showed cytotoxic effects on PC-3. In essential oil-treated cells, the viable MCF-7 cells diminished to 6.9%. They concluded that *M. recutita* oil may have a potential of anticancer activity (97).

Ogata et al. examined the cytotoxic activity of bisabololoxide A which was cytometrically investigated on rat thymocytes using suitable fluorescent dyes. Bisabololoxide A, at concentrations of 30 μM or higher, markedly increased the number of dead cells, shrunken cells, and cells with phosphatidylserine exposed on membrane surface. It should be underlined that phosphatidylserine accumulation over to the surface of the cell was stimulated through apoptosis. In addition, the number of cells with hypodiploid DNA was significantly elevated by bisabololoxide A. However, this increment completely attenuated by benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone, a pan-caspase inhibitor, indicating the involvement of caspase activation (98).

Lefort and Blay studied the chemopreventive role of apigenin on gastrointestinal cancers. They reported that apigenin may further contribute beyond the available chemotherapeutics in slowing the emergence of metastatic diseases through blocking the chemokine signaling pathways, inhibiting cell adhesion molecules and remodeling of the extracellular matrix (99).

#### 2.2.1.4.10. Antimicrobial Activity

*M. recutita* polyphenols were investigated by Varoni et al. to present their protective role on oral health. The pre-clinical studies have shown its beneficial effects in the treatment of general oral diseases such as caries, periodontitis, candidiasis and oral cancer (100).

Disc diffusion method was used by Roby et al. to evaluate antimicrobial activity of *M. recutita* essential oil and its various solvent extracts on microorganisms using *Escherichia coli, Salmonella tyhpi, Staphylococcus aureus, Aspergillus flavus* and *Candida albicans*. They concluded that both essential oil and extracts dose-dependently exhibit antimicrobial activities (7.5, 10, 12.5, 15, 20 μg). Both the lowest and highest concentrations of the methanolic extract presented potent activity against *A. flavus*, while those of the ethanolic and the *n*-hexane extracts showed the highest activity against *S. aureus*. Besides, diethyl ether extract showed the highest antimicrobial activity against *C. albicans* at the lowest and the highest concentrations. Minimum inhibitory concentrations (MIC) demonstrated that *B. cereus* and *A. flavus* were the most sensitive microorganisms analyzed. On the other hand, the test materials were reported to show only a weak activity against *E. coli* (101).

#### 2.2.1.4.11. Hepatoprotective Activity

Albino rats having hepatic damage were examined by Gupta and Misra to evaluate the hepatoprotective activity of hydroalcoholic *M. recutita* extract on different biochemical parameters such as blood and liver GSH, liver $Na^+K^+$-ATPase activity, serum marker enzymes i.e. serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), serum bilirubin and liver thiobarbituric acid reactive. Rats were fed on extract (400 mg/kg b.w.) and after 5 days hepatic damage was generated by paracetamol (200 mg/kg b.w.). Consequently, the concentration of GSH in liver and in blood and liver $Na^+K^+$-ATPase activity increased whereas the level of liver thiobarbituric acid reactive substances as well as the levels of serum ALT, AST, ALP, and bilirubin decreased in extract-treated group. They further suggested the possible mechanism of extract against paracetamol-induced liver damage that interception of free radicals involved in paracetamol metabolism by microsomal

enzymes and elevation of the GSH level in blood providing an efficient protection for liver tissue against antioxidative stress (102).

#### 2.2.1.4.12. Antidiabetic Activity

Kato et al. investigated antihyperglycemic activity of aqueous *M. recutita* extract and its components such as umbelliferone, esculetin, luteolin, and quercetin. The results of the study showed that esculetin ($IC_{50}$ = 0.9 mg/mL) and quercetin ($IC_{50}$ = 72 and 71 μM) exerted moderate inhibition of sucrase enzyme. In a sucrose-loading assay, after 15 and 30 min esculetin (50 mg/kg b.w.) suppressed hyperglycemia, whereas the extract (500 mg/kg b.w.) and quercetin (50 mg/kg b.w.) were found to be less effective. On the other hand, long-term administration of the extract and quercetin provided significant inhibition of blood glucose levels. It was also found that these samples increased the liver glycogen levels. Moreover, extract ($IC_{50}$ = 16.9 μg/mL) showed potent supression against aldose reductase. In addition, umbelliferone, esculetin, luteolin and quercetin markedly inhibited the aggregation of sorbitol in human erythrocytes. Accordingly they concluded that aqueous *M. recutita* extract contributes to the prevention of the progress of hyperglycemia and diabetic obstacles (103).

STZ-induced (70 mg/kg, i.p.) diabetic rats were practiced by Cemek et al. to investigate possible antihyperglycemic and antioxidative activities of ethanolic *M. recutita* extract. The animals were grouped into four: (I) control, (II) group treated with STZ plus distilled water, (III) group administered with STZ plus 5 mg/kg glibenclamide and (IV) group treated with STZ plus 20, 50, 100 mg/kg extract. As a result, the data revealed that extract significantly reduced postprandial hyperglycemia and oxidative stress, and augmented the antioxidant status in all doses. The histological evaluation also evidenced that extract protected the majority of the pancreatic islet cells comparing to the control group. Consequently, *M. recutita* extract exhibits significant antihyperglycemic effect through protected β-cell damage stimulated by STZ, dose-dependently and restoration of hyperglycemia-related oxidative stress (104).

### 2.2.2. Literature Review on *Cynara scolymus*

#### 2.2.2.1. Phytochemical Studies

Main chemical constituents in *C. scolymus* can be divided into two groups which are phenolic and terpenic compounds **(Figure 2)**.

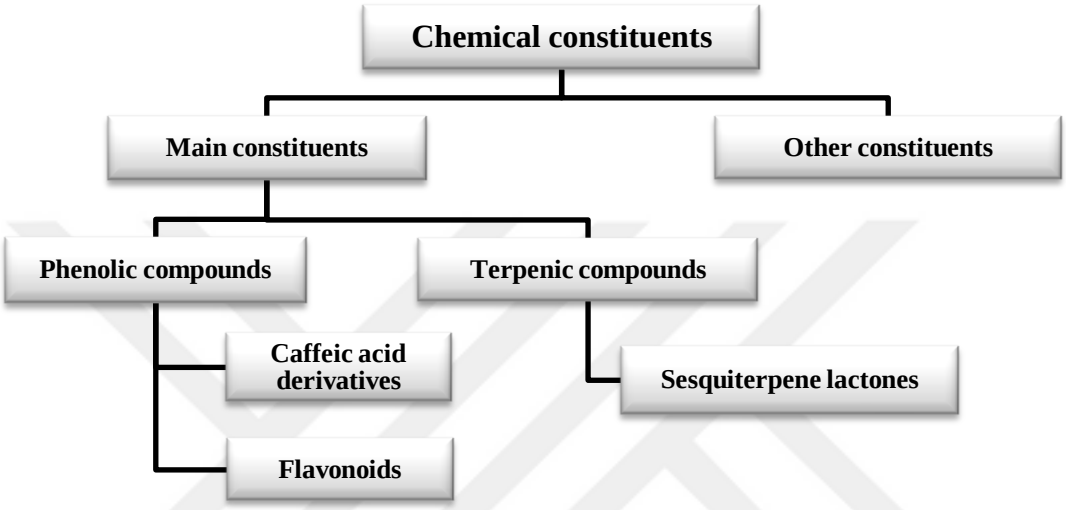



**Figure 2.** Classification of *C. scolymus* secondary metabolites

In **Table 6-12**, mostly found phenolic and terpenic compounds in *C. scolymus* are summarized.

### 2.2.2.1.1. Main Constituents

#### 2.2.2.1.1.1. Phenolic Compounds

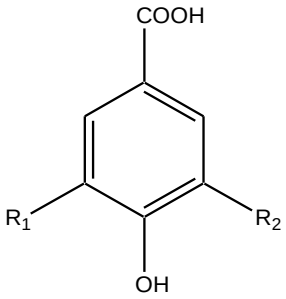



**Table 6.** Phenolic acids identified in *C. scolymus*

| **Compound** | **$R_1$** | **$R_2$** | **Ref** |
|---|---|---|---|
| Gallic acid | OH | OH | (105) |
| Protocatechuic acid | H | OH | (105) |
| Vanillic acid | OMe | H | (106) |

OMe: Methoxy

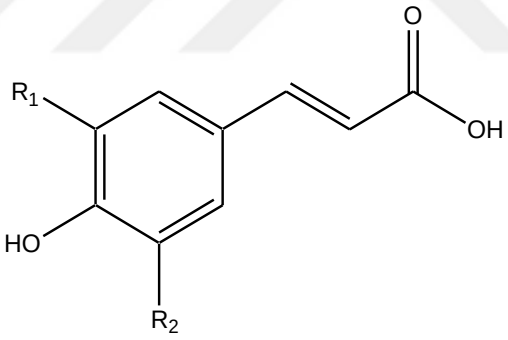



**Table 7.** Caffeic acid derivatives identified in *C. scolymus*

| **Compound** | **$R_1$** | **$R_2$** | **Ref** |
|---|---|---|---|
| Caffeic acid | OH | H | (105–108) |
| Ferulic acid | OMe | H | (106) |
| *p*-coumaric acid | H | H | (105,106) |
| Sinapic acid | OMe | OMe | (109) |

OMe: Methoxy

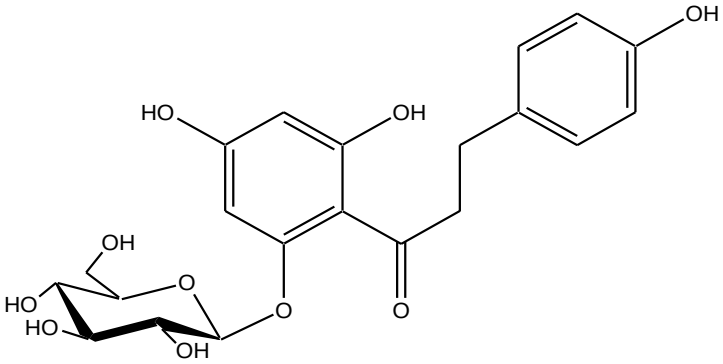



**Table 8.** Chalcone derivatives identified in *C. scolymus*

| **Compound** | **Ref** |
|---|---|
| Phloretin 2-*O*-glucoside (Phloridzin) | (105) |

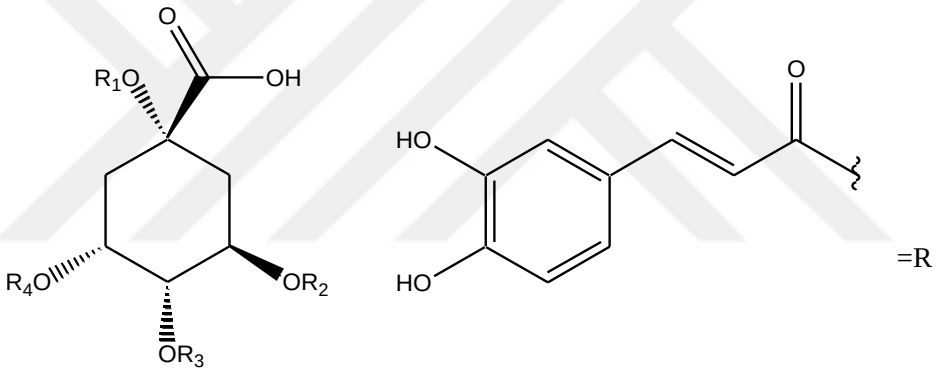



**Table 9.** Quinic acid derivatives identified in *C. scolymus*

| **Compound** | **$R_1$** | **$R_2$** | **$R_3$** | **$R_4$** | **Ref** |
|---|---|---|---|---|---|
| 1-*O*-caffeoylquinic acid | R | H | H | H | (107,110) |
| 3-*O*-caffeoylquinic acid (Neochlorogcnic acid) | H | R | H | H | (107,108,110) |
| 4-*O*-caffeoylquinic acid (Cryptochlorogenic acid) | H | H | R | H | (107,108,110) |
| 5-*O*-caffeoylquinic acid (Chlorogenic acid) | H | H | H | R | (105,107,108,110) |
| 1,3-*O*-dicaffeoylquinic acid (Cynarin) | R | R | H | H | (107,108,110) |
| 1,4-*O*-dicaffeoylquinic acid | R | H | R | H | (111) |
| 1,5-dicaffeoylquinic acid | R | H | H | R | (107,108,110) |
| 3,4-dicaffeoylquinic acid | H | R | R | H | (107,110) |
| 3,5-dicaffeoylquinic acid | H | R | H | R | (107,110) |
| 4,5-dicaffeoylquinic acid | H | H | R | R | (107,110) |
| Syringic acid | H | Me | H | Me | (106) |

Me: Methyl; R: Caffeoyl

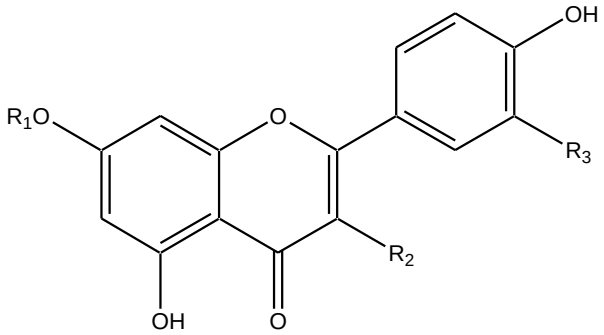



**Table 10.** Flavonoids identified in *C. scolymus*

| **Compound** | **$R_1$** | **$R_2$** | **$R_3$** | **Ref** |
|---|---|---|---|---|
| Apigenin | H | H | H | (105) |
| Chrysoeriol | H | H | OMe | (105) |
| Luteolin | H | H | OH | (105,108) |
| Quercetin | H | OH | OH | (105) |
| Apigenin 7-*O*-glucoside | Glc | H | H | (105,107) |
| Apigenin 7-*O*-glucuronide | GluA | H | H | (105,107) |
| Apigenin 7-*O*-rutinoside (Isorhoifolin) | Rut | H | H | (105,107) |
| Luteolin 7-*O*-galactoside | Gal | H | OH | (105) |
| Luteolin 7-*O*-glucoside (Cynaroside) | Glc | H | OH | (105,107,108) |
| Luteolin 7-*O*-(6`-malonyl)glucoside | Glc-Mal | H | OH | (112,113) |
| Luteolin 7-*O*-glucuronide | GluA | H | OH | (105,107,108) |
| Luteolin 7-*O*-hexoside | Hexose | H | OH | (114) |
| Luteolin 7-*O*-neohesperidoside | Neo | H | OH | (105) |
| Luteolin 7-*O*-rhamnoside (Scolimoside) | Rha | H | OH | (105) |
| Luteolin 7-*O*-rutinoside | Rut | H | OH | (105,108) |
| Quercetin 3-*O*-arabinoside (Avicularin) | H | OAra | OH | (105) |
| Quercetin 3-*O*-galactoside (Hyperoside) | H | OGal | OH | (105) |
| Quercetin 3-*O*-glucoside (Isoquercitrin) | H | OGlc | OH | (105) |
| Quercetin 3-*O*-rhamnoside (Quercitrin) | H | ORha | OH | (105) |
| Quercetin 3-*O*-rutinoside (Rutin) | H | ORut | OH | (105) |

Ara: Arabinose; Gal: Galactose; Glc: Glucose; GluA: Glucuronide; Mal: Malonyl; Neo: Neohesperidoside; OMe: Methoxy; Rha: Rhamnose; Rut: Rutinose

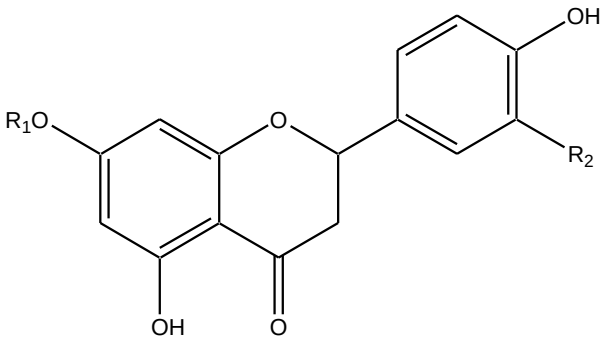



**Table 10.** Continued.

| Compound | $R_1$ | $R_2$ | Ref |
|---|---|---|---|
| Eriodictyol | H | OH | (105) |
| Naringenin | H | H | (105) |
| Naringenin 7-*O*-glucoside (Prunin) | Glc | H | (105,107) |
| Naringenin 7-*O*-neohesperidoside (Naringin) | Neo | H | (105) |
| Naringenin-7-*O*-rutinoside (Narirutin) | Rut | H | (107,110) |

Glc: Glucose; Neo: Neohesperidoside; Rut: Rutinose

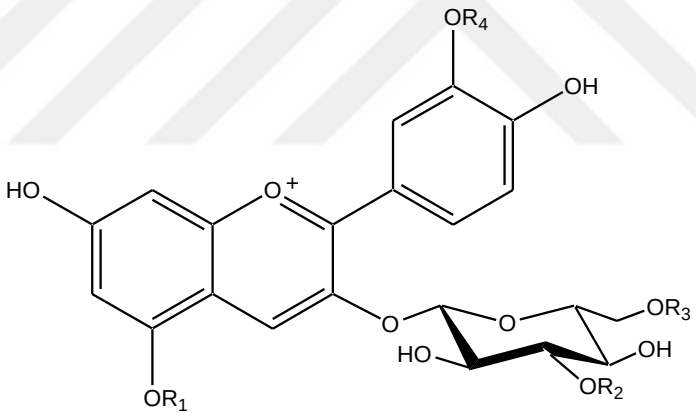



**Table 11.** Anthocyanins identified in *C. scolymus*

| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Ref |
|---|---|---|---|---|---|
| Cyanidin 3-*O*-glucoside | H | H | H | H | (115) |
| Cyanidin 3-*O*-(3''-malonyl)glucoside | H | Mal | H | H | (115) |
| Cyanidin 3-*O*-(6''-malonyl)glucoside | H | H | Mal | H | (115) |
| Cyanidin 3,5-*O*-diglycoside | Glc | H | H | H | (115) |
| Peonidin 3-*O*-glucoside | H | H | H | Me | (115) |
| Peonidin 3-*O*-(6''-malonyl)glucoside | H | H | Mal | Me | (115) |

Glc: Glucose; Mal: Malonyl; Me: Methyl

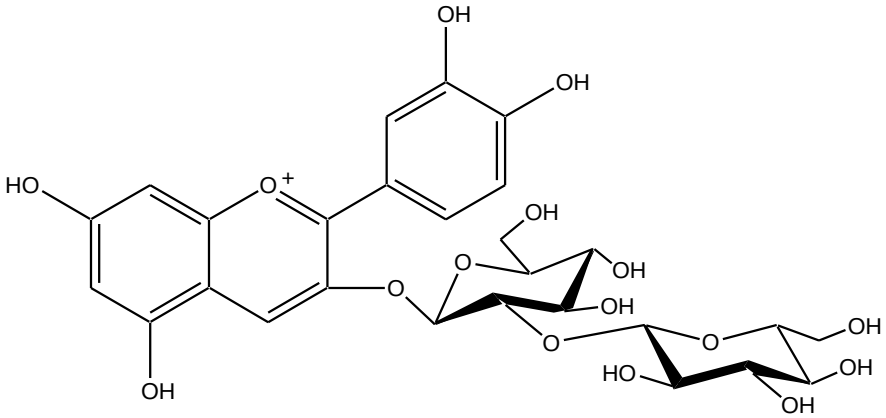



**Table 11.** Continued.

| Compound | Ref |
|---|---|
| Cyanidin 3-*O*-sophoroside | (115) |

### 2.2.2.1.1.2. Terpenic Compounds

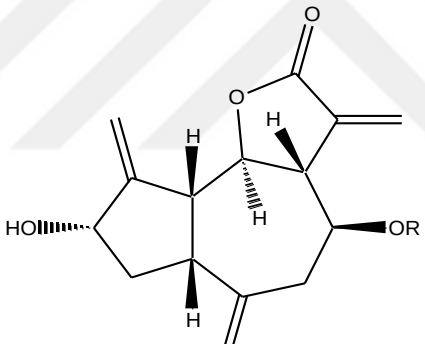
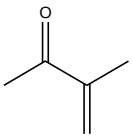
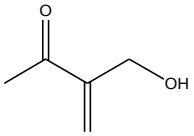
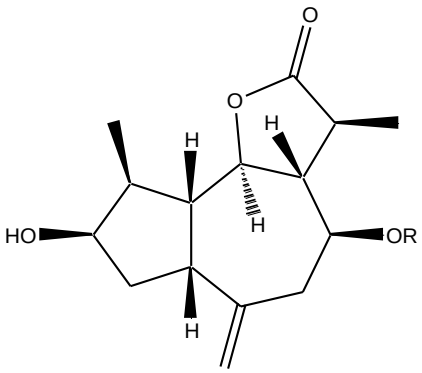
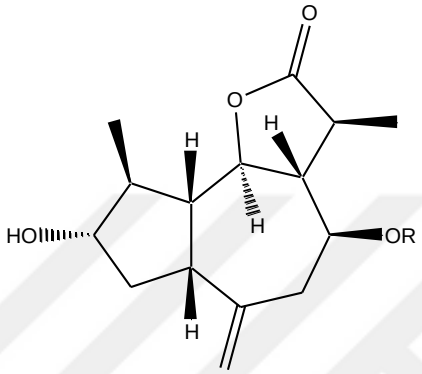



**Table 12.** Terpenic compounds identified in *C. scolymus*

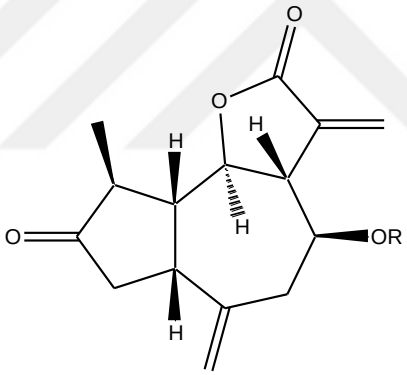
| Compound | Chemical Group | R | Ref |
|---|---|---|---|
| Auerin | Sesquiterpene lactone |  | (116) |
| Cynaropicrin | Sesquiterpene lactone |  | (116,117) |




**(1)**




**(2)**

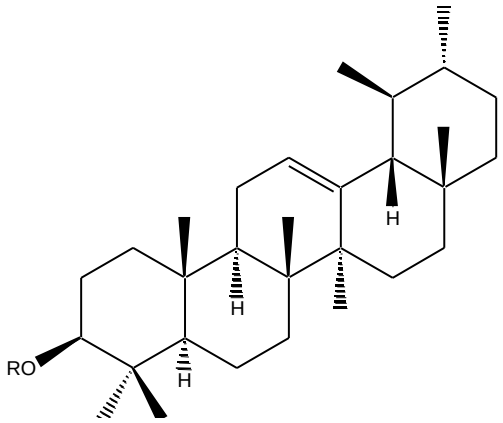



**(3)**

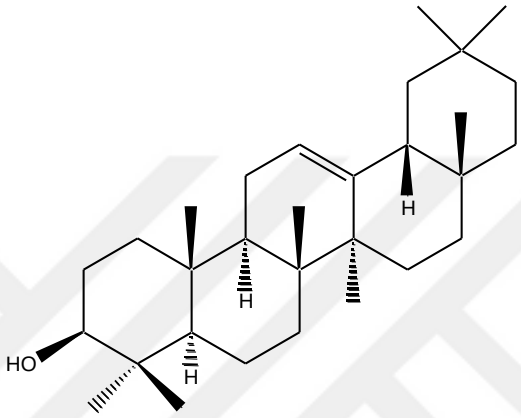
**Table 12.** Continued.

| Compound | Chemical Group | R | Ref |
|---|---|---|---|
| Cynarascolide (1) | Sesquiterpene lactone | H | (116) |
| Isolipidiol (2) | Sesquiterpene lactone | H | (116,117) |
| Grosheimin (3) | Sesquiterpene lactone | H | (116) |
| Cynarascoloside A (1) | Sesquiterpene glycoside | Glc | (116) |
| Cynarascoloside B (2) | Sesquiterpene glycoside | Glc | (116) |
| Cynarascoloside C (3) | Sesquiterpene glycoside | Glc | (116) |

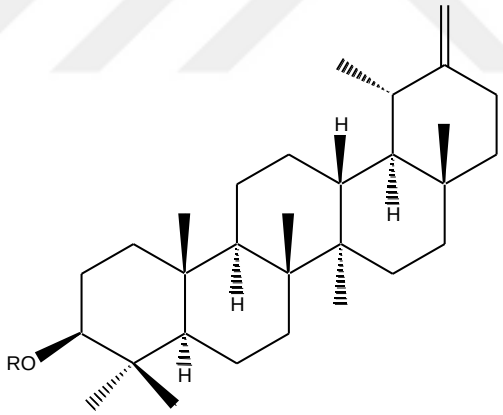
Glc: Glucose




**(1, 2)**




**(3)**




**(4, 5)**

**Table 12.** Continued.

| Compound | Chemical Group | R | Ref |
|---|---|---|---|
| α-amyrin (1) | triterpene | H | (118) |
| α-amyrin acetate (2) | triterpene | Ac | (118) |
| β-amyrin (3) | triterpene | - | (118) |
| Taraxasterol (4) | triterpene | H | (118,119) |
| Taraxasterol acetate (5) | triterpene | Ac | (118) |

Ac: Acetyl

#### 2.2.2.2. Analytical Studies

*C. scolymus* components particularly caffeoylquinic acid derivatives such as caffeic acid, ChA and cynarin and flavonoids mostly L7G have been investigated either in its leaf or other plant parts (bract, stem and receptacle) or dietary supplements (dragées, effervescent tablets, juice) by several qualitative and quantitative methods using different techniques. These methods are based on HPLC or UPLC coupled with UV (109,120,121) or DAD (110,122) or MS (108,112–115,117,123–125) or $MS^2$ (105,107,115,126–131), TLC (57,132,133) and micellar electrokinetic capillary chromatography (MEKC) detections (134).

Moreover, volatile components of *C. scolymus* leaf have been determined and quantified as percentage composition by GC-MS (135–137).

Analytical studies on *C. scolymus* are briefly summarized in **Table 13**.

**Table 13.** Analytical studies on *C. scolymus*

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **1** | Methanolic leaf extract | HPLC-UV/Vis<br>λ = 280 nm | Hypersil ODS $C_{18}$ column (4 µm, 250 mm x 4.6 mm, i.d) | **A:** acetonitrile<br>**B:** water (0.2% sulphuric acid)<br>**Gradient system:** 15% A (0-12 min), 40% A (12-14 min), 60% A (14-18 min), 80% A (18-20 min), 90% A (20-24 min), 100% A (24-28 min)<br>**Flow rate:** 0.5 mL/min | **Qualitative analysis**<br>gallic acid, sinapic acid, ChA, epicatechin, syringic acid, vanillic acid, rosmarinic acid, *p*-coumaric acid, ferulic acid, quercitrin, quercetin, *trans*-cinnamic acid, apigenin, amentoflavone | (109) |
| **2** | Acetone-ethanol-methanol (70:15:15) leaf head, bract and receptacle extracts | HPLC-UV<br>λ = 280 nm | Khromasil KR 100-5 $C_{18}$ column (250 mm x 4.6 mm, i.d) | **A:** water (0.1% TFA)<br>**B:** 95% acetonitrile (0.1% TFA)<br>**Gradient system:** 10% B (0 min), 50% B (30 min) 100% B (5 min), hold at 100% B for 2 min, 10% B (2 min) and back to the initial 10% B (10 min)<br>**Flow rate:** 1 mL/min | **Qualitative and quantitative analysis**<br>ChA, *p*-coumaric acid, ferulic acid, cynarin, luteolin, apigenin<br>**Major component(s):** ChA (inner bracts from Tondo di Paestum, Bianco di Pertosa and Violet de Provence, 8.14, 6.79 and 5.5 µM/g, respectively). | (120) |
| **3** | Aqueous and hydroalcoholic mature and baby head extracts | HPLC-UV/Vis<br>λ = 326 nm | Lichrospher $C_{18}$ column (5 µm, 250 mm x 4.0 mm, i.d) | **A:** water (pH 2.4)<br>**B:** acetonitrile<br>**Gradient system:** 92% A and 8% B to 30% A and 70% B within 50 min<br>**Flow rate:** 0.4 mL/min | **Qualitative and quantitative analysis**<br>caffeic acid, ChA and cynarin<br>**Major component(s):** ChA (456.30 mg/100 g in baby and cooked extract).<br>Note: effects of cooking were investigated. | (121) |

**Table 13.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **4** | Hydroalcoholic dietary supplements | HPLC-DAD<br>λ = 280-350 nm | Phenomenex Hydro-Synergi $C_{18}$ (4 µm, 150 x 3.0 mm i.d.) | **A:** water (2% acetic acid)<br>**B:** water (5% acetic acid-acetonitrile (50:50, v/v))<br>**Gradient system:** 10-18% B (20 min), 18-24% B (10 min), 24-30% B (15 min), 30% B isocratic (20 min), 30-55% B (5 min), 55-100% B (5 min), 100% B isocratic (8 min), 100-10% B (2 min)<br>**Flow rate:** 0.4 mL/min | **Qualitative and quantitative analysis**<br>1-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, ChA, 1,3-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid, L7G, luteolin 7-*O*-glucuronide, luteolin 7-*O*-rutinoside, A7G, apigenin 7-*O*-glucuronide, apigenin 7-*O*-rutinoside, naringenin 7-*O*-glucoside, and narirutin<br>**Major component(s):** ChA (36.7-67.8%) and cynarin (34.2-51.6%). | (110) |
| **5** | Hydroalcoholic leaf extract | HPLC-DAD<br>λ = 210 and 330 nm | Phenomenex Luna $C_{18}$ column (5 µm, 250 mm x 2 mm, i.d) | **A:** water (0.03% TFA)<br>**B:** acetonitrile<br>**Gradient system:** 90A:10B to 64A:36B (35 min)<br>**Flow rate:** 0.2 mL/min | **Qualitative and quantitative analysis**<br>pseudochlorogenic acid, neochlorogenic acid, ChA, cryptochlorogenic acid, cynarin, 3,4-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid<br>**Major component(s):** monocaffeoylquinic acids expressed as ChA (4.24 %). | (122) |
| **6** | Aqueous and hydroalcoholic commercial and leaf extracts | HPLC-DAD<br>λ = 326 nm<br>HPLC-APCI-MS | Chrompack Intersil C3 (3 µm, 150 x 3.0 mm i.d.)<br>Pre-column: $C_3$ (5 µm, 10 mm x 2 mm, i.d) | **A:** water (pH 2.4)<br>**B:** acetonitrile<br>**Gradient system:** 92% A to 30%A (50 min)<br>**Flow rate:** 0.4 mL/min | **Qualitative and quantitative analysis**<br>2-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, ChA, 4-*O*-caffeoylquinic acid, caffeic acid, 1,5-*O*-dicaffeoylquinic acid, luteolin *O*-monoglucoside, L7G, luteolin 7-*O*-rutinoside, 1,3-*O*-dicaffeoylquinic acid, luteolin 7-*O*-glucuronide, luteolin<br>**Major component(s):** cynarin (30985 mg/kg) and ChA (24088 mg/kg). | (108) |

**Table 13.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **7** | Hydroalcoholic leaf, outer bract, head and stem extracts | HPLC-DAD<br>λ = 254-350 nm<br>HPLC-APCI-MS | Merck LiChrosorb $C_{18}$ column (5 µm, 250 mm x 4.6 mm, i.d) | **A:** water (pH 3.2)<br>**B:** acetonitrile<br>**Gradient system:** 11% B (0-5 min), 20% B (10-15 min) and 100% (25-33 min)<br>**Flow rate:** 0.8 mL/min | **Qualitative analysis**<br>1-*O*-caffeoylquinic acid, ChA, 1,5-*O*-dicaffeoylquinic acid, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, caffeic acid, luteolin 7-*O*-rutinoside, luteolin 7-*O*-glucuronide, L7G, apigenin 7-*O*-glucuronide, luteolin malonylglucoside, luteolin | (112) |
| **8** | Hydroalcoholic leaf extract | HPLC-DAD<br>λ = 254-350 nm<br>HPLC-APCI-MS | Phenomenex Luna $C_{18}$ column (5 µm, 150 mm x 4.6 mm, i.d) with a security guard $C_{18}$ (4 mm x 3 mm, i.d) | **A:** water (pH 3.2)<br>**B:** acetonitrile<br>**Gradient system:** 20% B (0-5 min), 30% B (7-13 min), 100% B (20-30 min)<br>**Flow rate:** 0.6 mL/min | **Qualitative and quantitative analysis**<br>1-*O*-caffeoylquinic acid, ChA, 1,5-*O*-dicaffeoylquinic acid mono and disuccinyl dicaffeoylquinic acid derivatives, luteolin 7-*O*-rutinoside, L7G, luteolin-7-*O*-malonylglucoside, luteolin aglycone, A7G, apigenin 7-*O*-glucuronide<br>**Major component(s):** ChA (73.68 µmol/g). | (113) |
| **9** | Hydroalcoholic dietary supplements | HPLC-DAD<br>λ = 254 nm<br>HPLC-ESI-MS | LiChrospher $C_{18}$ column (5 µm, 250 mm x 4.6 mm, i.d) | **A:** isopropyl alcohol-acetonitrile-methanol-0.3 % aqueous formic acid (18:30:12:40, v/v/v/v)<br>**B:** water (0.3% formic acid)<br>**Gradient system:** 8% (A) (0 min) to 48% (A) (35 min)<br>**Flow rate:** 1 mL/min | **Qualitative and quantitative analysis**<br>8-deoxy-11-hydroxy-13-chlorogrosheimin, ChA, cryptochlorogenic acid, neochlorogenic acid, cynarin, cynaratriol (tentatively), grosheimin, 8-deoxy-11,13-dihydroxygrosheimin, luteolin-7-*O*-rutinoside, L7G, and cynaropicrin<br>**Major component(s):** ChA (0.35-18.34 mg/g); cynaropicrin (up to 22.6%). | (117) |

**Table 13.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **10** | Residue derived from hydroalcoholic head during industrial processing | HPLC-DAD<br>λ = 280, 320 and 365 nm<br>HPLC-ESI-MS-MS | Phenomenex Luna $C_{18}$ column (3.5 µm, 50 mm x 2.1 mm, i.d)<br>Pre-column: $C_{18}$ (4 mm x 3 mm, i.d) | **A:** water (0.1% formic acid)<br>**B:** acetonitrile (0.1% formic acid)<br>**Gradient system:** 94% A (0 min), 83.5% A (5 min), 82.5% (7 min), 81.5% A (12.5 min), 0% A (21 min), 94% (23 min)<br>**Flow rate:** 0.4 mL/min | **Qualitative analysis**<br>Caffeoylquinic acid derivatives, gallic acid, protocatechuic acid, esculin, *p*-coumaric acid-*O*-glucoside, ChA, caffeic acid, eriodictyol-glucuronide, quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, luteolin 7-*O*-rutinoside, L7G, quercetin 3-*O*-glucoside, luteolin 7-*O*-glucuronide, luteolin 7-*O*-galactoside, quercetin 3-*O*-arabinoside, apigenin 7-*O*-rutinoside, quercetin *O*-pentoside, quercetin 3-*O*-rhamnoside, luteolin 7-*O*-neohesperidoside, naringenin 7-*O*-glucoside, naringenin 7-*O*-neohesperidoside, luteolin 7-*O*-rhamnoside, apigenin 7-*O*-glucuronide, phloretin 2-*O*-glucoside, luteolin, quercetin, naringenin, apigenin, chrysoeriol | (105) |
| **11** | Hydroalcoholic head and pomace extracts and juice | HPLC-DAD<br>λ = 280-350 nm<br>HPLC-ESI-MS-MS | Phenomenex Hydro-Synergi $C_{18}$ (4 µm, 150 x 3.0 mm i.d.) | **A:** water (2% acetic acid)<br>**B:** water (5% acetic acid)-acetonitrile (50:50, v/v)<br>**Gradient system:** 10-18% B (20 min), 18-24% B (10 min), 24-30% B (15 min), 30% B isocratic (20 min), 30-55% B (5 min), 55-100% B (5 min), 100% B isocratic (8 min), 100-10% B (2 min)<br>**Flow rate:** 0.4 mL/min | **Qualitative and quantitative analysis**<br>1-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, ChA, cynarin, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid, L7G, luteolin 7-*O*-glucuronide, luteolin 7-*O*-rutinoside, A7G, apigenin 7-*O*-glucuronide, apigenin 7-*O*-rutinoside, naringenin 7-*O*-glucoside, narirutin<br>**Major component(s):** 1,5-*O*-dicaffeoylquinic acid (3889.9 mg/kg in head and 3268.8 mg/kg in pomace), ChA (3143 mg/kg in head) and apigenin 7-glucuronide (1318.6 mg/kg in pomace). | (107) |

**Table 13.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **12** | Methanolic leaf extract and dietary supplements | HPLC-DAD<br>λ = 280 and 320 nm<br>HPLC-ESI-MS-MS | Phenomenex Gemini $C_{18}$ column (5 μm, 250 x 3.0 mm i.d.) | **A:** acetonitrile<br>**B:** water-formic acid (100:0.1, v/v)<br>**Gradient system:** 20% A (0 min), 25% A (10 min), 25% A (20 min), 50% A (40 min), 100% A (42–47 min), 20% A (49–55 min)<br>**Flow rate:** 0.4 mL/min | **Qualitative and quantitative analysis**<br>3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 1,3-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic, luteolin 7-*O*-rutinoside, luteolin 7-*O*-hexoside, luteolin 7-*O*-glucuronide, luteolin *C*-hexoside, luteolin *O*-diacetylhexoside, luteolin *O*-acetylhexoside, luteolin *O*-acylated, apigenin 7-*O*-rutinoside, apigenin 7-*O*-hexoside, apigenin 7-*O*-glucuronide, quercetin 7-*O*-rutinoside, quercetin 7-*O*-neohesperidoside<br>**Major component(s):** ChA (32.5 mg/100g dragées) and cynarin (23.5 mg/100g leaf). | (114) |
| **13** | Aqueous acetone head extract | HPLC-DAD<br>λ = 520 nm<br>HPLC-ESI-MS-MS | Phenomenex Hydro-Synergi $C_{18}$ column (4 μm, 150 mm×3.0 mm)<br>Pre-column: $C_{18}$ (4 mm×3.0 mm) | **A:** water-formic acid-acetonitrile (87:10:3, v/v/v)<br>**B:** water-formic acid-acetonitrile (40:10:50, v/v/v)<br>**Gradient system:** 5-12% B (7 min), 12-18% B (8 min), 18-25% B (5 min), 25-40% B (10 min), 40-100% B (2 min), 100% B (8 min), 5% B (5 min)<br>**Flow rate:** 0.4 mL/min | **Qualitative and quantitative analysis**<br>cyanidin 3,5-diglucoside, cyanidin 3-sophoroside, cyanidin 3-glucoside, cyanidin 3,5-malonyldiglucoside, cyanidin 3-(3"-malonyl)glucoside, delphinidin glycoside, cyanin pendoside, cyanidin 3-(6"-malonyl)glucoside, peonidin 3-(6"-malonyl)glucoside<br>**Major component(s):** cyanidin 3-(6"-malonyl)glucoside (1234.3 mg/kg) in cultivar ‘Camus’. | (115) |

**Table 13.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **14** | Hydroalcoholic immature inflorescence extract | HPLC-DAD<br>λ = 280, 310 and 350 nm<br>HPLC-ESI-MS-MS | Zorbax Eclipse XDB $C_{18}$ (1.8 µm, 50 mm x 4.6 mm, i.d) | **A:** water (1% formic acid)<br>**B:** acetonitrile<br>**Gradient system:** 5-10% B (5 min), 40% B (20 min), 90% B (25-29 min)<br>**Flow rate:** 0.5 mL/min | **Qualitative and quantitative analysis**<br>1-caffeoylquinic acid, 5-caffeoylquinic acid, 1,5-caffeoylquinic acid, luteolin 7-*O*-rutinoside, L7G, luteolin 7-*O*-glucuronide, luteolin, apigenin 7-*O*-rutinoside, A7G, apigenin 7-*O*-glucuronide, apigenin<br>**Major component(s):** apigenin 7-*O*-glucuronide (7068 mg/kg) in Altilis cultivar. | (123) |
| **15** | Hydroalcoholic receptacle | HPLC-DAD<br>λ = 230-330 nm<br>HPLC-ESI-QTOF-MS-MS | Agilent Zorbax $C_{18}$ column (1.8 µm, 150 mm x 4.6 mm, i.d) | **A:** water (0.5% acetic acid)<br>**B:** acetonitrile<br>**Gradient system:** 0% B (0 min), 20% B (10 min), 30% B (15 min), 50% B (20 min), 75%, B (25 min), 100% B (30 min), 0% B (37 min) and finally, the initial conditions were maintained for 3 min<br>**Flow rate:** 0.8 mL/min | **Qualitative analysis**<br>protocatechuic acid 4-*O*-hexoside, syringic acid *O*-hexoside, ChA, several caffeoylquinic acid derivatives, *p*-coumarylglucose, dihydroxypropiophend-hexoside, caffeoyl-hexoside, *p*-coumaric acid, 3-*p*-coumarylquinic acid, 3-*O*-feruloylquinic acid, 3-*p*-coumaroyl-4-caffeoylquinic acid, quercetin 3-*O*-rutinoside, apigenin 4-*O*-glucoside, A7G, apigenin 7-*O*-rutinoside, apigenin 7-*O*-glucuronide, apigenin 7-*O*-(6″-*O*-acetyl)glucoside, apigenin 7-(6″-*O*-apiosyl)-glucoside, apigenin 4-*O*-(6″-O-malonyl)glucoside, L7G, luteolin 7-*O*-rutinoside, luteolin 7-*O*-glucuronide, naringenin 7-*O*-rutinoside, lignan derivatives. | (124) |

**Table 13.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **16** | Edible parts prone to different cooking methods | HPLC-UV/Vis $\lambda$ = 280 and 330 nm HPLC-APCI-MS-MS | Phenomenex Prodigy ODS $C_3$ column (5 µm, 250 × 4.6 mm, i.d) | **A:** water (0.2% formic acid) **B:** acetonitrile-methanol (60:40, v/v) **Gradient system:** 20-30% B (6 min), 30-40% B (10 min), 40-50% B (8 min), 50-90% B (8 min), 90-90% B (3 min), 90-20% B (3 min) **Flow rate:** 0.8 mL/min | **Qualitative and quantitative analysis** luteolin 7-*O*-rutinoside, L7G, luteolin 7-*O*-glucuronide, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, narirutin, apigenin 7-*O*-rutinoside, 1,5-*O*-dicaffeoylquinic acid, naringenin 7-*O*-glucoside, A7G, 4,5-*O*-dicaffeoylquinic acid, apigenin 7-*O*-glucuronide **Major component(s):** ChA (3050 mg/kg) and 1,5-di-*O*-caffeoylquinic acid (3180 mg/kg) in raw. Note: caffeoylquinic acid content was increased in cooked samples due to isomerization. Dicaffeoylquinic content of steamed and fried samples was similar and higher than the boiled samples. Flavonoid content was decreased in all cooking methods, especially frying. | (126) |
| **17** | Hydroalcoholic leaf and stem extracts | HPLC-DAD $\lambda$ = 280, 310 and 350 nm HPLC-ESI-MS-MS | Zorbax Eclipse XDB $C_{18}$ (1.8 µm, 50 mm x 4.6 mm, i.d) | **A:** water (1% formic acid) **B:** acetonitrile **Gradient system:** 5-10% B (5 min), 40% B (20 min), 90% B (25-29 min) **Flow rate:** 0.5 mL/min | **Qualitative and quantitative analysis** 1-caffeoylquinic acid, 3-caffeoylquinic acid, 5-caffeoylquinic acid, 3,5-caffeoylquinic acid, 1,5-caffeoylquinic acid, monosuccinyldicaffeoylquinic acid, luteolin rutinoside, luteolin glucoside, luteolin glucuronide, luteolin malonylglucoside, luteolin, apigenin rutinoside, apigenin glucoside, apigenin glucuronide, apigenin malonylglucoside, apigenin **Major component(s):** ChA (13.3 g/kg) and luteolin glucoside (4.2 g/kg) in stem and leaf from Violetto di Sicilia, respectively. | (128) |

**Table 13.** Continued.

| No. | Extract | Method | Column | Mobile Phase | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|
| **18** | Acetone-ethanol-methanol (70:15:15) leaf and head extracts | HPLC-DAD<br>λ = 280-350 nm<br>HPLC-ESI-MS-MS | Phenomenex Luna $C_{18}$ (5 µm, 250 mm x 4.6 mm, i.d) | **A:** water (0.01% TFA)<br>**B:** 95% acetonitrile (0.01% TFA)<br>**Gradient system:** 10 B (0 min), 50% B (30 min), 100% B (35 min)<br>**Flow rate:** 1 mL/min | **Qualitative and quantitative analysis**<br>1-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, ChA, 1,3-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, luteolin 7-*O*-rutinoside, L7G, luteolin 7-*O*-glucuronide, luteolin, apigenin 7-*O*-rutinoside, A7G<br>**Major component(s):** L7G 34043.69 mg/kg and 69658.27 mg/kg in head and leaf from Violet Provence genotype, respectively. | (129) |
| **19** | Hydroalcoholic leaf, stem, receptacle and bract extracts | HPLC-DAD<br>λ = 280-350 nm<br>HPLC-ESI-MS-MS | Phenomenex Hydro-Synergi $C_{18}$ (4 µm, 150 mm x 3.0 mm, i.d)<br>Pre-column: $C_{18}$ (4 mm x 3.0 mm, i.d) | **A:** water (2% acetic acid)<br>**B:** water (0.5% acetic acid)-acetonitrile (50:50, v/v)<br>**Gradient system:** 10-18% B (20 min), 18-24% B (10 min), 24-30% B (15 min), 30% B isocratic (20 min), 30-55% B (5 min), 55-100% B (5 min), 100% B isocratic (8 min) and 100-10% B (2 min)<br>**Flow rate:** 0.4 mL/min | **Qualitative and quantitative analysis**<br>1-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, ChA, 4-*O*-caffeoylquinic acid, caffeic acid, cynarin, luteolin 7-*O*-rutinoside, L7G, luteolin 7-*O*-glucuronide, narirutin, naringenin 7-*O*-glucoside, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, apingenin 7-*O*-rutinoside, A7G<br>**Major component(s):** apigenin 7-*O*-glucuronide (6298 mg/kg in 'Romanesco clone C3' receptacle) and ChA (14841 mg/kg in the inner bracts of Violetto di Sicilia). | (130) |
| **20** | Hydroalcoholic bract and receptacle extracts | HPLC-DAD<br>λ = 280, 310 and 350 nm<br>HPLC-ESI-MS-MS | Agilent Zorbax Eclipse XDB-$C_{18}$ (1.8 µm, 50 mm x 4.6 mm, i.d) | **A:** water (1% formic acid)<br>**B:** acetonitrile<br>**Gradient system:** 5-10% B (5 min), 40% B (20 min), 90% B (25-29 min)<br>**Flow rate:** 0.5 mL/min | **Qualitative and quantitative analysis**<br>1-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, ChA, 1,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, cynarin, luteolin 7-*O*-rutinoside, L7G, luteolin 7-*O*-glucuronide, luteolin, A7G, apigenin 7-*O*-glucuronide, apigenin malonyl-glucoside, apigenin<br>**Major component(s):** ChA (4158 mg/kg in inner bracts of Violetto di Sicilia). | (131) |

**Table 13.** Continued.

| No. | Extract | Method | Stationary Phase | Developing Solvent System | Derivatization | Detection | Analyzed compounds | Ref |
|---|---|---|---|---|---|---|---|---|
| **1** | Methanolic leaf extract | TLC | TLC silica gel 60 $F_{254}$ | dichloromethane-acetone (60:20, v/v) | Anisaldehyde reagent | Visualizer: Vis | **Qualitative analysis** sesquiterpene lactones | (57) |
| **2** | Methanolic leaf extract | TLC | TLC silica gel 60 $F_{254}$ | ethyl acelate-formic acid-acetic acid-water (100:11:11:26, v/v/v/v) | NP/Polyethylene glycol (PEG) 400 | Visualizer: 366 nm | **Qualitative analysis** caffeoylquinic acids and flavonoids | (57) |
| **1** | Hydroalcoholic dietary supplements | HPTLC | HPTLC silica gel 60 $F_{254}$ | dichloromethane-acetone (75:25, v/v) | Anisaldehyde reagent | Visualizer: Vis | **Qualitative analysis** sesquiterpene lactones | (117) |
| **2** | Aqueous leaf extract | HPTLC | HPTLC silica gel 60 $F_{254}$ | ethyl acelate-formic acid-acetic acid-water (100:11:11:26, v/v/v/v) | NP/PEG 400 | Scanner: absorption (313 nm) or fluorescence (cutoff filter: 395 nm) modes Visualizer: 366 nm | **Qualitative and quantitative analysis** ChA and cynarin | (132) |

### 2.2.2.3. Traditional Usage

Traditionally, *C. scolymus* leaf is frequently used as tea either by simmering or boiling and its dry or soft extracts has been practiced for their several health benefits. Moreover, *C. scolymus* other plant parts such as bracts, stem and receptacle have been eaten as a healthy vegetable for centuries **(Table 14)**.

**Table 14.** Traditional usage of *C. scolymus*

| Part(s) used | Administration; Preparation; Dosage | Usage |
|---|---|---|
| Leaf | Internally;<br>-Infusion; 1.5 g powdered dried leaf in 150 mL or 3 g in 150 ml of boiling water, 4 times or 1-2 times daily;<br>-Powdered leaf, 600-1500 mg daily (138);<br>-Aqueous extract, 600-1320 mg daily;<br>-Aqueous extract of fresh leaf, 900-2400 mg daily;<br>-Soft aqueous extract of fresh leaf, 600-1200 mg daily;<br>-Soft ethanolic extract 20% (v/v), 2.1 g daily (138) | Cholagogue, diuretic (14,138), hepatoprotective, cholesterol-reducing, relieve digestive complaints (stomach ache, feeling of fullness and flatulence (138). |
| Leaf and flower | Internally; infusion (14) | Appetizer, diuretic, cholagogue (14), stomach ache (64). |
| | Internally; decoction; drink one teacup 3 times a day for 3-4 weeks (67) | Anorexia, appetizer, diuretic, cholagogue, nephralgia, kidney stones, cancer (67). |
| Receptacle | Internally; cooked; eaten (64) | - |
| Whole plant | Internally; fresh; eaten (139) | Liver diseases, diabetes (139). |

#### 2.2.2.4. Bioactivity Studies

*C. scolymus* leaf as an herbal drug has long been used in folk medicine mostly for its hepatoprotective and cholagogue activities. In addition to its folkloric practice, *C. scolymus* has antihyperlipidemic, hepatoprotective, antidyspeptic, antihyperglycemic, cardioprotective, anticancer, antibacterial, antioxidant and antihypertensive effects.

##### 2.2.2.4.1. Antihyperlipidemic Activity

Gebhardt observed that aqueous *C. scolymus* leaf extract at 1 mg/mL significantly supressed cholesterol biosynthesis from $^{14}$C-acetate in primary cultured rat hepatocytes in a biphasic manner. Inhibitory mechanism of the extract could be because of indirectly modulating 3-hydroxy-3-methylglutaryl-CoA reductase activity. Among the several constituents of *C. scolymus* extracts, L7G and particularly its aglycone luteolin were considered to be main components responsible for the activity. However, ChA was found to be less effective and caffeic acid, cynarin and other dicaffeoylquinic acids did not have any significant influences. Also, the effect of the insulin on cholesterol biosynthesis was effectively inhibited by luteolin. These results evidenced that *C. scolymus* extracts suppress hepatic cholesterol biosynthesis in an indirect but effective manner. Therefore, extract may provide normal lipid profile in man (140).

Shimoda et al. comparatively investigated the serum triglyceride (TG) levels in olive oil-loaded mice which were either treated with methanolic *C. scolymus* leaf extract (125-500 mg/kg, p.o.) or Orlistat (250 mg/kg, p.o.), a lipase inhibitor, or Clofibrate (500 mg/kg, p.o.), a hypolipidemic drug. In all cases, serum TG elevation remarkably suppressed. Further bioassay-guided procedures yielded the sesquiterpenes i.e. cynaropicrin, aguerin B, grosheimin and cynarascolosides A, B and C as the active components of *C. scolymus* leaf (116).

Adult volunteers with mild to moderate hypercholesterolemia were used by Bundy et al. to evaluate the effect of standardised *C. scolymus* leaf extract on plasma lipid levels. During 12 weeks, adults were administered either 1280 mg of extract or placebo, daily. Plasma total cholesterol level decreased in the treatment group from 7.16 (SD 0.62) mmol/L to 6.86 (SD 0.68) mmol/L, whereas it increased in the control group from 6.90 (SD 0.49) mmol/L to 7.03 (0.61) mmol/L. However, low density lipoprotein

(LDL)-cholesterol, high density lipoprotein (HDL)-cholesterol or TG levels not significantly improved. On the other hand, since general well-being remarkably improved in both the treatment (11%) and control groups (9%), researchers concluded that 12 weeks *C. scolymus* leaf extract consumption provided a modest effect in total cholesterol level (141).

Hypercholesterolemic rats were analyzed by Kucukgergin et al. to investigate *C. scolymus* leaf extract (containing 6.5% caffeoylquinic acids and 1% flavonoids) efficacy either on serum and hepatic lipid levels or pro-oxidant-antioxidant balance. Test animals were treated with 4% (w/w) cholesterol and 1% cholic acid (w/w) supplemented diet plus extract (1.5 g/kg/day) which was given during the last 2 week-period for 1 month. Consequently serum and liver cholesterol and TG levels markedly increased due to high cholesterol diet. Besides, MDA and diene conjugate (DC) levels increased while hepatic vitamin E levels, hepatic and cardiac GSH-Px activities reduced. However SOD and glutathione transferase (GST) activities, GSH, and vitamin C levels did not change. The serum cholesterol and TG levels declined and the ratio of cholesterol to HDL-cholesterol increased, whereas liver cholesterol and TG levels remained unchanged in extract-treated hypercholesterolemic rats. Besides, hepatic and cardiac MDA and DC levels markedly decreased whereas hepatic vitamin E and GSH-Px activities increased. Consequently, *C. scolymus* leaf extract may provide reduction in serum lipids and hypercholesterolemia-induced pro-oxidant status (142).

Golden Syrian hamsters were used to study the effects of *C. scolymus* leaf extract on lowering plasma total and non-HDL cholesterol. For 6 weeks, test animals were fed on control diet or a similar diet containing extract (4.5 g/kg diet). After 42 days of treatment, extract-fed male and female hamsters exhibited remarkably lower total cholesterol (15 and 15%), non-HDL-cholesterol (30 and 29%) and TG (22 and 29%) levels, respectively. As a result, higher excretion of fecal bile acids and neutral sterols decreases plasma cholesterol levels (143).

#### 2.2.2.4.2. Hepatoprotective Activity

*In vitro* studies performed by Gebhardt confirmed that *C. scolymus* extracts had important antioxidant and hepatoprotective activities. Cumene hydroperoxide or *tert*-butylhydroperoxide (*t*-BHP) was used to trigger the MDA production in primary rat

hepatocyte cultures. Consequently, the basal MDA production did not change in extract-treated cultures; however MDA level suppressed dose-dependently in *t*-BHP-induced cultures. Besides, the cellular level of GSH did not affect whereas the loss of total GSH and the cellular leakage of glutathione disulfide caused by *t*-BHP reduced (144). Gebhardt and Fausel also demonstrated both MDA production and LDH leakage were suppressed by addition of aqueous *C. scolymus* extracts to *t*-BHP-exposed rat hepatocytes either priorly or simultaneously with $EC_{50}$ values of about 95 and 12 µg extract powder/mL, respectively. In addition, various polyphenolic and flavonoid components i.e. caffeic acid, ChA, cynarin and cynaroside of extract were found to reduce MDA production with the $EC_{50}$ values of 8.1, 12.5, 15.2 and 28 pg/mL, respectively. These results suggested that *C. scolymus* extract has a marked antioxidative and hepatoprotective potentials (145).

To study antihepatotoxic/hepatoprotective activity of the drugs, carbon tetrachloride ($CCl_4$) is frequently applied as a model. Mehmetçik et al. studied the effect of *C. scolymus* leaf extract pretreatment on $CCl_4$-stimulated oxidative stress and hepatotoxicity. For 2 weeks prior to $CCl_4$ (1 mL/kg; i.p.) induced hepatotoxicity, rats were fed on extract (1.5 g/kg/day) by gavage and 24 h after induction they were sacrificed. The hepatic necrosis, plasma transaminase activities as well as hepatic MDA and DC levels markedly increased after $CCl_4$ administration in the liver of rats. Moreover, GSH and vitamin C levels diminished, while vitamin E level increased. The hepatic SOD activities remained unchanged, however GSH-Px and GST activities decreased. On the other hand, extract treatment markedly normalized the plasma transaminase activity which was also histopathologically verified. In addition, hepatic MDA and DC levels decreased, while GSH levels and GSH-Px activities increased, other antioxidant parameters did not alter. They concluded that *C. scolymus* extract may be useful in prevention of oxidative stress-induced hepatotoxicity (146).

Aktay et al. comparatively investigated hepatoprotective effects of ethanolic *C. scolymus* extracts from bracts, receptacles and stems against $CCl_4$-induced hepatotoxicity model in male Sprague-Dawley rats. Histopathological studies and also serum MDA, AST and ALT levels were evaluated. Consequently microscopic examinations revealed that the highest reduction on severe hepatic lesions induced by $CCl_4$ was monitored with the *C. scolymus* bracts extract. In addition, the highest

inhibitory effect on MDA concentration was observed with *C. scolymus* receptaculum and bract extracts, provided 71.66 and 69.64% protection comparing to control group, respectively. AST and ALT levels were also suppressed by *C. scolymus* stem, receptaculum and bract extracts, protective effects were 64.37, 66.14 and 66.10%, respectively (147).

Male Sprague-Dawley rats were used to investigate whether ChA activities related to the inhibition of toll-like receptor 4 (TLR4) signaling pathway on liver inflammation and fibrosis stimulated by $CCl_4$. For 8 weeks, test animals were administrated $CCl_4$ together with or without ChA. In conclusion, $CCl_4$-induced liver damage and symptoms of liver fibrosis significantly reduced after ChA-treated rats. Besides, reduction in serum transaminase levels, collagen I and α-smooth muscle actin expression was observed. The expression levels of TLR4, myeloid differentiation factor 88 (MyD88), iNOS and COX-2 reduced in the treatment group of $CCl_4$ and ChA when compared to $CCl_4$-treated group. On the other hand, bone morphogenetic protein and activin membrane-bound inhibitor expressions increased. It also suppressed $CCl_4$-stimulated NF-κB activation. Moreover, it diminished the hepatic mRNA expression and serum levels of TNF-α, IL-6 and IL-1β in $CCl_4$-treated rats. These results indicated that ChA may inhibit TLR4/MyD88/NF-κB signaling pathway and, thus, prevents liver fibrosis in $CCl_4$-treated rats (148).

#### 2.2.2.4.3. Antidyspeptic Activity

*C. scolymus* leaf extract usage in digestive system disorders is mainly based on its strong antidyspeptic activities which are mediated by its choleretic effect. In a randomised placebo-controlled double-blind cross-over study, the choleretic action of *C. scolymus* extract was investigated by Kirchhoff et al. The volume of bile secretion was measured to investigate the effects of single dose administration of standardized *C. scolymus* extract, Hepar SL forte®. After 30 minutes, bile secretion increased up to 127.3% in the extract-administered group. Besides, 1 h later the secretion was detected as 151.5%, and after additional 1 h it was 94.3%. For the placebo group the highest increase was recorded as 139.5% after 30 minutes. At 120 and 150 minutes, the volume of bile secretion under the active treatment was also markedly higher than the placebo ($p < 0.05$). However, bile secretion decreased below the initial level in the placebo

group after 3 hours. Approximately, 120-150 minutes were found to be effective period when extract was given postprandially. Researchers concluded that *C. scolymus* extract can be recommended for the treatment of dyspepsia, particularly due to dyskinesia of the bile ducts or problems in digestion of fat (149).

Wistar rats were practiced by Rodriguez et al. to comparatively study the effects of *C. scolymus* leaf extract with dehydrocholic acid, a synthetic bile acid, on bile flow and bile composition after acute and repeated (twice a day for 7 consecutive days) oral administration. Consequently, they observed remarkable increment in bile flow which was found to be similar as the reference agent. Single and repeated administration of the extract at the highest dose (400 mg/kg), bile acid-increasing effect of extract was more pronounced than that of the reference, while cholesterol and phospholipid contents remained unchanged. These results have clearly demonstrated that *C. scolymus* leaf extract significantly increase bile secretion supporting its efficacy in relieving dyspesia symptoms (150).

A recent post-marketing study was performed by Marakis et al. to search whether standardized *C. scolymus* leaf extract may alleviate dyspeptic symptoms and improve quality of life or not. During 2 months, the extract in daily 320 or 640 mg doses was randomly administered to volunteers. Results were assessed by the Nepean Dyspepsia Index and the State-Trait Anxiety Inventory. Comparing the baseline, significant reduction in all dyspeptic symptoms was observed, with an average reduction of 40% in global dyspepsia score. However, differences in the primary outcome measures between the two dosage groups were not remarkable, relief from anxiety, a secondary outcome, was greater with the higher dosage ($p = 0.03$). Moreover, comparing to baseline status, quality of life significantly improved in both groups. Researchers concluded that *C. scolymus* leaf extract recovers upper gastro-intestinal symptoms and improves the quality of life in subjects suffering from dyspepsia otherwise healthy (151).

Holtman et al. investigated the *C. scolymus* leaf extract efficacy in the treatment of patients with functional dyspepsia. In a double-blind, randomized controlled trials, 247 patients with functional dyspepsia were treated with either extract (2 x 320 mg t.d.s.) or a placebo. The patient's weekly self-rating the overall change in their dyspeptic

complaints was the primary efficacy variable. Besides, secondary variables were the scores of each dyspeptic symptom and the quality of life as evaluated by the Nepean Dyspepsia Index. Data from 244 patients (129 active treatments, 115 placebos) were found to be suitable for involvement in the statistical analysis. Over the 6 weeks of treatment, the overall symptom improvement was remarkably greater with extract than placebo-treated group (8.3 ± 4.6 versus 6.7 ± 4.8, $p < 0.01$). Furthermore, extract-treated patients showed markedly greater improvement in the global quality-of-life scores compared to that of placebo-treated patients (-41.1 ± 47.6 versus -24.8 ± 35.6, $p < 0.01$). They suggested that *C. scolymus* extract may be used in alleviating symptoms and improving the disease-specific quality of life in patients with functional dyspepsia (152).

#### 2.2.2.4.4. Antihyperglycemic Activity

Overweight, dyslipidemic and diabetic rats were used to evaluate ChA activity on fasting plasma glucose, plasma and liver triacylglycerols and cholesterol concentrations. In addition, selected mineral concentrations in plasma, spleen and liver were investigated. For 3 weeks, ChA (5 mg/kg b.w.) was daily injected to rats implanted with jugular vein catheters. ChA decreased the postprandial glucose peak response when compared to the previously ChA-injected rats; however it did not improve hypoglycemia. In ChA-injected rats, fasting plasma cholesterol and triacylglycerols concentrations markedly diminished by 44% and 58% respectively. The concentration of adipose triacylglycerols remained unchanged ($p > 0.05$). The differences ($p > 0.05$) in the plasma, liver, and spleen concentration of selected minerals were found to be significant in ChA-treated rats. Eventually ChA was found to develop glucose tolerance, reduced some plasma and liver lipids, and improve mineral pool distribution (153). Moreover, the effects of ChA on glucose tolerance, insulin sensitivity, hepatic gluconeogenesis, lipid metabolism and skeletal muscle glucose uptake in Leprdb/db mice were evaluated by Ong et al. ChA activity on hepatic glucose production and fatty acid synthesis was investigated using Hepatoma HepG2. In an oral glucose tolerance test, acute treatment with ChA reduced glucose area under the curve in Leprdb/db mice. In addition, chronic ChA treatment supressed hepatic glucose-6-phosphatase expression and activity as well as hepatic steatosis, whereas improved lipid profiles and skeletal muscle glucose uptake. Eventually fasting glucose level, glucose

tolerance, insulin sensitivity and dyslipidemia in Leprdb/db mice improved. ChA activated AMP-dependent kinase (AMPK) such as suppression of hepatic glucose production and fatty acid synthesis. Inhibition and knockdown of AMPK terminated these metabolic alterations. As a result, ChA improved glucose and lipid metabolism via activating AMPK (154).

Rondanelli et al. studied the effects of dietary supplement of *C. scolymus* extract on satiation. For 2 months, a randomized, double-blind, placebo-controlled clinical trial has been carried out in 39 overweight subjects (20 supplemented group, 19 placebo group). Finally, in the extract-treated group the net change of the Haber's mean score notably increased. The net change of the glycaemia and of the dietary restriction score of the three factor eating questionnaire (TFEQ), reduced significantly only in the extract-treated group. Moreover, the homeostasis model assessment, the body mass index and the susceptibility-to-hunger score of the TFEQ reduced remarkably; these parameters remained unchanged in controls. In conclusion, *C. scolymus* dietary supplements potentially useful in the management of overweight and dysglycaemia (155).

Rondanelli et al. also studied *C. scolymus* extract as a dietary supplement on the glucose pattern in a group of patients with impaired fasting glycaemia (IFG). A randomized, double-blind, placebo-controlled trial has been performed in 55 overweight volunteers with IFG (fasting blood glucose [FBG]: 6.11 ± 0.56mmol/L). For 8 weeks, these volunteers were randomly treated either with extract (600 mg/d) (26 subjects) or placebo (29 matched subjects). Consequently, FBG (-9.6%), Homeostatic Metabolic Assessment (HOMA) (-11.7%), glycosylated haemoglobin (-2.3%), A1c-Derived Average Glucose (ADAG) (-3.1%) and lipidic pattern markedly decreased in the extract-treated group. However, the placebo group did not show any differences. The extract-treated group showed a significant difference in FBG, HOMA and lipidic pattern when compared with that of the placebo. In conclusion, these data showed *C. scolymus* supplement effects on the reduction of glycometabolic parameters in overweight subjects with IFG (156).

#### 2.2.2.4.5. Cardioprotective Activity

Li et al. investigated the effects of *C. scolymus* leaf extract belong to organic subfraction on endothelial nitric oxide synthase (eNOS) gene expression and NO production using EA.hy 926 cells, a cell line derived from human umbilical vein endothelial cells (HUVECs). The extract was found to be responsible for this eNOS-up-regulating action. It was also increased eNOS mRNA and eNOS protein expressions both in EA.hy 926 cells and in native HUVECs. Moreover, the NO-mediated vasodilator response to acetylcholine improved by *ex vivo* incubation of rat aortic rings with the extract, indicating that the up-regulated eNOS remained functional. Further investigations showed that this extract mainly composed of caffeoylquinic acids (cynarin and ChA) and flavonoids (luteolin and L7G). Luteolin and L7G increased eNOS promoter activity and eNOS mRNA expression, on the other hand cynarin and ChA were found to be ineffective. In conclusion, an increase in eNOS gene transcription may also contribute to *C. scolymus* cardioprotective activity in addition to lipid lowering and antioxidant properties. Besides, *C. scolymus* flavonoids were found to be responsible for mediating eNOS up-regulation (157).

Juzyszyn et al. studied to endothelial protective effects of *C. scolymus* extract containing 0.49% cynarin, 5.4% polyphenolic compounds, 5.0% caffeoylquinic acids derivatives, 0.72% L7G and luteolin 7-*O*-rutinoside. Preincubation of HUVECs with extract at concentrations of 25–100 μg/ml for 24 hours suppressed ROS generation induced by LPS and oxidized-LDL. Besides, the reduction kinetics of cytochrome C in reference to ascorbate showed dose-dependently reductive properties of the *C. scolymus* extract (158).

#### 2.2.2.4.6. Anticancer Activity

Yasukawa et al. identified a hydroxy taraxastane-type triterpene called taraxasterol from methanolic *C. scolymus* flower extract. Researchers also showed its significant inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice. At 0.2 μmol/mouse, this compound markedly reduced the tumour-promoting effect of TPA (1 μg/mouse) on skin tumour formation following initiation with 7,12-dimethylbenz[α]anthracene (50 μg/mouse) (159).

Miccadei et al. demonstrated that treatment of human hepatoma HepG2 cells for 24 hours with *C. scolymus* extract rich in polyphenolic compounds dose-dependently reduced cell viability. However, ChA did not show an effect on the cell death rate. Additionally, the extract rather than ChA induced apoptosis in HepG2 cells (160). Similarly, Mileo et al. investigated the antitumor effect of *C. scolymus* edible part of using the human breast cancer cell line, estrogen receptor negative, MDA-MB231. They concluded that that *C. scolymus* edible part reduced cell viability, inhibited cell growth, induced apoptotic mechanisms, and showed inhibitory properties against the invasive behavior of MDA-MB231 cancer cell line. As a result, these data indicate the potential chemopreventive activity of *C. scolymus* polyphenolic extracts (161).

#### 2.2.2.4.7. Antimicrobial Activity

Zhu et al. investigated antimicrobial activity of *C. scolymus* leaf extracts obtained by using chloroform, ethyl acetate and *n*-butanol solvents. Researchers performed disk-diffusion tests to each extract, respectively. As a result, *n*-butanol fraction showed the most effective antimicrobial activity against seven bacteria species including *Bacillus subtilis*, *S. aureus*, *Agrobacterium tumefaciens*, *Micrococcus luteus*, *E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, four yeasts such as *C. albicans*, *C. lusitaniae*, *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*, and four molds as *Aspergillus niger*, *Penicillium oxalicum*, *Mucor mucedo* and *Cladosporium cucumerinum*. Further, they also analyzed caffeoylquinic acid derivatives containing ChA, cynarin, 3,5-di-*O*-caffeoylquinic acid and 4,5-*O*-di caffeoylquinic acid and flavonoids including luteolin 7-*O*-rutinoside, L7G, apigenin 7-*O*-rutinoside and A7G using the above microorganisms. Among them, ChA, cynarin, luteolin 7-*O*-rutinoside and L7G showed a considerably higher activity than the others. Moreover, these compounds were more effective against fungi than bacteria. The MICs of these compounds were ranged from 50 to 200 μg/mL (162). Subsequently, they investigated chloroform, ethanol, and ethyl acetate fractions of not only *C. scolymus* leaf but also its different parts such as head and stem. Consequently, leaf extract was found to be most effective against all of the tested organisms, followed by head and stem extracts. Besides, its ethanol fraction remarkably showed an antimicrobial effect than the other fractions. The MICs of extracts determined by the agar and broth dilution method were ranged from 1.25 to 10.0 mg/mL (163).

The antimicrobial activity of *C. scolymus* extract from its bracts was also studied by Kukić et al. They extracted bracts with ethanol and then it was further partitioned with dichloromethane, ethyl acetate and *n*-butanol, respectively until leaving the water extract as a residue. Then, microdilution method was applied to each extract using *S. typhimurium, E. coli, B. subtilis, Staphylococcus epidermidis, S. aureus,* as well as micromycetes such as *A. niger, Aspergillus ochraceus, A. flavus, Penicillium ochrochloron, Penicillium funiculosum, Trichoderma viride, Fusarium tricinctum* and *Alternaria alternata*. As a result, ethyl acetate extract of *C. scolymus* showed the most significant (with MICs of 1.0–1.5 mg/mL and MBCs 1.5–2.0 mg/mL) antimicrobial activity, followed by ethanol, chloroform, water and *n*-butanol extracts (164).

**2.2.2.4.8. Antioxidant Activity**

Oxidative stress is defined as an imbalance between local ROS production and antioxidant mechanism. Pérez-García et al. concluded that *C. scolymus* leaf extract dose-dependently inhibited oxidative stress when human leukocytes were triggered with agents such as $H_2O_2$, phorbol-12-myristate-13-acerate and N-formyl-methionyl-leucyl-phenylalanine that generate ROS. Further examination showed that cynarin, caffeic acid, ChA and luteolin components of *C. scolymus* were responsible for the contribution to the antioxidant activity of the extract in human neutrophils (165).

Zapolska-Downar et al. studied the influence of aqueous and ethanolic *C. scolymus* extracts on intracellular oxidative stress stimulated by inflammatory mediators (TNF-α and LPS) and oxidized-LDL in endothelial cells and monocytes. The oxidation of 2',7'-dichlorofluorescin to 2',7'-dichlorofluorescein were monitored to detect oxidative stress. Both extracts inhibited basal and stimulated ROS production in endothelial cells and monocytes, dose-dependently. In endothelial cells, oxidized-LDL triggered intracellular ROS production suppressed by 50 µg/mL ethanolic and aqueous extracts up to 60% 43%, respectively. Moreover, the intracellular ROS production in monocytes diminished by ethanolic extract till 76%. These results demonstrated that *C. scolymus* extracts have protective efficacies against oxidative stress induced by inflammatory mediators and oxidized-LDL in cultured endothelial cells and monocytes (166).

Llorach et al. studied the antioxidant phenolics of *C. scolymus* by-products such as raw, blanched (thermally treated) and blanching waters by both methanol and water extractions. *C. scolymus* extracts from industrial by-products decreased the lipid peroxidation. Consequently, *C. scolymus* by-products usage could be suggested due to their health-promoting properties (167).

Jiménez-Escrig et al. determined the antioxidant activity of aqueous-organic *C. scolymus* extracts by using three methods which were DPPH˙ radical scavenging assay, ferric-reducing antioxidant power (FRAP), and inhibition of copper(II)-catalyzed *in vitro* human LDL oxidation. In addition, the ability of the edible portion of *C. scolymus* to alter *in vivo* antioxidative defense was investigated in male rats using selected biomarkers of antioxidant status. One gram (dry matter) had a DPPH activity and a FRAP value in vitro equivalent to those of 29.2 mg and 62.6 mg of vitamin C and to those of 77.9 mg and 159 mg of vitamin E, respectively. *C. scolymus* extracts inhibited LDL oxidation *in vitro*. Both ferric-reducing ability and 2,2˙-azinobis(3-ethylbenzothiazolin-6-sulfonate) radical scavenging activity did not alter in the plasma of the *C. scolymus* group compared to the control group. Among different antioxidant enzymes measured such as SOD, GSH-Px, gluthatione reductase and CAT in erythrocytes, only GSH-Px activity increased in the *C. scolymus* group compared to the control group. 2-aminoadipic semialdehyde, a protein oxidation biomarker, decreased in plasma proteins and hemoglobin in the extract-fed group versus the control group (168).

**2.2.2.4.9. Antihypertensive Activity**

Roghani-Dehkordi and Kamkhah leaded a randomized, placebo-controlled trial to illuminate the therapeutic efficacy of concentrated leaf juice of *C. scolymus* in patients with mild hypertension (systolic/diastolic blood pressure, 140-159/90-99 mmHg). Subjects were randomized into a placebo and two drug groups that treated with 50 and 100 mg of *C. scolymus* juice, respectively. When compared to the baseline data in case groups after 12 weeks, systolic blood pressure markedly diminished. Besides, diastolic blood pressure was notably lower from the baseline when compared to the placebo group. Consequently, *C. scolymus* juice may have a blood pressure-lowering effect in mild hypertension (169).

### 2.2.3. Chromatography

The term chromatography is derived from two Greek words which are ‘chroma’ and ‘graphein’ meaning color and to write, respectively. It was first presented by Russian Botanist Michael Semyonovich Tswett in 1906 to define his work which was separation of chlorophylls and carotenoids by column chromatography (1).

The International Union of Pure and Applied Chemistry (IUPAC) define chromatography as follow (170):

> *“Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction*.” (Ettre, 1993: 823)

Chromatography can be categorized into three general groups: (I) physical state of mobile phase phase, (II) separation mechanism and (III) applied technique. According to the first classification, mobile phase can be liquid, as in HPLC, paper chromatography and TLC, or it can be supercritical fluid and also gas, as in GC **(Figure 3)**. Second group comprises adsorption, partition, size-exclusion, ion-exchange, affinity and hydrophobic interaction chromatographies, because these are based on the typical reactions of the compounds, stationary and mobile phases each other. Lastly, chromatography can be categorized as column (HPLC, GC), capillary (GC) and planar chromatography (paper chromatography, TLC) as shown in **Figure 4** according to the applied technique (1,171,172).

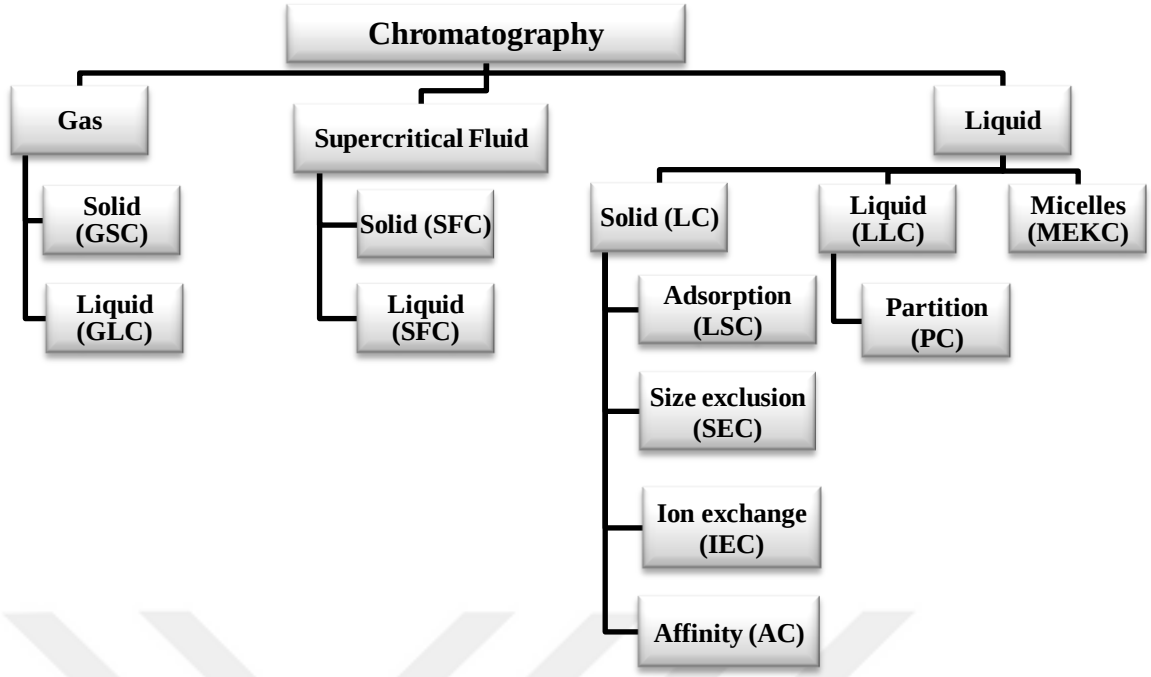



**Figure 3.** Family tree of chromatographic methods. GSC: Gas Solid Chromatography; GLC: Gas Liquid Chromatography; SFC: Supercritical Fluid Chromatography; LC: Liquid Chromatography; LLC: Liquid Liquid Chromatography; MEKC: Micellar Electrokinetic Chromatography LSC: Liquid Solid Chromatography; SEC: Size Exclusion Chromatography; IEC: Ion Exchange Chromatography; AC: Affinity Chromatography; PC: Partition Chromatography (171).

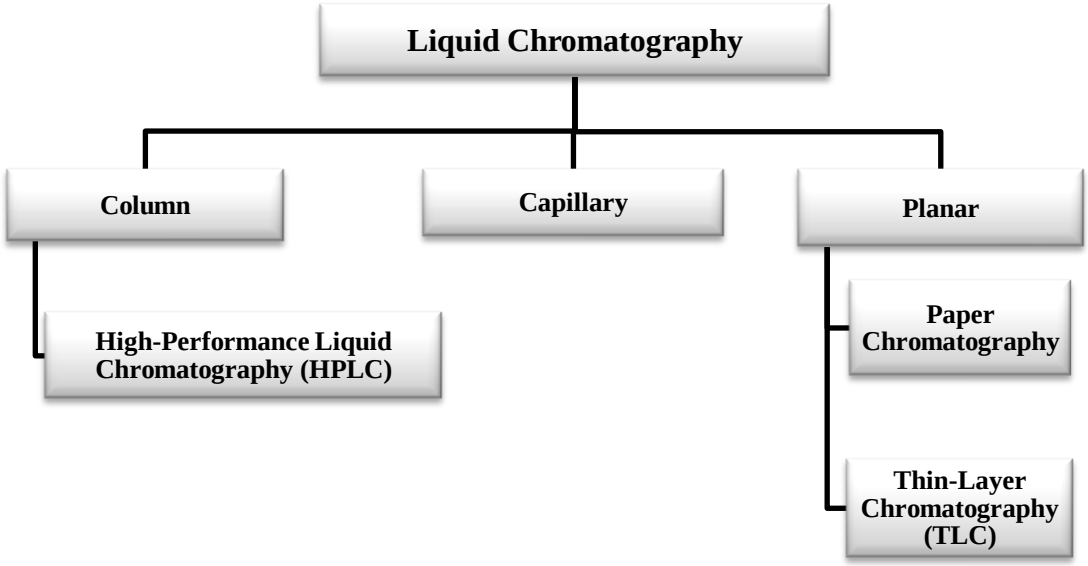



**Figure 4.** Liquid chromatography related to separation techniques (1)

### 2.2.3.1. High Performance Thin-Layer Chromatography

TLC is one of the mostly used analytical techniques mostly in qualitative analysis. It is performed on a solid support covered with a thin-layer of adsorbent material using a developing solvent system. It was first introduced by Egon Stahl through his publication named 'Thin-Layer Chromatography' in 1956 (1).

Up to now, various innovations on TLC have been done to improve its performance, particularly on stationary phases i.e. standardized and modified layers have been introduced. In addition, instrumentation such as semi- or fully automatic sample applicator, automatic developing chamber, densitometer, coupling TLC with MS and automation i.e. software for computer controlled operation of each TLC step have been renewed during years. Consequently, applications on TLC have been started to be replaced by HPTLC (*syn*. contemporary or modern or instrumental TLC) methods (173,174).

Nowadays, the application of HPTLC covers a wide range of fields such as herbal product, pharmaceutical industry, clinical chemistry, forensic chemistry and food analysis **(Figure 5)** (175).

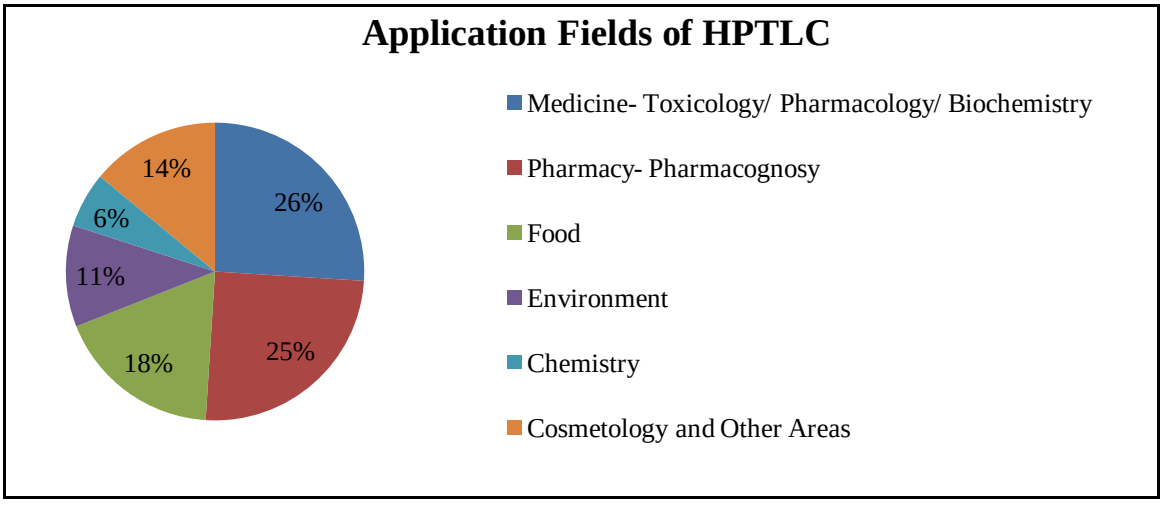



**Figure 5.** HPTLC application fields [Adapted from Hahn-Deinstrop (175)]

Among these fields, basic applications of HPTLC in plant analysis are (176):

- Identification and authentication of the materials
- Determination and quantification of the marker compounds
- Quality control of both raw materials and finished products
- Detection of adulteration
- Stability control and evaluation of degradation products
- Standardization of marketed products
- Bioautography studies

HPTLC is an efficient, instrumentalized and quantitative method that can produce comparable results with column liquid chromatography i.e. HPLC. In addition, each technique has its own advantages and drawbacks. In **Table 15**, comparative features of HPTLC and HPLC techniques are briefly discussed (171,177–179).

**Table 15**. Comparative features of HPTLC and HPLC techniques (171,177–179)

| **HPTLC** | **HPLC** |
|---|---|
| Liquid chromatography. | Liquid chromatography. |
| Planar chromatography. | Column chromatography. |
| Adsorption chromatography is common. | Partition chromatography is common. |
| Open system. | Closed system. |
| Semi automated, computer controlled system. | Fully automated, computer controlled system. |
| Steps of analysis such as sample application, separation and detection are segmented. | All steps are continuously processed. |
| No need to equilibrate the system before of the analysis; replacement of the either mobile or stationary phases can be performed simultaneously. | Entire system should be equilibrated before analysis; re-equilibration is needed when the parameters are changed which is time consuming. |
| Several samples can be analyzed concurrently. | Single sample at a time. |
| Duration of analysis for multiple samples depends *n* times required for a single sample. | *n* times require to analyze *n* samples (*n* x *n* times). |
| Simultaneously separate several components using various eluants by vario chambers. | Not possible. |
| Easy to handle the two-dimensional separation. | Not practical. |
| Single use of the plate. | Same column can be used many times. However, excessively retained sample constituents may accumulate and alter either specifications of the column or create artifact peaks. |
| Visual detection is possible. | Not possible. |
| Derivatization is simple by various non-specific or specific derivatization reagents after development on the plate. | Not practical. |
| Sample development ends when the solvent reaches to front. | Overruns until the last compound eluted from the column. |

**Table 15.** Continued.

| HPTLC | HPLC |
|---|---|
| Limitless choices of solvents can be used in mobile phase without considering UV-absorbing property. Since mobile phase is removed before processing, there would be no risk for interactions with solvents during measurement. | Restriction in solvents due to their cut-off wavelengths. Moreover, column may be sensitive to very low (< 2) or high pH (> 8) values of mobile phases. |
| Pretreatment of mobile phase is not necessary; analytical grade solvents can be used. | Need to prior treatment i.e. filtration, degassing of the mobile phase; high purity and labeled as HPLC grade solvents are necessary. |
| Low amount consumption of mobile phase. | High amount consumption of mobile phase. |
| Mobile phase flow through the stationary phase at atmospheric pressure with capillary forces. | External force (pressure). |
| Since separated compounds are immobilized on the plate, scanning or visualization can be repeated at any time with changed parameters after development. | No repetition. |
| Sample preparation is easy and clean-up is not necessary; choice of solvent is not critical. | Complicated, requires proper purification to prevent damaging of the column. |
| Choice of solvent for sample preparation is not critical. | Solvents for sample preparation should be compatible with the mobile system. |
| Difficulties in operation highly volatile samples or substances sensitive to oxygen or light. | Difficulties in operation of irreversibly interacted substances to the adsorbent. |
| Low resolution and limited separation capacity. | Superior resolution due to applied high pressure is resulted in continuous and constant flow elution. |
| Affected by the environmental conditions such as relative humidity, temperature and air pollutants. | Less affected by the environmental parameters. |
| Operating is cost effective. | Operating is expensive. |

#### 2.2.3.1.1. Theoretical Concepts

##### 2.2.3.1.1.1. General Chromatographic Principles

###### 2.2.3.1.1.1.1. Distribution Coefficient

Among all separation mechanisms, adsorption and partition chromatography play important role in TLC:

**Adsorption chromatography:** Separation relys on the different adsorption affinities between compounds and the active surface of the stationary phase. The distribution coefficient also known as the repartition coefficient can be stated as (174):

$$K = \frac{c_s[g/g]}{c_m\,[g/cm^3]}$$

$K$ = repartition coefficient (g/cm$^3$)
$c_s$ = substance concentration in the stationary phase
$c_m$ = substance concentration in the mobile phase

**Partition chromatography:** The separation depends on the relative solubility of the components in two immiscible solvents. The partition coefficient can be expressed as (174):

$$K = \frac{c_s}{c_m} = \frac{V_m m_s}{V_s m_m}$$

$K$ = Partition coefficient
$c_s$ = Substance concentration in the stationary phase
$c_m$ = Substance concentration in the mobile phase
$V_{s,m}$ = Volume of the stationary or mobile phase
$m_{s,m}$ = Substance mass in the stationary or mobile phase

#### 2.2.3.1.1.1.2. Retardation Factor

Retardation factor (retention factor or $R_F$) is used to state the placement of a separated compound in a chromatogram **(Figure 6)** (180). The $R_F$ values should be between 0 and 1, '$0 < R_F < 1$'. This can be described as (175):

$$R_F = z_i/(z_f - z_o)$$

$R_F$ = Retardation factor

$z_i$ = Migration distance of the spot (mm)

$z_f$ = Migration distance of the solvent front measured from the immersion line (mm)

$z_o$ = Distance between immersion line and sample application position (mm)

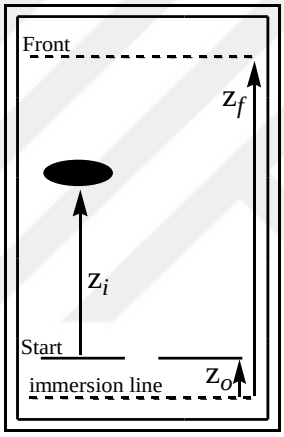



**Figure 6.** A sample zone in a TLC chromatogram (174)

#### 2.2.3.1.1.1.3. Capacity Factor

The retention of a component by the stationary phase is defined by capacity factor ($k'$) or in other words retention factor. This can be determined as (181):

$$k' = \frac{1 - R_F}{R_F}$$

#### 2.2.3.1.1.1.4. Selectivity Factor

The ability of a chromatographic system to produce different $R_F$ values for the separated compounds is called selectivity or separation factor (181):

$$\alpha = \frac{k'_B}{k'_A}$$

$k'_B$ = Capacity factor of B

$k'_A$ = Capacity factor of A

#### 2.2.3.1.1.1.5. Resolution

Resolution ($R_s$) defines how well the two peaks are separated from each other. This can be stated as (180):

$$R_s = \frac{1.18a(R_{F2} - R_{F1})}{w_{h1} + w_{h2}}$$

$R_{F1}$, $R_{F2}$ = Retardation factors of the peaks

$w_{h1}$, $w_{h2}$ = Peak widths at half-height

$a$ = Migration distance of the solvent front

#### 2.2.3.1.1.1.6. Theoretical plate number and height

Theoretical plate number (N) and height (*H* or height equivalent to theoretical plate (*HETP*)) are parameters to evaluate the efficiency and narrowness of a peak. This is defined as (181):

$$N = 16(MD/W)^2$$

*MD* = Migration distance of spot

*W* = Peak width

#### 2.2.3.1.1.1.7. Band Broadening

The overall dispersion or widening of a sample peak when it moves through a separation system is described as band-broadening. Due to the diffusion, the efficiency of the separation is reduced resulting in broadening of the investigated peak (178).

#### 2.2.3.1.1.1.8. Flow Constant

Flow constant or velocity constant is a measure of the solvent movement speed to the front (182).

#### 2.2.3.1.1.1.9. Flow through Porous Layers

The mobile phase moves through the layer via capillary action, forced flow, and electroosmosis (174,183,184):

**Capillary flow:** Since stationary phases have microporous structure, they have high specific surface areas (cellulose: 50 $m^2/g$; silica: 500 $m^2/g$) with high surface energy, migration of the solvents in the mobile phase through the thin-layer is triggered by capillary forces. They also contain cavities and channels between the particles of the layer packing. Briefly, there are two reasons for the capillary flow: (I) the high surface energy of the stationary phase is reduced when come in touch with the mobile phase and (II) solvent molecules supress its own energy when they move through cavities.

**Forced flow:** The forced-flow development allows controlled the mobile phase flow rate through the layer using either centrifugal force or pressure.

**Electroosmotic flow:** Electroosmotic solvent flow arises from the formation of an electrical double layer at a solid-liquid interface in the applied electric field.

#### 2.2.3.1.1.2. Stationary Phases

Stationary phase is an immobile adsorbent which is composed of either inorganic or organic materials having porous structure and relatively high specific surfaces. These materials are connected to aluminum or plastic sheets or glass packing supports with the help of various binders to prepare plates.

In early stages, TLC plates were made by hand or special tools; however by emerging technologies commercial precoated glass TLC plates were first introduced to the market in 1961. Moreover, the first silica gel 60 HPTLC plates were presented in 1975. In addition, chemically modified precoated HPTLC plates such as reversed-phase (RP-2, RP-8, and RP-18), amino-, cyano-, diol-bonded and chiral plates were introduced to the market in the course of time. Lately, ultra thin-layer chromatography (UTLC) plates were reported and commercialy available since 2002 (185). The comparative characteristics of TLC, HPTLC and UTLC plates are given in **Table 16** (174,186).

**Table 16.** Characteristic properties of TLC, HPTLC and UTLC layers (174,186)

| **Parameter** | **TLC** | **HPTLC** | **UTLC** |
|---|---|---|---|
| Plate size (cm) | 20 x 20 | 10 x 10; 20 x 10 | 6 x 3.6 |
| Plate thickness (μm) | 100-250 | 100-200 | 10 |
| Particle size (μm) | 8-10 | 6-8 | 1-2 |
| Pore size (nm) | 6 | 6 | Mesopores 3-4 nm<br>Macropores 1-2 μm |
| Application volume (μL) | 1-5 | 0.1-5 | 0.01-0.1 |
| Migration distance (cm) | 7-15 | 3-7 | 1-3 |
| Solvent consumption (mL) | 100 | 20 | 1-4 |
| Separating time (min) | 30-200 | 3-20 | 1-5 |
| Separating distance (cm) | 10-15 | 3-8 | 1-3 |
| Plate height (μm) | 35-75 | 23-25 | - |
| Detection limit in reflectance (ng) | 1-5 | 0.5-1 | 0.5 |
| Detection limit in fluorescence (pg) | 50-100 | 5-10 | 5 |

The stationary phases can be grouped as silica based layers, non-silica based layers, mixed stationary phases and dual phases **(Table 17)** (187,188).

**Table 17.** Types of stationary phases (187,188)

| **Types of stationary phases** | **Examples** |
|---|---|
| Silica based layers | Silica gel |
| | Modified silica gel<br>• amino-, cyano-, diol-bonded layers<br>• Reversed-phase layers<br>• Chiral and impregnated layers |
| Non-silica based layes | Cellulose<br>Aluminum oxide<br>Kieselguhr<br>Polyamide<br>Miscellaneous stationary phases |
| Mixed stationary phases | Mixed phases of silica gel-kieselguhr; silica gel-alumina; silica gel-cellulose |
| Dual phases | Having two stationary phases mostly one normal and the other is reversed phases on one plate i.e. silica gel/RP-18 |

Among these various phases, silica gel is one of the most practically preferred in TLC **(Figure 7)** (189).

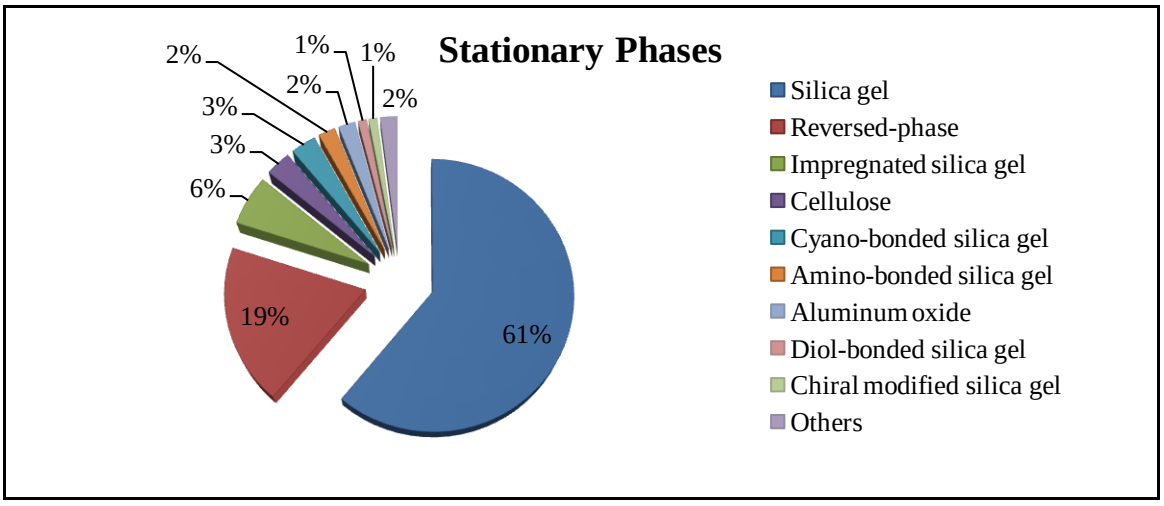



**Figure 7.** The practical usage of the stationary phases [Adapted from Berezkin et al. (189)]

### 2.2.3.1.1.2.1. Silica Based Adsorbents

I. **Silica gel:** Silica gel is a hydrated silicon dioxide, $SiO_2.(H_2O)_n$. The structure of silica gel as shown in **Figure 8**, each silicon atom is surrounded by four oxygen atoms, enables specific interactions on the surface of the layer with the functional groups of the sample such as proton donor or acceptor interaction, dipole-dipole interaction, interaction with induced dipoles or interaction based on dispersion forces (173).

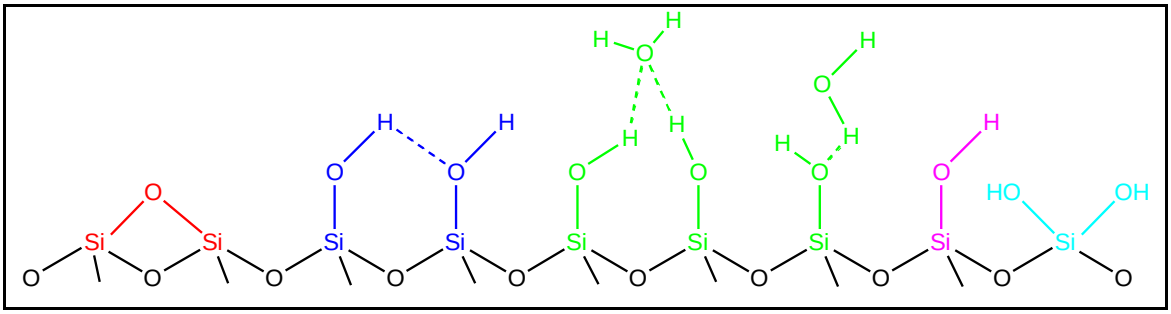



**Figure 8.** Chemical functionality of the silica gel surface. Siloxane group (red); vicinal silanol groups (dark blue); adsorbed water on silica gel (green); free silanol group (pink); geminal silanol group (light blue); dotted lines: H-bonds [Adapted from Reich and Schibli (173)]

## II. Modified silica gels:

- **Reversed-phase:** Organosilanes with various chain lengths are used to modify silica gel to produce $C_2$, $C_8$, $C_{12}$ and $C_{18}$ and also aromotic ring bonded phases.
- **Amino-, cyano-, and diol-bonded phases:** Aminopropyl groups, cyanopropyl groups and vicinal diol alkyl ether groups are bonded to siloxane linkages to produce amino, cyano and diol bonded phases, respectively.
- **Chiral-bonded phases:** These are mostly made from impregnation with a chiral selector.
- **Impregnated layers:** These layers are mostly prepared by spraying or immersing of silica gel plates in a solution of a corresponding reagent such as metal cations, boric acid and caffeine (188).

In addition, the stationary phases based on silica gel are briefly schematized in **Figure 9** (177).

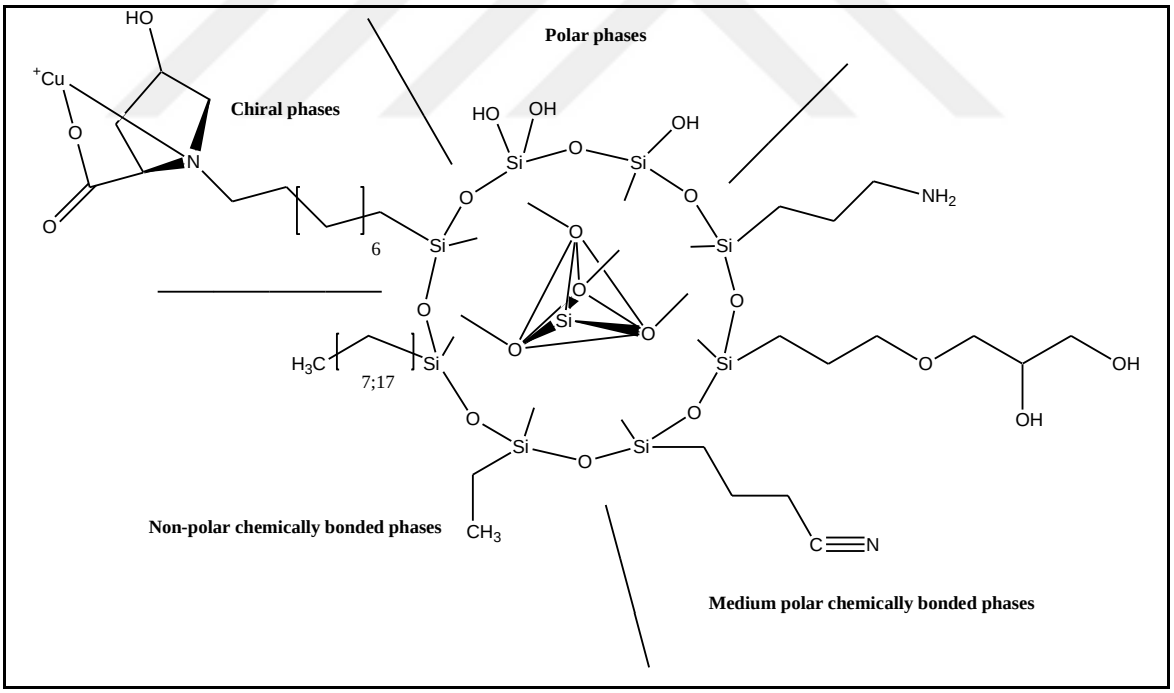



**Figure 9.** Stationary phases based on silica gel (177)

#### 2.2.3.1.1.2.2. Non-silica Based Adsorbents

- **Cellulose:** It is a glucose polymer. As a stationary phase, it plays role to support for water. Therefore, separation is mostly achived by partitioning (188).
- **Aluminum oxide or alumina ($Al_2O_3$):** It is made by dehydration of aluminum hydroxide, $Al(OH)_3$, at high tempreratures (500ºC). The active sites on the surface comprise acidic and basic aluminum ions and also polar Al-OH groups, among them basic alumina is widely used.
- **Kieselguhr:** It is a natural and highly porous diatomaceous earth consists of 90% silicic acid.
- **Polyamide:** It consists of amide and carbonyl groups which have capability to do hydrogen-bonding, mostly used for separation of phenolic compounds.
- **Miscellaneous stationary phases:** These are florisil, chitin and sephadex (187).

The selection of stationary phases for different classes of compounds is explained in **Table 18**, (188).

**Table 18.** Stationary phases and separated compound groups (188)

| Types of stationary phases | Separated compound groups |
|---|---|
| Silica gel | Constituents from all classes |
| Aluminium oxide | Basic compounds, steroids, terpenes, aromatic and aliphatic hydrocarbons |
| Cellulose | Amino acids, food dyes, antibiotics, carbohydrates |
| Kieselguhr | Carbohydrates, aflatoxins, herbicides, tetracyclines |
| Polyamide | Phenols, flavonoids and nitro-compounds |
| Amino-bonded silica gel | Many classes of constituents (carbohydrates, sulfonic acids, phenols, carboxylic acids) |
| Cyano-bonded silica gel | Many classes of compounds (pesticides, steroids, preservatives) |
| Diol-bonded silica gel | Many classes of compounds (steroids, hormones) |
| Reversed-phase silica gel | Steroids, tetracyclines, antioxidants, lipids, barbiturates, capsaicins, aminophenols, fatty acids |
| Chiral modified silica gel | Enantiomers of amino-acids, halogenated, N-alkyl, simple peptides |
| Silica gel impregnated with silver nitrate | Lipids |
| Silica gel impregnated with caffeine | Mostly used for polyaromatic hydrocarbons |
| Silica gel impregnated with boric acid/phosphate | Especially selective for carbohydrates |

### 2.2.3.1.1.3. Mobile Phases

There are two basic roles of developing solvents: (I) separation of the compounds in a mixture and (II) transportation. Therefore, selected solvents should dissolve the sample for migration.

Eluotropic series in which solvents are arranged in order of increasing elution power ($\varepsilon^o$) is one of the mostly used systems during the selection of the solvents according to their properties shown in **Table 19**, (177).

**Table 19.** Eluotropic series for solvents used in silica gel layer [Adapted from Reich and Schibli (173)]

| Solvent | Solvent strength ($\varepsilon^o$) | Polarity index P' | Selectivity group[a] |
|---|---|---|---|
| Pentane | 0.00 | 0.0 | - |
| Hexane | 0.00-0.01 | 0.1 | - |
| Cyclohexane | 0.03 | 0.2 | - |
| Carbon tetrachloride | 0.11 | 1.6 | - |
| Toluene | 0.22 | 2.4 | VII |
| Benzene | 0.25 | - | VII |
| Chloroform | 0.26 | 4.1 | VIII |
| Dichloromethane | 0.32 | 3.1 | V |
| Diethyl ether | 0.38-0.43 | 2.8 | I |
| Ethyl acetate | 0.38-0.48 | 4.4 | VI |
| Acetone | 0.47-0.53 | 5.1 | VI |
| Methy *t*-butyl ether | 0.48 | 2.5 | I |
| Acetonitrile | 0.50-0.52 | 5.8 | VI |
| Tetrahydrofuran | 0.44-0.53 | 4.0 | III |
| Isopropyl alcohol | 0.60 | 3.9 | II |
| Methanol | 0.70-0.73 | 5.1 | II |

[a]I: Aliphatic ethers; II: Aliphatic alcohols; III: Tetrahydrofurane; IV: Glycols, acetic acid; V: Dichloromethane; VI: Aliphatic ketones and esters, nitriles; VII: Aromatic hydrocarbons; VIII: Chloroform, water

In addition to the systematic approach, trial and error testing of reported solvent mixtures with suitable strengths (optimum $R_F$ values should be 0.2-0.8) assists to find the appropriate developing solvent system(s).

#### 2.2.3.1.2. Practical Aspects of HPTLC

##### 2.2.3.1.2.1. Sample Preparation and Application

Neatly prepared sample is one of the most important parameters for the successful analysis. The selection of the extraction types such as homogenization, vortex-mixing, soxhlet extraction, supercritical fluid extraction, microwave and ultrasonic assisted extraction should be carefully considered according to the interested compounds particularly in plant analysis (190).

In addition, solvent used in the sample preparation should meet with the necessary criteria such as it should be pure as possible, safe, stable and not react with the sample compounds as well as extract the interested compounds in the sample i.e. toluene, dichloromethane are used for apolar compounds whereas methanol, ethanol are good choice for polars. Moreover, some solvents i.e. dimethysulfoxide, acids or bases may cause degradation of the compounds in the sample; therefore their usage should be avoided as possible. Optimal volatility of the extraction solvent is another important parameter, except acetone that is because it may cause formation of acetylacetone by the condensation reaction with the active surfaces of the adsorbent. In addition, solvents should have a low solvent strenght to prevent chromatography during application (173).

Application of the prepared sample test solution could be done either direct contact or spray-on techniques on the stationary phase. For reproducible results some key points should be highlighted such as sample application volume should not be overloaded on stationary phase through excessive applications and adsorbent material should not be damaged especially during manual application. Moreover, samples should be applied as bands by semi or fully automatic instruments to supply precise positioning and application volume which are crucial parameters for quantitative analysis. Lastly, it should also be noted that application positions should be properly dried before development (175).

### 2.2.3.1.2.2. Development

The development is a process in which sample components are separated by mobile phase movement through the stationary phase in a developing chamber. There are various types of chambers used for development which are listed as below (188):

- Flat-bottom chamber (N-chamber)
- Twin-trough chamber (two compartment tank)
- Horizontal chamber
- U-chamber (circular)
- Automatic development chamber (ADC)
- Automated multiple development (AMD)
- Forced flow development chamber (development under pressure)
- Vario chamber (using six different solvent on one sorbent layer)

In general, there are two main development techniques: (I) one-dimensional and (II) two-dimensional (2D) developments. The operating modes are schematized in **Figure 10**, (177).

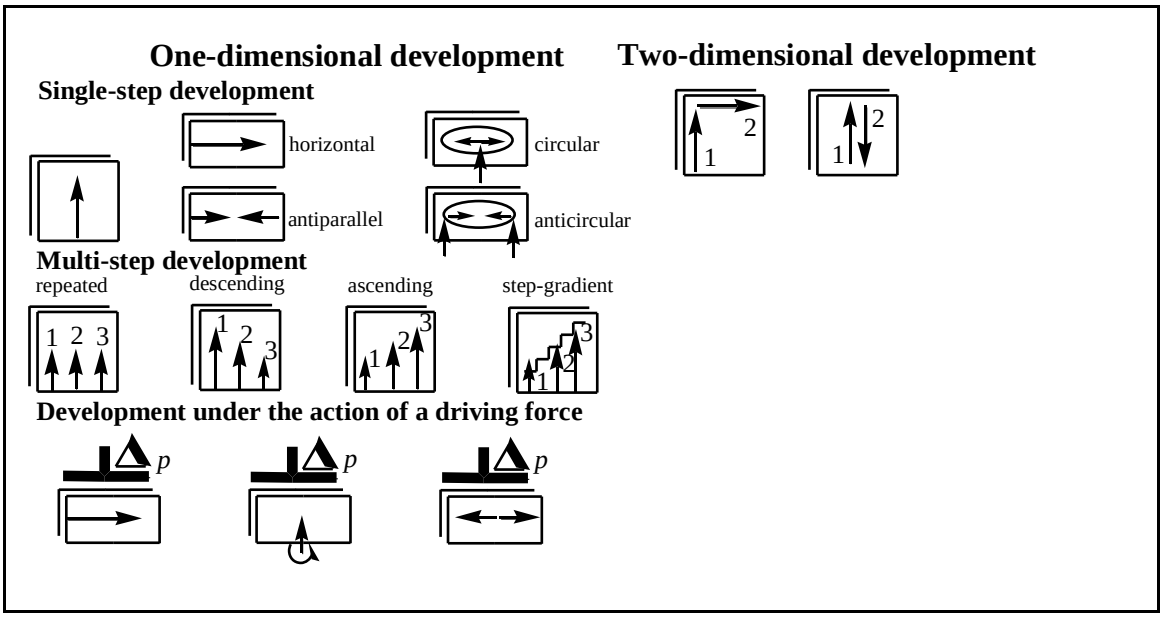



**Figure 10.** Development techniques [Adapted from Krasikov (177)]

#### 2.2.3.1.2.3. Densitometry

Densitometry means evaluating the amount of the separated zones directly on the plate layer by an instrument called densitometer or scanner, using either in absorption or fluorescence measurements **(Figure 11)** (173).

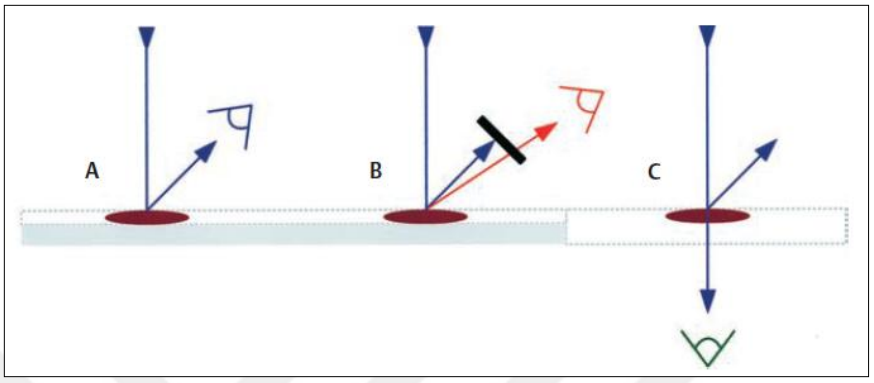



**Figure 11.** Densitometric measurements. (a) absorption in remission mode (b) fluorescence (c) absorption in transmission mode [Adapted from Reich and Schibli (173)]

The most generally accepted theory in densitometry is Kubelka-Munk model instead of Beer-Lamber Law (Beer's Law) in spectrometry. In general, Beer's Law shows direct relationship between the absorbance and the sample concentration. That is because, the absorbed light by the sample solution is directly proportional to its concentration (173). In case of TLC, relationship between the intensity of the reflected light and the concentration of the separated zone on the plate may not be linear due to either opaque surface of plate or sample distribution throughout the layer depth may not be homogenous. (191). In contrast, the amount of light emitted is proportional to the sample concentration in the fluorescence measurement (178).

##### 2.2.3.1.2.3.1. Densitometer

The densitometer is also called slit-scanning densitometer, spectrodensitometer or scanner **(Figure 12)**, (171). Lamp sources, deuterium lamp (D2), tungsten lamp (W) and a high pressure mercury vapor lamp (Hg) supply a beam of monochromatic light. Then, the optical system which is composed of lenses, mirrors and a monochromator assists a narrow light beam of a specified wavelenght to reach the plate. This light beam can be absorbed, reflected or scattered due to irregularly shaped silica gel particles,

during the precise movement of the plate track by track. Therefore, certain amount of light returns to a photomultiplier (detector) and it is measured and also recorded. In addition, photomultiplier may be placed beneath the plate for transmission measurement (188).

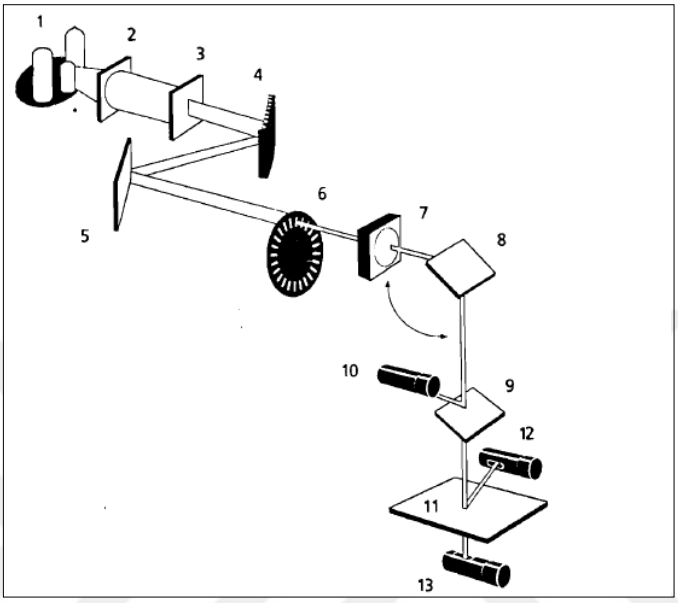



**Figure 12.** A schematized densitometer. 1: lamp selector; 2: entrance lens slit; 3: monochromator entry slit; 4: grating; 5: mirror; 6: slit aperture disk; 7: lens system; 8: mirror; 9: beam splitter; 10: reference photomultiplier; 11: TLC plate; 12: measuring photomultiplier; 13: photodiode for transmission measurements [Adapted from Poole (171)]

In general two types of measurements are common in densitometry for the quantitative analysis: (I) absorption and (II) fluorescence measurements. Absorption measurements are generally done by using D2 (190-450 nm) or W (350-800 nm) lamp sources at absorption maximum(s) of the analyzed compound(s) in a sample. On the other hand, Hg lamp (254-578) is used in the fluorescence measurements. In addition, D2 lamp from 190 nm to 380 nm and a K400 cutoff filter could also be used.

Spectral evaluation of the specified zones is useful either to evaluate the absorption maximum or to identity of the unknown compound when compared with the reference spectra. Besides, it is also practical to evaluate the peak purity (173).

#### 2.2.3.1.2.4. Video Densitometry and Derivatization

Video densitometry can be performed by an imaging system consists of a high resolution charge-coupled device camera and software-based asssesment.

The chromatogram can be visualized after removing the developing solvents on the surface of the plate. If the separated components are colorful, the chromatogram can be evaluated by naked eye in the visible wavelenght range (390-700 nm). For colorless components, UV lamps with an excitation wavelengths 254 nm or 366 nm may be useful. If the compounds are not visible under white light or UV lights then chemical derivatization is performed by specific or non-specific reagents. The commonly used techniques for applying such reagents are spraying on the plate, dipping the plate inside the reagent or exposed to the vapor of the reagent (192). Mostly used reagents for plant components are listed in **Table 20**.

Moreover, this should be also highlighted that quantitative measurements can be performed either in absorption or fluorescence modes using video densitometry. However, spectral evaluation is not possible in this case (172,173,193).

**Table 20.** Derivatization reagents used in plant analysis [Adapted from Reich and Schibli (173)]

| Reagent | Examination | Detection |
|---|---|---|
| Acetic anhydride | UV 254 and 366 nm | Ginkgolides |
| Acetic anhydride- sulphuric acid (Liebermann-Burchard reagent) | White light | Sterols, terpenoids |
| Aluminum chloride | UV 366 nm | Flavonoids, anthraquinones |
| Ammonia vapor | White light, UV 366 nm | Opiates, mycotoxins, flavonoids, sennosides, valepotriates, anthracenes |
| Aniline-diphenylamine-phosphoric acid | White light | Sugar, glycosides |
| Anisaldehyde- sulphuric acid | White light, UV 366 nm | Terpenoids, saponins, sterols, iridoids, most lipophilic compounds |
| Antimony (III) chloride (Carr-Price reagent) | White light, UV 366 nm | Double bonds in carotinoids, sterols, saponins, flavonoids |
| Copper (II) sulfate | White light | Universal reagent |
| 2,6-dibromoquinone-4-chloroimide (Gibbs' reagent) | White light | Arbutin, phenols, coumarins, thiols, thiones, antioxidants, capsaicin, amines |
| *p*-dimethyamino-benzaldehyde (Erlich' s reagent) | White light | Iridoids, proazulenes |
| 2,4-dinitrophenylhydrazine | White light | Ketones, aldehydes, alkaloids, silimarin |
| Dragendorff' s reagent | White light | Alkaloids, heterocyclic nitrogen compounds |
| Fast blue salt B | White light, UV 366 nm | Phenolic compounds, tannins, cannabinoids |
| Hydrochloric-acetic acid | White light, UV 366 nm | Valepotriates |
| Iodine vapor | White light, UV 366 nm | Conjugeted double bonds, alkaloids, purine derivatives |
| Iodine-hydrochloric acid | White light, UV 254 nm | Purine derivatives |
| Iron (III) chloride | White light | Phenols, flavonoids, tannins, plant acids, ergot alkaloids, Hop bitter principles, hypericines |
| Marquis' reagent | White light | Opiates |
| Natural products/polyethylene glycol 400 (NP/PEG 400) | UV 366 nm | Flavonoids, carbonhydrates, anthocyanines, plant acids |
| Nitric acid | UV 254 and 366 nm | Ephedrine derivatives |
| Ninhydrine | White light | Amino acids, biogenic amines, ephedrine |
| Phosphomolybdic acid | White light | Fatty oils, reducing substances, steroids, essential oils compounds, morphine |
| Potassium hydroxide | White light, UV 366 nm | Antraquinones, antrones, cumarins, flavonoids |
| Sulphuric acid | White light, UV 366 nm | General reagent |
| Tin (II) chloride-hydrochloric acid | UV 366 nm | Aristolochic acids |
| Vanillin-inorganic acid | White light, UV 366 nm | Terpenoids, sterols, salicin, ergot alkaloids, most lipophilic compounds |

#### 2.2.3.1.3. Bioautography with HPTLC

Bioautography is a biological activity detection method which could be combined with HPTLC method to provide information if separated compounds on the plate are biologically active or not. Therefore, HPTLC-bioautography plays an important role in the analysis of plant samples and mostly performed to identify antimicrobial, antiestrogenic, antimutagenic, antibacterial, antioxidant, antifungal compounds (176,194).

##### 2.2.3.1.3.1. Detection of Antioxidants by HPTLC-bioautography

HPTLC combined with $DPPH^{\cdot}$ is commonly applied to detect potential antiradical compounds. Developed HPTLC plate is either sprayed by or dipped into a $DPPH^{\cdot}$ solution with characteristic intense purple color. In the presence of an antioxidant, $DPPH^{\cdot}$ is reduced to diphenyl picryl hydrazine by which characteristic purple color of $DPPH^{\cdot}$ changes into pale yellow. Therefore, active antiradical constituents appear on the HPTLC plate as yellow zones on the purple background (176).

#### 2.2.3.1.4. Hyphenation with HPTLC

Hyphenation is an approach of combining spectrometers with chromatographic systems aiming to get a wide range of information about the compound in a short time. The combinations of HPTLC having UV/Visible (Vis)/Fluorescence detectors with MS, high-resolution mass spectrometry (HRMS), fourier transform infrared spectroscopy (FTIR) and surface enhanced raman spectroscopy (SERS) (195).

### 2.2.4. Development and Validation of HPTLC Methods

Method development and its optimization are very challenging processes particularly in plant analysis due to their chemical complexity. The method optimization contains several steps such as sample preparation and its application, selection of the type of the stationary phase, composition of the developing solvents, chamber type, development, derivatization, detection and laboratory conditions (173).

International Conference on Harmonization (ICH) guideline states the required validation characteristics according to the type of the analytical method either for qualitative or quantitative analysis (**Table 21)** (196).

**Table 21.** Analytical methods and required validation characteristics [Adapted from ICH (196)]

| Characteristics | Qualitative methods | Quantitative methods |
|---|---|---|
| Accuracy | - | + |
| Precision | - | + |
| Specificity | + | + |
| Detection limit | - | - |
| Quantitation limit | - | - |
| Linearity | - | + |
| Range | - | + |

#### 2.2.4.1. Pre-validation

##### 2.2.4.1.1. Stability

One of the drawbacks of HPTLC is being an open system. The application positions are exposed to air, light, temperature, humidity or dust in general, these conditions can be affect the sample compound(s) or standard(s), and therefore they may undergo degradation. Therefore, stability should be checked prior validation (197).

##### 2.2.4.1.2. Robustness

Robustness evaluates the analytical method to stay unaffected according to some alterations in method parameters and indicates its reliability under normal conditions (196).

**Table 22** briefly summarizes the some factors that may have critical impact on HPTLC methods (198).

**Table 22.** Potential factors to be examined in the robustness testing (198)

| Method | Factors |
|---|---|
| HPTLC | Sample extraction and its duration<br>The type of the stationary phase (TLC or HPTLC)<br>The stationary phase from different manufacturers<br>Batch of the plates<br>Chamber saturation<br>Development distances<br>Amount of the mobile phase<br>Drying conditions (temperature, time)<br>Detection wavelength<br>Derivatization: heating time and temperature<br>Temperature<br>Stability |

### 2.2.4.2. Validation

#### 2.2.4.2.1. Specificity

Specificity of an analytical method is the ability to evaluate the analyte which is under investigation in the presence of other compounds (196).

**HPTLC criteria:** A reference plant material(s) or standard(s) and test sample solution(s) are applied on the same plate and the chemical fingerprinting profiles of the zones under investigation should be comparable with respect to number, location, color and intensity (199).

#### 2.2.4.2.2. Limit of Detection and Quantitation

The lowest amount of a compound under investigation in a sample is called limit of detection (LOD). It can be monitored whereas quantification is not needed (196).

**HPTLC criteria:** A signal to noise (S/N) ratio should be 3 or 2:1 (196).

The limit of quantitation (LOQ) is the lowest amount of an investigated compound in a sample which can be quantitatively calculated (196).

**HPTLC criteria:** S/N should be 10:1 (196).

#### 2.2.4.2.3. Linearity and Range

The linearity of an analytical method is its capacity to produce the results directly proportional to the content of interested compound in the sample (196).

One of the crucial parameters in HPTLC methods is to determine the calibration curve. In densitometry, the working range is notably narrow because measurements are performed in reflectance mode by using Kubelka-Munk equation. Consequently, the quasi-linear working range for linear regression should be determined (197).

**HPTLC criteria:** The plotting of residuals is one of the ways of testing linearity. In addition, statistical analyses such as Mandel's and Lack-of-fit tests may be used to predict the linearity (200).

#### 2.2.4.2.4. Precision

The precision defines the closeness of compatibility between series of calculations obtained from multiple sampling of the same homogeneous sample under the described conditions (196).

Precision can be grouped as repeatability, intermediate precision and reproducibility.

##### 2.2.4.2.4.1. Repeatability

Repeatability means the precision under the same operating conditions over a short period of time. Repeatability is also called as intra-assay precision (196).

**HPTLC criteria:** Relative standard deviation (RSD) < 10% (201); All fingerprints must be identical. The difference of the $R_F$ values of 3 markers across each plate should be ≤ 0.02 (199).

#### 2.2.4.2.4.2. Intermediate Precision

Intermediate precision indicates variations within laboratories such as different days, analysts and equipments (196).

**HPTLC criteria:** 1.3 to 1.7 × RSD of repeatability (197); The difference of the $R_F$ values of 3 markers should be $\leq 0.05$ (199).

#### 2.2.4.2.4.3. Reproducibility

Reproducibility states the precision between laboratories (196).

**HPTLC criteria:** The difference of the $R_F$ values of 3 markers should be $\leq 0.07$ (199).

#### 2.2.4.2.5. Accuracy

The accuracy of an analytical procedure defines the closeness of compatibility between the value which is accepted as a true or reference value and the calculated value (196).

Since it is impossible to reconstitute a blank matrix in plant materials, the accuracy of the proposed analytical method may be calculated as recovery of standards inserted to the pre-analyzed samples (202).

**HPTLC criteria:** RSD < 10%; Relative recovery of the mean value should be within 80-120% (197).

# 3. MATERIALS and METHODS

## 3.1. Materials

### 3.1.1. Plant Materials

#### 3.1.1.1. *Matricaria recutita*

Bodegold type *M. recutita* cultivar was obtained from Ataturk Central Horticultural Research Institute in Yalova province of Turkey.

Several Asteraceae plants resembling to *M. recutita* flowers were collected by local people asumming to be *M. recutita* from various localities in Turkey and named as '*M. recutita*-like materials'. All plant materials were identified by Prof.Dr. Galip Akaydın (Faculty of Education, Hacettepe University, Ankara, Turkey), Assist.Prof.Dr. Gizem Bulut (Faculty of Pharmacy, Marmara University, Istanbul, Turkey), Dr. M. Ufuk Özbek (Faculty of Science, Gazi University, Ankara, Turkey).

The voucher specimen of materials has been kept in the Herbarium of the Yeditepe University, Faculty of Pharmacy, Department of Pharmacognosy (YEF), Turkey. The identified *M. recutita*-like materials, collection regions and years are listed in **Table 23**.

**Table 23.** *M. recutita* and *M. recutita*-like materials

| No. | Plant Materials | Region | Date | Part used | YEF |
|---|---|---|---|---|---|
| 1 | *Matricaria recutita* L. | Kurtkoy, Istanbul | 03/06/2012 | Flower | 12020 |
| 2 | *Matricaria recutita* L. | Intepe, Canakkale | 13/05/2012 | Flower | 12009 |
| 3 | *Anthemis coelopoda* Boiss. var. *coelopoda* Boiss. | Odemis, Izmir | 10/05/2012 | Flower | 12018 |
| 4 | *Anthemis austriaca* Jacq. | Ankara | 08/07/2011 | Flower | 11014 |
| 5 | *Anthemis auriculata* Boiss. | Osmangazi, Bursa | 17/06/2011 | Flower | 11013 |
| 6 | *Anthemis cretica* L. | Kayısdagi, Istanbul | 25/04/2012 | Flower | 12008 |
| 7 | *Anthemis cotula* L. | Sile, Istanbul | 11/06/2011 | Flower | 11011 |
| 8 | *Anthemis altissima* L. | Osmangazi, Bursa | 17/06/2011 | Flower | 11012 |
| 9 | *Anthemis tinctoria* L. var. *discoidea* (All) D.C | Kurtkoy, Istanbul | 03/06/2012 | Flower | 12019 |
| 10 | *Anthemis scariosa* Banks et Sol. | Adana | 20/06/2012 | Flower | 12022 |
| 11 | *Bellis perennis* L. | Bayramic, Canakkale | 21/04/2012 | Flower | 12007 |
| 12 | *Bellis sylvestris* Cyr. | Amasra, Bartin | 29/09/2012 | Flower | 12023 |
| 13 | *Chrysanthemum coronarium* L. | Mugla | 08/04/2012 | Flower | 12006 |
| 14 | *Tanacetum parthenium* L. | Kayisdagi, Istanbul | 10/06/2011 | Flower | 11010 |

In addition, 5 different brands of *M. recutita* tea bags coded as M1-5 were purchased from food stores in Istanbul, Turkey. The expired dates were 2016/10, 2015/08, 2016/05, 2015/05, and 2015/09 (year/month), respectively. Moreover, bulk or packaged crude flowers (A1-11) sold as *M. recutita* were obtained from 11 different spice shops in Istanbul and named as 'crude flowers' **(Figure 13)**.

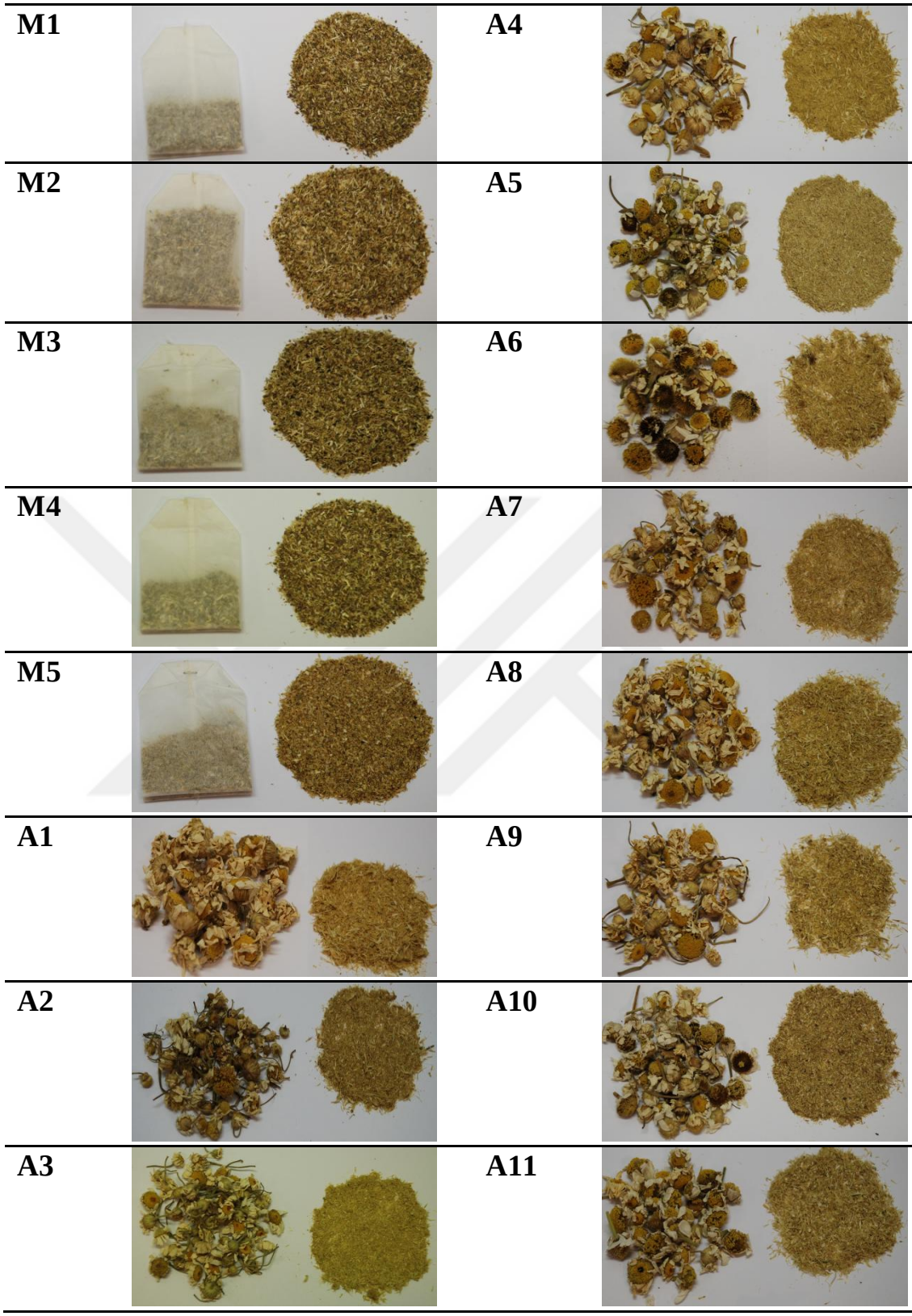



**Figure 13.** Commercial *M. recutita* products

### 3.1.1.2. *Cynara scolymus*

The young basal *C. scolymus* leaf, without central floral bud, was harvested on March 22, 2014 in Bayramic, Canakkale province of Turkey. The plant material was identified by Prof.Dr. Erdem Yesilada (Faculty of Pharmacy, Yeditepe University, Istanbul, Turkey). The voucher specimen of *C. scolymus* (YEF 12005) was deposited at the Herbarium of Yeditepe University.

On April 21, 2014, five *C. scolymus* central heads (CH) including stems (diameter of 6.5-7 cm, at early stage of maturity) were randomly picked from the same area. The stems were cut 1 cm under the head and thinly sliced. Then, each head was separated into outer bracts (~15 bracts), intermediate bracts (~12 bracts), inner bracts (rest) and receptacle. The receptacles were also cut into small pieces.

On June 2, 2014, five *C. scolymus* axillary-heads (AH) as well as their stems (diameter of 7-8 cm, at maturity) were randomly harvested from the same area. Besides, five *C. scolymus* central heads containing stems with the diameter of 12-13 cm (at maturity) were purchased from a local bazaar in Istanbul and the same procedures were also followed as previous.

In addition, 2 different capsules containing *C. scolymus* extract (C1) and *C. scolymus* enriched with *Silybum marianum* L. (C2) extracts containing at least 5% ChA were purchased from a pharmacy in Istanbul. Another brand of food supplement which was also in capsule form encoded as C3 including *C. scolymus* extract with 10% caffeoylquinic acid derivatives as ChA was obtained from a spice shop in Istanbul. Their expired dates were 2016/10, 2015/04 and 2019/04 (year/month), respectively.

The plant materials belong to *M. recutita* and *C. scolymus* were protected from direct sunlight and dried at room temperature for two weeks. In addition, dried raw materials stored in a refrigerator at -25ºC and ground to powder in a mechanic grinder and sieved before extraction. The sieve numbers according to the Ph. Eur. were 500 (*M. recutita* flower) and 1000 (*C. scolymus* leaf) (180).

### 3.1.2. Chemicals and Solvents

**Table 24.** Chemicals, solvents, distributors and lot numbers

| **Chemicals and Solvents** | **Distributors and Lot numbers** |
|---|---|
| 2-aminoethyl diphenylborinate | Fluka[a], 1358059 |
| 2,2-diphenyl-1-picrylhydrazyl | Sigma Aldrich[b], STBC5115V |
| 5,7-dihydroxy-4-methylcoumarin, ≥ 98.0% | Sigm Aldrich[b], 07922MHV |
| Apigenin, ≥ 97.0% | Sigm Aldrich[b], 096K1426 |
| Apigenin 7-*O*-glucoside, ≥ 97.0% | Fluka[a], BCBH5843V |
| Acetic acid | Riedel-de Haen[a], UN 2789 |
| Acetone | Sigma-Aldrich[b], SZBDO775V |
| Acetonitrile | J. T. Baker[c], SZBB3145V |
| Benzol | Riedel-de Haen[a], 53410 |
| Butylated hydroxytoluene | Sigma Aldrich[b], MKBD8339 |
| Caffeic acid, ≥ 98.0% | Sigma Aldrich[b], 086K1885 |
| Chlorogenic acid, ≥ 95% | Sigma Aldrich[b], SLBF3987V |
| Cynarin, ≥ 98% | Sigma Aldrich[b], SLBH0368V |
| Dichloromethane | Analar Normapur[d], K43296150 214 |
| Ethanol | Sigma Aldrich[b], SZBD0920V |
| Ethyl acetate | Sigma Aldrich[b], SZBD315SV |
| Ethyl methyl ketone | Merck[e], K38981914 |
| Ethyl formate | Merck[e], S5271191 031 |
| Formic acid | Sigma Aldrich[b], SZBB1250V |
| Gallic acid | Fluka[a], 1126284 |
| Isopropyl alcohol | Riedel-de Haen[a], 33310 |
| Luteolin 7-*O*-glucoside, ≥ 98% | Sigma Aldrich[b], BCBG6221V |
| Methanol | Sigma Aldrich[b], SZBD1270V |
| Magnesium chloride-6-hydrate | Merck[e], A914133 838 |
| *n*-hexane | Analar Normapur[f], 11H050501 |
| *o*-phosphoric acid | Merck[e], K36723873 646 |
| Polyethlene glycol 400 | Merck[e], S6041785 019 |
| Rutin hydrate, ≥ 95.0% | Sigma Aldrich[b], 09421JB |
| Sodium hydroxide | Sigma Aldrich[b], 82730 |
| Tetrahydrofuran | HiPerSolv Chromanorm[g], I 1009923 132 |
| Triethylamine | Merck[e], S5123752 823 |
| Ultrapure water | Merck Millipore, Simplicity UV[e] |

[a]Seelze, Germany; [b]Steinheim, Germany; [c]Deventer, the Netherlands; [d]Muarrie, Australia; [e]Darmstadt, Germany; [f]Muarrie, Australia; [g]Lutterworth, England

### 3.1.3. Chromatographic Plates

**Table 25.** Plates, manufacturer and lot numbers

| **Plates** | **Manufacturer** | **Lot numbers** |
|---|---|---|
| HPTLC glass 20 cm x 10 cm, Si 60 $NH_2$ $F_{254}$s | Merck | HX177941 |
| HPTLC glass 20 cm x 10 cm, Si 60 $F_{254}$ | Merck | HX377581 |
| HPTLC glass 20 cm x 10 cm, Si 60 RP-18 $F_{254}$s | Merck | HX731629 |
| TLC aluminum 20 cm x 20 cm, Si 60 RP-18 $F_{254}$s | Merck | HX42376159 |
| TLC aluminum 20 cm x 20 cm, Si 60 $F_{254}$ | Merck | 0B473478 |

### 3.1.4. HPLC Column

**Table 26.** Column, manufacturer and lot number

| **Columns** | **Manufacturer** | **Lot numbers** |
|---|---|---|
| Zorbax Eclipse Plus $C_{18}$ ODS column (5 µm, 250 mm x 4.6 mm, i.d.) | Agilent | 959990-902 |

### 3.1.5. Equipments

**Table 27.** Equipments and manufacturers

| **Equipment** | **Manufacturer** |
|---|---|
| Automatic developing chamber 2 | Camag, Switzerland |
| Balance | Ohaus Explorer, USA |
| Centrifuge tubes (10, 50 mL) | Isolab, Turkey |
| Chemstation software | Agilent, USA |
| Chromatogram immersion device III | Camag, Switzerland |
| Filter paper | Munktell, Sweden |
| Flask (10, 20, 50 mL) | Isolab, Turkey |
| Fume hood | Flores Valles, Spain |
| Glass basic laboratory equipments | Isolab, Turkey |
| Glass weighing scoops (3 and 5 mL) | Isolab, Turkey |
| Grinding mill | IKA, USA |
| Hair dryer | Rowenta, USA |
| HPLC vacuum degasser | Agilent G1379A, USA |
| HPLC quaternary pump | Agilent G1311A, USA |
| HPLC auto-sampler | Agilent G1313A, USA |
| HPLC column compartment | Agilent G1316A, USA |
| HPLC diode array detector | Agilent G1315B, USA |
| Kettle | Arcelik, Turkey |
| Linomat 5 | Camag, Switzerland |
| Lyophilizer | Christ Alpha 2-4 LD, Germany |
| Micropipette (100-1000 µL) | Rainin, USA |
| Micropipette (500-5000 µL) | Rainin, USA |
| Micropipette tips (1000, 5000 µL) | Rainin, USA |
| Oven | Binder, Germany |
| RC-disk filter, 0.45 µm | Sartorius stedim biotech, Germany |
| RC-syringe filter, 0.45 µm | Sartorius stedim biotech, Germany |
| Refrigerator | Arcelik, Turkey |
| Rotary evaporator | Heidolph, Germany |
| Sample dosage syringe 100 µL | Hamilton, Switzerland |
| Screw caps | Agilent, USA |
| Single-use syringe | HSW Soft-ject, Germany |
| Test tubes | Isolab, Turkey |
| TLC scanner 3 | Camag, Switzerland |
| TLC plate heater | Camag, Switzerland |
| TLC visualizer and Reprostar 3 | Camag, Switzerland |
| Twin-trough chamber | Camag, Switzerland |
| Ultrasonic bath | Sonorex RK156BH, Germany |
| Vacuum filtration apparatus | Sartorius stedim biotech, Germany |
| Vial (2 mL) | Agilent, USA |
| Vortex | IKA, USA |
| WinCATS and VideoScan TLC Evaluation software | Camag, Switzerland |

## 3.2. Methods

### 3.2.1. *Matricaria recutita*

#### 3.2.1.1. Preparation of Standard Solutions

##### 3.2.1.1.1. HPTLC Analysis

A7G and L7G stock solutions (50 µg/mL) were separately prepared in methanol and used for the preparation of standard mixture (Mix2a, 25 µg/mL) and applied during the method development.

Each apigenin and A7G stock solution (100 µg/mL) were prepared in methanol and utilized for the preparation of the standard mixture (Mix2b, 10 µg/mL), which was used for the preliminary HPTLC analysis.

Additional A7G stock solution (250 µg/mL) was prepared in methanol and further diluted with the same solvent to prepare the working solutions of 2.5, 5 and 10 µg/mL.

##### 3.2.1.1.2. HPLC Analysis

Stock solution of A7G (100 µg/mL) was prepared in methanol and 2.5 mL of the stock solution was further diluted to 20 mL with the initial mobile phase mixture which was water-*o*-phosphoric acid (99.5:0.5, v/v) and acetonitrile-*o*-phosphoric acid (99.5:0.5, v/v) in proportion of 75:25 (v/v) to prepare the working solution. It was encoded as reference solution A, 12.5 µg/mL.

Stock solution of 5,7-dihydroxy-4-methylcoumarin (100 µg/mL) was prepared in methanol and 5 mL of the stock solution was further diluted to 20 mL with the initial mobile phase mixture. Then, 4 mL of this solution was added to 4 mL of reference solution A and diluted to 10 mL with the initial mobile phase mixture to prepare the reference solution B, 10 µg/mL (180).

#### 3.2.1.2. Preparation of Sample Test Solutions

##### 3.2.1.2.1. HPTLC Analysis

###### 3.2.1.2.1.1. *Matricaria recutita* Cultivar

*M. recutita* tea is the most preferred form in folkloric use. Therefore, all materials used in this study were extracted with hot water. The powdered flowers (2 g) in the beaker were poured by 100 mL of freshly boiled water and it was then enclosed by watch glass and kept at room temperature for 5 min for brewing (2% infusion). After filtration through a filter paper, the filtrate was cooled and lyophilized (yield: 32.47%). Ten milligrams of lyophilizate was then accurately weighed in triplicate and dissolved with 10 mL of methanol in an ultrasonic bath for 15 min. Suspended particles were removed by filtration through a 0.45 μm RC-membrane filter. Finally, the sample test solutions were diluted 2 times and used for the method validation.

###### 3.2.1.2.1.2. *Matricaria recutita*-like Materials

The same extraction procedure was performed as described in section 3.2.1.2.1.1. The extract yields after lyophilization are given in **Table 28**. Ten milligrams of each lyophilizate was then accurately weighed and processed as described above. The final concentration of each sample test solution was 1 mg/mL.

**Table 28.** *M. recutita*-like materials and extract yields

| **Plant Materials** | **Yield (%)** |
|---|---|
| *M. recutita* (Istanbul) | 17.58 |
| *M. recutita* (Canakkale) | 20.47 |
| *A. coelopoda* var. *coelopoda* | 23.49 |
| *A. austriaca* | 19.98 |
| *A. auriculata* | 13.90 |
| *A. cretica* | 26.13 |
| *A. cotula* | 32.45 |
| *A. altissima* | 16.81 |
| *A. tinctoria* var. *discoidea* | 19.71 |
| *A. scariosa* | 20.65 |
| *B. perennis* | 27.08 |
| *B. sylvestris* | 30.60 |
| *C. coronarium* | 21.30 |
| *T. parthenium* | 20.70 |

### 3.2.1.2.1.3. Commercial *Matricaria recutita* Products

Six tea bags from each batch of the tea brand were randomly selected due to variations in their weight (1.3-2 g) and mixed in a beaker for homogeneous sampling. Then, the same extraction procedure was performed both for different tea brands (M1-5) and crude flowers (A1-11) as described in section 3.2.1.2.1.1. The extract yields after lyophilization are given in **Table 29**. Twenty milligrams of each lyophilizate was then accurately weighed and extracted as described before. The final concentration of each sample test solution was 2 mg/mL.

**Table 29.** Commercial *M. recutita* products and extract yields

| Products | Yield (%) |
|---|---|
| M1 | 17.49 |
| M2 | 20.24 |
| M3 | 24.10 |
| M4 | 19.82 |
| M5 | 28.37 |
| A1 | 24.07 |
| A2 | 28.63 |
| A3 | 19.14 |
| A4 | 20.35 |
| A5 | 14.98 |
| A6 | 15.97 |
| A7 | 17.64 |
| A8 | 21.62 |
| A9 | 20.04 |
| A10 | 12.70 |
| A11 | 13.87 |

#### 3.2.1.2.2. HPLC analysis

##### 3.2.1.2.2.1. *Matricaria recutita* Cultivar

Powdered cultivar *M. recutita* flowers (1 g) were extracted with 100 mL ethanol (96%) and heated under a reflux condenser on a heating mantle for 15 min. After cooling and filtering through a filter paper, 10 mL of freshly prepared sodium hydroxide solution (0.085 g/mL in water) was added to the filtrate. The mixture was heated again under a reflux condenser on a heating mantle for 1 h and after cooling it was diluted to 125 mL with ethanol (96%). Citric acid (0.25 g) was added to 25 mL of this solution. After shaking for 5 min, the solution was filtered through a 0.45 µm RC-membrane filter. Finally, 5 mL of the solution was diluted to 10 mL with the initial mobile phase mixture to prepare test solution A, 4 mg/mL (180).

##### 3.2.1.2.2.2. Commercial *Matricaria recutita* Products

The same extraction procedure was performed for randomly selected samples of M1-5, A1-2 and A8 as described in section 3.2.1.2.2.1.

### 3.2.1.3. HPTLC Method

HPTLC analyses were performed on 20 cm x 10 cm HPTLC plates coated with silica gel 60 $NH_2$ $F_{254}$s. Sample and standard solutions were applied on the plates as 8 mm bands, 8 mm from the bottom edge and 15 mm from the left edge by means of Linomat 5 sample applicator equipped with a 100 µL syringe. The plates were pre-conditioned with vapor of the developing solvent system of ethyl acetate-formic acid-acetic acid-water (30:1.5:1.5:3, v/v/v/v) for 10 min and then, developed up to 7 cm in the saturated (20 min) ADC2. The relative humidity was fixed to 33% by a saturated magnesium chloride-6-hydrate ($MgCl_2.6H_2O$) solution. After development and 5 min of automatic drying quantitative evaluation of the plates was performed by TLC Scanner 3 in absorption/reflectance mode at 340 nm, using slit dimensions 6 mm x 0.30 mm the scanning speed 20 mm $s^{-1}$ and the data resolution 100 µm/step. The quantitative evaluations were established through peak area via polynomial regression. For the visual documentation, the plates were heated at 100˚C on the Camag TLC plate heater for 3 min and dipped into NP and PEG 400 solutions, sequentially. After derivatization, documentation of the plates was performed by the Camag TLC Visualizer at 366 nm. All these instruments were operated by winCATS program (version 1.4.8).

### 3.2.1.4. HPLC Method

HPLC system, HP1100 series equipped with a vacuum degasser, a quaternary pump, an auto-sampler, a thermo-stated column compartment and a DAD operated by Chemstation 10.01 software was used for the analyses following the method described in the 'Matricaria flower' monograph (Ph. Eur.) (180). The separations were performed on an Agilent Zorbax Eclipse Plus $C_{18}$ ODS column (5 µm, 250 mm x 4.6 mm, i.d.) The mobile phases A and B used in this study were water-*o*-phosphoric acid (99.5:0.5, v/v) and acetonitrile-*o*-phosphoric acid (99.5:0.5, v/v), respectively. The mobile phases were degassed and filtered before use. The following gradient pattern was used: 25% B (0-9 min), 25-75% B (9-19 min), 75% B (19-24 min). The flow rate was 1 mL/min, injection volume was 20 µL and the detection was monitored at 340 nm. Identification of the peaks was performed by the retention time ($t_R$) and the DAD spectrum. The percentage content of A7G was calculated by using the following expression (180):

$$\left(\frac{A_1 \times m_2}{A_2 \times m_1}\right) \times P \times 0.625$$

$A_1$: A7G peak area obtained with test solution A

$A_2$: A7G peak area obtained with reference solution A

$m_1$: mass of the drug in test solution, in grams

$m_2$: mass of A7G in reference solution A, in grams

$P$: percentage content of A7G in reagent.

### 3.2.2. *Cynara scolymus*

#### 3.2.2.1. Preparation of Standard Solutions

##### 3.2.2.1.1. HPTLC Analysis

Standard mixture solution (Mix2c) containing 0.01% (w/w) ChA and gallic acid used in HPTLC-DPPH˙ assay was prepared by methanol.

ChA, cynarin, caffeic acid, rutin and L7G stock solutions (100 µg/mL) were separately prepared in methanol. Equal volume of each solution was used for the preparation of standard mixture (Mix5, 20 µg/mL).

##### 3.2.2.1.2. HPLC Analysis

Stock solution of ChA (100 µg/mL) was prepared in methanol and 5 mL of the stock solution was added to 5 mL of methanol. Then, it was further diluted to 20 mL with water. The final concentration was 25 µg/mL.

Stock solution of cynarin (100 µg/mL) was prepared in methanol and further diluted to 0.5, 1, 1.5, 2.5, 3, 5, 7.5 10, 15, 20 and 40 µg/mL with methanol-water (3:7, v/v) mixture to prepare working solutions.

#### 3.2.2.2. Preparation of Sample Test Solutions

##### 3.2.2.2.1. HPTLC Analysis

###### 3.2.2.1.1.1. *Cynara scolymus*

Aqueous solvent extraction was applied to *C. scolymus* leaf, outer, intermediate and inner bracts, receptacle and stem as suggested in the ‘Artichoke leaf dry extract’ monograph (Ph. Eur.) (180). The suggested method was adapted based on the traditional usage. Therefore, powdered *C. scolymus* leaf (2 g) was poured by 100 mL of freshly boiled water and it was then enclosed by watch glass and kept at room temperature for 5 min for brewing (2% infusion). After filtration through a filter paper, the filtrate was cooled and lyophilized (yield: 36.23%). The same extraction procedure was performed for *C. scolymus* outer, intermediate and inner bracts, receptacle and stem collected at

maturity and those of in early stage of maturity**.** The extract yields after lyophilization are given in **Table 30**.

Then, twenty milligrams of each lyophilizate was accurately weighed and dissolved with 10 mL of methanol in an ultrasonic bath for 15 min. Suspended particles were removed by filtration through a 0.45 μm RC-membrane filter. The concentration of the each final sample test solution was 2 mg/mL.

**Table 30.** *C. scolymus* materials and extract yields

| **Materials** | **Early maturity (CH) Yield (%)** | **Mature (CH) Yield (%)** | **Mature (AH) Yield (%)** |
|---|---|---|---|
| Outer bracts | 33.03 | 20.86 | 26.98 |
| Intermediate bracts | 35.07 | 30.41 | 45.48 |
| Inner bracts | 36.02 | 33.54 | 33.10 |
| Receptacle | 46.56 | 50.49 | 58.02 |
| Stem | 39.29 | 42.19 | 33.03 |

#### 3.2.2.2.1.2. *Cynara scolymus* Food Supplements

Five capsules from each batch of the brand (C1-3) were randomly selected and mixed in a beaker for homogeneous sampling. Hundred milligrams of each was then accurately weighed and processed as described before. The final concentration of each sample test solution was 10 mg/mL.

#### 3.2.2.2.2. HPLC Analysis

Thirty milligrams of each leaf, bract, receptacle and stem extract and C1-3 were dissolved with 25 mL of methanol-water (3:7, v/v) mixture in an ultrasonic bath for 15 min. Suspended particles were removed by filtration through a 0.45 μm RC-membrane filter. Final concentration of each sample test solution was 1.2 mg/mL.

### 3.2.1.3. HPTLC-DPPH˙ Method

HPTLC-DPPH˙ assay was used to screen *C. scolymus* leaf components for the presence of the active antioxidative constituents. *C. scolymus* leaf sample test solution (25 µg/band) and Mix2c were applied on the silica gel 60 $F_{254}$ HPTLC plate. Then, the plate was developed with a developing solvent system of ethyl acetate-formic acid-acetic acid-water (100:11:11:27, v/v/v/v) up to 7 cm in a 20 min saturated twin-trough chamber (180). After development, the plate was dried (cold air) and sprayed with freshly prepared 0.05% methanolic DPPH˙solution. The plate was stored in dark for 50 min (203). Then, image capturing and data evaluation steps were performed with Camag Video documentation system in conjuction with Reprostar 3 UV/Vis analysis lamps. The plate was captured under combined direct and transmitted white light. The images were evaluated by Camag VideoScan TLC evaluation software (version 1.02.00).

### 3.2.1.4. HPTLC Method

HPTLC analyses were performed on 20 cm x 10 cm HPTLC plates coated with silica gel 60 $F_{254}$. The plates were pre-developed with methanol-dichlorometane (1:1, v/v) containing 0.5% triethylamine (TEA). Then, they were activated in 120˚C for 20 min. Sample and standard solutions were applied on the plates as 8 mm bands, 8 mm from the bottom edge and 15 mm from the left edge by means of Linomat 5 sample applicator equipped with a 100 µL syringe. The plates were developed with the solvent system of ethyl acetate-formic acid-acetic acid-water (35:2:2:5, v/v/v/v) up to 7 cm in a saturated (20 min) ADC2. After development and 5 min of automatic drying qualitative evaluation of the plates was performed by TLC scanner 3 in absorption/reflectance mode at 330 nm, using slit dimensions 6 mm x 0.30 mm the scanning speed 20 mm $s^{-1}$ and the data resolution 100 µm/step. For the visual documentation, the plates were heated at 100˚C on the Camag TLC plate heater for 3 min and dipped into NP and PEG 400 solutions, respectively. After derivatization, documentation of the plates was performed by the Camag TLC visualizer at 366 nm. All these instruments were operated by winCATS program (version 1.4.8).

### 3.2.1.5. HPLC Method

HPLC system, HP1100 series equipped with a vacuum degasser, a quaternary pump, an auto-sampler, a thermo-stated column compartment, and a DAD operated by Chemstation 10.01 software was used for the analyses following the method described in the 'Artichoke leaf dry extract' monograph in the Ph. Eur. (180). The separations were performed on an Agilent Zorbax Eclipse Plus $C_{18}$ ODS column (5 µm, 250 mm x 4.6 mm, i.d.) The mobile phases A and B used in this study were water-*o*-phosphoric acid (99.5:0.5, v/v) and acetonitrile-*o*-phosphoric acid (99.5:0.5, v/v), respectively. The mobile phases were degassed and filtered before use. The following gradient pattern was used: 8% B (0-1 min), 8-25% B (1-20 min), 25% B (20-33 min), 25-100% B (33-35 min). The flow rate was 1.2 mL/min, injection volume was 25 µL and the detection was monitored at 330 nm. Identification of the peaks was performed by the $t_R$ and the DAD spectrum. The percentage content of ChA was calculated by using the following expression (180):

$$\frac{A_1 \times m_2 \times P \times 0.125}{A_2 \times m_1}$$

$A_1$: ChA area obtained with test solution

$A_2$: ChA area obtained with reference solution

$m_1$: mass of the extract to be examined in test solution, in milligrams

$m_2$: mass of ChA in reference solution, in milligrams

$P$: percentage content of ChA in reagent

Moreover, each peak area of cynarin standard solution versus related concentrations in the ranged from 0.5 to 40 µg/mL were used for the calibration curve to calculate cynarin content in sample test solutions.

### 3.2.3. Preparation of Detection Reagents

NP detection reagent was prepared by dissolving 1 g of 2-aminoethyl diphenylborinate in 200 mL of ethyl acetate. To prepare PEG 400, 10 g of polyethlene glycol 400 was dissolved in 200 mL of dichloromethane (173). Moreover, 0.05% (w/w) $DPPH^{\cdot}$ spraying solution was prepared by dissolving 2,2-diphenyl-1-picrylhydrazyl radical in methanol (204).

### 3.2.4. Statistical Analysis

The applications of each analyzed solution were performed in triplicate. The results were presented as mean ± standard deviation (SD). Statistical comparisons of several mean values were done by using one-way analysis of variance (ANOVA), taking the appropriate condition as a single factor. When the ANOVA leads to significant results, least significant difference (LSD) test is performed to identify where the differences occur. The evaluation of curve estimation, lack-of-fit test, and Pearson correlation coefficients ($r$) calculation were also performed. Statistically significant difference was defined as $p < 0.05$. These analyses were carried out using SPSS Data Editor (version 20.0).

# 4. RESULTS

## 4.1. *Matricaria recutita*

### 4.1.1. HPTLC Analysis and Validation

#### 4.1.1.1. Method Optimization

During the evaluation of the developing solvent systems, Mix2a was used due to close chemical structures of A7G and L7G which would help to develop conditions to provide the optimal separation.

First step was to test the developing solvent systems reported in the previous studies for the separation of phenolic compounds from co-existing components (55,173,205) **(Figures 14a-e)**. The second step was to test the combinations of the neat solvents i.e. *n*-hexane, 2-propanol, tetrahydrofuran, ethyl acetate, ethyl methyl ketone, acetone, methanol with either formic acid or acetic acid to prevent fronting of the peaks on silica gel (firstly used due to its low cost and widespread usage) and amino plates. The obtained results assisted to establish various developing solvent mixtures in different ratios **(Figures 14f-l)**. In all analyses, 20 min saturated twin-trough chamber was used. The best result was established by ethyl acetate-formic acid-acetic acid-water (30:1.5:1.5:3, v/v/v/v) using amino plates. After assessments of the stationary phase and developing solvent system some modifications have been done such as the plate was pre-conditioned and humidity was controlled (33%) in order to get better resolution.

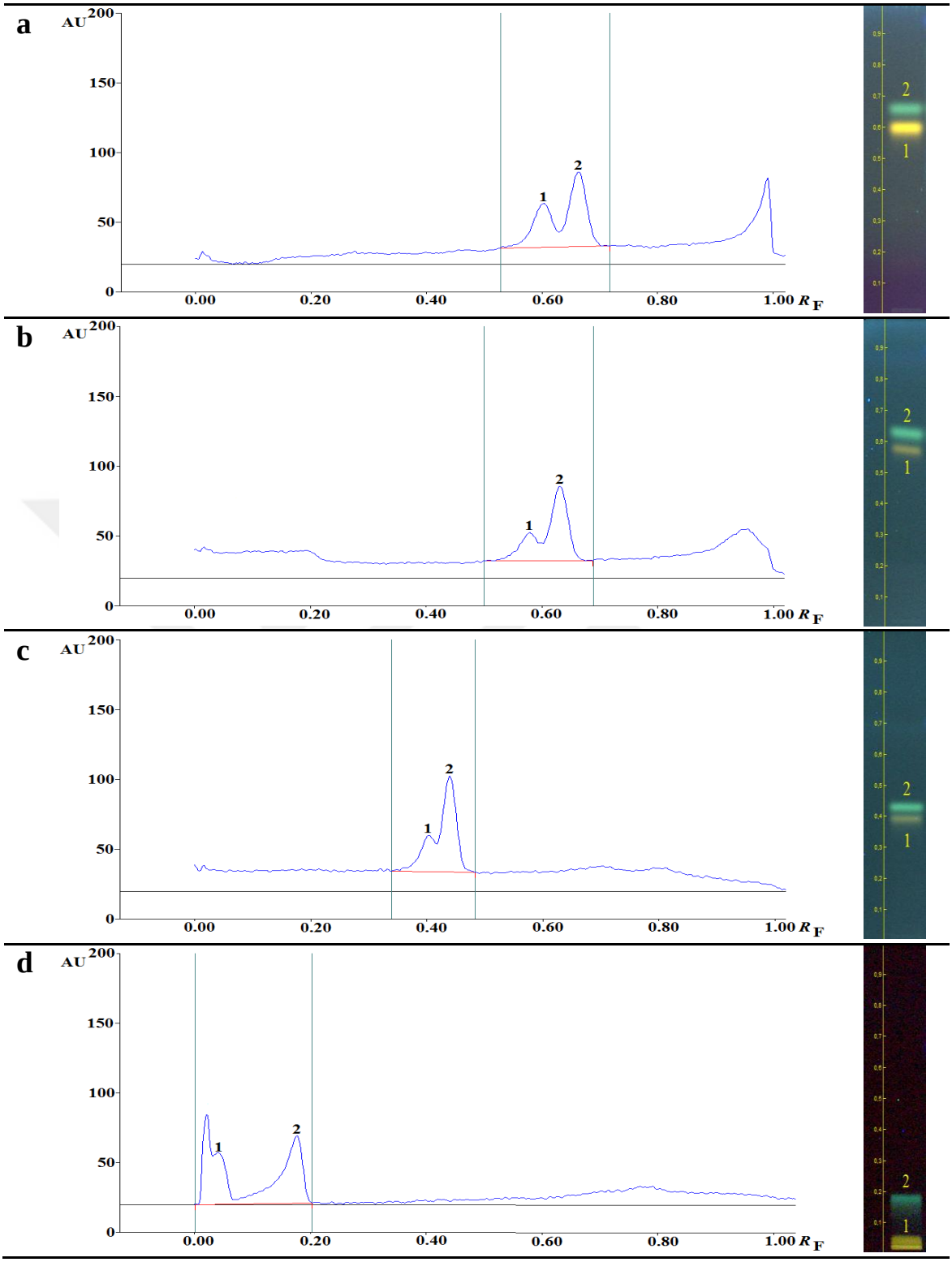



**Figure 14.** Method optimization on *M. recutita* flower extract. HPTLC densitograms (at 340 nm) and chromatograms (at 366 nm) of Mix2a (50 ng/band) separated on silica gel 60 $F_{254}$ HPTLC plate using the following developing solvent systems: (a) ethyl acetate-acetic acid-formic acid-water (100:11:11:26, v/v/v/v) (173); (b) ethyl acetate-ethyl methyl ketone-formic acid-water (5:3:1:1, v/v/v/v) (173); (c) tetrahydrofuran-toluene-formic acid-water (16:8:2:1, v/v/v/v) (173); (d) benzol-ethyl methyl ketone-methanol (5.5:3:1.5, v/v/v) (55); derivatization: NP/PEG 400; 1: L7G; 2: A7G.

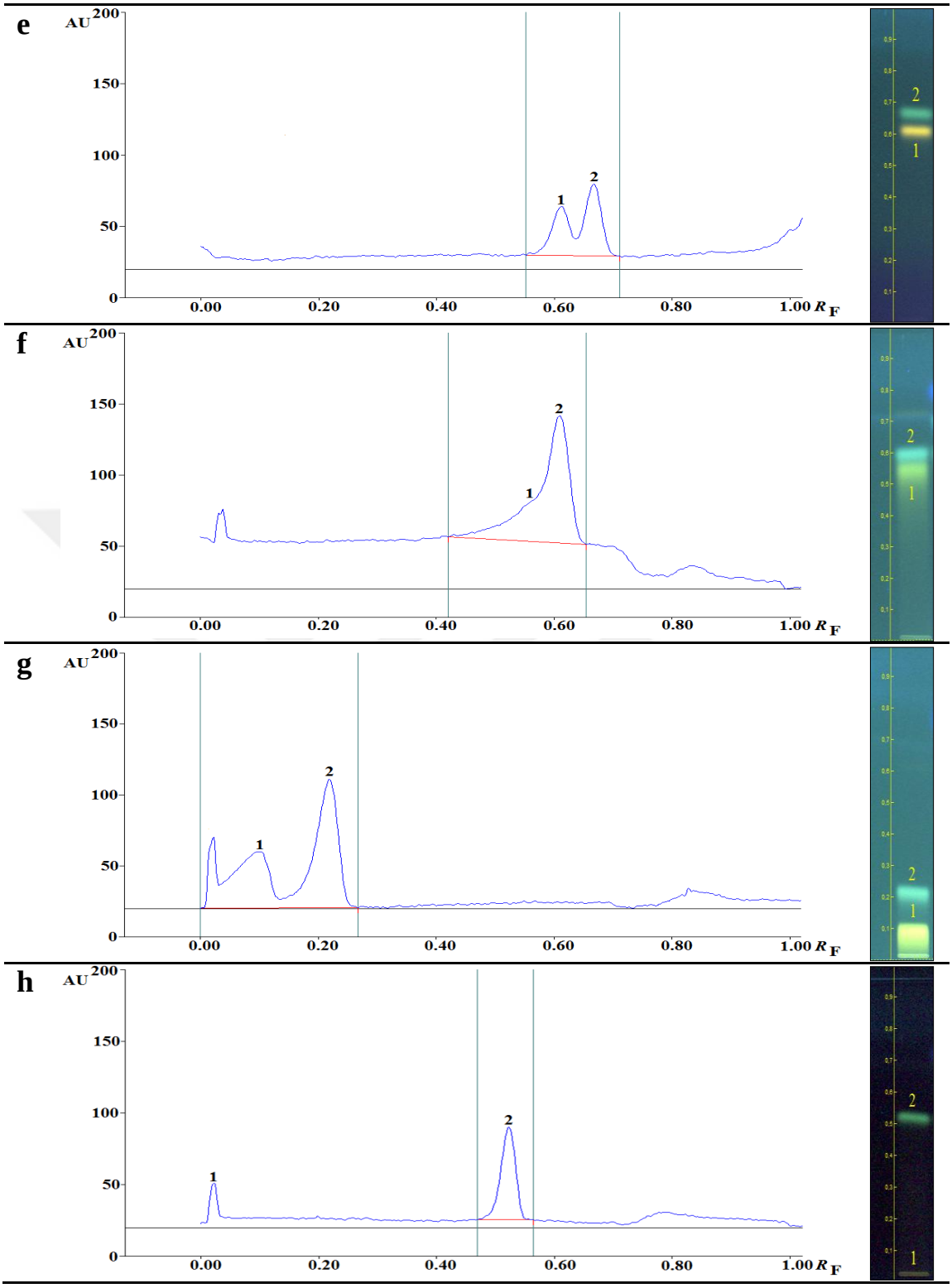



**Figure 14.** Continued. (e) ethyl acetate-acetic acid-formic acid-water (100:11:11:26, v/v/v/v) and toluene-ethyl acetate-acetic acid (50:45:5, v/v/v), incrementally developed (205); (f) acetone-*n*-hexane-2-propanol-formic acid (40:10:10:1, v/v/v/v); (g) acetone-*n*-hexane-2-propanol-acetic acid (45:15:1:1, v/v/v/v); (h) tetrahydrofuran-acetic acid (25:0.5, v/v).

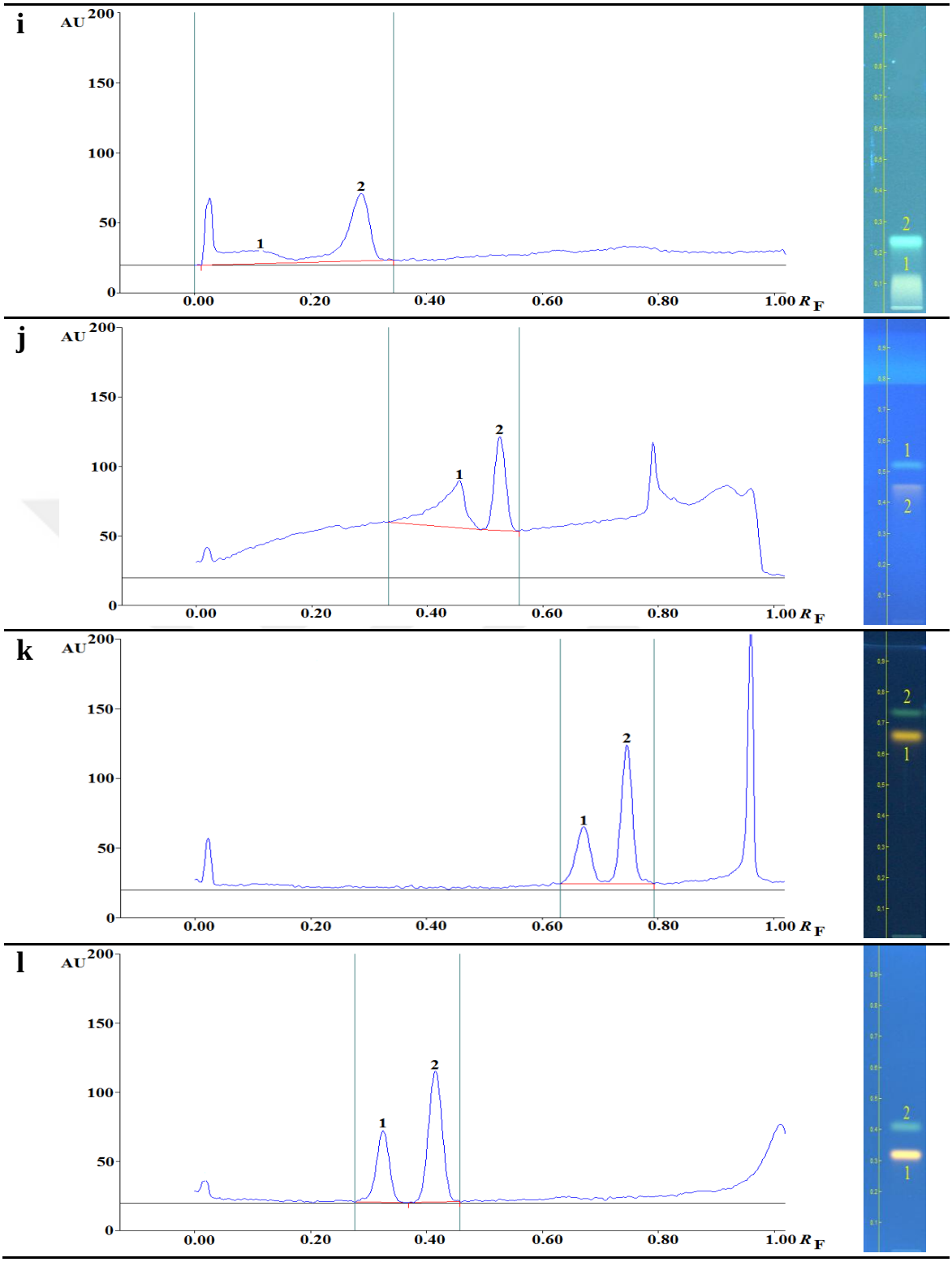



**Figure 14.** Continued. (i) ethyl methyl ketone-water-acetic acid (49:0.5:0.5, v/v/v); (j) ethyl methyl ketone-water-acetic acid-formic acid (40:5:4:1, v/v/v/v); Mix2a (50 ng/band) was developed on silica gel 60 $NH_2$ $F_{254}$s HPTLC plate using the following developing solvent systems: (k) ethyl acetate-acetic acid-formic acid-water (100:11:11:26, v/v/v/v); (l) ethyl acetate-acetic acid-formic acid-water (30:1.5:1.5:3, v/v/v/v).

### 4.1.1.2. Preliminary HPTLC Analysis

The presence of both apigenin and A7G was initially investigated in aqueous *M. recutita* flower extract. The sample test solution, apigenin and Mix2b standard solutions were developed on silica gel 60 $NH_2$ $F_{254}$s HPTLC plate using the developing system of ethyl acetate-acetic acid-formic acid-water (30:1.5:1.5:3, v/v/v/v). Then, the developed plate was derivatized with NP and PEG 400 detection reagents, respectively and evaluated at 366 nm. As a result, the $R_F$ of apigenin (Track 2) was found close to 0.80. Comparison of tracks 1, 2 and 3 belong to sample test solution, apigenin and Mix2b standard solutions, respectively resulted that not apigenin but A7G ($R_F$ = 0.38) found in the investigated sample test solution **(Figure 15)**.

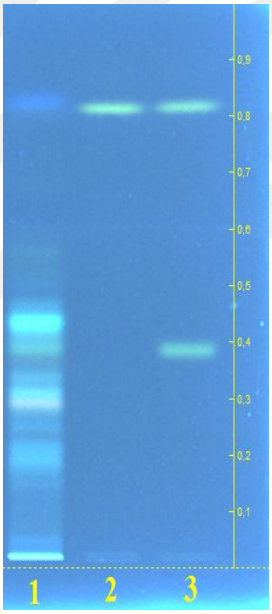



**Figure 15.** A preliminary HPTLC analysis on *M. recutita* flower extract. HPTLC chromatogram (at 366 nm) of (1) *M. recutita* sample test solution (2 ng/band), (2) apigenin (50 ng/band) and (3) Mix2b (20 ng/band) standard solutions on silica gel 60 $NH_2$ $F_{254}$s HPTLC plate developed with ethyl acetate-acetic acid-formic acid-water (30:1.5:1.5:3, v/v/v/v); derivatization: NP/PEG 400.

### 4.1.2. HPLC Analysis

Initially, the system suitability was verified for the further HPLC analyses. According to the Ph. Eur., the system suitability should be controlled by the reference solution B and the $R_s$ between the peaks belong to A7G and 5,7-dihydroxy-4-

methylcoumarin standards should be minimum 1.8 (180). In this study, $R_s$ was found as 7.54 ± 0.03 ($n$ = 3), indicating suitability of the HPLC system **(Figure 16a)**.

The identity of the A7G in test solution A was confirmed by comparing the $t_R$ with reference solution A and it was found as 6.50 ± 0.01 **(Figure 16b)**. Moreover, the percentage content of total A7G in cultivar *M. recutita* was determined to be 0.70% which was higher than the official limit (0.25%) (180). Eventually, cultivar *M. recutita* was taken into account as a reference plant material for further analyses.

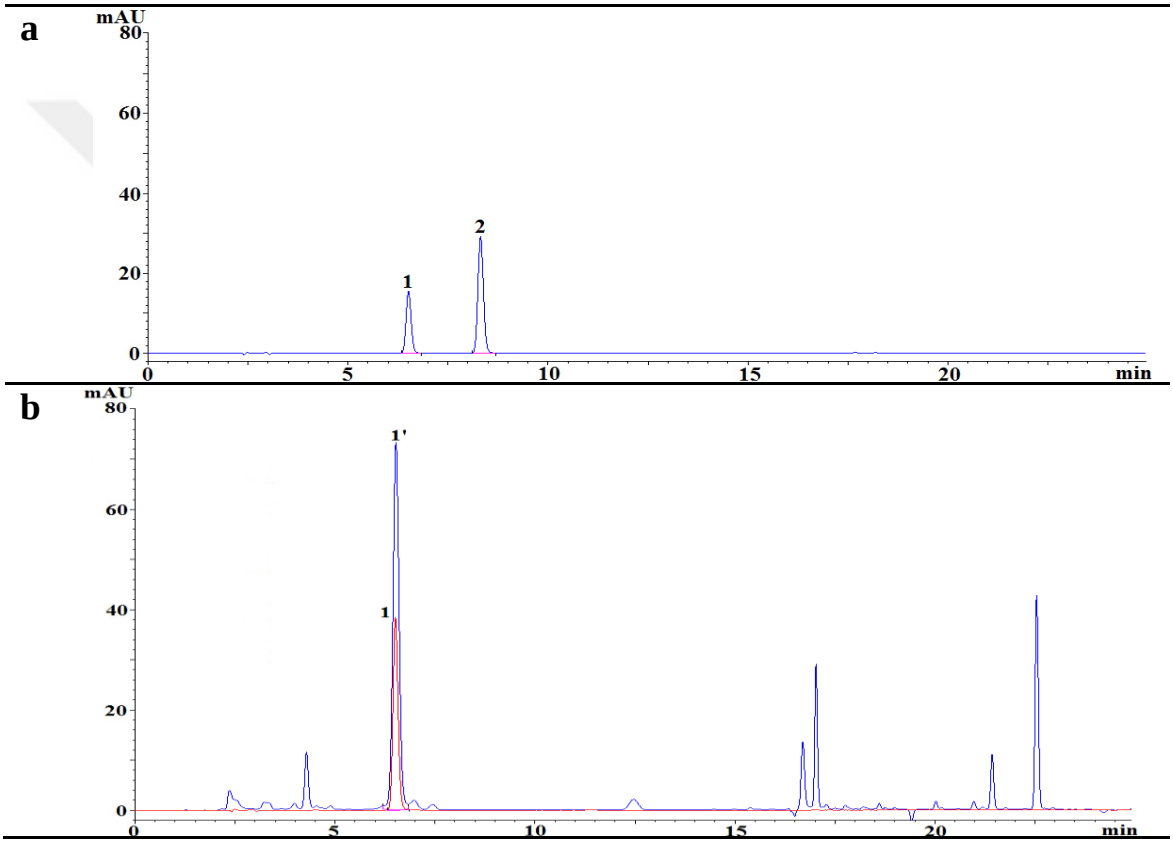



**Figure 16.** HPLC analysis on *M. recutita* flower extract. HPLC chromatograms of (a) reference solution B and (b) *M. recutita* sample test solution at 340 nm. 1 and 1': A7G; 2: 5,7-dihydroxy-4-methylcoumarin.

It should be noted that in the Ph. Eur. instead of A7G content, total A7G amount is taken into account in *M. recutita* flower extract. After extraction of *M. recutita* powdered flowers with 96% ethanol, further steps especially before and after addition of sodium hydroxide, which causes ester hydrolysis by heat, were followed by HPTLC.

As shown in HPTLC chromatogram at 366 nm, the zones around $R_F$ = 0.60 and $R_F$ = 0.70 values might be acetylated A7G derivatives because of their characteristics color similar to A7G. Besides, they have lower polarity than A7G **(Figure 17a)**. In addition, inspection of HPTLC densitograms at 340 nm also supported these findings that the peaks coded as 2 and 3 which were considered to be acetylated A7G **(Figure 17b)** disappeared after addition of sodium hydroxide **(Figure 17c)**.

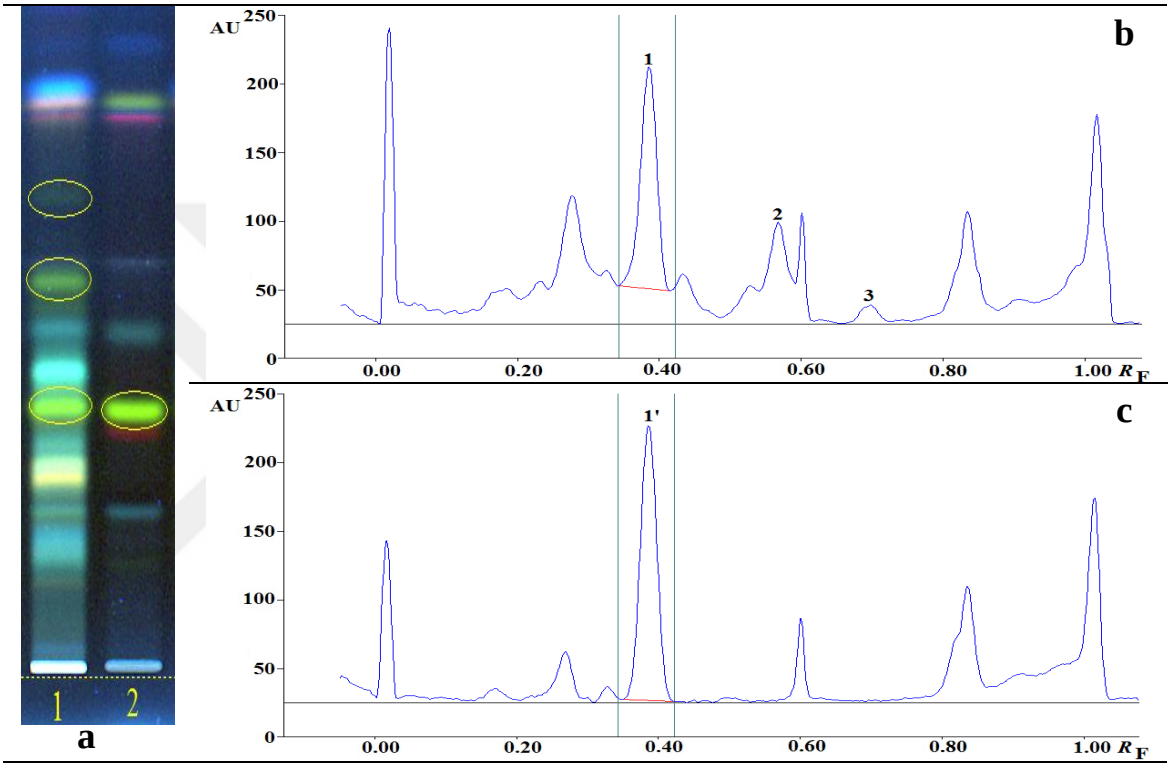



**Figure 17.** Evaluation of *M. recutita* sample preparation for HPLC analysis by HPTLC method. HPTLC chromatogram (at 366 nm) and densitograms (at 340 nm) of *M. recutita* sample test solution (a1 and b) before; (a2 and c) after ester hydrolysis; derivatization: NP/PEG 400; 1: A7G; 1': total A7G; 2 and 3: acetylated A7G derivatives.

It could be concluded that after addition of the sodium hydroxide, A7G derivatives in *M. recutita* flower extract may undergo ester hydrolysis yielding total A7G.

#### 4.1.2.3. Methodological Comparison of Developed HPTLC and HPLC

The separation power of the HPTLC method in this study was compared with the HPLC method described in the Ph. Eur. in order to demonstrate the applicability of the developed HPTLC method.

Firstly, the test solution A and A7G standard solutions were applied to HPTLC. After development, the zones belong to A7G were marked and scraped from the surface of the plate layer. A7G was extracted from the layer by using ethanol 96% and filtered through a 0.45 µm RC-membrane filter and then, solvent was evaporated by rotavapor. The residue was dissolved in 1 mL of ethanol-initial mixture of the mobile phase (1:1, v/v) and applied to both methods. Consequently, a co-existing compound with very low resolution eluted together with A7G in the HPLC method **(Figures 18a and b)**, whereas this compound could not clearly detected in the HPTLC method **(Figure 18c)**. On the other hand, comparative evaluations of the HPTLC densitogram with that of HPLC chromatogram **(Figure 18d)** found at the website of the European Directorate for the Quality of Medicines and Health Care (EDQM), gives detailed information on Ph. Eur. analysis such as type of the column and obtained chromatogram, revealed that this compound was co-eluted with A7G (206). Consequently, due to the close resolution patterns between HPTLC densitogram and HPLC chromatogram, separation power of the developed HPTLC method was found to be appropriate for further analysis.

On the other hand, HPLC chromatogram in our study **(Figure 18b)** indicated that superior separation was achieved to that of the HPLC chromatogram described by EDQM (206). This could be due to the difference in selectivity of $C_{18}$ ODS columns. In our study, Zorbax Eclipse Plus $C_{18}$ ODS column (5 µm, 250 mm x 4.6 mm, i.d.) was used whereas Nucleosil $C_{18}$ ODS column (5 µm, 250 mm x 4.6 mm, i.d.) was used in the method of Ph. Eur. which was stated in the EDQM website (206).

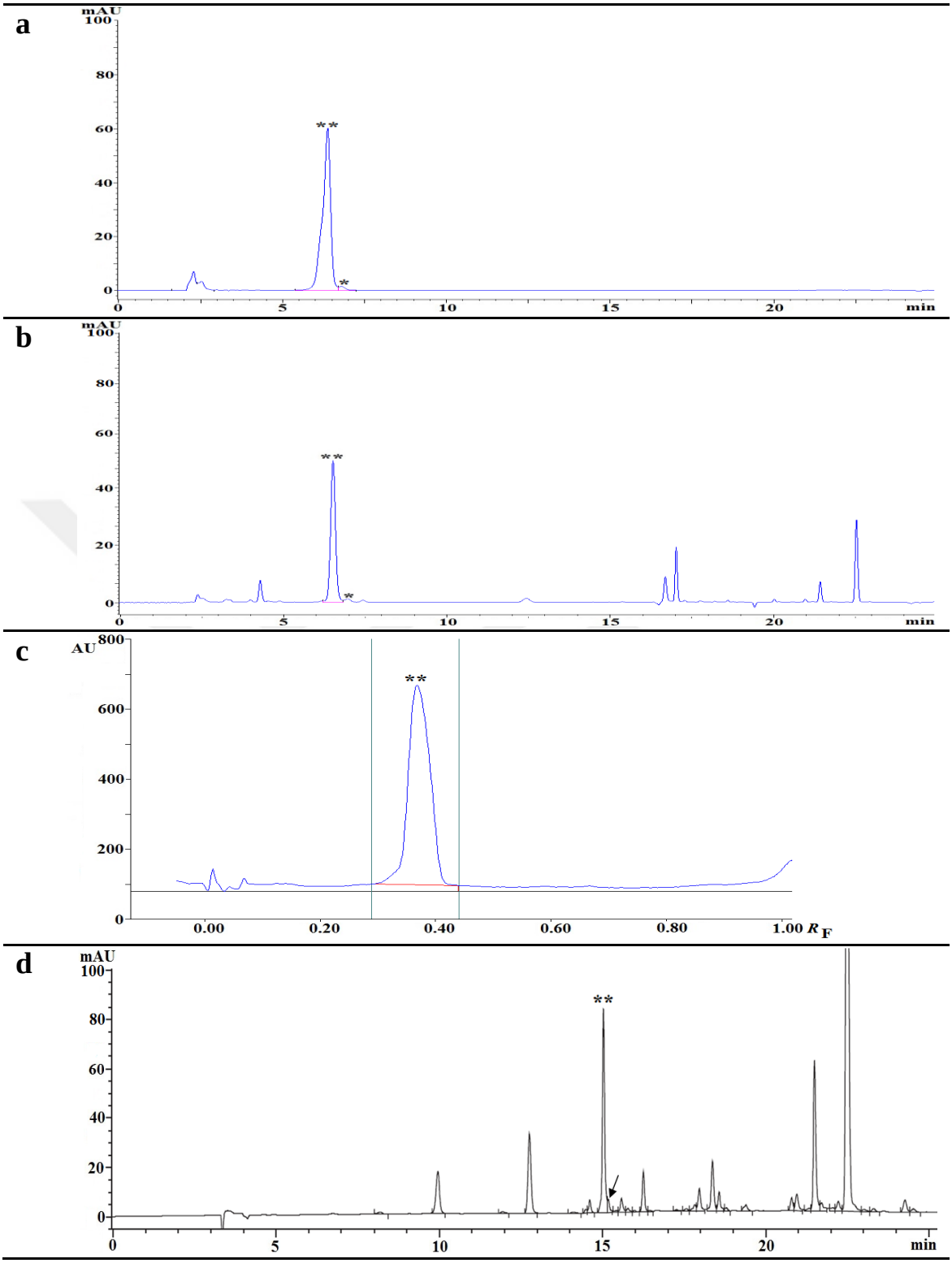



**Figure 18.** Comparative evaluation of developed HPTLC and HPLC methods. HPLC chromatograms of (a) scraped A7G in test solution A and (b) test solution A (4 mg/mL) at 340 nm; (c) HPTLC densitogram of scraped A7G in test solution A at 340 nm; (d) HPLC chromatogram of test solution A (4 mg/mL) at 340 nm stated at the website of the EDQM; '**': A7G; '*' and black arrow: co-existing compound.

#### 4.1.2.4. Validation

Following the ICH guidelines, the developed HPTLC method was validated for stability, robustness, specificity, LOD, LOQ, linearity, precision and recovery (196).

##### 4.1.2.4.1. Stability

For the evaluation of the stability, the following parameters were tested: stability of the A7G in methanol at different storing temperatures, stability of the A7G standard solution on the plate before development, and stability of the sample solution during the chromatography.

The standard solutions of A7G (0.01 mg/mL) were stored at -80˚C, -20˚C and 4˚C during three weeks. As a result of statistical evaluation by one-way ANOVA, significant changes in mean areas were not established in samples kept at -80˚C, -20˚C, 4˚C and the freshly prepared standard solutions after three weeks, $F_{(3,8)} = 0.306$, $p = 0.821$ ($F_{crit(3,8)} = 4.066$).

In addition, A7G standard solution (0.01 mg/mL) was freshly prepared and applied on the plate in 30 min intervals from 0 to 120 min before development. During these intervals the plate was left in an open area, exposed to air and light. Interruptions in time intervals were evaluated by one-way ANOVA. As a result, the mean areas were found to be different across time intervals, $F_{(4,10)} = 3.989$, $p = 0.035$ ($F_{crit(4,10)} = 3.478$). The LSD multiple comparisons performed at the 0.05 significance level found that the mean area of freshly applied A7G standard solution ($M = 587.33$, $SD = 10.75$, $n = 3$) was significantly different than the average area of the A7G standard solutions, applied in 60 min ($M = 541.97$, $SD = 33.45$, $n = 3$), 90 min ($M = 534.42$, $SD = 22.14$, $n = 3$) and 120 min ($M = 528.48$, $SD = 23.27$, $n = 3$), but not significantly different than the mean area of the A7G standard solutions, applied in 30 min ($M = 576.30$, $SD = 18.82$, $n = 3$).

Moreover, stability of the *M. recutita* sample test solution on the plate during chromatography was investigated by 2D-development. All components in the sample were detected on the diagonal line connecting the application position and the intersection of the 2 solvent fronts, which means that components were stable during chromatography.

#### 4.1.2.4.2. Robustness

The robustness of the method was evaluated by considering the effects of some alterations. The following items were investigated during the testing of robustness: duration of the plate preconditioning time, plate prewashing, chamber humidity, as well as saturation. The ratios of the area of 0.01 mg/mL of A7G standard and 0.5 mg/mL sample solutions were used during the evaluation of the variations described. The evaluated ratios were compared statistically by using one-way ANOVA. As a result, the mean proportions were not found to be different across variations, $F$ (4, 10) = 0.242, $p$ = 0.908.

#### 4.1.2.4.3. Specificity

The specificity of the method was ascertained by analyzing standard and test solutions. The identity of the A7G in *M. recutita* test solution was evaluated by comparison of the $R_F$ value with the standard solution of A7G, 0.38 ± 0.01 **(Figure 19)**. The HPTLC chromatogram at 366 nm and the spectrum of A7G belongs to standards and samples were also used for the confirmation.

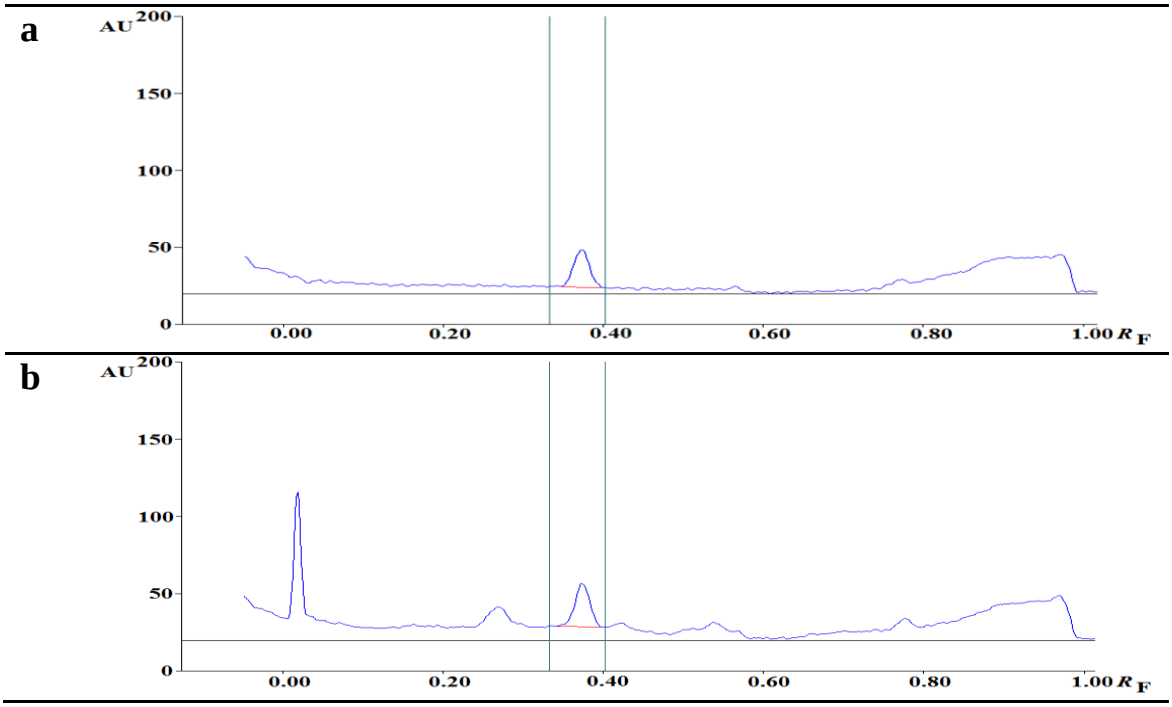



**Figure 19.** Specificity of the developed HPTLC method. HPTLC densitograms of (a) A7G standard (10 ng/band) and (b) *M. recutita* sample test solutions (1 µg/band) at 340 nm.

#### 4.1.2.4.4. Limit of Detection and Quantitation

A7G standard solutions of 0.5 µg/mL, 1 µg/mL and 2 mg/mL of were applied on the different plates in triplicate with an increased application volume (2-5 µL) to evaluate the LOD and LOQ. The visual and semi-quantitative evaluation of the S/N ratios 3:1 for the LOD and 10:1 for the LOQ was assisted to found 1.5 and 5 ng/band, respectively.

#### 4.1.2.4.5. Linearity and Calibration Curve

To avoid adverse effects of overloading on the application position in HPTLC, starting near the LOQ and selecting the calibration ranges as low as possible are recommended (202). In this study, each 2.5, 5 and 10 µg/mL of the working standard solutions was applied to HPTLC plates to obtain 5, 10, 15, 20, 25 and 50 ng/band.

The curve estimation option in SPSS compared linear or quadratic regression models, plotting the residuals and Lack-of-fit tests to evaluate how well the sets of data in the range 5 to 25 ng/band and 5 to 50 ng/band fitted either with linear or quadratic models. The results were shown in **Table 31**. The high values of *F*- test for a significant correlation, probability was lower than 0.05 for both models in both ranges, indicated that the variation explained by each model was not due to chance. Secondly, the coefficient of determination, $R^2$ values indicated that there was a strong relationship between both models and ranges. However, quadratic model of the $R^2$ ranged between 5 and 50 ng/band was higher than the linear model. The scatter plot of residuals by fit values for the linear and quadratic models was the key point for the selection of the model in this study. In the range from 5 to 50 ng/band the scatter plot of residuals by fit values for the linear model showed an 'inverted U' shaped pattern **(Figure 20a)**, indicated that there was a pattern in the data that was not captured by the linear model, whereas the scatterplot of residuals by fit values for the quadratic model did not show a pattern **(Figure 20b)**, thus the quadratic model was acceptable in sense the residuals were independent of the fit values. On the other hand, in the range 5 to 25 ng/band the scatter plot of residuals by fit values for the linear and quadratic models also did not show a pattern **(Figures 20c and d)**. In addition, Lack-of-fit test illustrated the probability of the *F*-test statistic $F(3, 10) = 0.844$ was $p = 0.501$, greater than the alpha level of significance of 0.05. These findings also supported that linear regression model

was appropriate in the range from 5 to 25 ng/band. However, widening of the calibration curve by adding one more standard (50 ng/band) was decided in this study to be more useful due to the calculations of the recovery results.

**Table 31.** Linear and quadratic model comparisons

| Range (ng/band) | Model Summary | | | | | | Parameter Estimates | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Models[a] | $R^2$ | $F$ | df[b]1 | df[b]2 | Sig.[c] | a | b | c | $n$ |
| 5-25 | Linear | 0.996 | 2925.50 | 1 | 13 | 0.00 | 16.07 | 33.21 | - | 15 |
| | Quadratic | 0.996 | 1620.91 | 2 | 12 | 0.00 | -10.64 | 37.78 | -0.15 | 15 |
| 5-50 | Linear | 0.997 | 6169.48 | 1 | 16 | 0.00 | 48.98 | 30.78 | - | 15 |
| | Quadratic | 0.999 | 8065.50 | 2 | 15 | 0.00 | -0.93 | 36.04 | -0.09 | 18 |

[a]Regression models: Linear equation: y= a + bx; Quadratic equation: y= a + bx + $cx^2$
[b]degree of freedom
[c]*p*-value

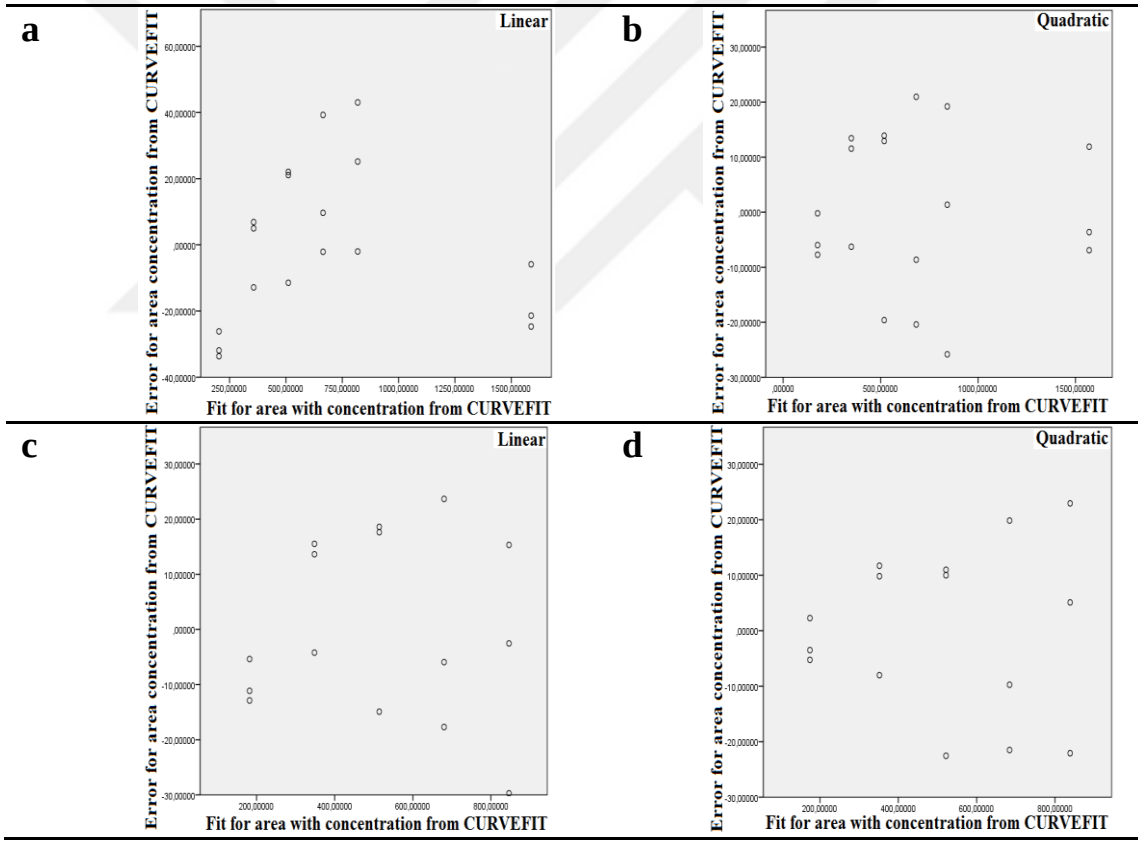



**Figure 20.** The scatter plot of residuals by fit values. A7G standard solution (a) and (b) ranged from 5 to 50 ng/ band; (c) and (d) 5 to 25 ng/ band.

#### 4.1.2.4.6. Precision

##### 4.1.2.4.6.1. Repeatability

The intraday precision of the method was performed by replicating the experiment in triplicate with three times freshly prepared samples during the day. The mean of the A7G amount in samples, SD and RSD was shown in **Table 32**.

##### 4.1.2.4.6.2. Intermediate Precision

The intermediate precision of the method was examined by freshly prepared samples in triplicate on consecutive three days. The determined intermediate precision showed similar deviations as intraday precision **(Table 32)**.

**Table 32.** Precision of the developed HPTLC method

| Intra and inter-days | A7G[a] (mg/g) | | |
|---|---|---|---|
| | **1. prepared** | **2. prepared** | **3. prepared** |
| intraday-1.[b] | 3.27 ± 0.11 | 3.20 ± 0.06 | 3.28 ± 0.06 |
| intraday-2.[b] | 3.31 ± 0.06 | 3.34 ± 0.11 | 3.21 ± 0.09 |
| intraday-3.[b] | 3.35 ± 0.11 | 3.30 ± 0.08 | 3.27 ± 0.15 |
| RSD (%) | 2.69 | 2.97 | 3.05 |
| interday | 3.32 ± 0.03 | 3.15 ± 0.15 | 3.27 ± 0.20 |
| interday | 3.35 ± 0.06 | 3.29 ± 0.12 | 3.33 ± 0.21 |
| interday | 3.30 ± 0.13 | 3.29 ± 0.07 | 3.28 ± 0.08 |
| RSD (%) | 2.37 | 3.82 | 4.65 |

[a]Mean ± SD, $n = 3$
[b]HPTLC applications

RSD values for repeatability and intermediate precision were found to be within the range of acceptance criteria (197).

#### 4.1.2.4.7. Recovery

Due to impossibility to reconstitute a blank matrix, the accuracy of the proposed method was evaluated as recovery and 2.5, 5, and 10 ng/band of standard A7G was added to the pre-analyzed sample, respectively. The average percentage of recovery of the added standard A7G was found to be in the range of acceptance criteria **(Table 33)**.

**Table 33.** Recovery results of the developed HPTLC method

| A7G[a,b] (ng/band) | Added A7G (ng/band) | Found A7G[c] (ng/band) | Recovery (%) |
|---|---|---|---|
| 10.31 ± 0.19 | 2.5 | 2.51 ± 0.33 | 100.54 |
| | 5 | 5.11 ± 0.28 | 102.11 |
| | 10 | 10.20 ± 0.50 | 102.0 |

[a]Mean ± SD, *n* = 3
[b]A7G amount in *M. recutita* sample test solution (1 µg/band)
[c]Mean ± SD, *n* = 6

### 4.1.3. Evaluation of *Matricaria recutita* and *M. recutita*-like Flower Extracts

The presence of A7G in wild *M. recutita* and *M. recutita*-like flower extracts was monitored by comparing the $R_F$ values and the spectra of the corresponding retention zones with that of the standard A7G. The amount of A7G evaluated in these samples was shown in **Table 34**.

**Table 34.** *M. recutita* and *M.recutita*-like materials and A7G contents

| Plant Materials | A7G (mg/g)[a] |
|---|---|
| *M. recutita* (Istanbul) | 0.95 ± 0.08 |
| *M. recutita* (Canakkale) | < LOQ |
| *A. coelopoda* var. *coelopoda* | 0.86 ± 0.03 |
| *A. austriaca* | 0.80 ± 0.03 |
| *A. auriculata* | < LOQ |
| *A. cretica* | < LOQ |
| *A. cotula* | ndt |
| *A. altissima* | ndt |
| *A. tinctoria* var. *discoidea* | ndt |
| *A. scariosa* | ndt |
| *B. perennis* | < LOQ |
| *B. sylvestris* | < LOQ |
| *C. coronarium* | ndt |
| *T. parthenium* | ndt |

ndt: A7G not detected; < LOQ: under the limit of quantitation
[a]Mean ± SD (*n* = 3)

In addition, comparative HPTLC chromatogram **(Figure 21)** and densitograms **(Figures 22a-n)** belong to plant materials were used for the interpretation of the results more efficiently during the discrimination of genuine specimen, *M. recutita* from *M. recutita*-like materials.

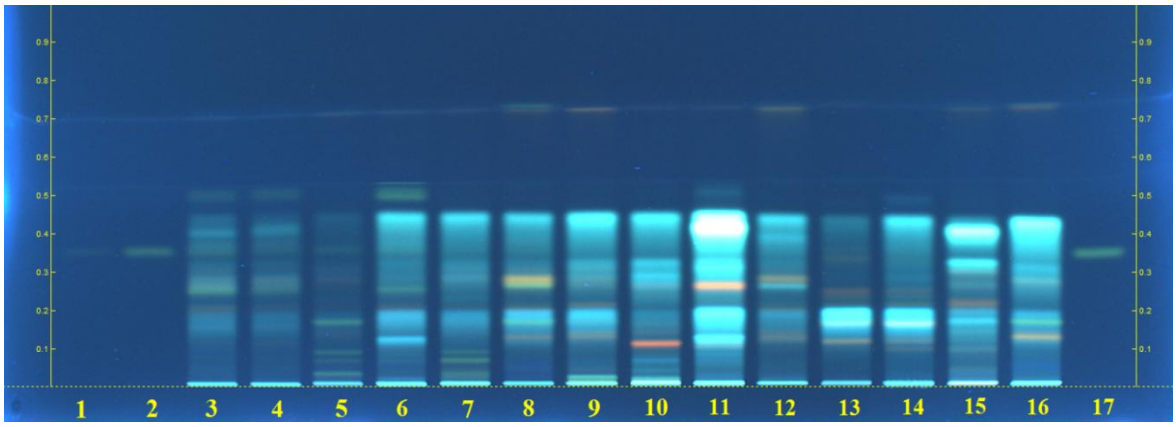



**Figure 21.** HPTLC chromatogram of *M. recutita* and *M. recutita*-like flower extracts at 366 nm. Applications 1: A7G (5 ng/band), 2: A7G (25 ng/band), 3: *M. recutita* (Istanbul), 4: *M. recutita* (Canakkale), 5: *A. coelopoda* var. *coelopoda*, 6: *A. austriaca*, 7: *A. auriculata*, 8: *A. cretica*, 9: *A. cotula*, 10: *A. altissima*, 11: *A. tinctoria* var. *discoidea*, 12: *A. scariosa*, 13: *B. perennis*, 14: *B. sylvestris*, 15: *C. coronarium*, 16: *T. parthenium*, 17: A7G (50 ng/band); applied sample test solution: 2 µg/band; derivatization: NP/PEG 400.

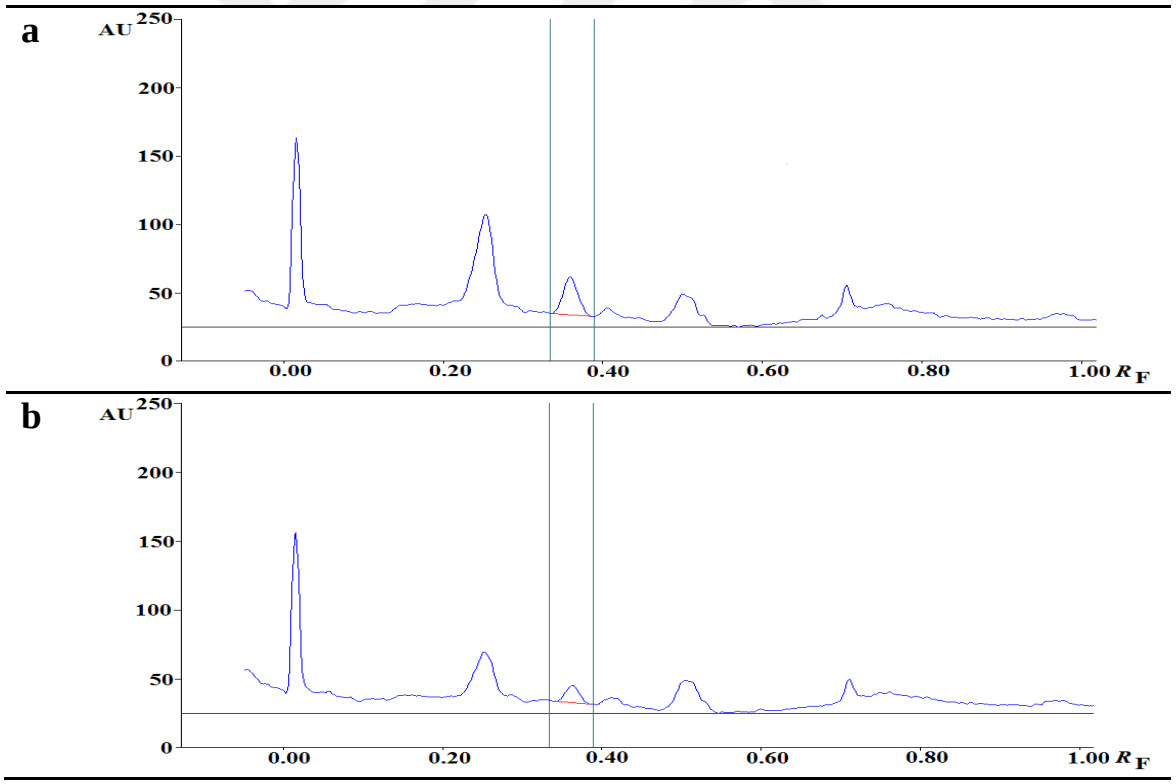



**Figure 22.** HPTLC densitograms of *M. recutita* and *M. recutita*-like flower extracts at 340 nm. (a) *M. recutita* (Istanbul) and (b) *M. recutita* (Canakkale) sample test solutions (2 µg/band).

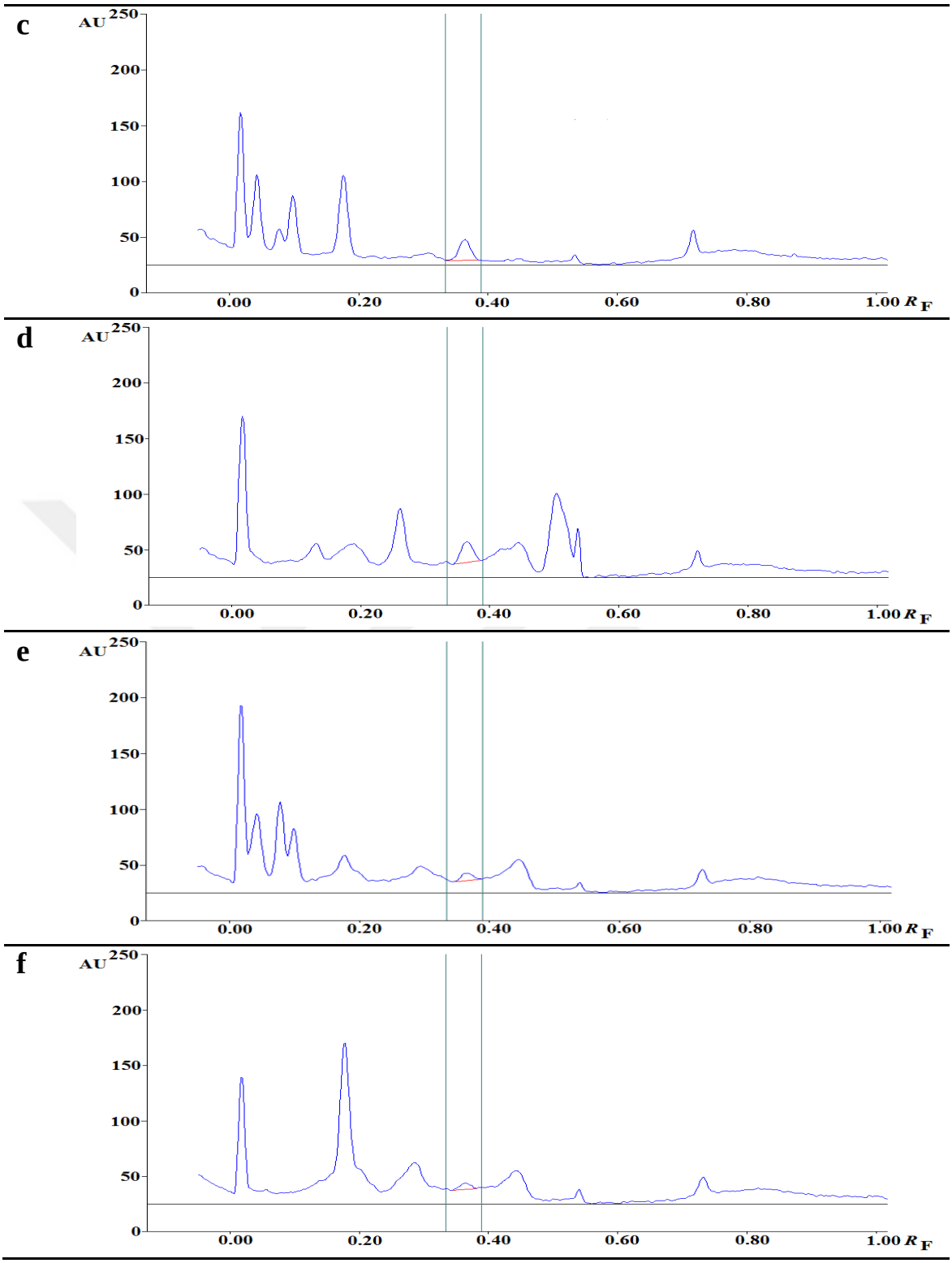



**Figure 22.** Continued. (c) *A. coelopoda* var. *coelopada*, (d) *A. austriaca*, (e) *A. auriculata* and (f) *A. cretica* sample test solutions (2 µg/band).

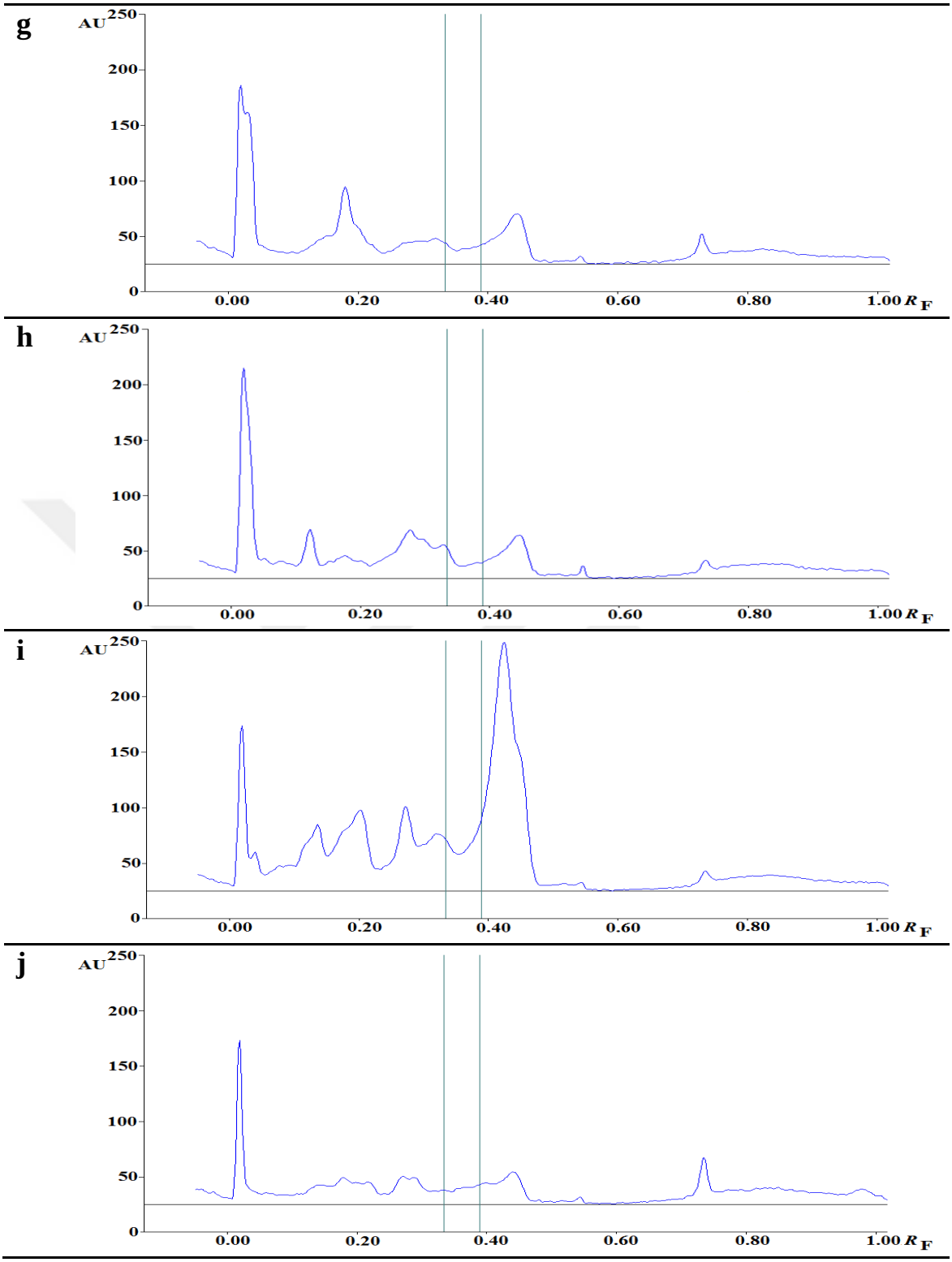



**Figure 22.** Continued. (g) *A. cotula*, (h) *A. altissima*, (i) *A. tinctoria* and (j) *A. scariosa* sample test solution (2 µg/band).

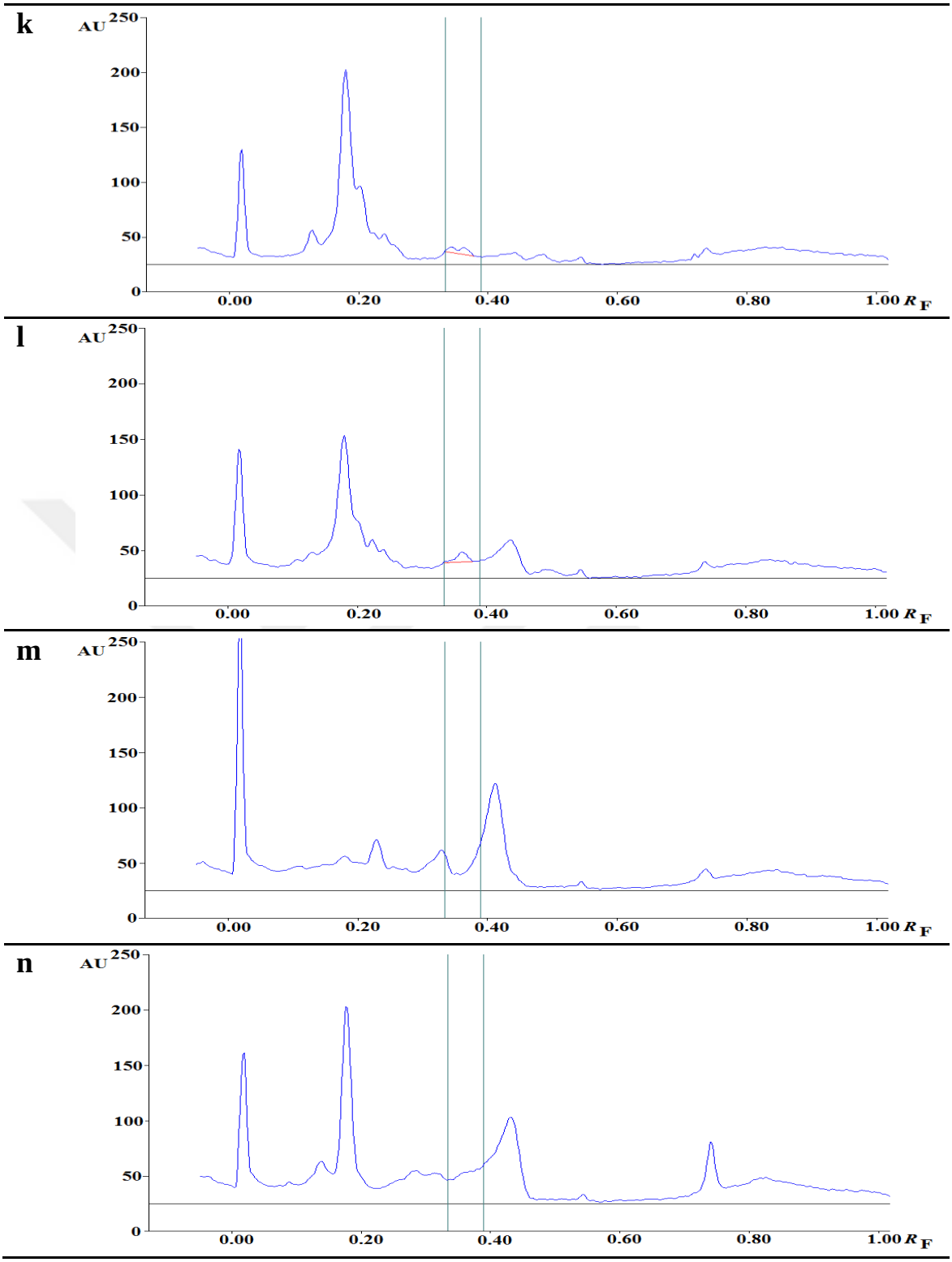



**Figure 22.** Continued. (k) *B. perennis*, (l) *B. sylvestris*, (m) *C. coronarium* and (n) *T. parthenium* sample test solutions (2 µg/band).

It was concluded that A7G was found in the wild *M. recutita* samples from two different localities along with *A. coelopoda* var. *coelopoda*, *A. austriaca*, *A. auriculata*, *A. cretica*, *B. perennis* and *B. sylvestris* samples. Subsequently, the amount of A7G was evaluated in these species and its content was ranged from 0.80 to 0.96 mg/g. Besides, in some plant species A7G was under the LOQ and therefore could not be calculated **(Table 34)**. When A7G content was compared in wild *M. recutita* samples, it was found that A7G concentration was quite different in these samples collected from different locations: Istanbul (0.96 mg/g) and Canakkale (below LOQ). On the other hand, A7G contents of these wild *M. recutita* samples were found to be lower than the reference *M. recutita* (3.29 mg/g). On the other hand, A7G was not identified in *A. cotula*, *A. altissima*, *A. tinctoria* var. *discoidea*, *A. scariosa*, *C. coronarium* and *T. parthenium* samples.

It should be underlined that although A7G is an active marker, it should not be considered as a chemotaxonomic marker for discriminating *M. recutita* from *M. recutita*-like materials. Therefore, the densitograms of all plant materials at 340 nm were compared. Consequently, chemical fingerprint profiles of *M. recutita* samples from Istanbul and Canakkale were matched with that of the reference *M. recutita*. On the other hand, fingerprint profiles of *M. recutita*-like flower extracts were not matched with that of the reference *M. recutita*. In other words, it was clearly seen that *M. recutita* has a unique chemical fingerprinting profile than that of *M. recutita*-like materials.

### 4.1.4. Evaluation of Commercial *Matricaria recutita* Products

#### 4.1.4.1. HPTLC Analysis

The presence of A7G was monitored by comparing the $R_F$ values with that of the standard A7G in commercial *M. recutita* products bought from different food stores and spice shops. Then, A7G content was quantified in these products as shown in **Table 35**.

**Table 35.** Commercial *M. recutita* products and A7G contents

| **Products** | **A7G (mg/g)[a]** |
|---|---|
| M1 | 0.49 ± 0.01 |
| M2 | 0.49 ± 0.03 |
| M3 | 0.80 ± 0.02 |
| M4 | 0.54 ± 0.01 |
| M5 | 0.43 ± 0.01 |
| A1 | 0.30 ± 0.02 |
| A2 | 0.51 ± 0.01 |
| A3 | < LOQ |
| A4 | ndt |
| A5 | < LOQ |
| A6 | < LOQ |
| A7 | 0.29 ± 0.01 |
| A8 | 0.26 ± 0.01 |
| A9 | ndt |
| A10 | ndt |
| A11 | < LOQ |

ndt: A7G not detected; < LOQ: under the limit of quantitation
[a]Mean ± SD ($n$ = 3)

In addition, HPTLC chromatogram **(Figure 23)** and densitograms **(Figures 24a-p)** were used for the interpretation of the results more efficiently during the evaluation of genuine specimen, *M. recutita* among marketed products.

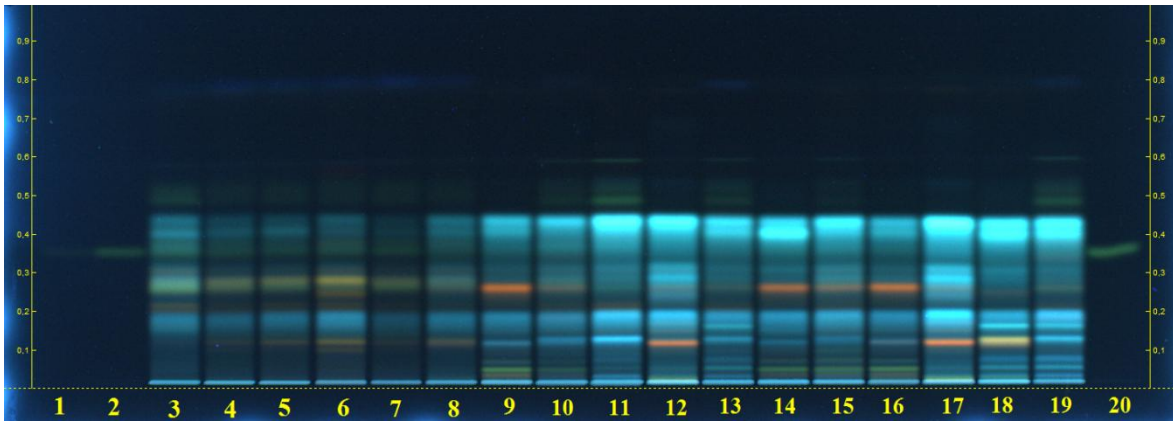



**Figure 23.** HPTLC chromatogram of commercial *M. recutita* products at 366 nm. Applications: 1: A7G (5 ng/band), 2: A7G (25 ng/band), 3: *M. recutita* (Istanbul), 4-8: M1-5 (4 µg/band), 9-19: A1-11 (4 µg/band), 20: A7G (50 ng/band); derivatization: NP/PEG 400.

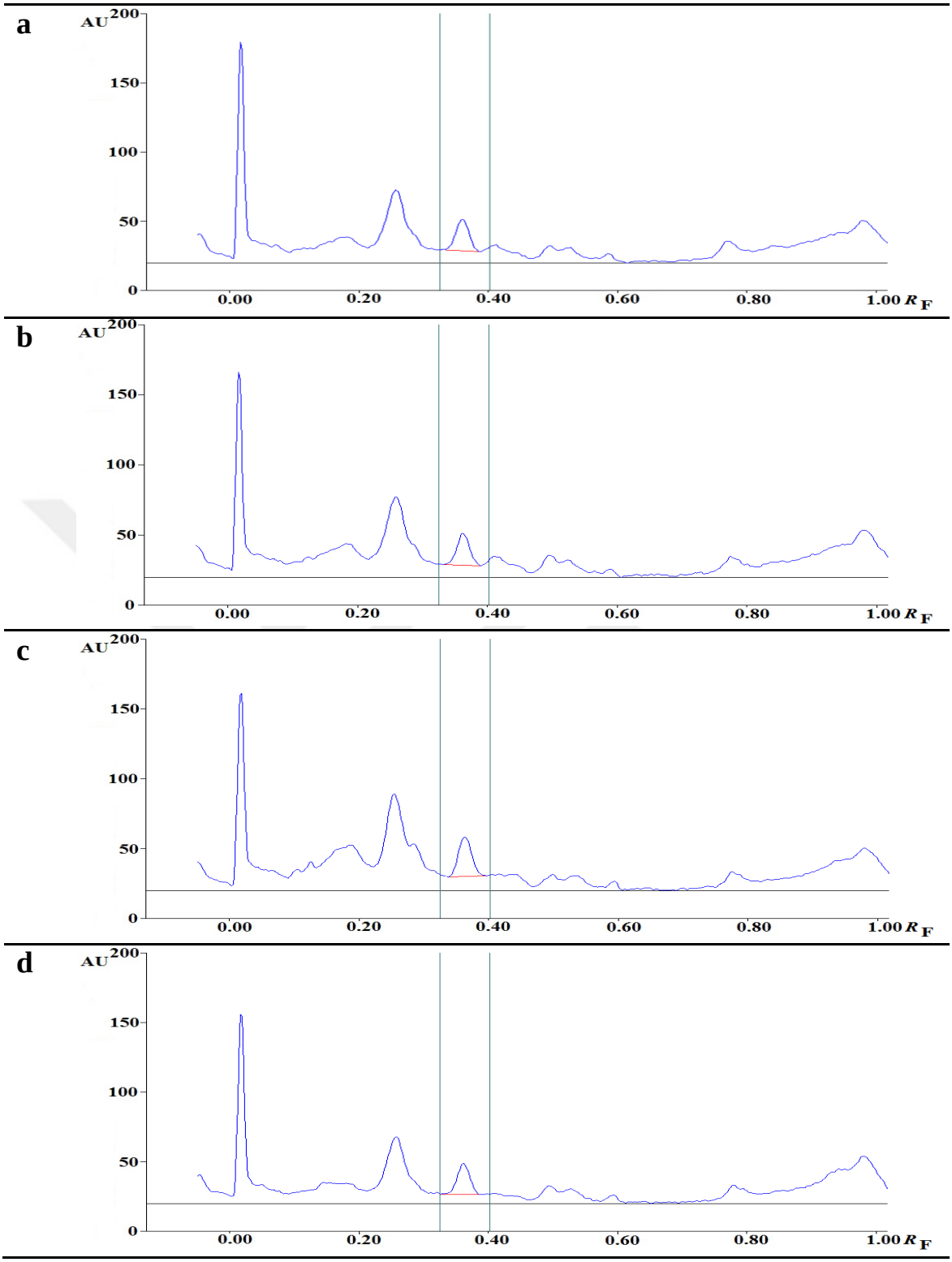



**Figure 24.** HPTLC densitograms of commercial *M. recutita* products at 340 nm. (a) M1, (b) M2, (c) M3 and (d) M4 sample test solutions (4 µg/band).

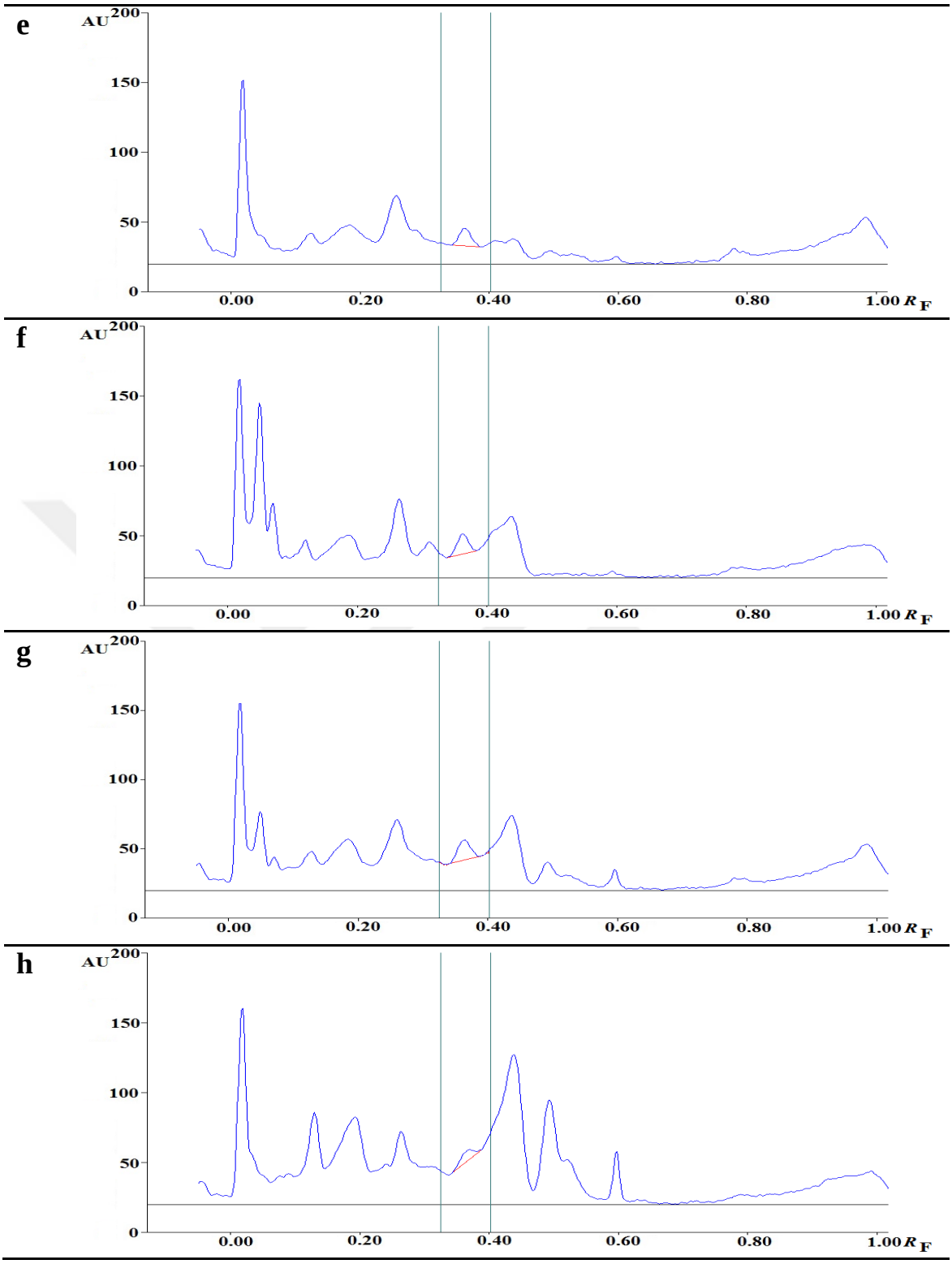



**Figure 24.** Continued. (e) M5, (f) A1, (g) A2 and (h) A3 sample test solutions (4 µg/band).

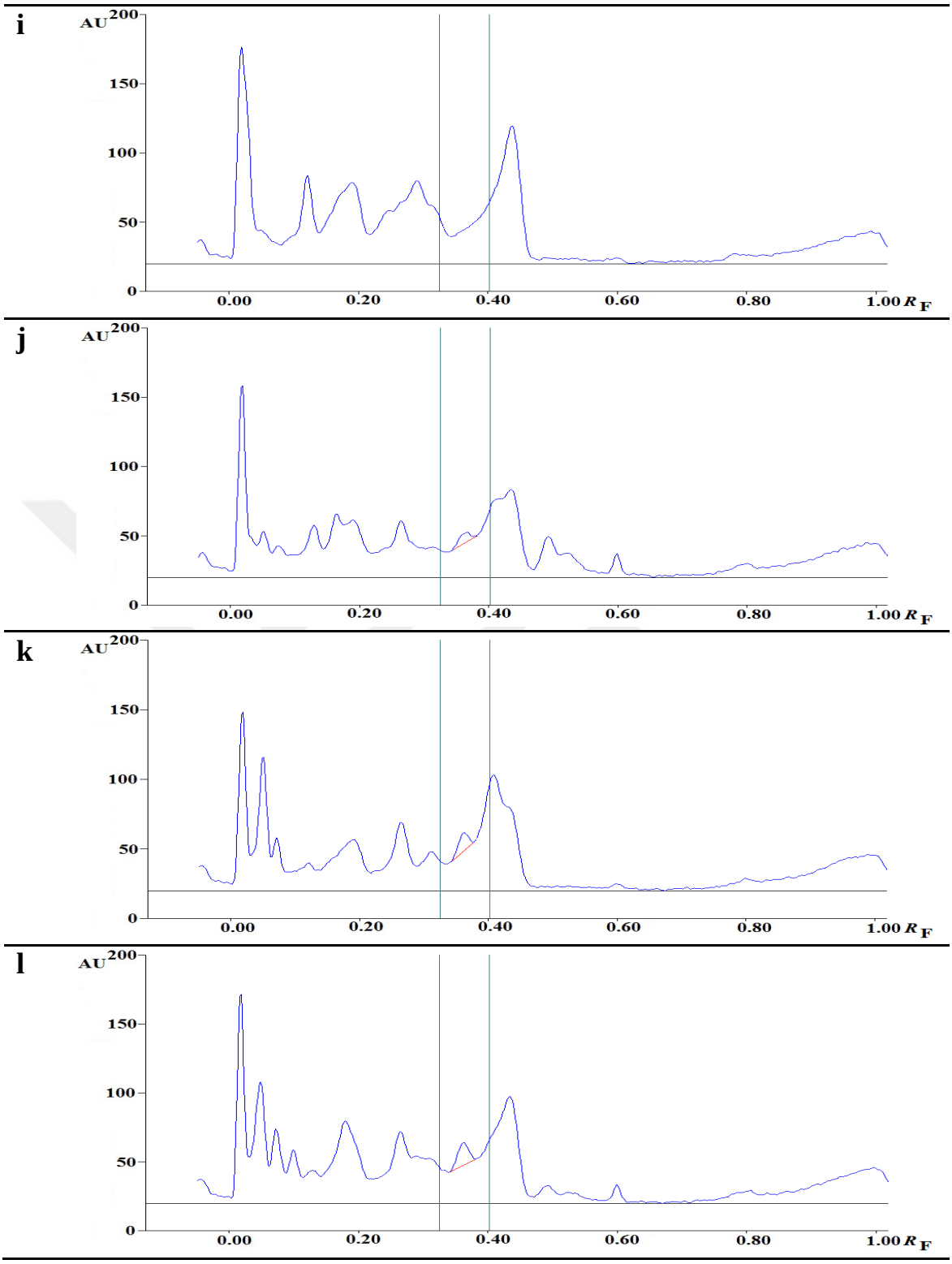



**Figure 24.** Continued. (i) A4, (j) A5, (k) A6 and (l) A7 sample test solutions (4 µg/band).

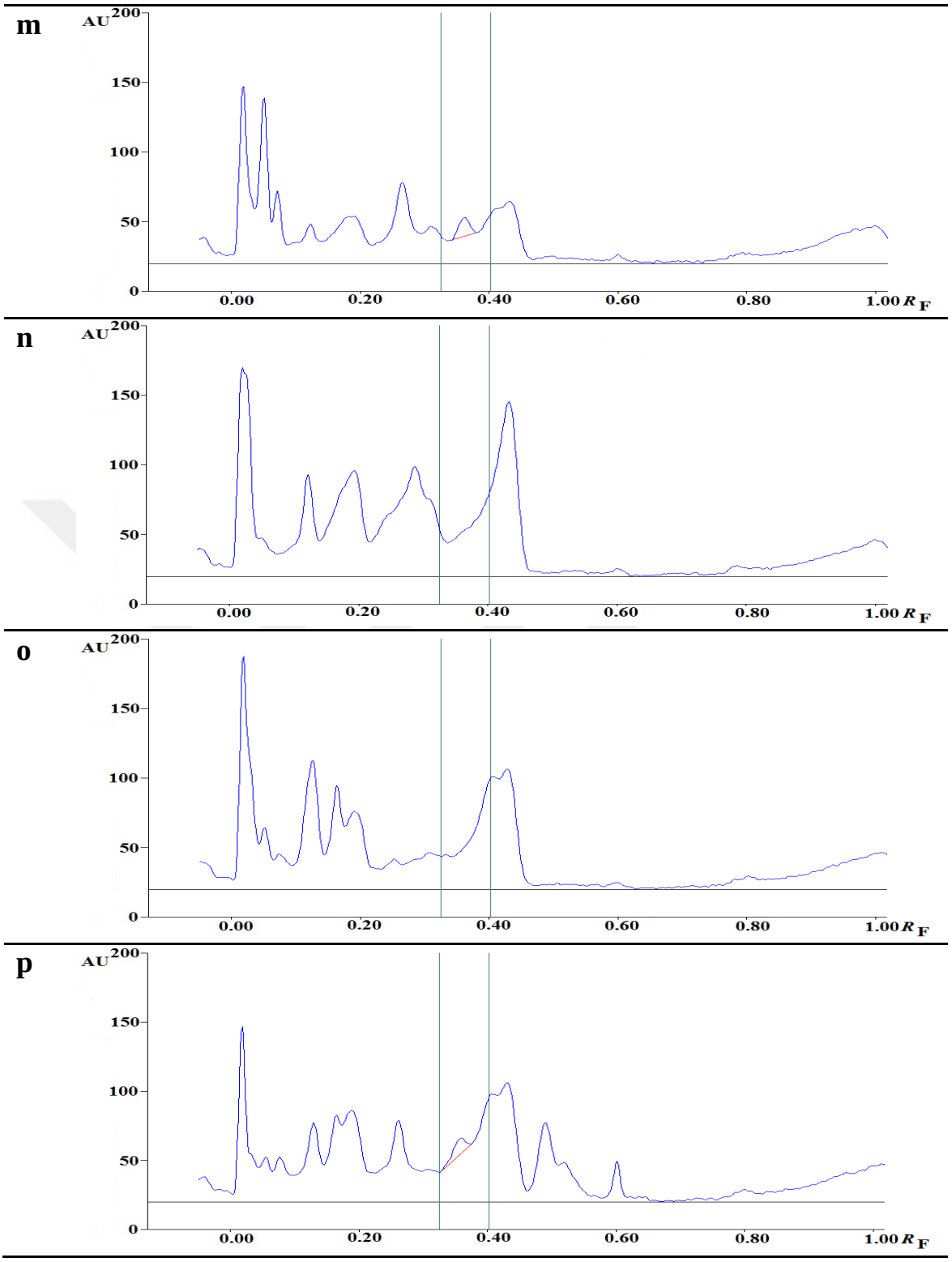



**Figure 24.** Continued. (m) A8, (n) A9, (o) A10 and (p) A11 sample test solutions (4 µg/band).

The presence of A7G was investigated in commercial *M. recutita* products. All tea bags from different brands (M1-5), A1-3, A5-8, as well as A11 samples were found to be containing A7G. Moreover, HPTLC densitograms of both wild *M. recutita* and the reference *M. recutita* samples were compared with that of HPTLC densitograms of all marketed samples. As a result, M1-5 samples showed almost identical chromatogram both with wild *M. recutita* samples and the reference *M. recutita* at 340 nm. On the other hand, different fingerprint densitograms were obtained from A1-11 samples, and none of their densitograms were matched with that of wild and the reference *M. recutita*. In addition, it is clearly postulated that the densitograms of A1, A6 and A8; A4 and A9; and also A5 and A11 were found to be identical, respectively. Although each sample was bought from different spice shops, sellers of these samples may be purchased them from the same suppliers or retailers.

The chromatograms of different species stated in the section 4.1.3 revealed that the intense blue bright zones at $R_F$ = 0.42-0.5 were seen in the *Anthemis* sp., *Bellis* sp., *Tanacetum* sp. flower extracts and also an intense orange zones close to $R_F$ = 0.1 and $R_F$ = 0.2-0.3 was also observed in the flowers of *A. altissima* L., *A. tinctoria* L. var. *discoidea* (All) D.C and *A. scariosa* L. These specified zones were also seen chromatograms of A2-3, A5, and A7-10. Eventually they might be adulterated with that of the mentioned species.

A7G content determined by HPTLC method in different tea brands was ranged from 0.43 to 0.80 mg/g, shown in **Table 35**. Besides, in crude flowers sold in spice shops, A7G was either not determined or its found amount was lower than the LOQ. On the other hand, A7G content in A2 was found as similar as M1-2 and M4 **(Table 35)**. However, it was found to be adulterated with other species.

#### 4.1.4.2. HPLC Analysis

Sample preparation for HPLC method stated in the Ph. Eur. was compared with proposed HPTLC method. Therefore, M1-5 which had identical chemical fingerprinting with that of the reference *M. recutita* and also A1-2 and A8 samples thought to be adulterated as a result of HPTLC analysis were used as an explanatory example. As mentioned before, in HPLC analysis instead of A7G content, total A7G content is determined after addition of sodium hydroxide to provide ester hydrolysis. In **Figures 25a-e**, HPLC chromatograms of M1-5 samples were compared with the HPLC chromatogram of reference *M. recutita* **(Figure 16b)**.

When chromatograms belong to M1-5 matched with that of the reference *M. recutita*, almost identical fingerprinting profiles were found. Further, chromatograms of A1-2 and A8 **(Figures 25f, g and h)** were also compared with that of the reference *M. recutita*. Consequently, not A1 and A8 samples but A2 showed a close fingerprinting profile with that of the reference *M. recutita*. This could be due to elimination of other components after ester hydrolysis which would be helpful in detection of the adulteration **(Figure 17a)**. Therefore, HPLC chemical fingerprinting profile was not enough selective to reveal adulteration observed by HPTLC profile.

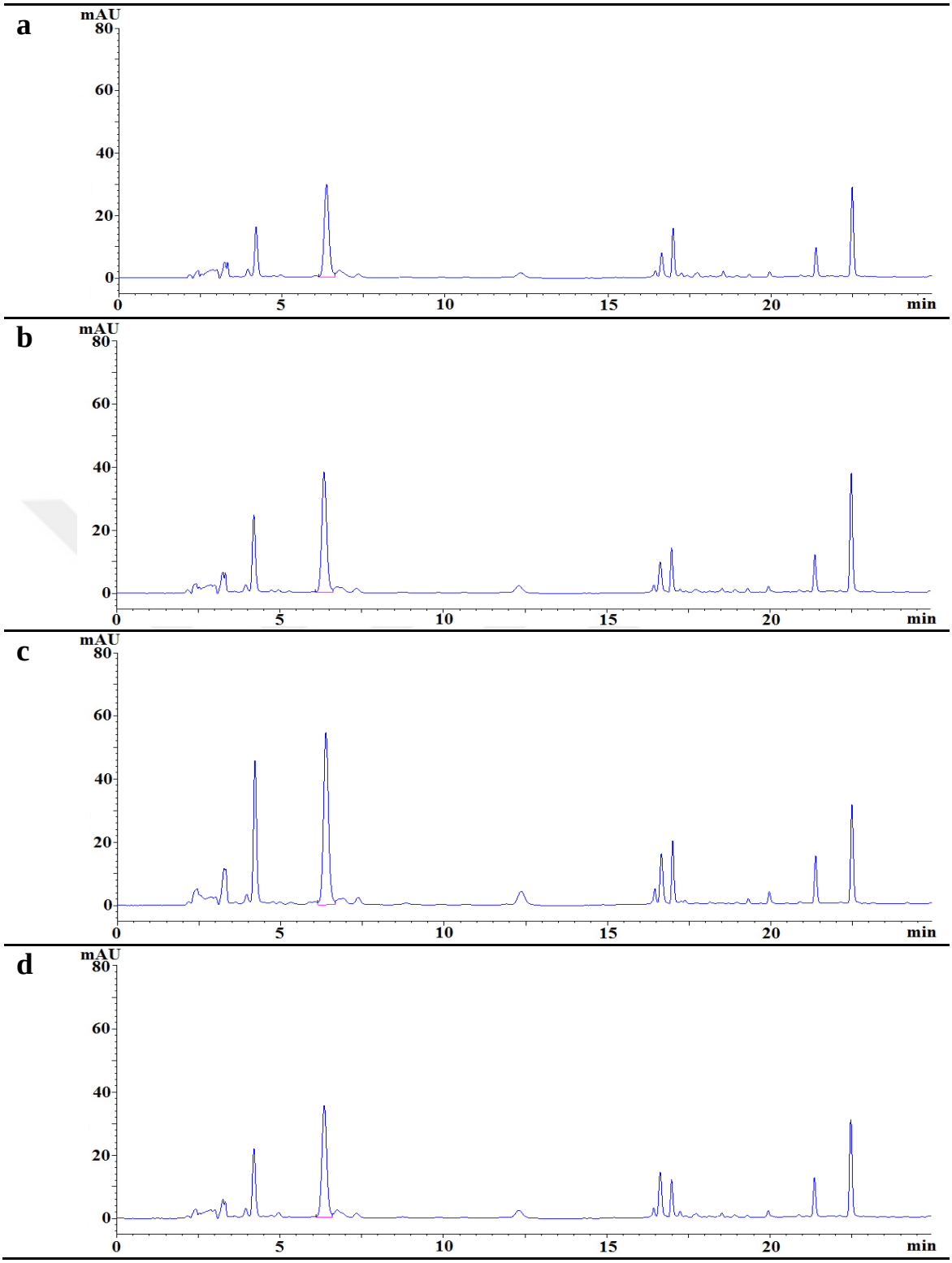



**Figure 25.** HPLC chromatograms of commercial *M. recutita* products at 340 nm. (a) M1, (b) M2, (c) M3 and (d) M4 sample test solutions (4 mg/mL).

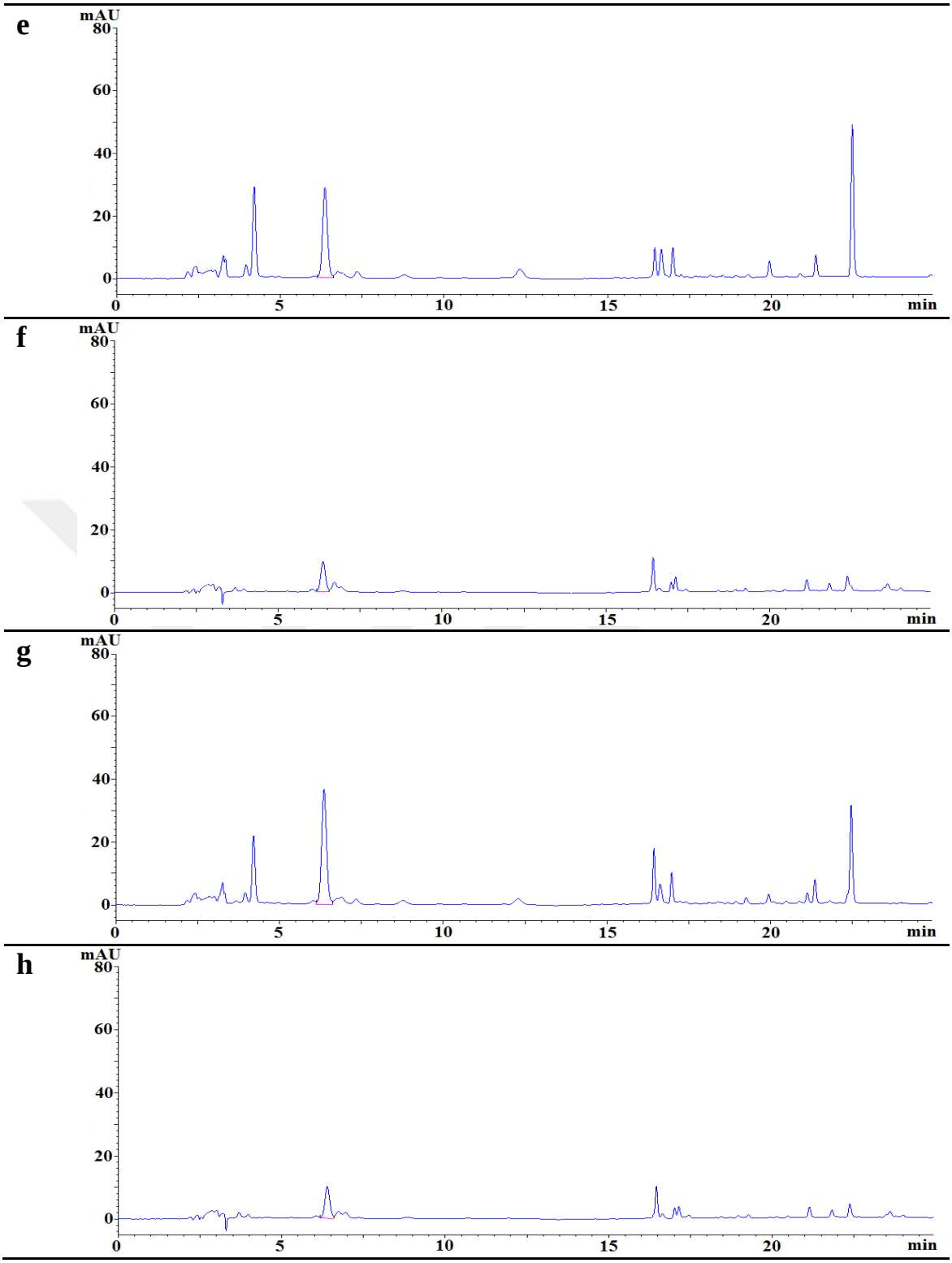



**Figure 25.** Continued. (e) M5, (f) A1, (g) A2 and (h) A8 sample test solutions (4 mg/mL).

## 4.2. *Cynara scolymus*

### 4.2.1. Preliminary Chromatographic Studies

#### 4.2.1.1. TLC Analysis and Size-Exclusion Chromatography

According to the Identification C section belong to 'Artichoke leaf dry extract' monograph (Ph. Eur.), it is stated that *C. scolymus* leaf extract should be developed on TLC silica gel plate using ethyl acetate-acetic acid-formic acid-water (100:11:11:27, v/v/v/v) as a developing solvent system (180). Additionally, ChA and L7G are used as identity markers to authenticate *C. scolymus* leaf **(Figure 26)**.

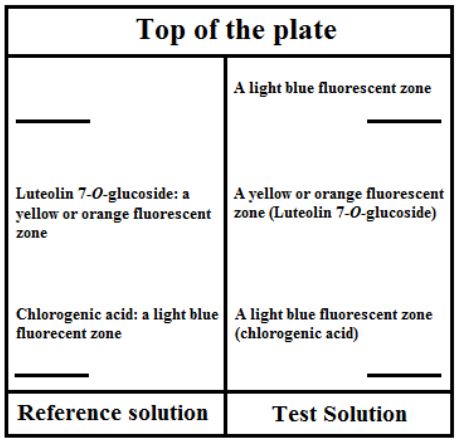



**Figure 26.** Schematized TLC chromatogram [Adapted from Ph. Eur. (180)]

To perform gel filtration or in other words sephadex column chromatography which separates low and high molecular weight molecules powdered *C. scolymus* leaf (3.5 g) was extracted with methanol by continuous rotary movement at 40˚C for 1 hour. After filtration through a filter paper, methanol moved away from the filtrate under reduced pressure at 40˚C by using rotary evaporator (yield: 700 mg). The same procedure was repeated to increase the yield of the extraction.

Before the chromatographic separation fifty grams of Sephadex LH-20 was mixed with methanol and kept at room temperature for 1 night. Then, it was filled inside a suitable glass column allowing the adsorbent to fit properly. Methanol was used as an eluent during the chromatography. Elutes were collected in test tubes and each was developed on silica gel 60 $F_{254}$ TLC plates using the developing sytem of ethyl acetate-

acetic acid-formic acid-water (100:11:11:27, v/v/v/v). Developed plate was then dried with cold air and sprayed with vanillin-sulphuric acid reagent for detection. Lastly, separated compounds were visualized using UV light at 366 nm **(Figure 27)**.

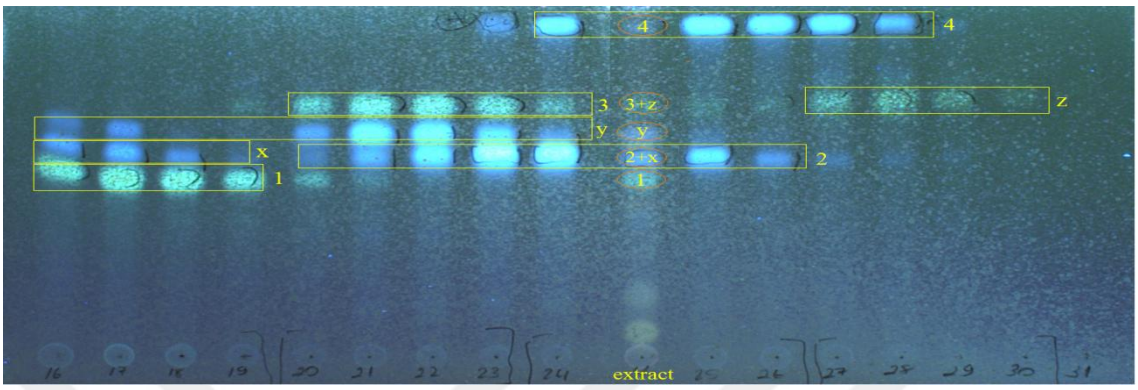



**Figure 27.** TLC chromatogram of *C. scolymus* leaf extract and fractions at 366 nm. The fractions were developed on silica gel 60 $F_{254}$ TLC plate using a developing solvent system of ethyl acetate-formic acid-acetic acid-water (100:11:11:27, v/v/v/v); derivatization: vanillin-sulphuric acid; (1): rutin; (2): ChA; (3): L7G; (4): cynarin.

TLC chromatogram **(Figure 27)** showed that at the same $R_F$ values of ChA ($R_F$ = 0.55) and L7G ($R_F$ = 0.68), there were also another co-existing compounds encoded as 'x' and 'z', respectively. Moreover, two more compounds were detected close to ChA ($R_F$ = 0.60), coded as 'y' in this study.

Moreover, due to similar color of 'x' and 'y' with that of ChA, could be possibly be caffeic acid derivatives. In addition, 'z' had also same color with that of L7G. Therefore, 'z' could be a luteolin derivative.

### 4.2.2. HPTLC-DPPH˙ Analysis

In order to screen free radical scavenging compounds present in *C. scolymus* leaf extract, the chromatographic zones developed on silica gel 60 $F_{254}$ HPTLC plates using a developing solvent system of ethyl acetate-acetic acid-formic acid-water (100:11:11:27, v/v/v/v) were sprayed with 0.05% methanolic DPPH˙ solution.

Chromatogram of Mix2c, containing ChA and gallic acid (antioxidant activity is often expressed in terms of gallic acid equivalents) confirmed the absence of gallic acid in *C. scolymus* leaf extract **(Figures 28a and b)**. After derivatization with DPPH˙ solution, the active antiradical constituents appeared on the HPTLC plate as yellow zones on the purple background. All separated compounds of *C. scolymus* leaf extract showed radical scavenging activity **(Figure 28b)**.

The applied quantitative methodology in this study was proportional to area of the individual peaks to total area which could be considered as the sum of antiradical activities of all compounds in the extract. Consequently, contribution of ChA and cynarin in total antioxidative activity was determined as 37.38% and 27.16%, respectively in *C. scolymus* leaf extract. Their assistance to antioxidant activity was remarkable when compared to rutin (11.53%) and L7G (18%), **Figure 28b**.

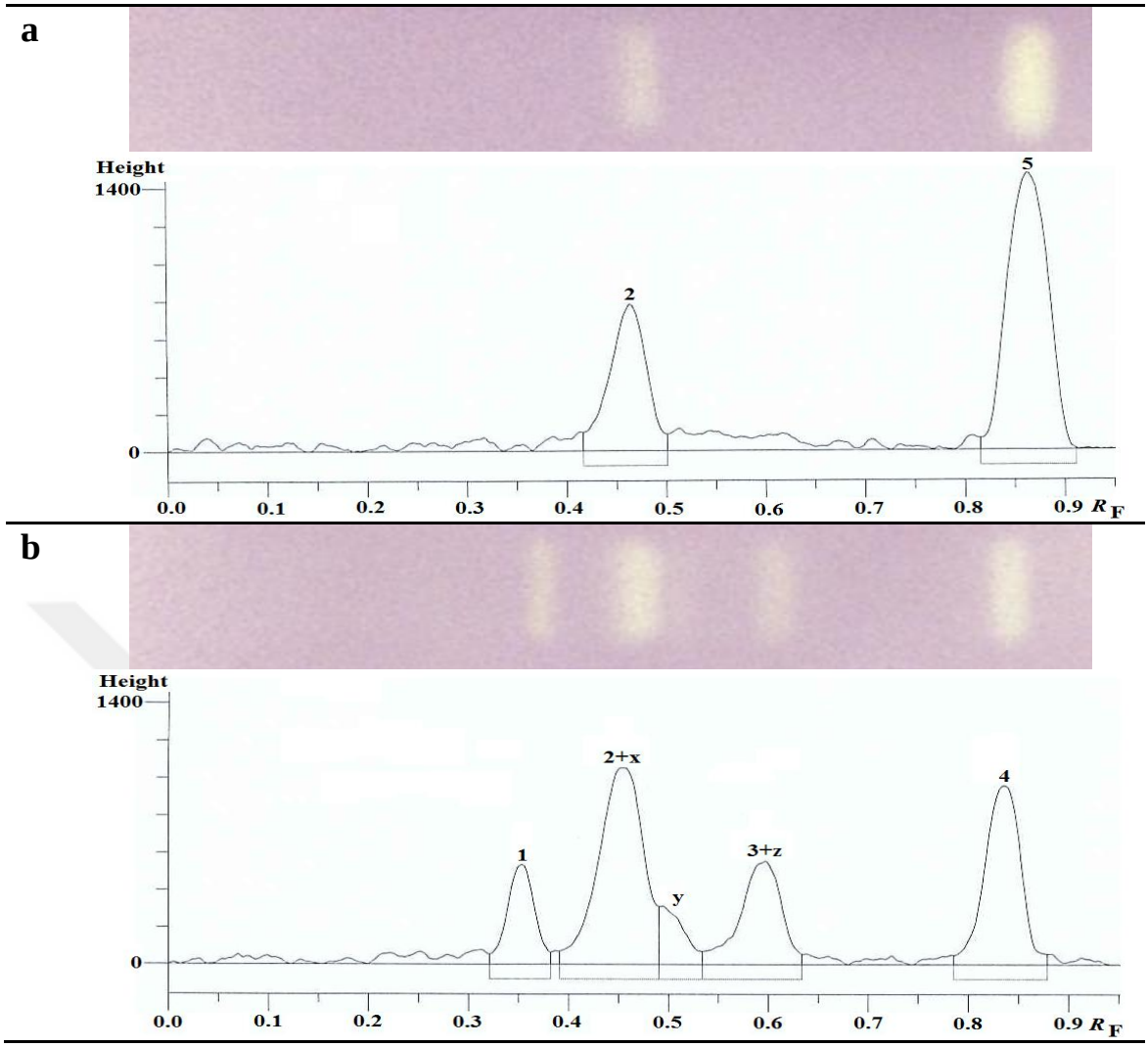



**Figure 28.** Antioxidant compounds of *C. scolymus* leaf extract. The chromatograms and videodensitograms of (a) Mix2c standard (0.3 µg/band) and (b) *C. scolymus* leaf sample test solutions (25 µg/band) developed on silica gel 60 $F_{254}$ HPTLC plate using a developing solvent system of ethyl acetate-acetic acid-formic acid-water (100:11:11:27, v/v/v/v); derivatization: $DPPH^{\cdot}$ solution; (1) rutin; (2) ChA; (3) L7G; (4) cynarin; (5) gallic acid.

### 4.2.3. HPTLC Analysis

#### 4.2.3.1. Method Optimization

In this study, Mix5 standard solution was applied during the evaluation of the developing solvent systems and assisted for the determination of the optimum separation.

First step was to test the developing solvent systems reported in the previous studies for the separation of phenolic compounds from co-existing components (173,207–212). The trails for the separation were presented in **Figures 29a-m**. In all analyses, 20 min saturated ADC2 was used. Besides, the relative humidity was fixed to 33% by a saturated $MgCl_2.6H_2O$ solution.

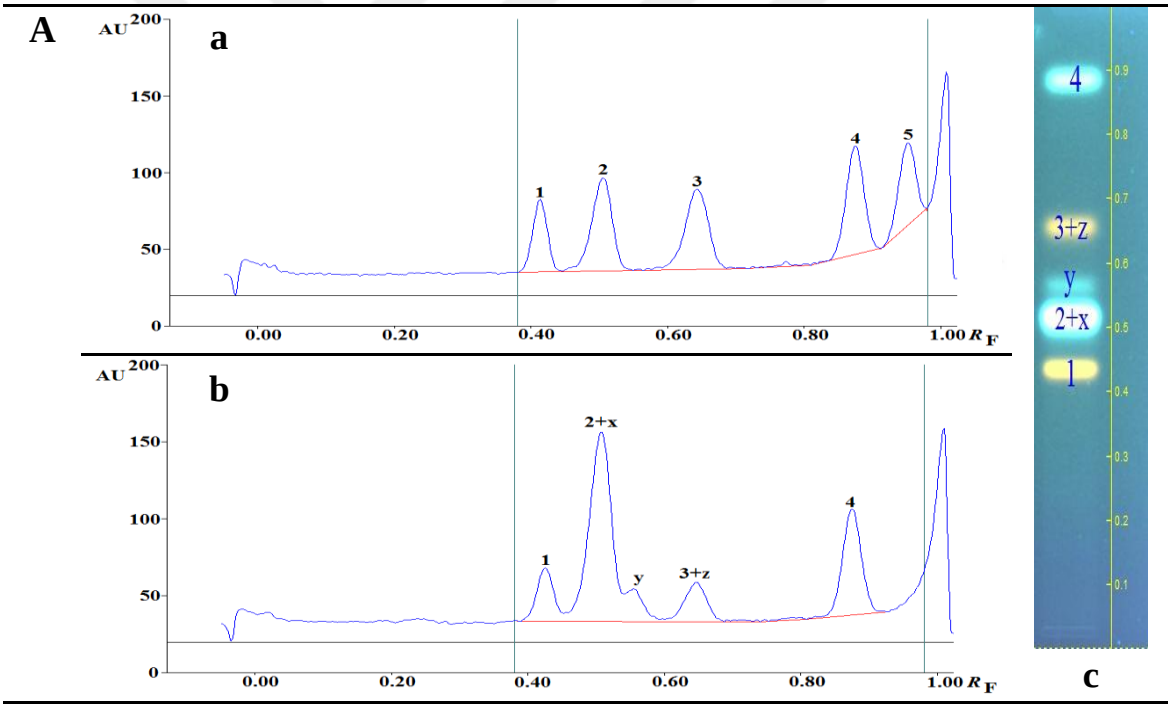



**Figure 29.** Method optimization on *C. scolymus* leaf extract. HPTLC densitograms (at 330 nm) and chromatogram (at 366 nm) of (a) Mix5 standard solution (80 ng/band), (b) and (c) *C. scolymus* sample test solution (4 µg/band) developed on silica gel 60 $F_{254}$ HPTLC plate using a developing solvent system of ethyl acetate-acetic acid-formic acid-water (100:11:11:26, v/v/v/v); derivatization: NP/PEG 400; (1) rutin; (2) ChA; (3) L7G; (4) cynarin; (5) caffeic acid (173,207–209,213).

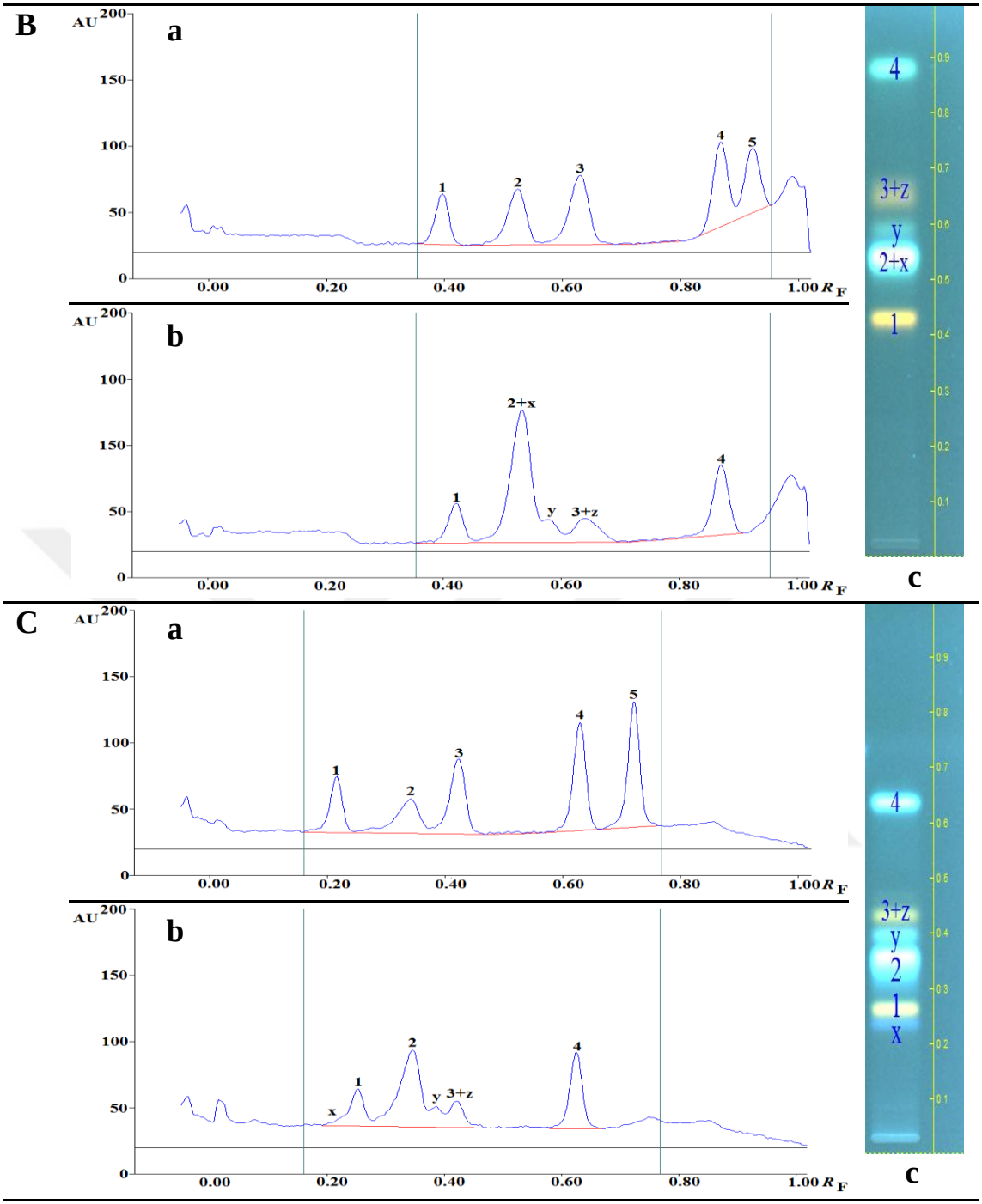



**Figure 29.** Continued. (b) ethyl acetate-ethyl methyl ketone-formic acid-water (50:30:10:10, v/v/v/v); (c) tetrahydrofuran-toluene-formic acid-water (16:8:2:1, v/v/v/v) (173).

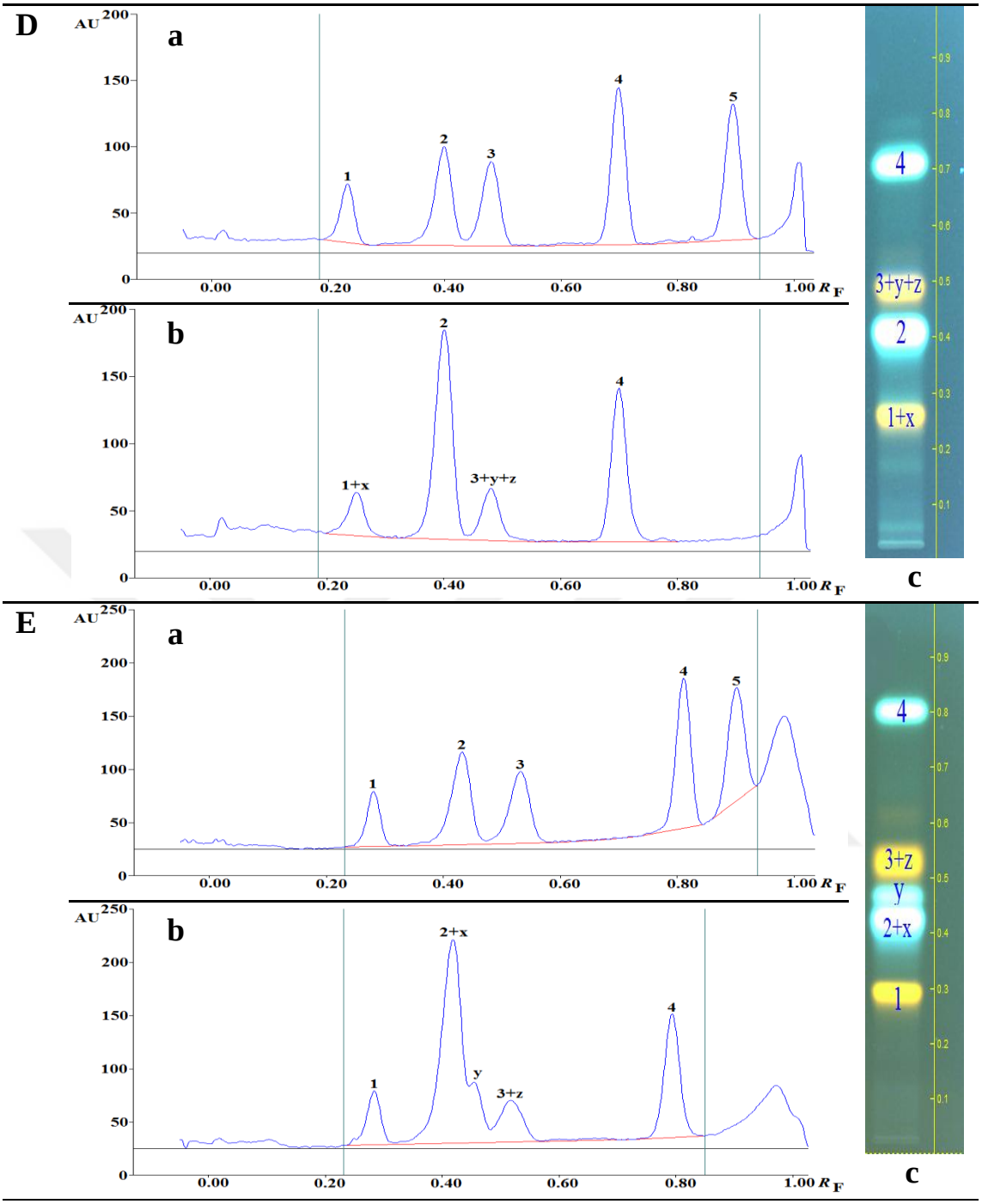



**Figure 29.** Continued. (d) ethyl formate-toluene-formic acid-water (30:1.5:4:3, v/v/v/v); (e) ethyl acetate-formic acid-water (40:5:5, v/v/v) (173).

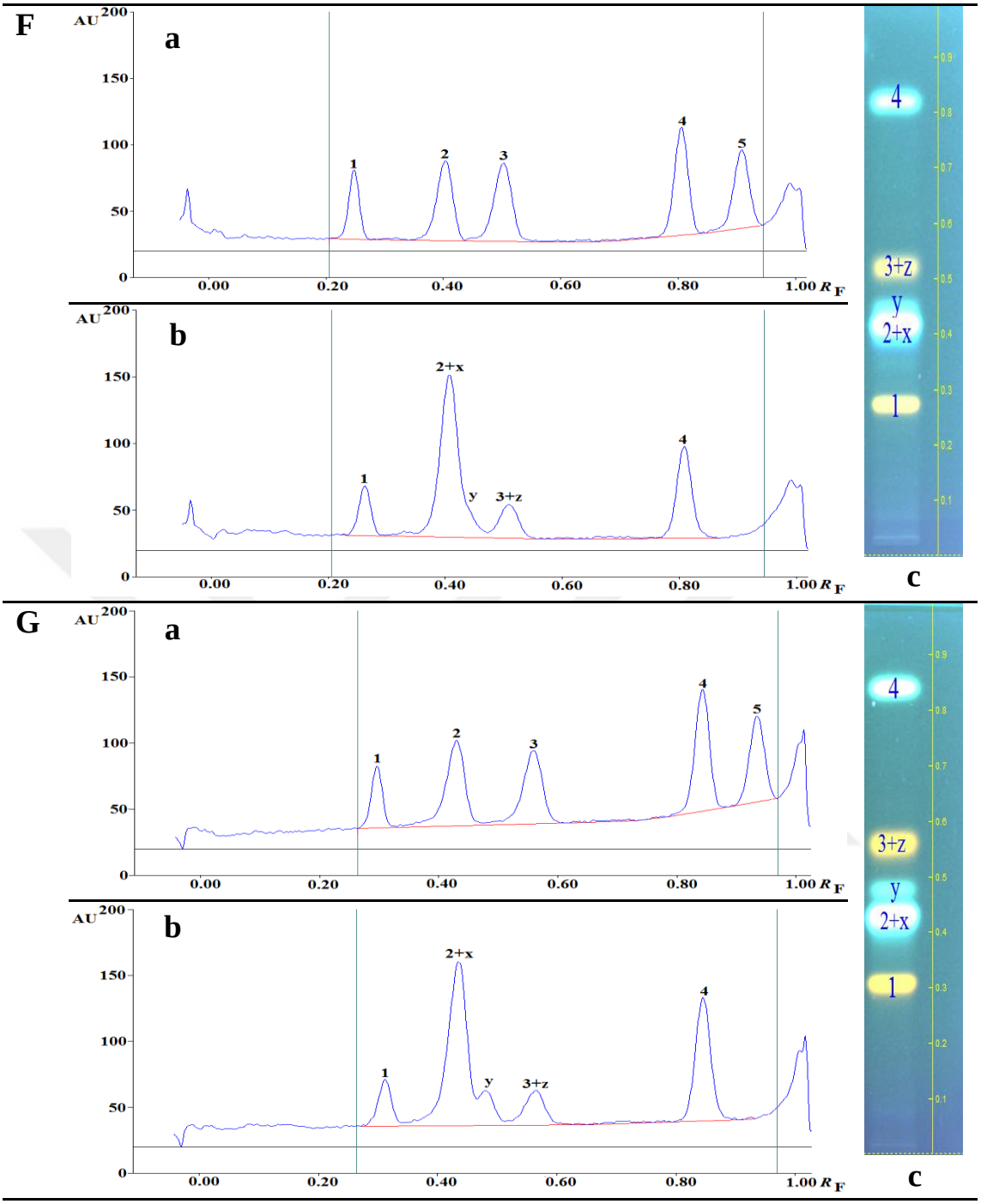



**Figure 29.** Continued. (f) ethyl acetate-formic acid-water (85:10:15, v/v/v/v) (210); (g) ethyl acetate-acetic acid-formic acid-water (34:1.5:3.5:7, v/v/v/v) (212).

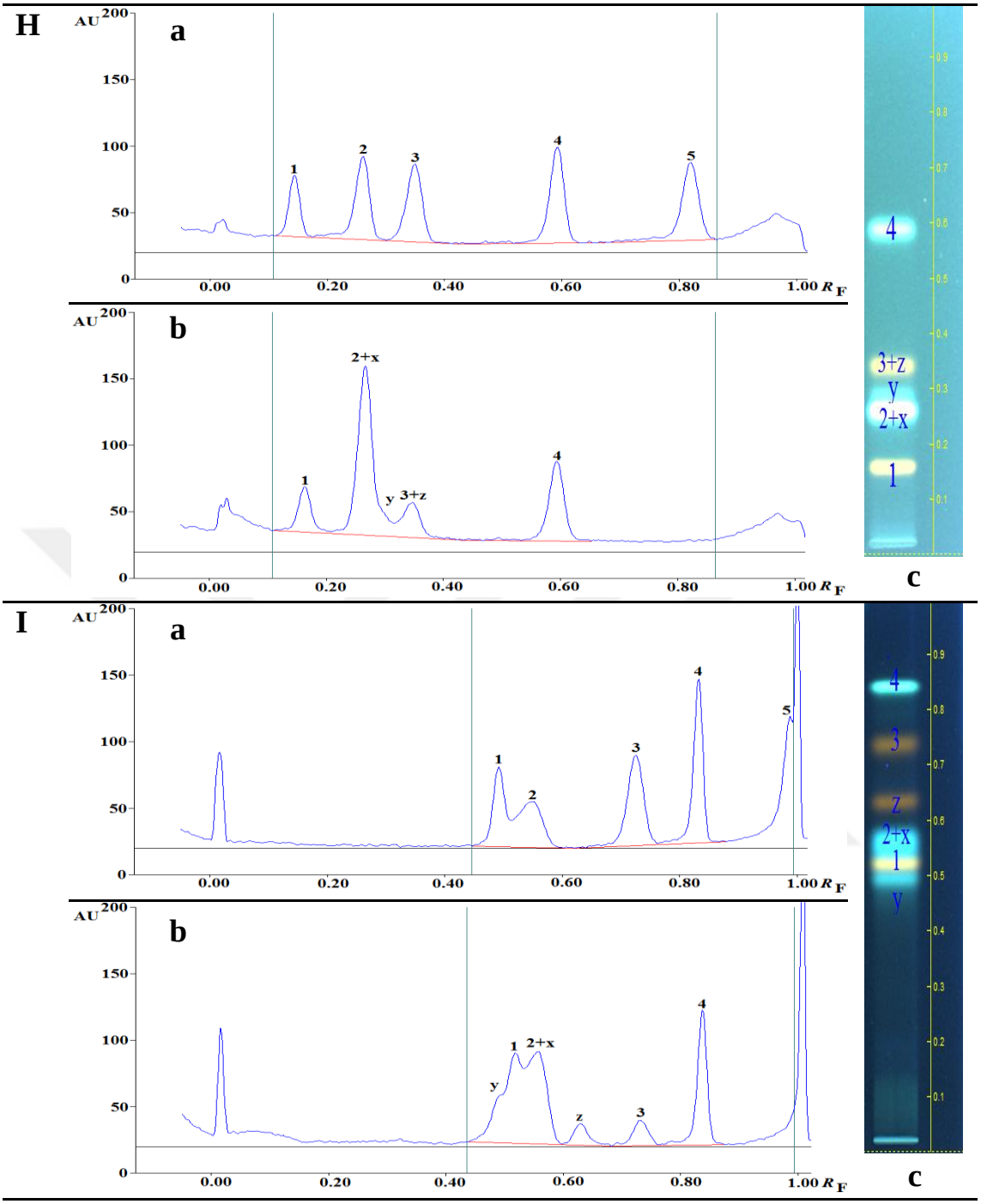



**Figure 29.** Continued. (h) ethyl acetate-dichloromethane-acetic acid-formic acid-water (100:25:10:10:11, v/v/v/v/v) (211); (i) silica gel 60 $NH_2$ $F_{254}$s HPTLC plate using ethyl acetate-acetic acid-formic acid-water (20:2:2:5, v/v/v/v).

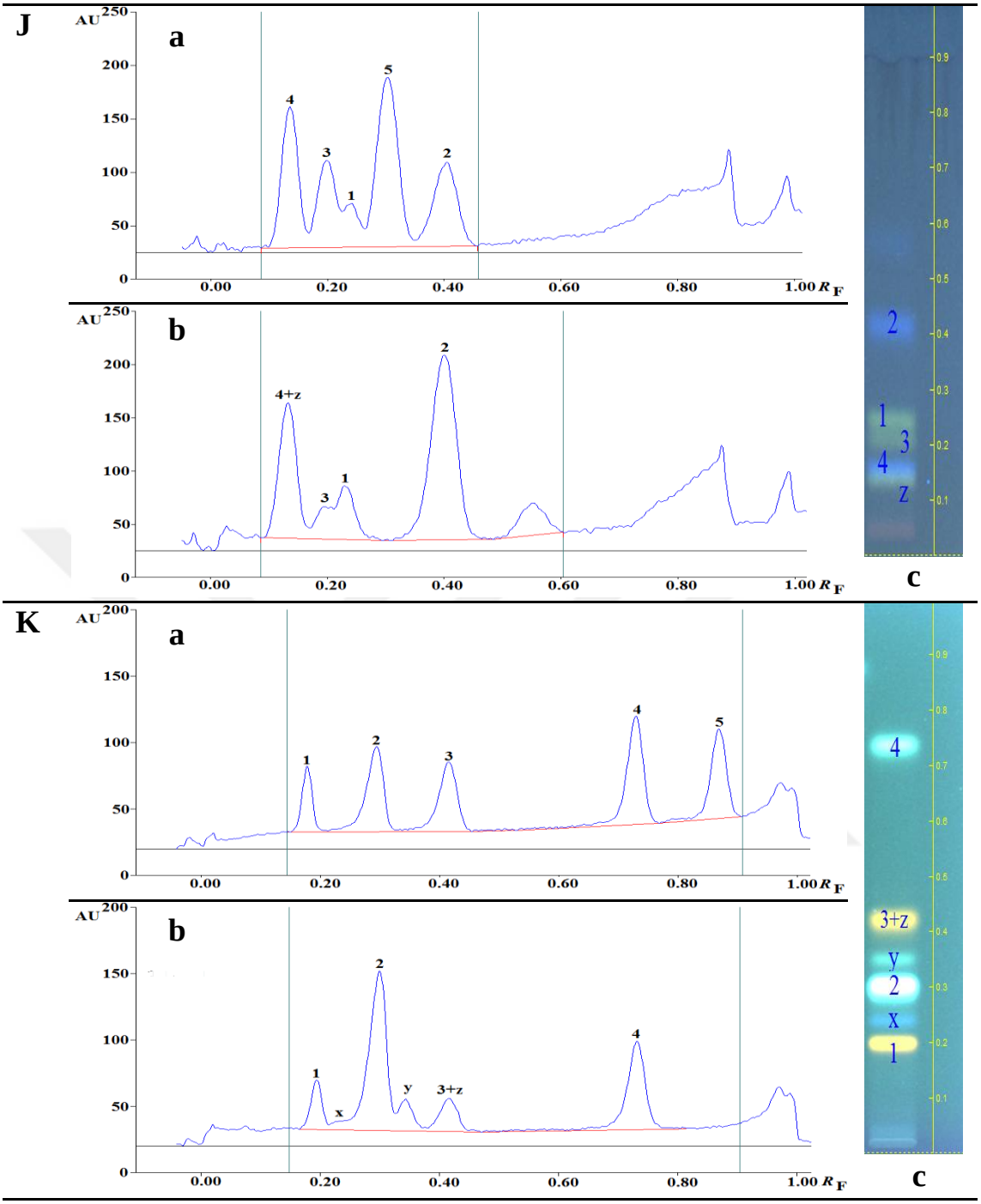



**Figure 29.** Continued. (j) silica gel 60 RP-18 $F_{254}$s HPTLC plate using water-acetonitrile-*o*-phosphoric acid (35:10:1, v/v/v); (k) silica gel 60 $F_{254}$ HPTLC plate using ethyl acetate-acetic acid-formic acid-water (35:2:2:5, v/v/v/v).

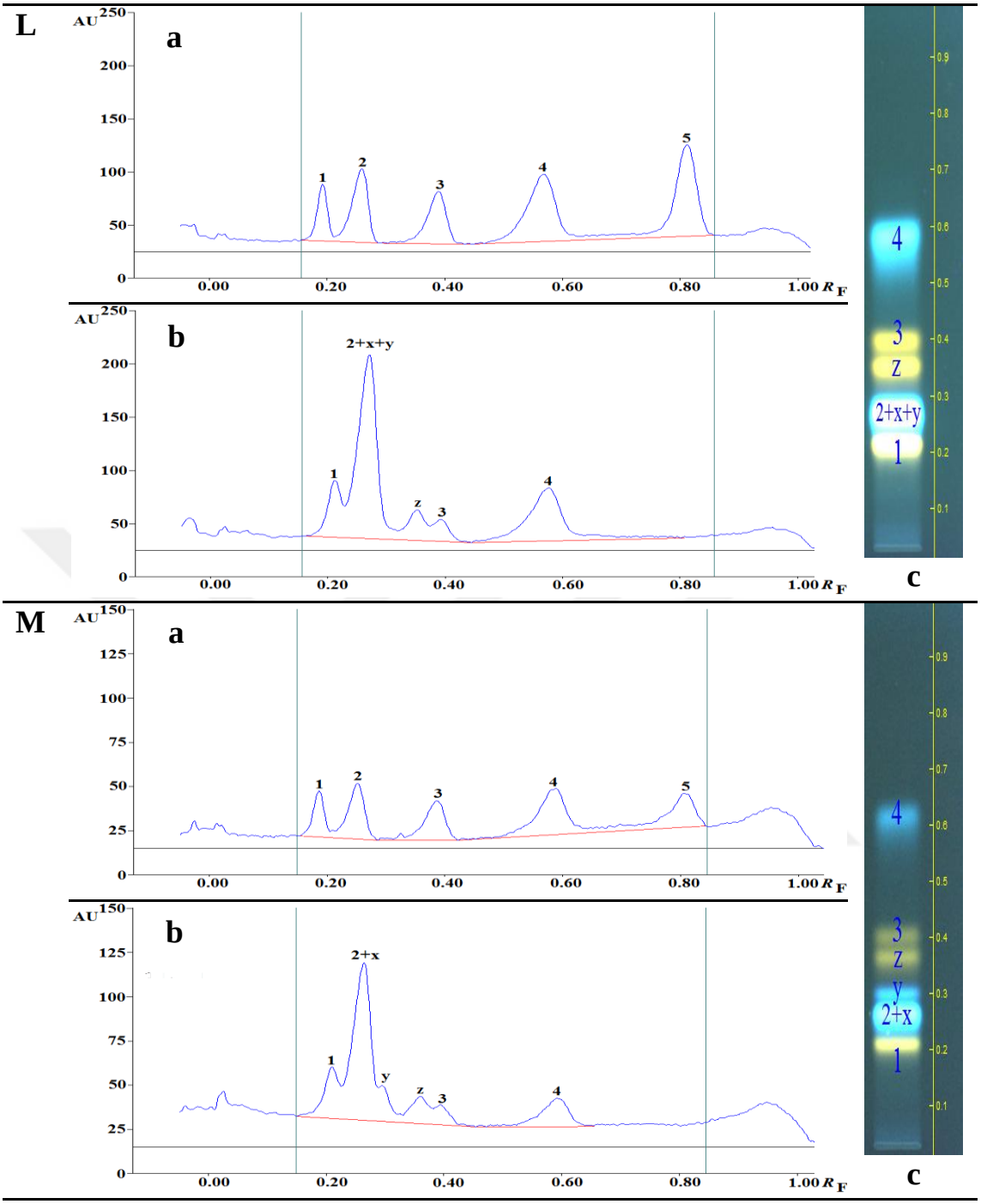



**Figure 29.** Continued. (l) silica gel 60 $F_{254}$ HPTLC plate, pre-developed by methanol-dichloromethane (1:1, v/v) containing 0.5% TEA, using a developing solvent system of ethyl acetate-formic acid-acetic acid-water (35:2:2:5, v/v/v/v); (m) Mix5 and sample solutions were diluted 2 times and developed as 'l' without humidity.

#### 4.2.3.2. Validation

##### 4.2.3.2.1. Stability

###### 4.2.3.2.1.1. Stability of the Sample during Chromatography

Stability of the components of *C. scolymus* leaf extract on the plate during the chromatography was investigated using 2D-development which facilitates the detection of compounds that decompose under the conditions used for the separation **(Figures 30a-f)**. Consequently, zones detected on the other regions of the diagonal line during 2D-development indicated that components in *C. scolymus* leaf extract prone to degradation on the plate during development with various developing solvent systems as shown in **Figures 30a-e**. However, components remained unchanged on silica gel 60 RP-18 $F_{254}$s HPTLC plate developed by a developing solvent system of water-acetonitrile-formic acid (35:10:1, v/v/v), shown in **Figure 30f**.

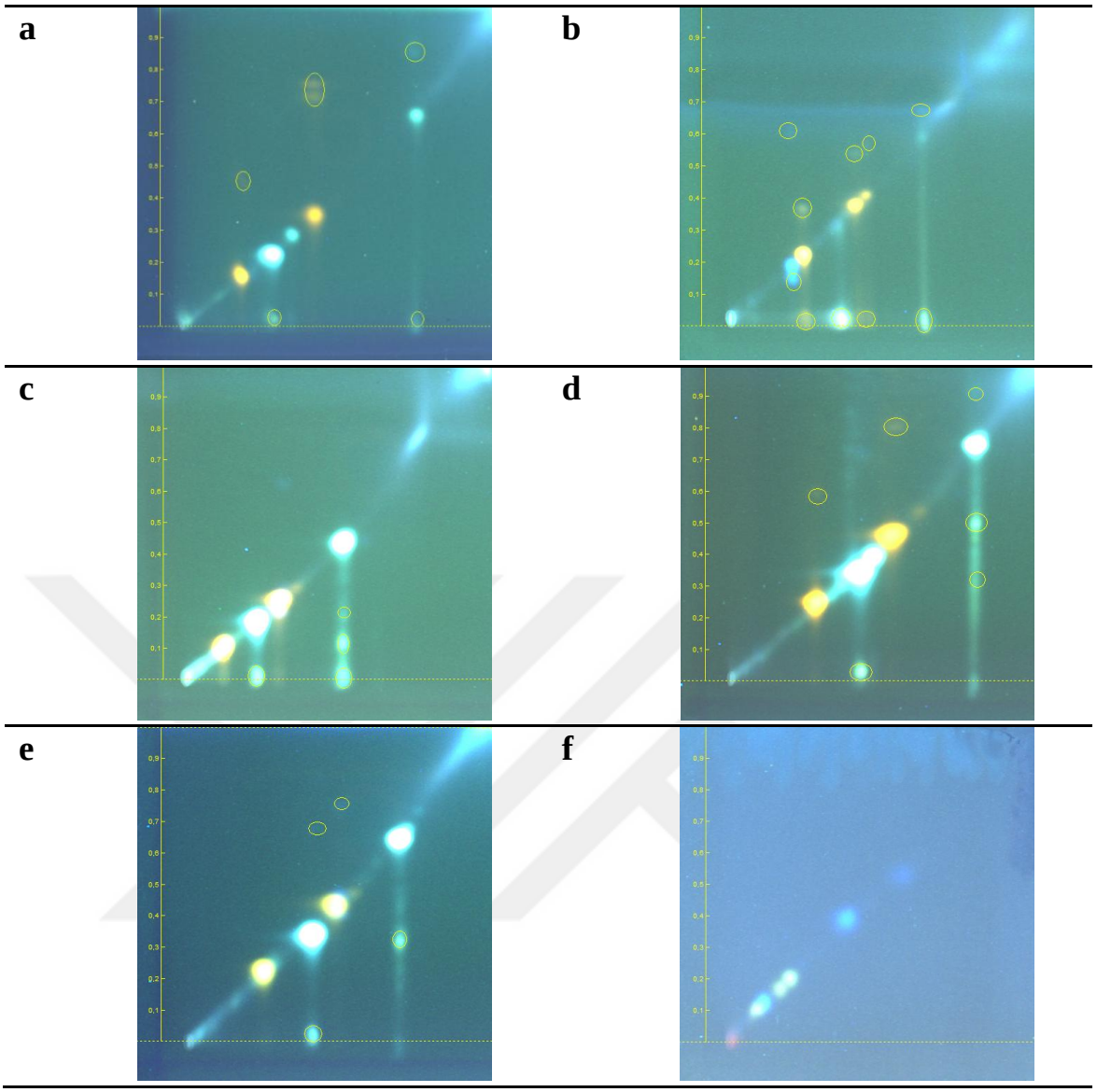



**Figure 30.** 2D-chromatograms of *C. scolymus* leaf extract at 366 nm. Sample test solution was developed on silica gel 60 $F_{254}$ HPTLC plate using the following developing solvent systems: (a) ethyl acetate-acetic acid-formic acid-water (35:2:2:5, v/v/v/v); (b) tetrahydrofuran-toluene-formic acid-water (24:12:3:1.5, v/v/v/v); (c) dichloromethane-ethyl methyl ketone-methanol-formic acid-water (20:15:5:2.5:2.5, v/v/v/v/v); (d) ethyl acetate-formic acid-water (40:5:5, v/v/v); (e) ethyl formate-toluene-formic acid-water (30:1.5:4:3 v/v/v/v); (f) sample test solution was silica gel 60 RP-18 $F_{254}$s HPTLC plate using a developing solvent system of water-acetonitrile-*o*-phosphoric acid (35:10:1, v/v/v); derivatization: NP/PEG 400.

For further studies, reasons of instability were evaluated as shown in **Figures 31a-f**. The following parameters were examined:

a) Sample test solution was prepared with the developing solvent sytem of ethyl acetate-acetic acid-formic acid-water (35:2:2:5, v/v/v/v) to evaluate the effects of acidic medium on compounds during sample preparation.
b) Plate was prewashed with methanol-dichlorometane (1:1, v/v) to remove possible impurities which may come on the plate either from the laboratory environment or packing material.
c) Developments performed on both silica gel 60 $F_{254}$ and silica gel 60 $NH_2$ $F_{254}$s HPTLC plates using ethyl acetate-acetic acid-formic acid-water (20:2:2:5, v/v/v/v) were compared to evaluate the effects of silica gel surface on compounds.
d) Developing solvent system containing an antioxidant (0.5% butylated hydroxytoluene-BHT) was applied to prevent oxidative degradation.
e) Developing solvent system without both acetic acid and formic acid was practiced to study the effects of acids on compounds.
f) Addition of high concentration of acid into the developing solvent system was investigated.

As a result of all these trials mentioned above, instability of compounds remained unchanged, except **Figure 31c**. When development performed on silica gel 60 $NH_2$ $F_{254}$s HPTLC plate using ethyl acetate-acetic acid-formic acid-water (20:2:2:5, v/v/v/v), not caffeic acid derivatives but flavonoids became stable.

As mentioned before all compounds developed on silica gel 60 RP-18 $F_{254}$s HPTLC plate using a developing solvent system of water-acetonitrile-formic acid (35:10:1, v/v/v) were also stable. However, resolution between cynarin and the compound encoded as ‘z’ was found to be not enough for further quantitative studies. Therefore, HPTLC was used only for identification analysis other than quantification due to instability of the components on the plate during chromatography.

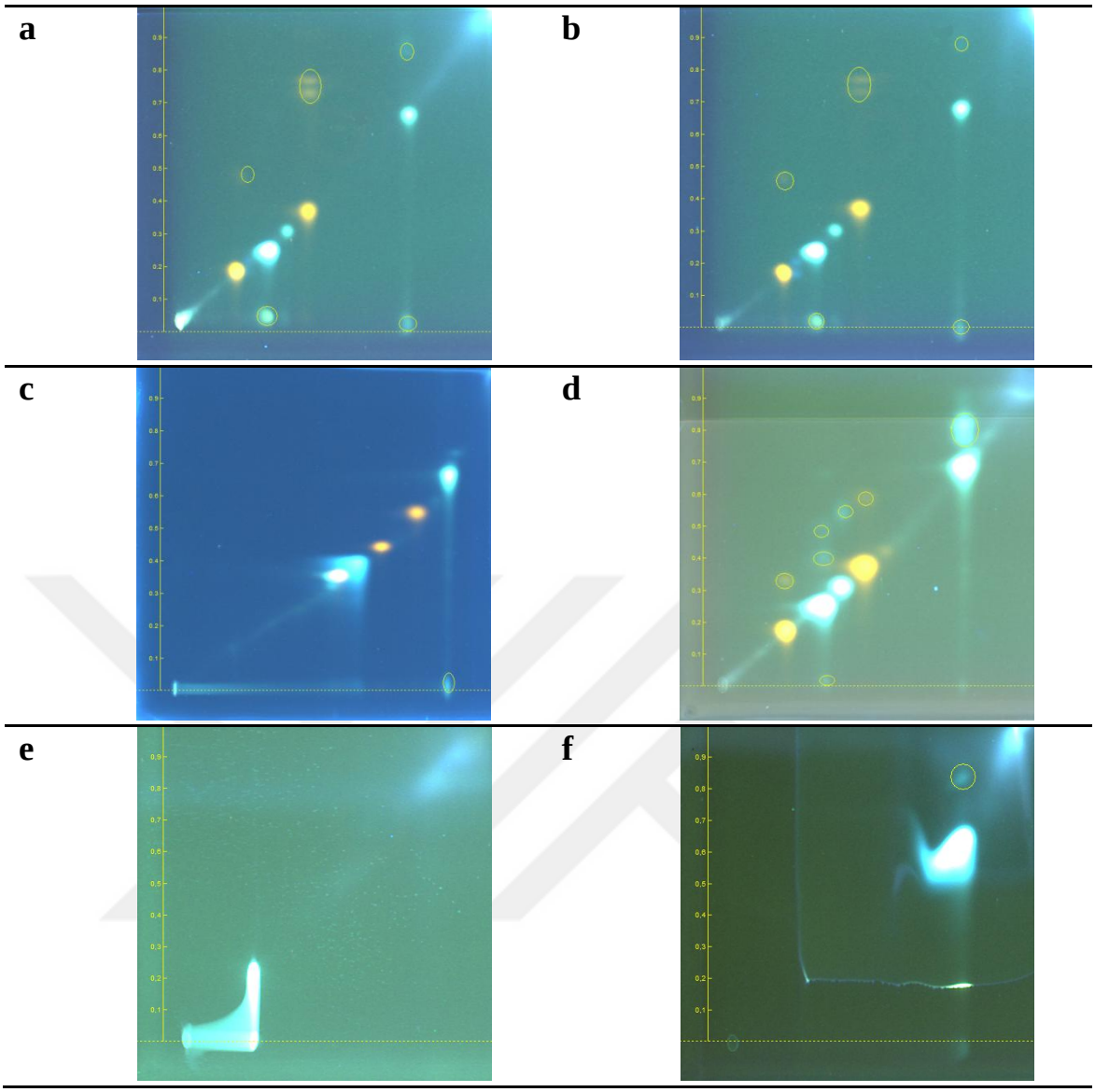



**Figure 31.** Evaluation of instability. (a) *C. scolymus* sample test solution was prepared by developing solvent system of ethyl acetate-acetic acid-formic acid-water (35:2:2:5, v/v/v/v) and developed on silica gel 60 $F_{254}$ HPTLC plate using the same developing solvent system; (b) the sample test solution was developed on prewashed (methanol-dichlorometane (1:1, v/v)) silica gel 60 $F_{254}$ HPTLC plate with the same developing solvent system as 'a'; (c) the sample test solution was developed on silica gel 60 $NH_2$ $F_{254}$s HPTLC plate using ethyl acetate-acetic acid-formic acid-water (20:2:2:5, v/v/v/v); (d) the sample test solution was developed on silica gel 60 $F_{254}$ HPTLC plate using ethyl acetate (containing 0.5% BHT)-acetic acid-formic acid-water (35:2:2:5, v/v/v/v); ChA standard solution was developed on silica gel 60 $F_{254}$ HPTLC plate using the following developing solvent systems: (e) ethyl methyl ketone-methanol-water (55:5:5, v/v/v); (f) ethyl methyl ketone-methanol-water-formic acid (55:5:5:10, v/v/v/v); derivatization: NP/PEG 400; documentation: 366 nm.

#### 4.2.3.2.2. Specificity

The specificity of the method was evaluated by analyzing standards and test solutions. The identity of the rutin, ChA, L7G and cynarin in *C. scolymus* sample test solution were evaluated by comparison of $R_F$ values with that of Mix5 standard solution. The $R_F$ values of rutin, ChA, L7G and cynarin were found as 0.18, 0.23, 0.38 and 0.58 respectively. Besides, the $R_F$ value of caffeic acid was as 0.82 **(Figure 32)**.

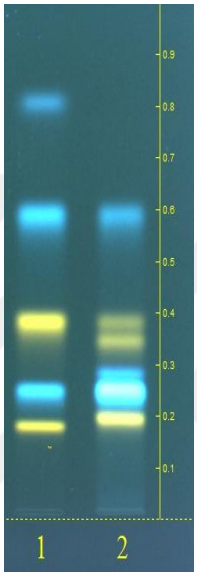



**Figure 32.** The specificity of the developed HPTLC method. HPTLC chromatogram of (1) Mix5 standard (40 ng/band) and (2) *C. scolymus* leaf sample test solutions (4 µg/band) at 366 nm; derivatization: NP/PEG 400.

#### 4.2.3.2.3. Precision

##### 4.2.3.2.3.1. Repeatability

The intraday precision of the method was performed by replicating the experiment in triplicate with three times freshly prepared plates by pre-developing with methanol-dichlorometane (1:1, v/v) containing 0.5% TEA during the day. It was concluded that all fingerprints in three different plates were identical. Beside, the variability of the $R_F$ values of rutin, ChA, L7G and cynarin across each plate were less than 0.02, indicates the repeatability of the developed HPTLC method.

### 4.2.4. Evaluation of *Cynara scolymus* Extracts and Food Supplements

#### 4.2.4.1. HPTLC Analysis

Initially, sample test solutions of *C. scolymus* leaf, bract, receptacle, stem and food supplements were evaluated using HPTLC. Eventually, it was clearly seen that *C. scolymus* leaf extract had a unique chemical fingerprinting profile when compared to *C. scolymus* bracts, receptacle and stem extracts. The compound encoded as 'z' was found only in *C. scolymus* leaf extract. In addition, ChA and cynarin were detected in all *C. scolymus* sample test solutions. When intensities of zones were compared, it was revealed that the most intense ChA zones were seen in both extracts of *C. scolymus* leaf and inner bracts at the early stage of maturity. Beside, the most intense cynarin zone was detected in extract of *C. scolymus* inner bracts (early stage of maturity) **(Figure 33)**.

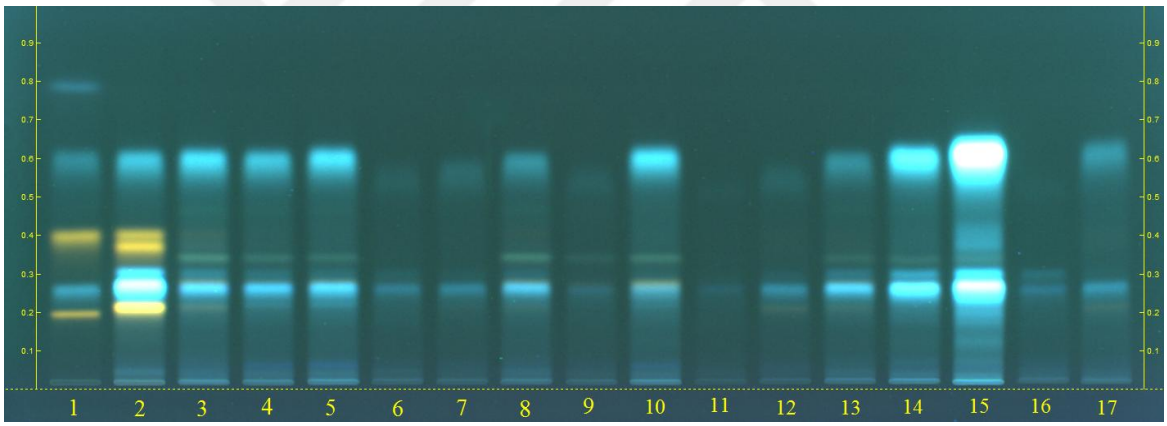



**Figure 33.** HPTLC chromatogram of *C. scolymus* leaf, bract, receptacle and stem extracts at 366 nm. Applications 1: Mix5 (40 ng/band), 2: leaf, 3: outer bract, 4: intermediate bract, 5: inner bract, 6: receptacle, 7: stem (CH at maturity), 8: outer bract , 9: intermediate bract, 10: inner bract, 11: receptacle, 12: stem (AH at maturity), 13: outer bract, 14: intermediate bract, 15: inner bract, 16: receptacle, 17: stem (CH at early stage of maturity); applied sample test solution: 4 µg/band; derivatization: NP/PEG 400.

In *C. scolymus* food supplements, ChA and cynarin were monitored whereas rutin, L7G and 'z' were not detected **(Figure 34)**. Additionally, caffeic acid was detected in C1 and C2 but not in C3. Contrarily to expected, fingerprinting profiles of C1 and C3 claimed to contain *C. scolymus* leaf extract were different than the profile of the *C. scolymus* leaf extract.

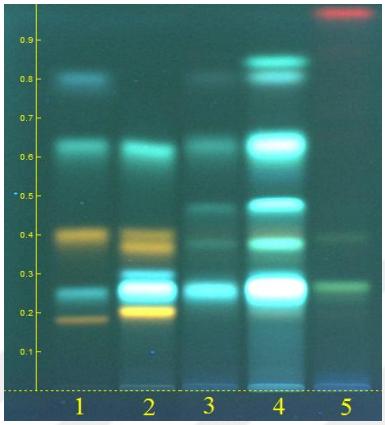



**Figure 34.** HPTLC chromatogram of *C. scolymus* leaf extract and food supplements at 366 nm. Applications 1: Mix5 (40 ng/band), 2: leaf (4 µg/band), 3: C1 (50 µg/band), 4: C2 (50 µg/band), 5: C3 (50 µg/band); derivatization: NP/PEG 400.

### 4.2.4.2. HPLC Analysis

As mentioned before, ChA and cynarin significantly contribute to *C. scolymus* antioxidant activity. Therefore, further quantitative analysis was performed by HPLC method stated in the Ph. Eur. to calculate both ChA and cynarin contents in extracts of *C. scolymus* leaf, *C. scolymus* other plant parts and also its food supplements.

Comparison of $t_R$ values belong to ChA (10.53 ± 0.01), caffeic acid (12.79 ± 0.02), rutin (17.84 ± 0.01), L7G (18.96 ± 0.02) and cynarin (21.72 ± 0.02) in Mix5 with $t_R$ values of chromatographic peaks of the *C. scolymus* sample test solution assisted to identify the compounds in *C. scolymus* leaf extract **(Figure 35)**. As a result, in accordance with HPTLC chromatogram ChA, rutin, L7G and cynarin were monitored in *C. scolymus* leaf extract.

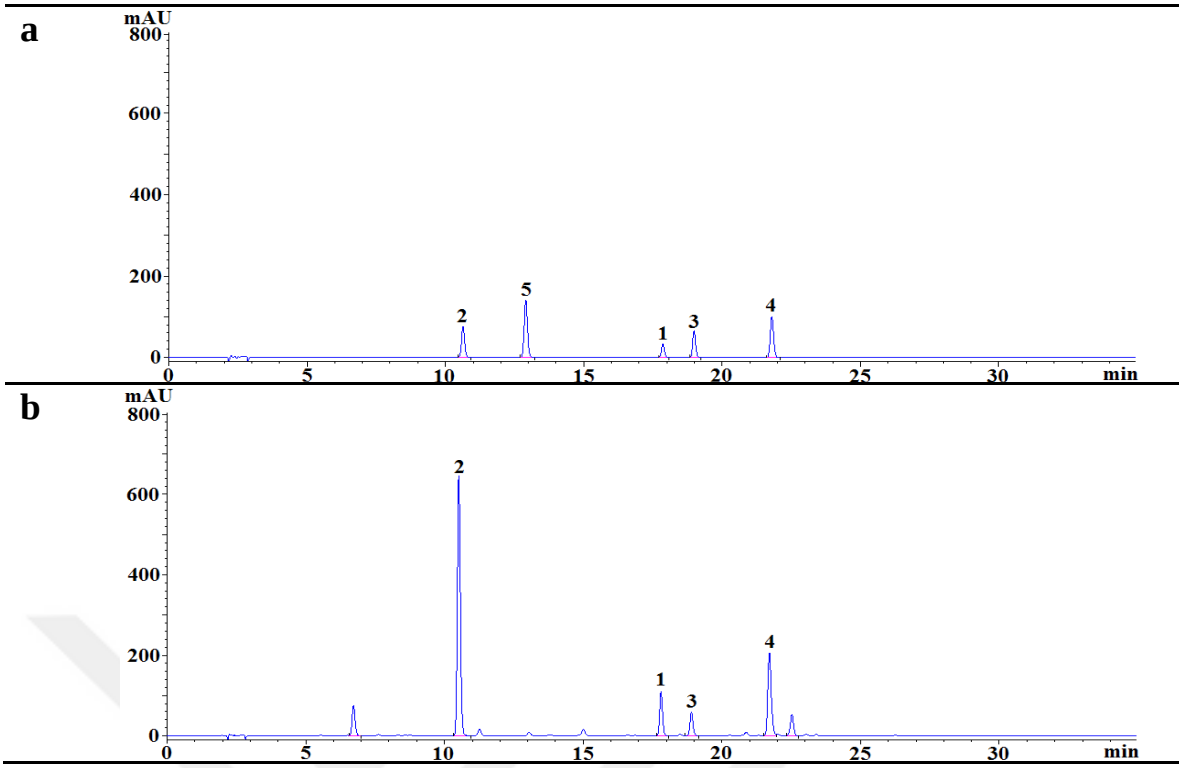



**Figure 35.** HPLC analysis on *C. scolymus* leaf extract. HPLC chromatograms of (a) Mix5 standard solution (5µg/mL) and (b) *C. scolymus* leaf sample test solution (1.2 mg/mL) at 330 nm.

In this study, ChA content in basal *C. scolymus* leaf extract was calculated as 6.05%, which was higher than the official limit stated in the Ph. Eur., 0.6% (180). Moreover, it was the highest ChA amount when compared to *C. scolymus* other plant part extracts, **Figure 36**.

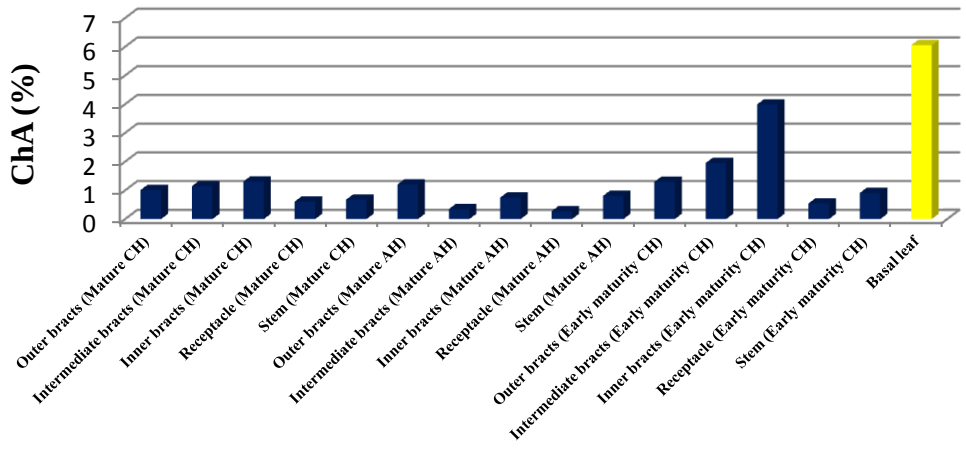



**Figure 36.** ChA contents in *C. scolymus* leaf, bract, receptaculum and stem

Moreover, the peak areas obtained from standard cynarin solutions in the range from 0.5 to 40 µg/mL were evaluated whether their peak areas fit either with linear or quadratic models **(Table 36)**. The high values of *F*-test for a significant correlation, probability was lower than 0.05 for both models in both ranges, indicated that the variation explained by each model was not due to chance. Secondly, the coefficient of determination, $R^2$ values indicated that there was a strong relationship between both models and ranges.

The scatter plot of residuals by fit values for the linear and quadratic models was the key point for the selection of the model in this study. In the range from 0.5 to 40 µg/mL the scatter plot of residuals by fit values for the linear model showed an 'inverted U' shaped pattern whereas the scatterplot of residuals by fit values for the quadratic model did not show a pattern, thus the quadratic model was acceptable in sense the residuals were independent of the fit values. In addition, Lack-of-fit test illustrated the probability of the *F*-test statistic $F$ (5, 14) = 225.867 was $p$ = 0.000, lower than the alpha level of significance of 0.05. These findings also supported that quadratic regression model was appropriate in the range from 0.5 to 40 µg/mL for the calibration curve.

**Table 36.** Linear and quadratic model comparisons

| Range (µg/mL) | Model Summary | | | | | | Parameter Estimates | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Models[a] | $R^2$ | *F* | df[b]1 | df[b]2 | Sig.[c] | a | b | c | *n* |
| 0.5-40 | Linear | 0.999 | 15160.90 | 1 | 19 | 0.00 | 13.27 | 81.60 | - | 21 |
| | Quadratic | 1.000 | 30121.35 | 2 | 18 | 0.00 | -20.30 | 90.93 | -0.24 | 21 |

[a]Regression models: Linear equation: $y = a + bx$; Quadratic equation: $y = a + bx + cx^2$
[b]degree of freedom
[c]*p*-value

In addition, independently prepared cynarin standard solutions of 1.5, 3, 7.5 and 15 µg/mL were used to evaluate the recovery of the calibration curve. The results were 103.48%, 97.04%, 94.12% and 97.27%, respectively. Eventually, each value was found to be between 80-120% indicating the suitability of the calibration curve for further analysis.

Results obtained from calibration curve belong to cynarin showed that the highest cynarin content was found as 12.06% in the inner bract extract belonged to CH at the early stage of the maturity **(Figure 37)**.

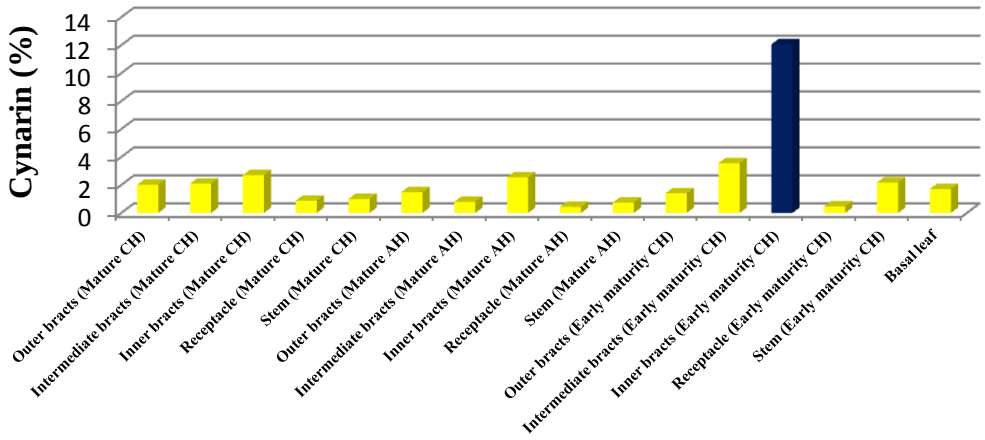



**Figure 37.** Cynarin contents in *C. scolymus* leaf, bract, receptaculum and stem

Moreover, ChA and cynarin contents in *C. scolymus* food supplements encoded as C1-3 were also evaluated. The amount of ChA was found as 0.58, 0.68 and 0.01% for C1, C2 and C3, respectively. Therefore, C1 and C2 but not C3 met the criterion stated in the Ph. Eur. In addition, cynarin concentrations were calculated as 0.44, 0.48 and 0.03, respectively **(Figure 38)**.

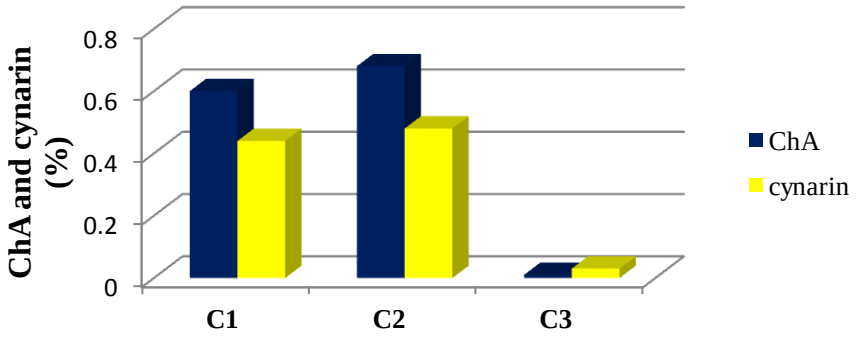



**Figure 38.** ChA and cynarin contents in *C. scolymus* food supplements

## 5. DISCUSSION

TLC is one of the most frequently used separation methods in chromatography especially for qualitative analysis of marker compounds in plant materials due to its simplicity, flexibility, and cost efficiency, parallel analysis of multiple samples on one plate, visual and rapid results. Advances particularly on stationary phases, instrumentation and automation led to the utilization of HPTLC methods which enables both qualitative and quantitative analyses concurrently.

TLC methods have long been stated in the pharmacopoeias such as Ph. Eur. which is officially approved in Turkey. Recently, conventional TLC methods have been started to be replaced by HPTLC methods in the American Herbal Pharmacopoeia (5). However, there has not yet referred an HPTLC method in the Ph. Eur. which has been accepted as an official source in Turkey. Therefore, we have selected two medicinal plants stated in the Ph. Eur., *M. recutita* and *C. scolymus* from the family Asteraceae to develop and validate qualitative and quantitative HPTLC methods.

Method development and also its optimization both in HPTLC and other analytical techniques are very challenging processes particularly for plant materials due to their chemical complexity. Method optimization in HPTLC contains several steps such as sample preparation and its application, selection of the type of the stationary phase, composition of the developing solvents, chamber type and its saturation, development, derivatization, detection and laboratory conditions (173).

As previously mentioned, sample preparation process such as selection of solvent type i.e. methanol, water, dichloromethane for extraction, addition of chemical reagents such as acids and sodium hydroxide is one of the most crucial parameters both in qualitative and quantitative analyses. In this study, powdered flowers of *M. recutita*, *M. recutita*-like materials and commercial *M. recutita* products were first brewed at 100˚C as frequently practiced in traditional medicines. Then, each aqueous part was lyophilized to obtain their dry extracts which were further dissolved by methanol to prepare sample test solutions.

The brewed tea of *M. recutita* flowers has been extensively utilized for centuries due to either its pleasant and calming taste or medicinal purposes. Recently, Harbourne

et al. optimized the extraction conditions for *M. recutita* flowers to obtain highest yield of A7G. Powdered flowers were steeped in 100 mL distilled water at 57, 70, 80, 90 and 100˚C, sequentially. Researchers observed that the concentration of A7G was incrementally increased between 57 to 90˚C and its content became stable from 90˚C to 100˚C (44). In the light of this data, it could be concluded that the preferred sample preparation in this study was a good choice for further studies. After selecting the optimum sample preparation, next step was to determine the type of the stationary phase and composition of the developing solvent system used during the chromatography. The standard mixture solution containing A7G and L7G was used due to close chemical structures of these compounds and assisted to determine the optimum separation. Initially, developing solvent systems reported in the previous studies were practiced for the separation of the phenolic compounds from co-existing components (55,173,205). Then, various combinations of the neat solvents were tested firstly on silica gel plate due to its low cost and widespread usage (187) and then amino plates. The results of trials in this study showed that the best separation for A7G and L7G was achieved in which these compounds were developed on silica gel 60 $NH_2$ $F_{254}$s HPTLC plate using ethyl acetate-formic acid-acetic acid-water (30:1.5:1.5:3, v/v/v/v) as a developing solvent sytem **(Figure 14l)**. After these assessments, some modifications have been also done such as pre-conditioning of the plate and adjusting relative humidity of the chamber as 33% in order to improve the resolution.

Bioassay-guided processing of *M. recutita* flowers has revealed that many of its healing benefits such as relieving painful gastrointestinal complaints, mild sleep disorders and inflammatory diseases are highly related with its phenolic content, in particular to apigenin and A7G (6). Recently, HPTLC methods to quantify apigenin content in alcoholic *M. recutita* extracts have been reported (54,56). Therefore, in the preliminary HPTLC analysis the existence of both apigenin and A7G were investigated in *M. recutita* extract. Consequently not apigenin but A7G was detected in *M. recutita* sample test solution **(Figure 15)**. This might be due to low solubility of free apigenin during brewing process. In addition to these findings, Srivastava and Gupta were also stated the absence of the apigenin in aqueous *M. recutita* extract using an HPLC method (48). On the other hand, A7G was found to be a major component in aqueous, alcoholic and hydroalcoholic *M. recutita* extracts (16,19,43,48,214). While, Guimarães et al. did not detect A7G both in aqueous and methanolic *M. recutita* extracts (45).

Additionally, preferred sample extraction process in this study was compared with the sample preparation for HPLC analysis stated in the Ph. Eur (180). It should be noted that in the Ph. Eur. instead of A7G content, total A7G amount is determined in hydroalcoholic *M. recutita* extract. That may be due to the existence of acetylated A7G derivatives such as apigenin 7-(2''-*O*-acetyl)-glucoside, apigenin 7-(4''-*O*-acetyl)-glucoside, apigenin 7-(3'',4''-*O*-diacetyl)-glucoside and apigenin 7-(4'',6''-*O*-diacetyl)-glucoside other than free A7G (20,22–24). Therefore, following extraction of *M. recutita* with 96% ethanol, before and after sodium hydroxide addition to hydrolyze esters was comparatively examined by HPTLC. According to the HPTLC chromatogram at 366 nm, the zones which were close to $R_F$ = 0.60 and $R_F$ = 0.70 could possibly be acetylated A7G derivatives, that is because their colors were similar to that of A7G. Besides, acetylated derivatives of A7G should have lower polarity then the glycoside itself **(Figure 17a)**. Moreover, HPTLC densitograms at 340 nm also supported these findings and it was clearly observed that the peaks coded 2 and 3 which were considered to be acetylated A7G **(Figure 17b)** disappeared after addition of sodium hydroxide **(Figure 17c)**. Haghi et al. also concluded that following hydrolysis, A7G yielded a larger peak area than that before (16). On the other hand, this suggested hydrolysis step to determine the total content of A7G eliminate many other components in the sample test solution which may be helpful in discrimination of the genuine specimen and detection of possible adulteration. Moreover, it should be underlined that although A7G is an active marker, it should not be considered as a chemotaxonomic marker for *M. recutita* authentication. Therefore, it is important to analyze not only A7G but also other components found in *M. recutita* extract.

Morlock and Schwack stated that the separation power of HPTLC due to its capillary force applied during chromatography may be lower than HPLC (215). So, it may be the only hesitation for resolution in complex matrices. Therefore, the separation power of the HPTLC method in this study was also compared with that of the HPLC method in the Ph. Eur. in order to show the efficiency of the developed HPTLC method. Consequently, a co-existing compound together with A7G was detected both in HPTLC and HPLC methods found at the website of the EDQM **(Figure 18d)**. Since close resolution patterns of developed HPTLC and HPLC methods, separation power of the HPTLC method was found to be appropriate for further analysis. Subsequently, the developed method was validated following the ICH guidelines for stability, robustness,

specificity, LOD, LOQ, linearity, precision and recovery using cultivar *M. recutita* as a reference plant material. The validated method was then applied to analyze numerous *M. recutita* samples such as nature-picking *M. recutita*, *M. recutita*-like materials and commercial *M. recutita* products. Several Asteraceae members have inadvertently been gathered from nature (different localities in Turkey) by unexperienced people assuming to be *M. recutita* and these were named as '*M. recutita*-like materials' in this study. The botanical identification of these plant materials have revealed that only 2 materials were identified as genuine specimen, *M. recutita.* The other species belonged to 4 different genera of Asteraceae, *Anthemis* L., *Bellis* L., *Chrysanthemum* L. and *Tanacetum* L. Addition to these samples, commercial *M. recutita* products including different brands of tea bags and bulk or wrapped crude flowers sold as *M. recutita* were purchased from food stores and spice shops, respectively.

Eventually, A7G was determined in the wild *M. recutita* samples from two different localities along with *A. coelopoda* var. *coelopoda*, *A. austriaca*, *A. auriculata*, *A. cretica*, *B. perennis* and *B. sylvestris* samples. Besides, A7G was detected in all tea bag samples (M1-5) and in various crude flowers as A1-3, A5-8 and A11. When HPTLC densitogram of the reference *M. recutita* **(Figure 19b)** was compared with these investigated samples, it was shown that wild *M. recutita* **(Figures 22a and b)** and M1-5 samples **(Figures 24a-e)** exerted almost identical densitogram with that of the reference plant material at 340 nm. On the other hand, different fingerprinting profiles were observed from *Anthemis* spp., *Bellis* spp., *Tanacetum* sp., *Chrysanthemum* sp. **(Figures 22c-n)** and A1-11 samples **(Figures 24f-p)**. In addition, none of them matched with the reference *M. recutita*. Moreover, chromatograms of these previously mentioned species **(Figure 21)** revealed that the intense blue bright zones at $R_F$ = 0.42-0.5 were seen in the *Anthemis* spp., *Bellis* spp. and *Tanacetum* sp. flower extracts and also intense orange zones close to $R_F$ = 0.1 and $R_F$ = 0.2-0.3 were also observed in the flowers of *A. altissima*, *A. tinctoria* var. *discoidea* and *A. scariosa*. These specified zones were also observed in the chromatograms of A2-3, A5, and A7-10 from spice shops **(Figure 23)**, which may indicate potential adulteration of these samples with *M. recutita*-like materials. Joharchi and Amiri also pointed out that *M. recutita* was the most adulterated and substituted plant with other members of Asteraceae due to close morphological characteristics (216).

On the other hand, samples M1-5 possessing identical chemical fingerprinting with that of the reference *M. recutita* and also A1-2 and A8 samples thought to be adulterated by HPTLC analysis were also analyzed by HPLC method in the Ph. Eur. as an explanatory example. Consequently, HPLC chromatograms belong to M1-5 **(Figures 25a-e)** showed almost identical fingerprinting with that of the reference *M. recutita* **(Figure 16b)**. Secondly, HPLC chromatograms of A1-2 and A8 samples (**Figures 25f, g and h**) were also compared with that of the reference *M. recutita*. Consequently, not A1 and A8 samples but A2 showed a close fingerprinting profile with that of the reference *M. recutita*. This could be due to elimination of other components after ester hydrolysis (**Figure 17a**) which would be helpful in detection of the adulteration. Eventually, HPLC chemical fingerprinting profile was not enough selective to reveal adulteration observed by HPTLC profile. Moreover, most of the *M. recutita* tea products available on market are in powdered form; therefore macroscopic analysis is quite difficult. On the other hand, due to similar microscopic characteristics of such *M. recutita*-like species from Asteraceae discrimination of genuine specimen may be problematic. Additionally, TLC method according to the 'Matricaria flower' monograph stated in the Ph. Eur. is only useful for its essential oil composition (180). Recently, Çalışkan performed microscopic analysis on commercial crude *M. recutita* flower products which were thought to be adulterated with *A. cretica* due to having lanceolate palea (receptacular bract). Consequently, microscopic characteristic properties belong to *M. recutita* such as outer and inner epidermis of ligulate flowers, glandular and covering trichomes, stigma extended to form rounded papillae and pollen grains were also observed in these adulterated products (217). Therefore, before performing HPLC analysis stated in the Ph. Eur. particularly for powdered *M. recutita* materials use of the HPTLC method developed in this study would be highly recommendable in order to determine the quality of the material.

The content of A7G was further evaluated after its identification in the investigated samples. When its content was compared in between two wild *M. recutita* samples, it was found that A7G concentration was quite different in these samples collected from different locations; Istanbul (0.96 mg/g) and Canakkale (below LOQ) **(Table 34)**. On the other hand, A7G contents of these wild *M. recutita* samples were found to be lower than the reference *M. recutita* (3.29 mg/g). This might be due to controlled growth of the reference *M. recutita* under cultivation which eliminates many

variations based on geographical conditions such as rainfall, temperature, soil, altitude and humidity. In addition, total A7G content was found as 7.0 mg/g in the hydroalcoholic *M. recutita* extract (reference) by HPLC analysis. It should be pointed out that this value reflects total A7G obtained through hydrolysis of the acetylated A7G derivatives. Moreover, A7G concentration in *A. coelopoda* var. *coelopoda* and *A. austriaca* samples was found as 0.86 and 0.80 mg/g, respectively **(Table 34)**. In the other species in which A7G was detected, its amount was lower than the LOQ; therefore further calculations could not be performed. Additionally, in different brands tea bags (M1-5) A7G content was ranged from 0.43 to 0.80 mg/g **(Table 35)**. On the other hand, in crude flowers thought to be adulterated with other species sold in spice shops, A7G either was not determined or its amount was found to be lower than the LOQ. Contrarily to expected, A7G content in adulterated A2 (0.51 mg/g) was found as similar as M1-2 and M4 **(Table 35)**.

Recently, Sagi et al. introduced an HPTLC method using dichloromethane-acetonitrile-ethyl formate-glacial acetic acid-formic acid (11:2.5:3:1.25:1.25, v/v/v/v/v) as a developing solvent system and amino plates as a stationary phase (similar to our study). They either identified or quantified some flavonoids such as A7G, L7G, rutin and chamaemeloside and one coumarin (umbelliferon) in methanolic extracts of cultivar *M. recutita*, *Anthemis nobilis* L., *Chrysanthemum morifolium* L. and commercial *M. recutita* products such as tea bags and capsules (53). Sagi et al. worked in between 50 and 200 ng/band for the calibration curve of A7G and they found LOQ value as 50 ng/band whereas in this study we worked in the ranged from 5 to 50 ng/band and LOQ was determined as 5 ng/band. The differences in the LOQ values may have various explanations such as extraction conditions, developing solvents, wavelength and derivatization. In both methods extraction of standards were performed by methanol. However, used mobile phase and development conditions in HPTLC analyses were different, that might be a reason to increase the intensity of the bands. In addition, the working wavelengths were different; Sagi et al. used 360 nm for scanning the investigated plate. On the other hand, as a result of spectral evaluation of the defined A7G standard zones the absorption maximum was found as 340 nm in the present study. Additionally, Sagi et al. determined the content of A7G in cultivar *M. recutita* samples in between 1 and 2.83 mg/g. In our study its content in cultivar *M. recutita* was found as 3.29 mg/g which was higher than these investigated samples. The difference in A7G

content in cultivar samples could be associated with some factors such as genetic background of the *M. recutita*, geographical origin, drying conditions, harvesting time as well as extraction procedure of the sample test solution. Besides, the amount of A7G in *M. recutita* tea bags from different brands was ranged from 0.19 mg/g to 6.10 mg/g in the study of Sagi et al. In other words, there was roughly 32-fold difference in between the lowest and highest amount of A7G in these marketed products. Moreover, in the present study A7G in commercial products were either not detected or its content was found up to 0.80 mg/g and also most of them found to be adulterated. Consequently, both studies highlight the necessity of the quality control of these products before marketing.

Moreover, sophisticated analytical methods such as HPLC, UPLC, CE and MS were also used to calculate A7G content in *M. recutita* flower extracts. Haghi et al. investigated both hydrolysed and non-hydrolysed A7G in aqueous, methanolic and hydroalcoholic *M. recutita* extracts using UPLC-UV method. They quantified A7G content as 0.28, 0.21 and 0.59 mg/g whereas total A7G was found as 0.68, 1.11 and 1.23 mg/g in aqueous, methanolic and hydroalcoholic extracts, respectively (16). In addition, Harbourne et al. found 2.9 mg/g of A7G in aqueous extract of *M. recutita* flowers prepared by brewing at 100˚C using HPLC-UV (44). Avula et al. investigated the content of methanolic extracts of *M. recutita* cultivars and its commercial products such as capsules, liquids and tea bags by HPLC-UV-MS. As a result, A7G was found to be within the range of 0.02 to 3.51 mg/g in *M. recutita* extracts. In other words, there was roughly 175-fold difference in between the lowest and highest amount of A7G in these samples. Additionally, in different brands of tea bags, its content was found in the range from 0.08 to 2.45 mg/g (46). Srivastava and Gupta found A7G in aqueous and methanolic *M. recutita* flower extracts as 58.61 and 52.76%, respectively using HPLC-ESI-MS-MS (48). Masci et al. used the HPLC-ESI-MS-MS method to quantify A7G in both aqueous extracts of *M. recutita* flowers and only its tubular flowers and found 0.26-0.32% A7G in flowers, whereas low amounts of A7G was detected as 0.02-0.09% in tubular flowers (59). In addition, using the same analytical technique Raal et al. investigated major phenolic compounds in tea bags from different brands and the ratio of apigenin glucosides were found to be between 0.6 to 10.2 mg/200 mL (49).

Quality assurance of plant materials may have a direct impact on their safety and efficacy. Therefore, qualitative and quantitative requirements for herbal drugs should be analytically studied before marketing. Differently from synthetic drugs, quality control of raw herbal materials should include not only quantification of the active or inactive ingredient(s) but also ascertainment of the chemical fingerprinting profile of the sample under investigation. That is because, quantitative analysis only focus on measuring the amount of marker component(s) of a given herbal drug. However, the content of the markers may not be satisfactory for quality assessment of herbal drugs due to their complex matrix. Therefore, qualitative analysis completes the quality analysis for establishing the identity of the plant material. HPTLC method developed in this study for *M. recutita* flower extract provides convenience for both qualitative and quantitative conclusions concurrently. Besides, developed HPTLC method has various advantages over most of the sophisticated methods reported so far for analysis of *M. recutita* samples, i.e. shorter run time, low solvent consumption, parallel analysis of several samples, rapid and visual results, easy discrimination of genuine *M. recutita* from other Asteraceae species resembling to *M. recutita* and also applicability on the quality assessment of commercial *M. recutita* products available on the market.

*C. scolymus* leaf is known to be rich in caffeoylquinic acid derivatives such as caffeic acid, ChA and cynarin and flavonoids including mostly L7G. Recently, Mulinacci et al. reported that use of water for extraction of *C. scolymus* leaf induces hydrolysis of dicaffeoylquinic acid derivatives, whereas hydroalcoholic extraction increases the amount of flavonoids (108). In addition, Pandino et al. extracted *C. scolymus* leaf with 70% methanol containing BHT to prevent compounds from decomposition during the extraction process (128). Wölkart et al. reported that polyphenol oxidase, an enzyme catalysing the oxidation, may be responsible for such oxidative degradation of caffeoylquinic acid derivatives (218). Moreover, Dawidowicz and Typek concluded that ChA may undergo transformations such as isomerization, esterification, hydrolysis and water molecule addition during its extraction through water at different pH conditions (219). On the other hand, Pistón et al. recently demonstrated the importance of *C. scolymus* consumption as infusion compared to its hydroalcoholic and decoction extracts due to its highest antioxidant activity which is related with its rich phenolic content (220). Moreover, in the Ph. Eur., it is suggested that *C. scolymus* leaf extract should be prepared using hot water of minimum 80˚C

(180). European Medicines Agency has also recommended that water extraction should be used to obtain *C. scolymus* dry extract for its herbal preparations (138). Eventually, in the light of recent studies and statements as well as traditional practice, *C. scolymus* leaf and *C. scolymus* other plant parts (bract, receptacle and stem) were brewed in hot water (100˚C) and then lyophilized to obtain their dry extracts in the present study.

According to the Identification section belong to 'Artichoke leaf dry extract' monograph (Ph. Eur.), *C. scolymus* leaf is determined by TLC analysis using ChA and L7G as identity markers (180). Initially, before adaption of the suggested TLC method to HPTLC, preliminary studies were conducted to screen its chemical composition through sephadex column chromatography. The obtained fractions were developed on silica gel 60 $F_{254}$ TLC plates using a developing solvent sytem of ethyl acetate-acetic acid-formic acid-water (100:11:11:27, v/v/v/v). As a result, at the same $R_F$ values belong to ChA and L7G, other components encoded as 'x' and 'z' were monitored, respectively **(Figure 27)**. When the *C. scolymus* leaf extract was developed with the same conditions, 'x' and 'z' were co-migrated with ChA and L7G, respectively. It was concluded that it is necessary to adjust the establish TLC method because of these co-migrated compounds with the identity markers of *C. scolymus* leaf extract.

DPPH˙ spectrophotometric assay is one of the most popular and widely used methods for determining the free radical scavenging capacity of the test samples. Recently, HPTLC-bioautography assay with DPPH˙ radical attracts attention of researchers not only because of screening antioxidant activity but also enabling to determine contribution of each separated antiradical compound to total antioxidant capacity. It is well known that DPPH˙ is a characteristic deep purple colored free radical. If DPPH˙ solution is sprayed (or dipped) to developed HPTLC plate, its color turns into pale yellow when reduced by antioxidants, which are separated on the plate (203). Total antioxidant activity of *C. scolymus* leaf extract was already determined by DPPH˙ spectrophotometric assay (109,114,121). However, in the present study HPTLC-DPPH˙ assay on *C. scolymus* leaf extract was performed to determine antioxidant activity of each separated compound on the plate. As a result of HPTLC-DPPH˙ assay, contribution of ChA together with a co-migrated compound and cynarin in total antioxidative activity of *C. scolymus* leaf extract was remarkable compared to other components **(Figure 28)**. Pérez-García et al. also demonstrated significant contributions

of both ChA and cynarin on antioxidant activity on human leukocytes induced with ROS generated agents using flow cytometry method (165).

According to the Ph. Eur., ChA is stated as an active marker for *C. scolymus* leaf extract. However, quantification of not only ChA but also cynarin was targeted in this study as a result of the HPTLC-DPPH˙ assay. It should be also highlighted that cynarin is a typical caffeoylquinic acid derivative in *C. scolymus* and has a significant contribution to its main pharmacological properties such as hepatoprotective, antihyperlidemic, antidyspeptic and antimicrobial activities (140,144,147,163). Afterwards, optimum separation conditions for *C. scolymus* leaf extract were assessed using the standard mixture of rutin, ChA, L7G, cynarin and caffeic acid. Successive trials showed that the solvent system of ethyl acetate-acetic acid-formic acid-water (35:2:2:5, v/v/v/v) seemed good enough to separate 'x' while not enough to separate 'z' on silica gel 60 $F_{254}$ HPTLC plate **(Figure 29k)**. Vice versa, the same solvent system managed to separate 'z' but not 'x' on silica gel 60 $F_{254}$ HPTLC plate which was pre-developed with methanol-dichloromethane (1:1, v/v) containing 0.5% TEA **(Figure 29m)**. In this study, although most of the phenolic compounds possessing conjugated chromophore groups which reflect blue-green fluorescence under UV excitation, silica gel plates with fluorescence indicator were preferred during the analysis. That is because, Reich and Anne recently investigated the effects of fluorescence indicator on resolution and concluded that in spite of very low ratio of indicator (2%), a better separation may be achieved comparing to that of the plate without indicator (173). Moreover, through TEA addition the chromatographic character of the silica gel plate could be modulated which lowers the acidity of silica gel plate surface and consequently adsorption of each component on the surface would be altered.

Pre-validation steps were performed following the method development. Initially, stability of the *C. scolymus* components on the plate during chromatography was tested by 2D-development. Eventually, it was monitored that compounds developed on silica gel plate using a developing system of ethyl acetate-acetic acid-formic acid-water (35:2:2:5, v/v/v/v) were not stable. Then, the other developing solvent systems were also tested using both silica gel and reversed-phase plates. Consequently, compounds were prone to decomposition on silica gel plates developed with various developing solvent systems. On the other hand, compounds remained unchanged when

they were developed on reversed-phase plate using water-acetonitrile-*o*-phosphoric acid (35:10:1, v/v/v) as shown in **Figure 30f**. However, 'z' was co-migrated with cynarin in this chromatographic system **(Figure 29j)**. Consequently, the resolution between 'z' and cynarin was not enough to perform further quantification of cynarin. It should be highlighted that the type of the stationary phase and composition of the mobile phase (acetonitrile, water and *o*-phosphoric acid) in HPTLC method in which compounds stay stable are similar with that of the HPLC method stated in the Ph. Eur. (180).

In further studies, reasons of instability were comparatively investigated by changing some parameters such as sample preparation, using prewashed plate, adsorbent types and developing solvent systems. Accordingly, following points were postulated:

- To test acid-degradation, sample test solution was prepared with a developing solvent system of ethyl acetate-acetic acid-formic acid-water (35:2:2:5 v/v/v/v). However, compounds in the sample did not yield any additional acid-degradated compounds **(Figure 31a)**.
- To investigate the effects of impurities probably come from laboratory environment or packing material to the plate surface. The plate was pre-develeped with methanol-dichlorometane (1:1, v/v) to remove possible impurities on the plate surface. However, same zones were observed out of the diagonal **(Figure 31b)**.
- To compare the adsorbent impacts, the sample test solution was applied and developed on both silica gel and amino plates using the same developing solvent system which was ethyl acetate-acetic acid-formic acid-water (20:2:2:5, v/v/v/v). Consequently, not flavonoids (rutin and L7G) but caffeic acid derivatives were subjected to decomposition on amino plate **(Figure 31c)**. Eventually, the large surface of silica gel may play a role catalyzing the degradation reactions and also compounds may undergo decomposition mostly on normal phases due to its highly active polar surfaces. On the other hand, flavonoids remained stable on amino plate; this may be due to having lower polarity surface than silica gel plate.
- To evaluate oxidative degradation, an antioxidant (0.5% BHT) was added to the developing solvent system to prevent decomposition during the chromatography.

However, additional degradation compounds other than previously observed were seen on the chromatogram **(Figure 31d)**.

- To study the effects of acids in the developing solvent system on compounds, mobile phase was prepared without acids. Then, the prepared solvent system was comparatively practiced with the solvent system containing concentrated formic acid **(Figures 31e and f)**. Accordingly, high levels of acidic medium during chromatography may cause disintegration of the ChA. However, phenolics are highly polar compounds and thus for their migration without tailing on the polar surface addition of highly polar solvents such as acids is highly required.
- Additionally, application positions were exposed to air and light during the application which may also affect the degradation.

Consequently, for the quantitative analysis of extracts of *C. scolymus* leaf and *C. scolymus* other plant parts HPTLC seems not a convenient method due to instability of the components during development while may be useful for its qualitative evaluation. Loescher et al. also monitored those caffeic acid derivatives degradated during chromatography practiced by HPTLC method in which silica gel plate and gradient mobile phase of ethyl acetate containing 2% acetic acid, water and hexane was used (221).

The chemical compositions were further evaluated in extracts of *C. scolymus* leaf and *C. scolymus* other plant parts such as bract, receptacle and stem belong to different development stages and *C. scolymus* food supplements. All samples were developed on pre-developed silica gel 60 $F_{254}$ HPTLC plate by methanol-dichlorometane (1:1, v/v) containing 0.5% TEA using a developing solvent system of ethyl acetate-formic acid-acetic acid-water (35:2:2:5, v/v/v/v). Eventually, not caffeic acid but rutin, ChA, L7G and cynarin were monitored in *C. scolymus* leaf extract. Additionally, both ChA and cynarin were the most representative compounds in extracts of *C. scolymus* outer, intermediate, inner bracts, receptacle and stem in the all development stages **(Figure 33)**. However, rutin, L7G and ‘z’ were not detected in extracts of these *C. scolymus* plant parts. In accordance with our findings, Romani et al. also characterized L7G only in a typical Italian variety of *C. scolymus* leaf extract other than that of *C. scolymus* outer bracts, head and stem using HPLC-DAD-MS (112). In

addition, Lombardo et al. could not find L7G in outer and inner bracts, receptacle and also stem extracts of Tema 2000 genotype of *C. scolymus* by HPLC-DAD-ESI/$MS^2$ (130). On the other hand, Sánchez-Rabaneda identified rutin using HPLC-DAD-$MS^2$ in *C. scolymus* bracts, receptable and stem extracts (105). Abu-Reidah et al. also monitored rutin in *C. scolymus* receptacle using HPLC-DAD-ESI-QTOF-MS (124).

In the present study, caffeic acid was not detected in extracts of *C. scolymus* leaf and *C. scolymus* other plant parts in accordance with previous findings (113,130,162,220). Contrarily to expected, caffeic acid was monitored in *C. scolymus* food supplements encoded as C1 and C2 **(Figure 34)**. Caffeic acid is a simple phenolic compound has widespread distribution in plant kingdom. The reason to detect this compound in *C. scolymus* food supplements other than the leaf material itself may be due to the decomposition of caffeoylquinic acid derivatives into caffeic and quinic acids during processing of the marketed samples. Schütz et al. also did not determine caffeic acid in fresh *C. scolymus* heads; however they monitored it in *C. scolymus* pomace and juice. They concluded that heating may cause either isomerisation or hydrolysis of caffeoylquinic acid derivatives (107). Lutz et al. also observed that cooking process has increased the caffeic acid content in *C. scolymus* heads (121). Therefore, it should be noted that both raw material and its finished products should be separately considered during the quality control. Particularly, care should be given whether the finished products maintain the features of the raw materials. In this study, additional components which was not detected in *C. scolymus* leaf extract were monitored in C1 and C2 products close to $R_F$ values of 0.4 and 0.5 and another zone was detected at $R_F$ = 0.80 in C2 **(Figure 34)**. As mentioned before, since *C. scolymus* leaf extract was mixed with *S. marianum* extract in C2 these compounds may be the components of this later extract. On the other hand, these components were also observed in C1 claming to contain only *C. scolymus* leaf extract. Moreover, rutin, L7G and 'z' were not determined in C1, C2 and C3, their contents in these samples may be lower than the LOD, although C1-3 sample test solutions were prepared 12.5-fold concentrated form compared to its leaf extract **(Figure 34)**. On the other hand, Mulinacci et al. (108) and Schütz et al. (110) monitored L7G in *C. scolymus* dietary supplements. Moreover, other food supplement coded as C3 showed exactly different chemical fingerprinting profile than that of *C. scolymus* leaf extract and also that of other food supplemets. In addition, a red colored zone close to the front was not monitored both in C1 and C2 while it was detected in C3

**(Figure 34)**. This zone might possibly due to chlorophyll residue mostly found in alcoholic plant extract other than water extract. Consequently, none of the food supplements have shown to possess identical chemical fingerprinting profile with that of *C. scolymus* leaf extract.

Following assessment of chemical composition in extracts of *C. scolymus* leaf, *C. scolymus* other plant parts and also its food supplements, HPLC analysis was performed to evaluate both ChA and cynarin contents in these extracts. Consequently, the highest ChA content was found in *C. scolymus* leaf extract as 6.05%, **Figure 36**. Pinelli et al. also concluded that ChA is the main component in various hydroalcoholic wild and cultivar *C. scolymus* leaf extracts. They calculated its content in between 11.69 and 73.68 µmol/g using HPLC-DAD (113). Moreover, among the caffeoylquinic acids ChA was also found to be a predominant compound in all parts of *C. scolymus* (110,121,128–130). Additionally, Gouveia and Castilho concluded that ChA was also the major compound in *C. scolymus* dietary supplements and they found its content as 7.31 mg/100 mL in juice and 32.4 mg/100g in dragées using HPLC-DAD-ESI-$MS^2$ (114). In this study it can be also pointed out that ChA content accummulated increasingly from outer to inner bracts particularly in both mature and at early maturity of *C. scolymus* heads **(Figure 36)**. Moreover, its content in all plant parts except receptacle and intermediate bracts belong to AH (at maturity) as well as receptacle of CH (at early stage of maturity) was higher than the criterion stated in the Ph. Eur., 0.6%. (180).

Recently, an HPLC-DAD analysis was performed by Özbilgin to quantify ChA concentration in cultivar *C. scolymus* leaf and its dry extracts. Eventually, researcher found ChA content as 1.06% and 0.02% in its leaf and dry extracts, respectively (222). However, in this study we found ChA content in *C. scolymus* leaf extract as 6.05%. The difference in ChA content in samples could be associated with genetic background of the *C. scolymus*, geographical origin, drying conditions as well as harvesting time. Moreover, as mentioned previously sample preparation is one of the crucial parameters for all analytical studies particularly in plant analysis. Özbilgin prepared the sample test solutions using a mixture of methanol-water (70:30) by continuous magnetic stirring for 15 hours. Eventually, long extraction duration may cause transformations such as isomerisation and decomposition of ChA, resulting in low yields. Moreover, *C.*

*scolymus* leaf dry extract was prepared using soxhlet apparatus first with petroleum ether for 8 hours-period and then with water extraction by magnetic stiring for 3 hours (222). Along with such a long extraction procedure, degradation of the particularly heat-sensitive compounds possibly occurs during its perpetual-motion. Therefore, extraction procedure may have a direct impact on yields of investigated components.

Cynarin mostly considered mainly responsible for *C. scolymus* therapeutic effects, its highest value was found as 12.06% in extract of *C. scolymus* inner bracts which was obtained from *C. scolymus* head in early stage of maturity. The other *C. scolymus* plant parts were also found to be very rich in cynarin content **(Figure 37)**. Its concentration in *C. scolymus* leaf extract was calculated as 1.71%. Gouveia and Castilho also determine remarkable cynarin content in methanolic *C. scolymus* leaf extract with a value of 23.5 mg/100g using HPLC-DAD-ESI-$MS^2$ (114). In accordance with previous findings stated by Fratiani et al. (120), cynarin content in inner bracts showed decreasing trend towards to the outer bracts of *C. scolymus* heads particularly in mature and early stage of maturity. It should be highlighted that not only *C. scolymus* leaf extract but also *C. scolymus* other plant part extracts especially inner bracts obtained from its head may be a good source as a health-promoting agent. Therefore, the effect of *C. scolymus* food supplements may be potentiated by addition of the other plant parts such as bract.

ChA and cynarin contents were also evaluated in *C. scolymus* food supplements. Eventually, the ratio of ChA in *C. scolymus* food supplements belong to two different brands encoded as C1 and C2 was found to be 0.58% and 0.68%, respectively which met the criterion stated in the Ph. Eur., while its amount was found as 0.01% in C3. Additionally, the labeled contents of these brands were found to be different than the calculated amounts in this study. Moreover, cynarin content in these samples was found to be as 0.44, 0.48 and 0.03% for C1, C2 and C3, respectively. Özbilgin also investigated six different brands of *C. scolymus* food supplements including either *C. scolymus* leaf extract or its extract enriched with *S. marianum* using HPLC-DAD method (222). However, none of the *C. scolymus* leaf extract enriched with *S. marianum* was met the criterion stated in the Ph. Eur. Additionally, only one food supplement containing *C. scolymus* leaf extract was met the criterion stated in the Ph.

Eur. In addition, Özbilgin also highlighted the differences of ChA contents in between found results and labeled contents.

As mentioned previously quantitative analysis only focus on measuring the amount of marker component(s) for a given plant material. Although C1 and C2 met the criterion stated in the Ph. Eur., their chemical fingerprinting profiles were found to be exactly different than the profile of the *C. scolymus* leaf extract. Therefore, quantification of marker compounds together with qualitative analysis should be considered for quality assessment of *C. scolymus* food supplements.

**Figure 39.** Summary of the highlighted points on *M. recutita*

**Figure 14l.**

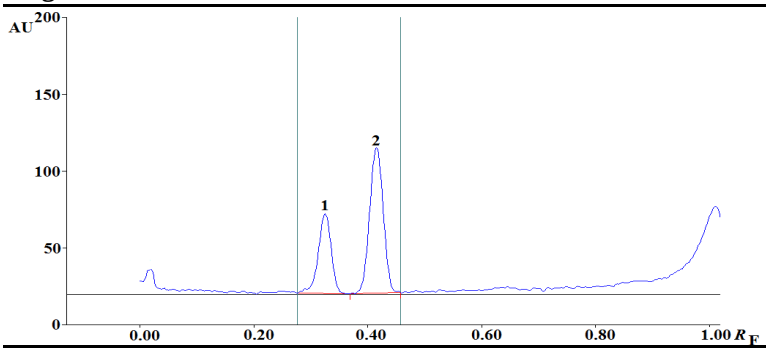



**Figure 15.**

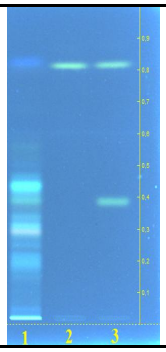



**Figure 17.**

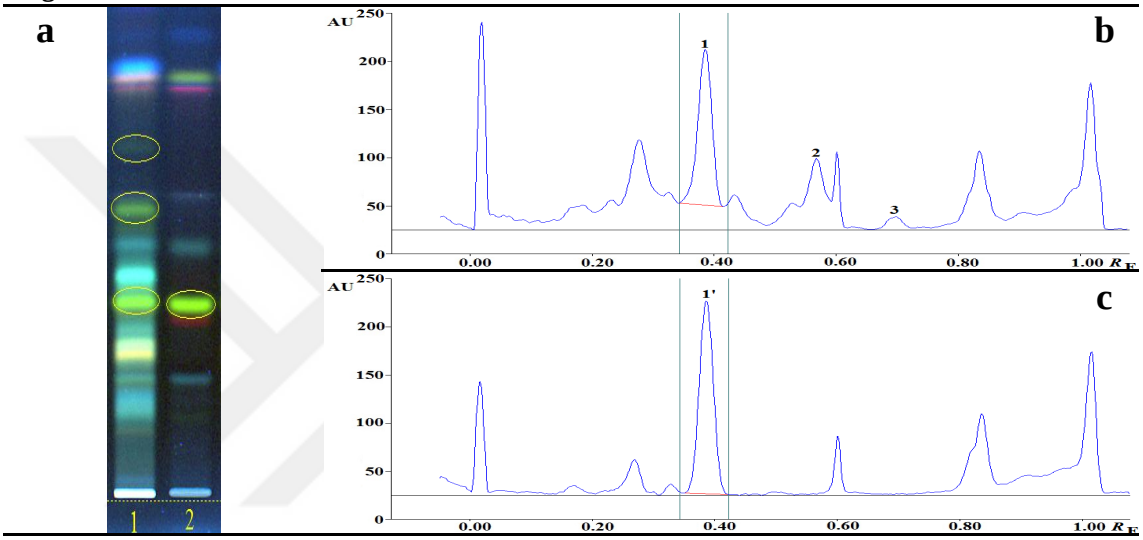



**Figure 18d.**

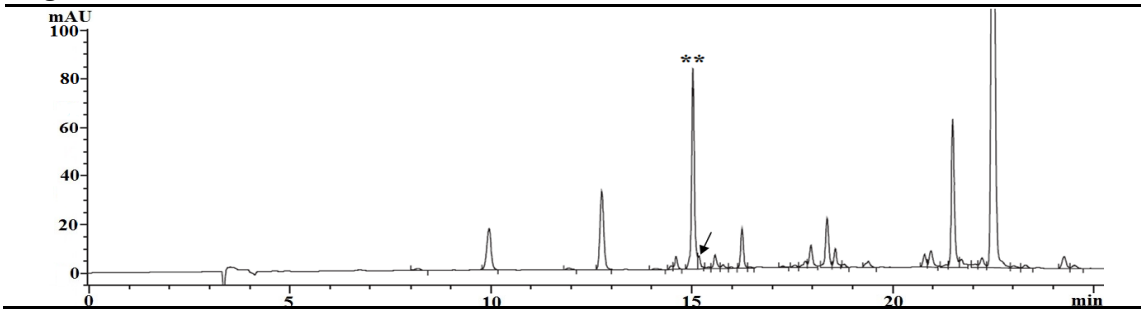


**Figure 39.** Continued.

**Figure 19b.** *M. recutita* (reference)

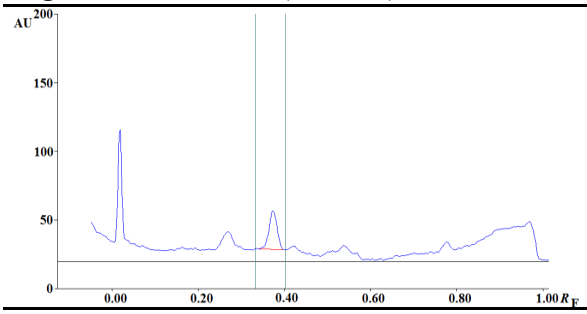



**Figure 22a.** *M. recutita* (Kurtkoy, Istanbul)

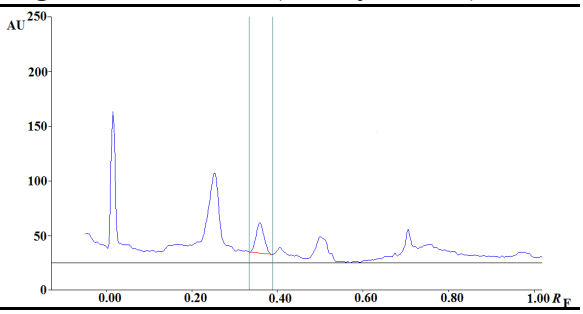



**Figure 22b.** *M. recutita* (Intepe, Canakkale)

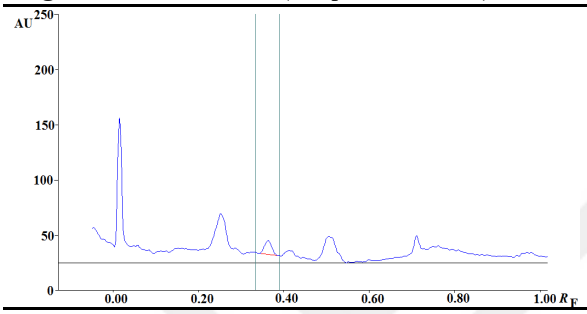



**Figure 24a.** M1

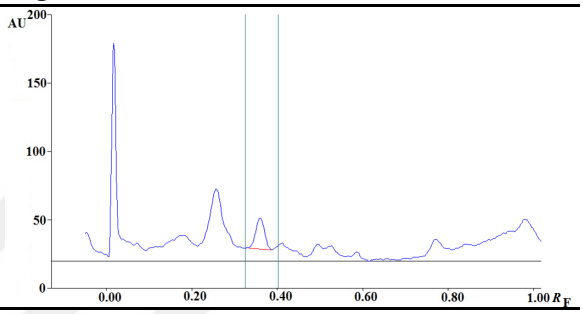



**Figure 24b.** M2

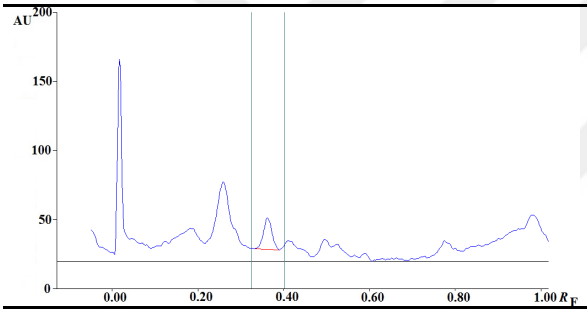



**Figure 24c.** M3

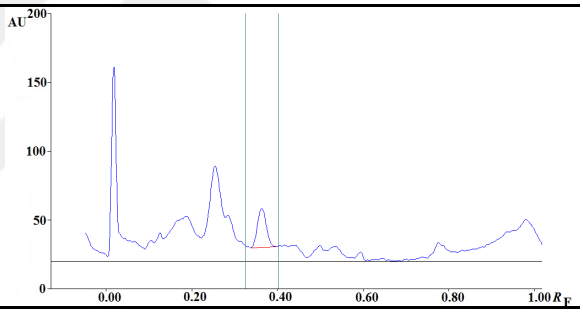



**Figure 24d.** M4

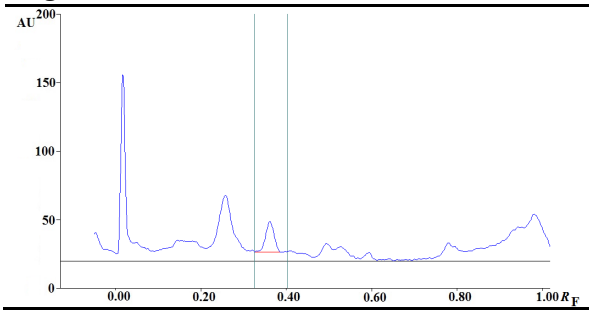



**Figure 24e.** M5

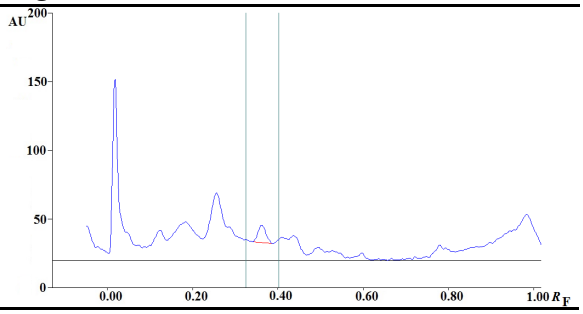


**Figure 39.** Continued.

**Figure 22c.** *A. coelopoda* var. *coelopada*

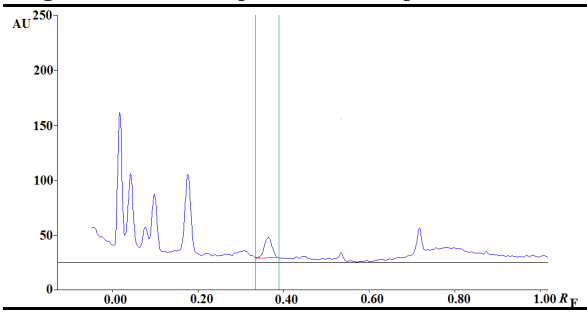



**Figure 22d.** *A. austriaca*

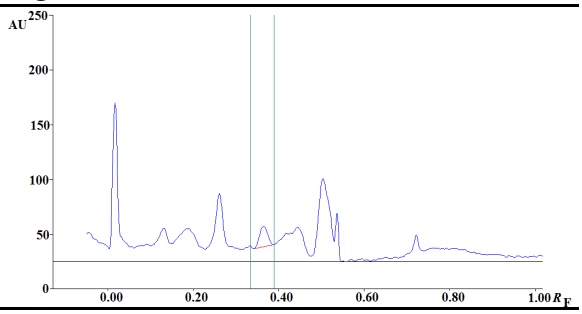



**Figure 22e.** *A. auriculata*

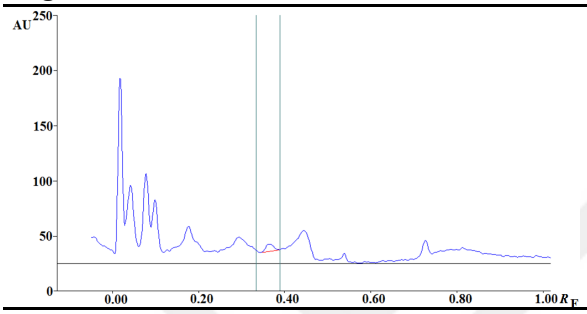



**Figure 22f.** *A. cretica*

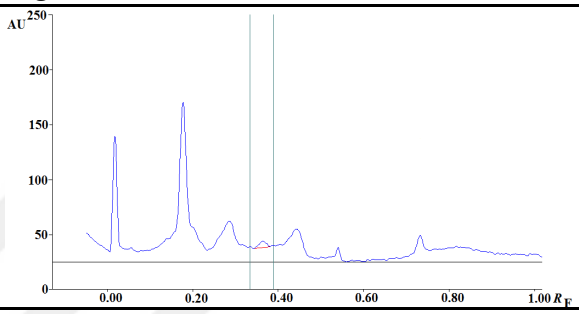



**Figure 22g.** *A. cotula*

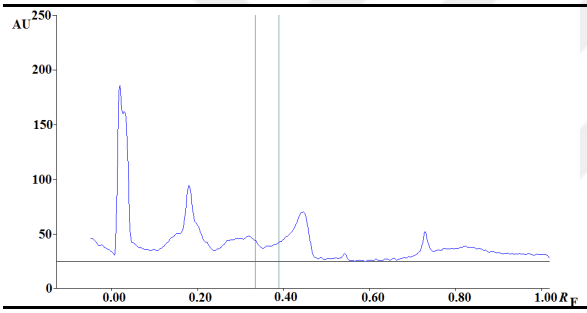



**Figure 22h.** *A. altissima*

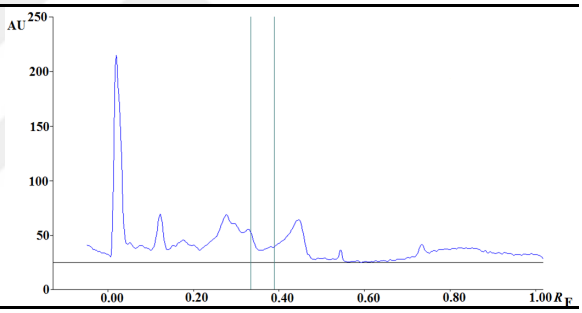



**Figure 22i.** *A. tinctoria*

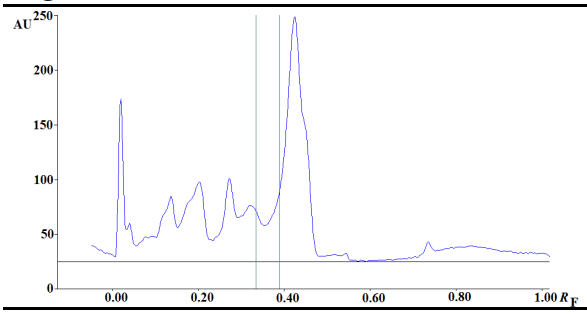



**Figure 22j.** *A. scariosa*

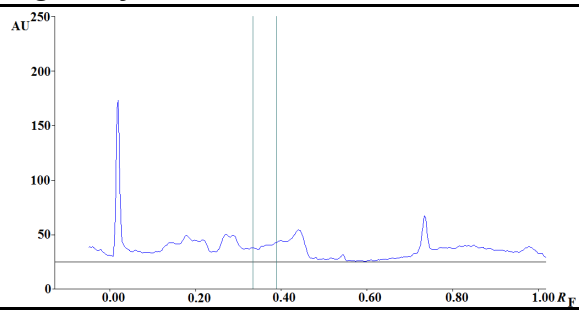



**Figure 22k.** *B. perennis*

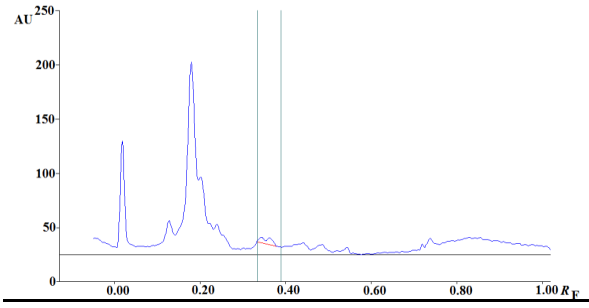



**Figure 22l.** *B. sylvestris*

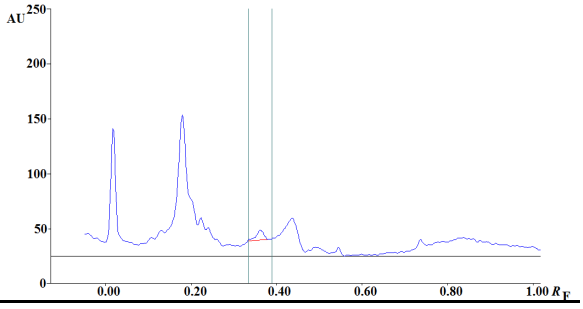


**Figure 39.** Continued.

**Figure 22m.** *C. coronarium*

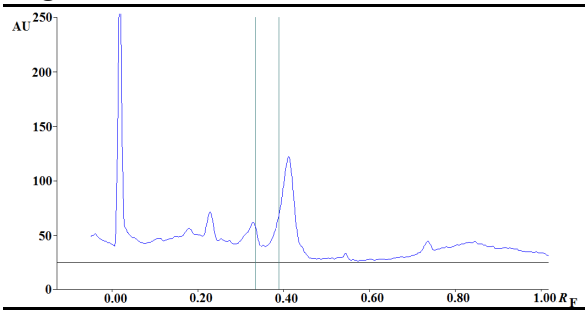



**Figure 22n.** *T. parthenium*

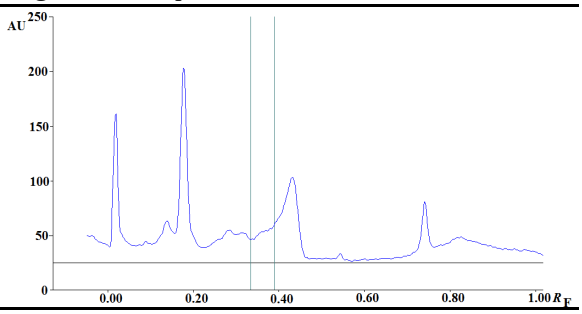



**Figure 24f.** A1

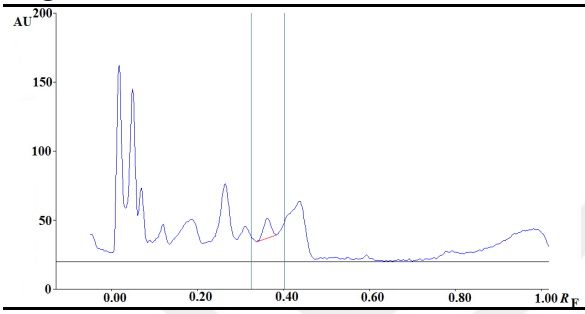



**Figure 24g.** A2

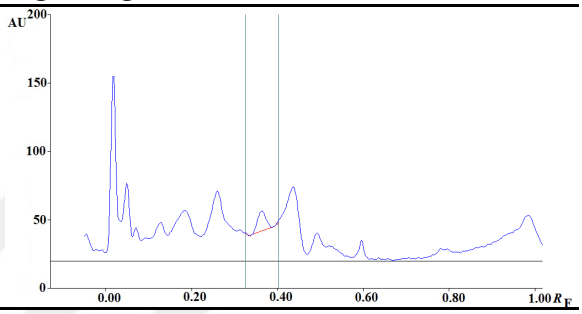



**Figure 24h.** A3

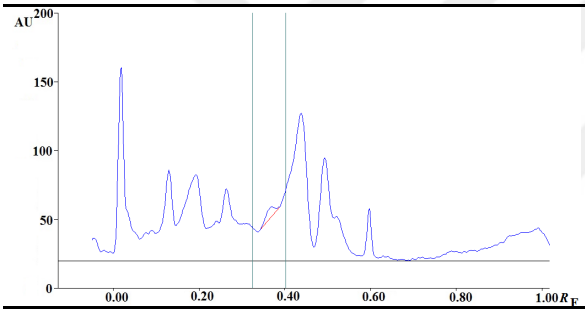



**Figure 24i.** A4

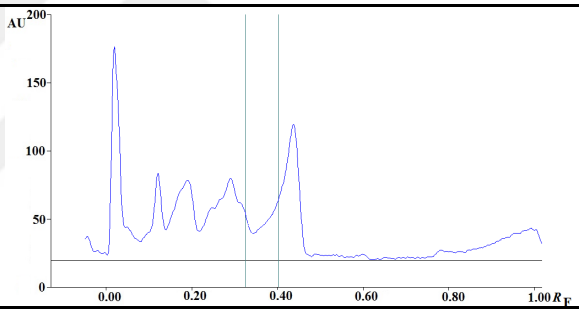



**Figure 24j.** A5

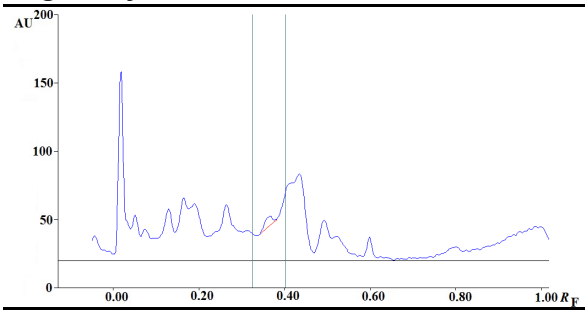



**Figure 24k.** A6

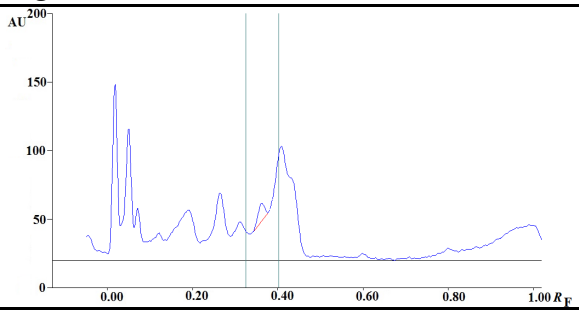



**Figure 24l.** A7

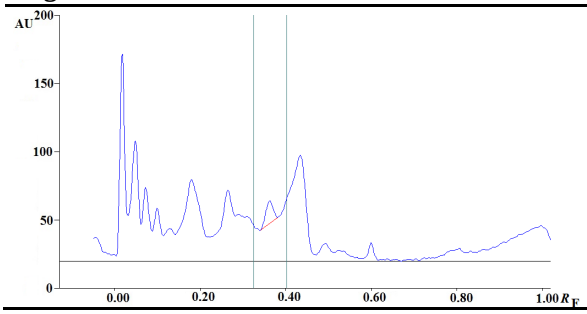



**Figure 24m.** A8

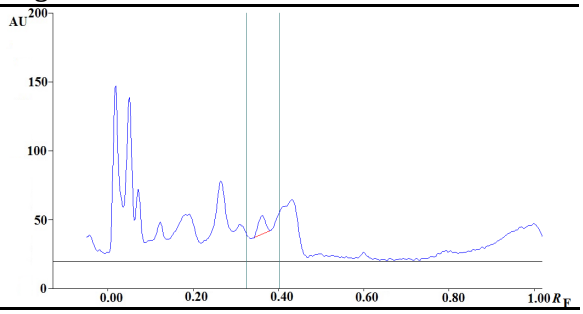


**Figure 39.** Continued.

**Figure 24n.** A9

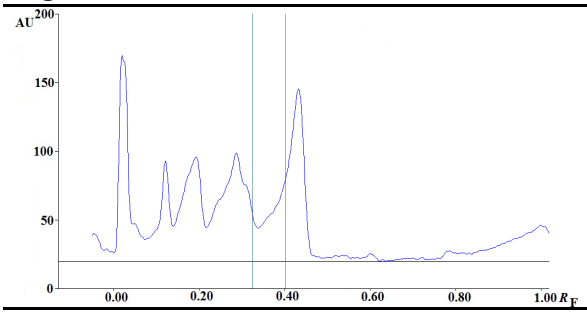



**Figure 24o.** A10

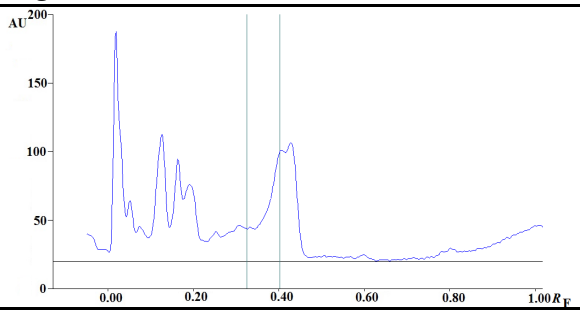



**Figure 24p.** A11

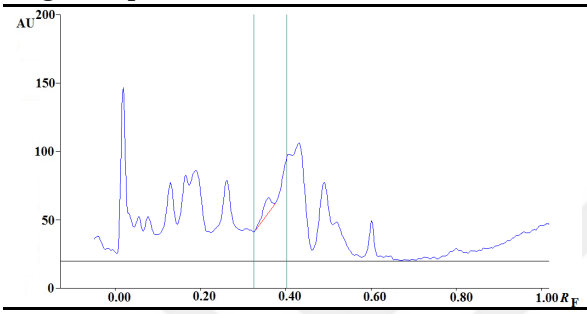



**Figure 21.**

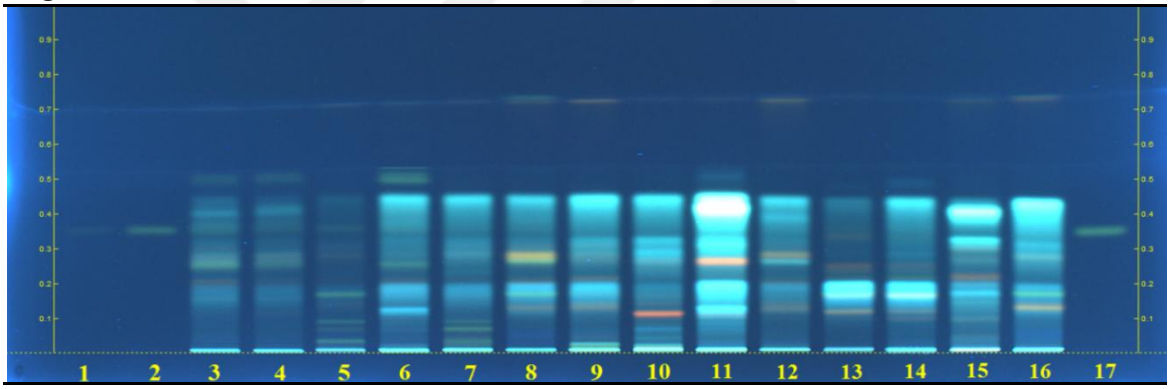



**Figure 23.**

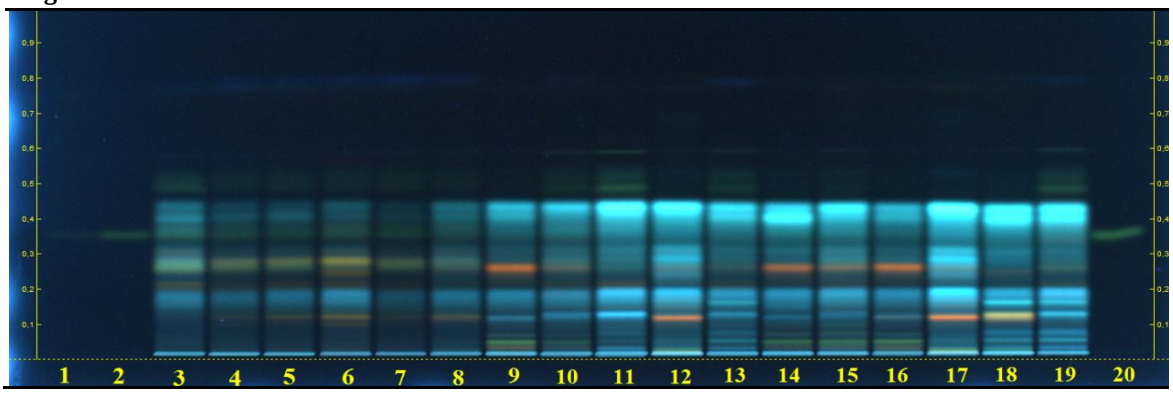


**Figure 39.** Continued.

**Figure 16b.** *M. recutita* (reference)

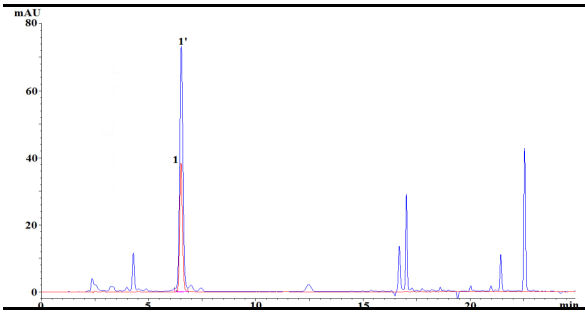



**Figure 25a.** M1

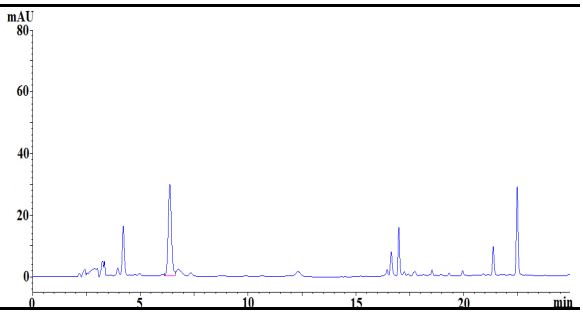



**Figure 25b.** M2

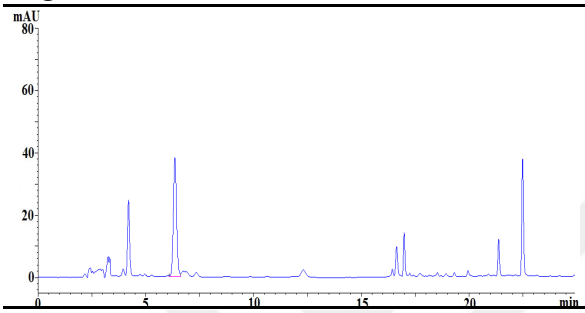



**Figure 25c.** M3

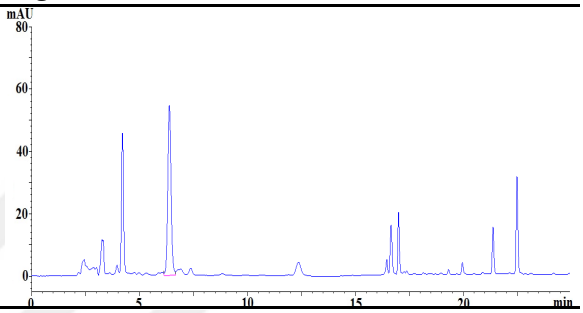



**Figure 25d.** M4

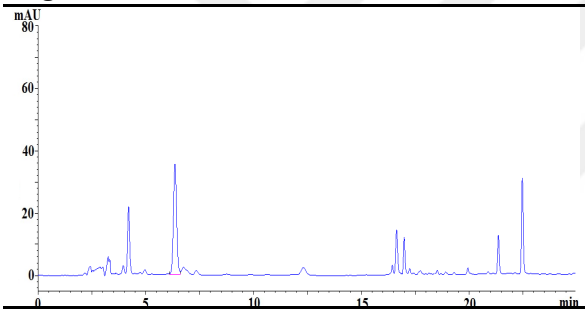



**Figure 25e.** M5

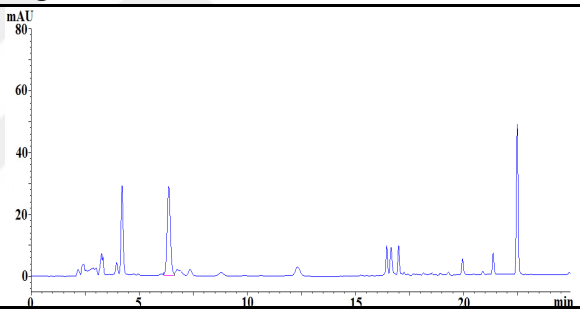



**Figure 25f.** A1

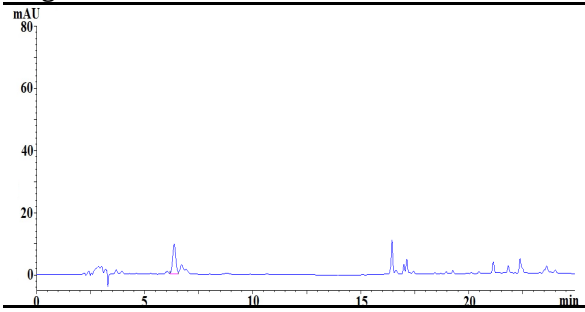



**Figure 25g.** A2

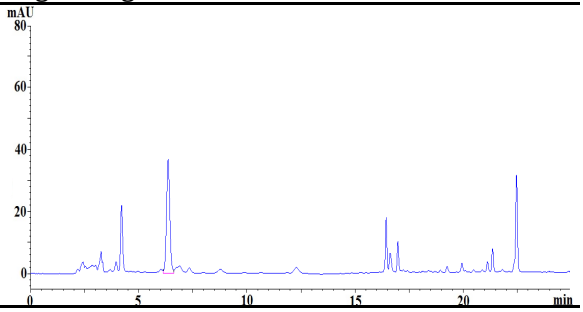



**Figure 25h.** A8

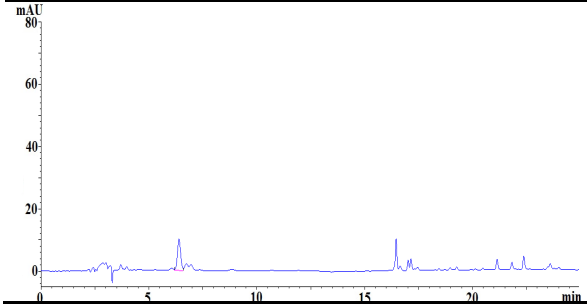


**Table 37.** Summary of the highlighted points on *M. recutita*

| **Table 34.** | | **Table 35.** | |
|---|---|---|---|
| **Plant Materials** | **A7G (mg/g)[a]** | **Products** | **A7G (mg/g)[a]** |
| *M. recutita* (Istanbul) | 0.95 ± 0.08 | M1 | 0.49 ± 0.01 |
| *M. recutita* (Canakkale) | < LOQ | M2 | 0.49 ± 0.03 |
| *A. coelopoda* var. *coelopoda* | 0.86 ± 0.03 | M3 | 0.80 ± 0.02 |
| *A. austriaca* | 0.80 ± 0.03 | M4 | 0.54 ± 0.01 |
| *A. cretica* | < LOQ | M5 | 0.43 ± 0.01 |
| *A. cotula* | ndt | A1 | 0.30 ± 0.02 |
| *A. altissima* | ndt | A2 | 0.51 ± 0.01 |
| *A. tinctoria* var. *discoidea* | ndt | A3 | < LOQ |
| *A. scariosa* | ndt | A4 | ndt |
| *B. perennis* | < LOQ | A5 | < LOQ |
| *B. sylvestris* | < LOQ | A6 | < LOQ |
| *C. coronarium* | ndt | A7 | 0.29 ± 0.01 |
| *T. partheniu* | ndt | A8 | 0.26 ± 0.01 |
| | | A9 | ndt |
| | | A10 | ndt |
| | | A11 | < LOQ |

**Figure 40.** Summary of the highlighted points on *C. scolymus*

**Figure 27.**

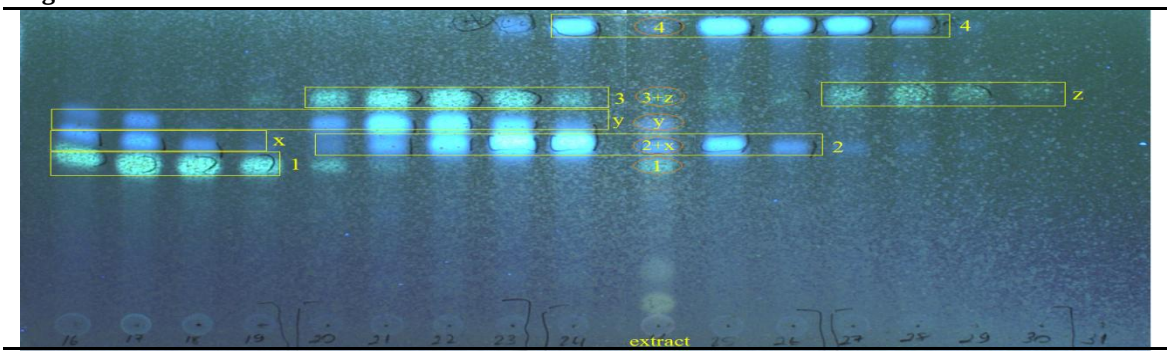
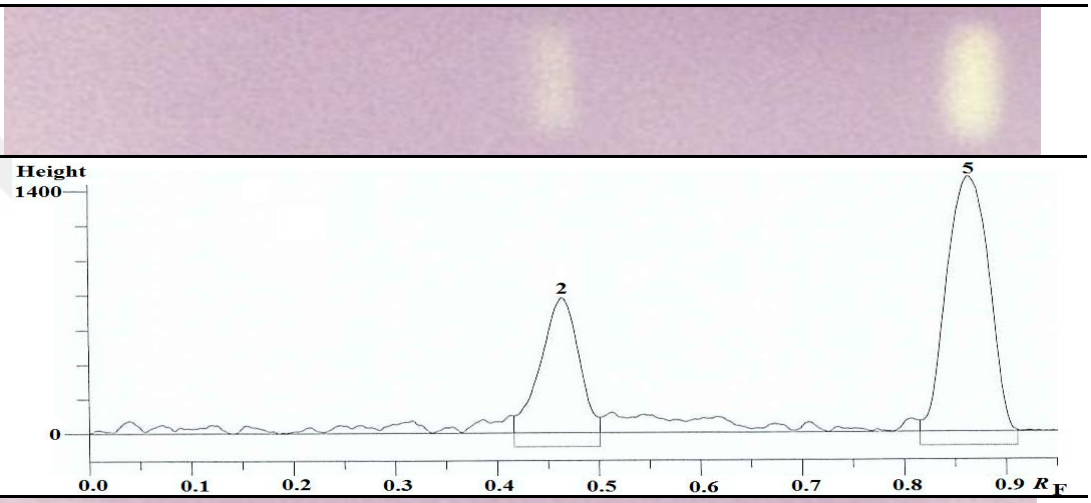



**Figure 28.**

**a**




**b**

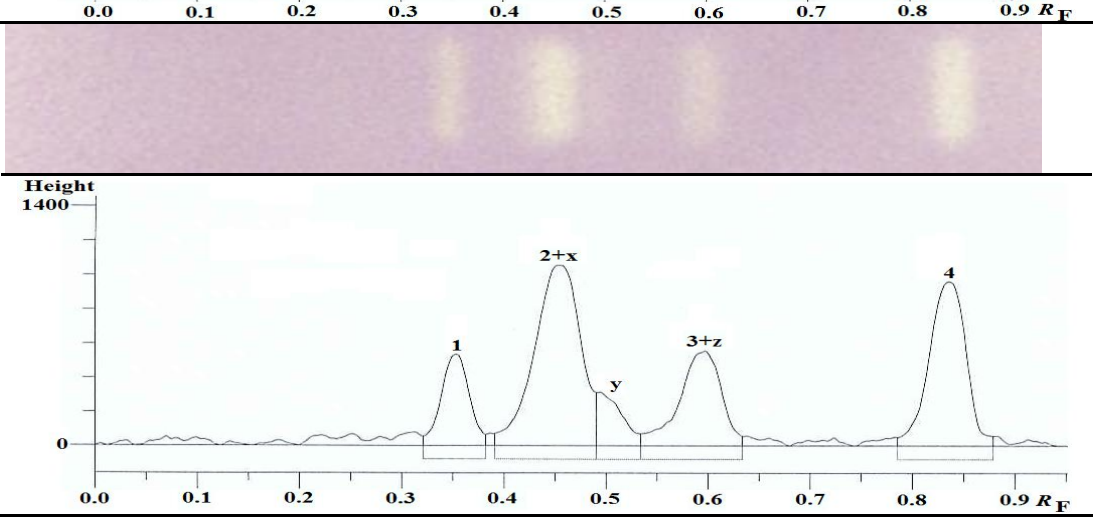


**Figure 40.** Continued.

**Figure 29k.**

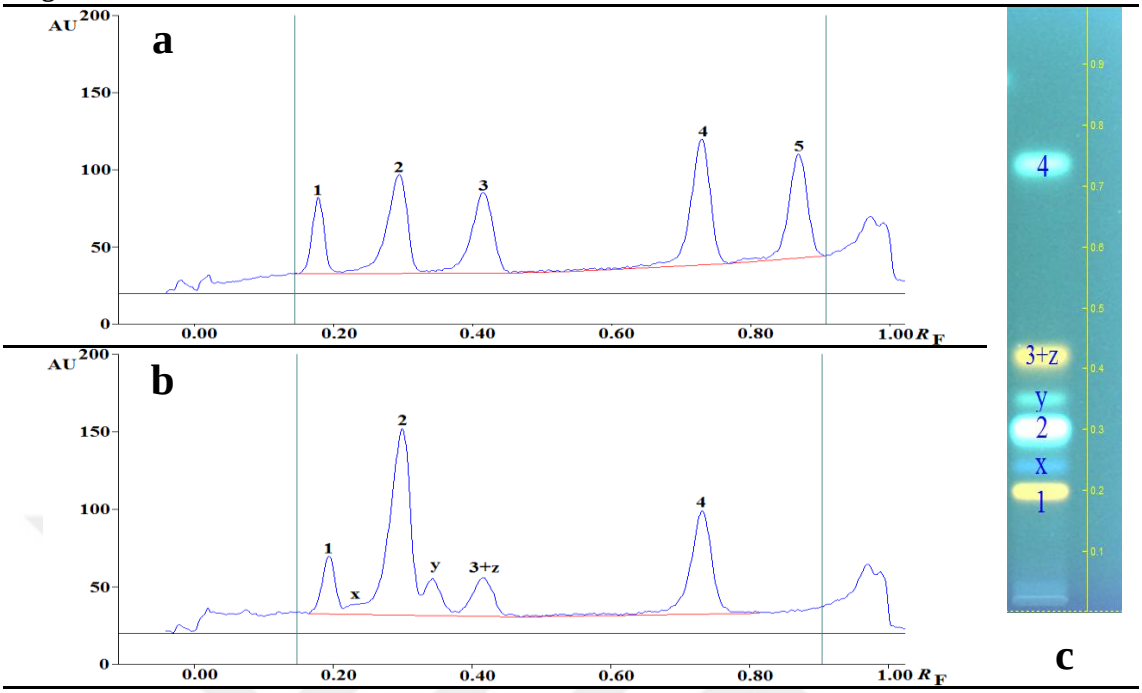



**Figure 29m.**

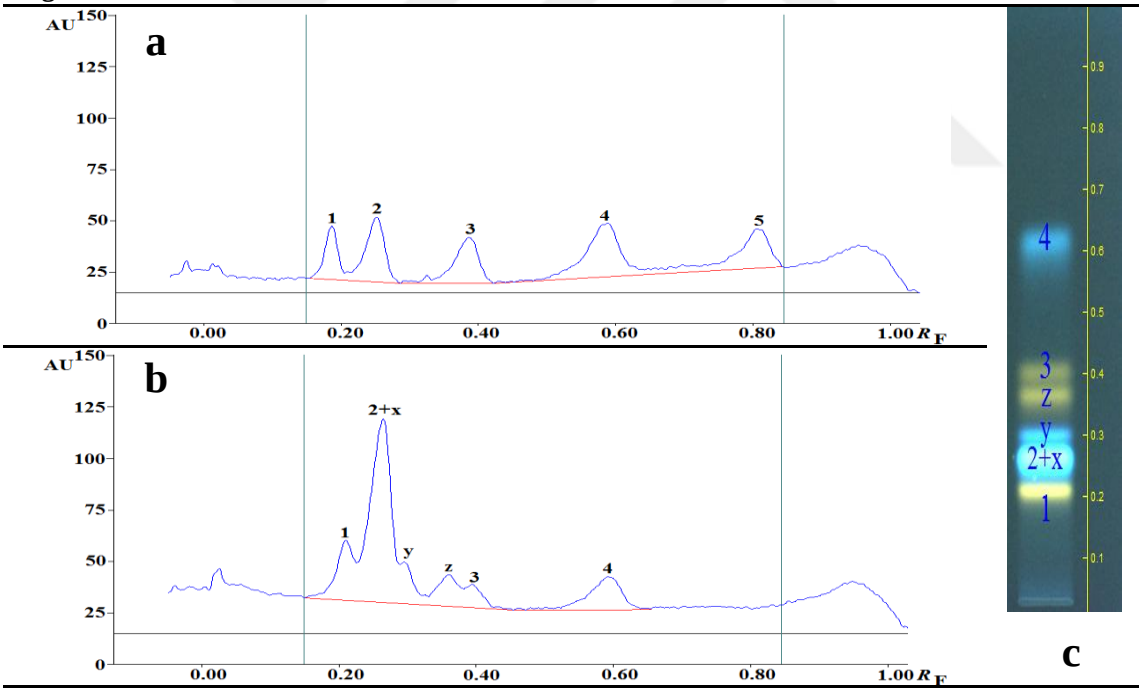


**Figure 40.** Continued.

**Figure 29j.**

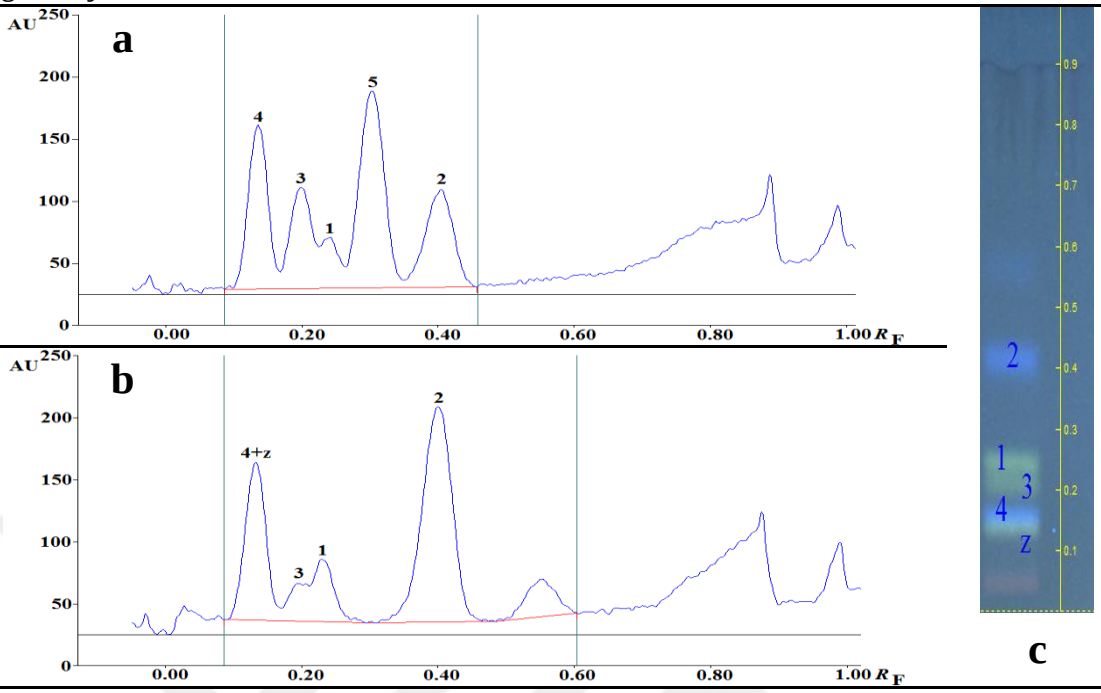



**Figure 30f.**

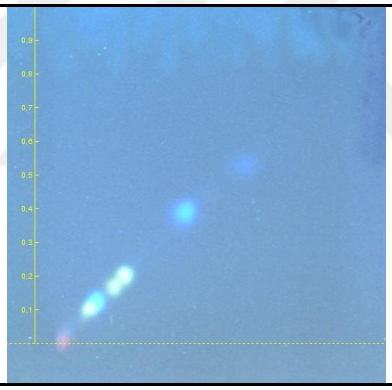


**Figure 40.** Continued.

**Figure 31.**

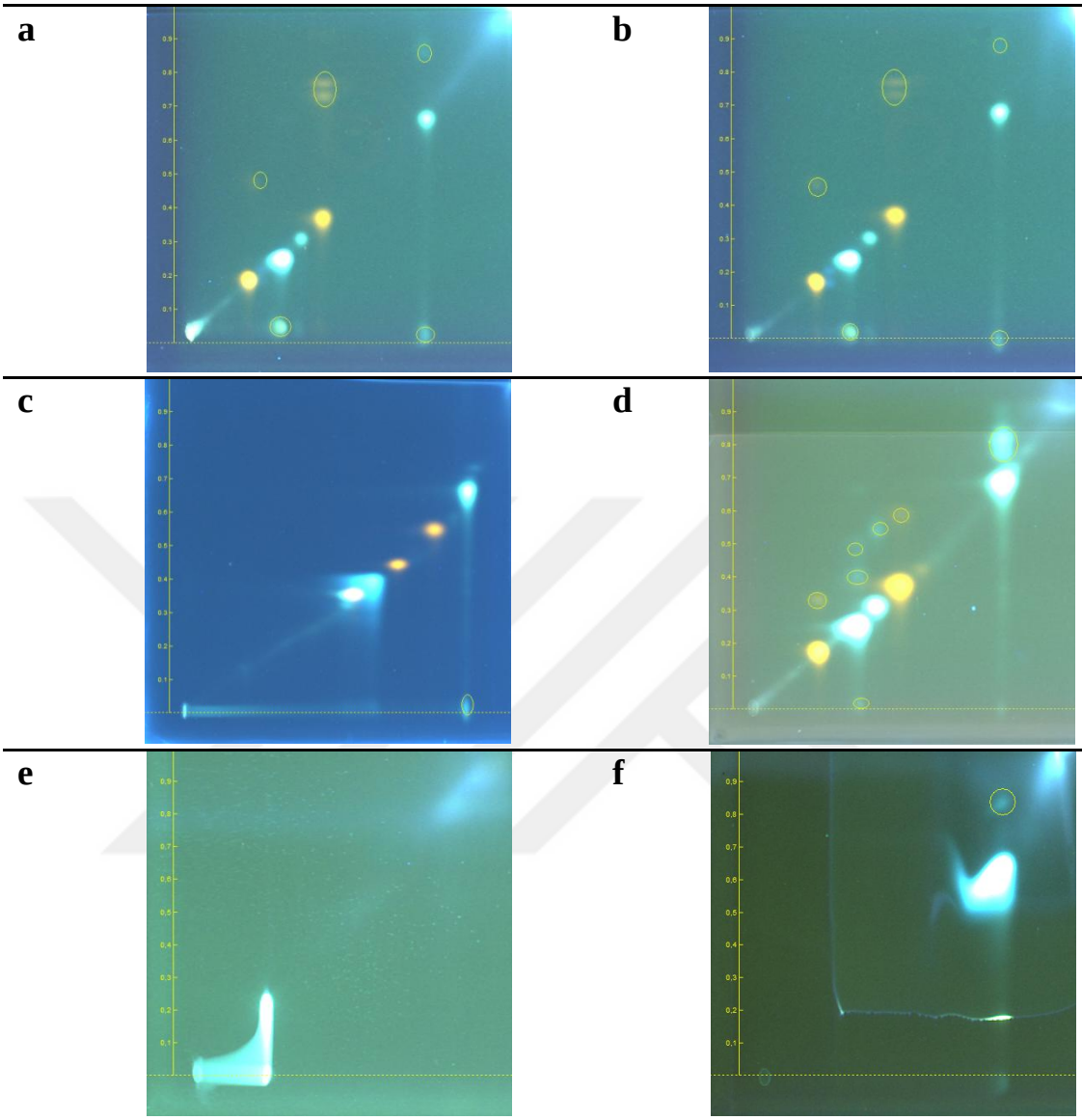



**Figure 33.**

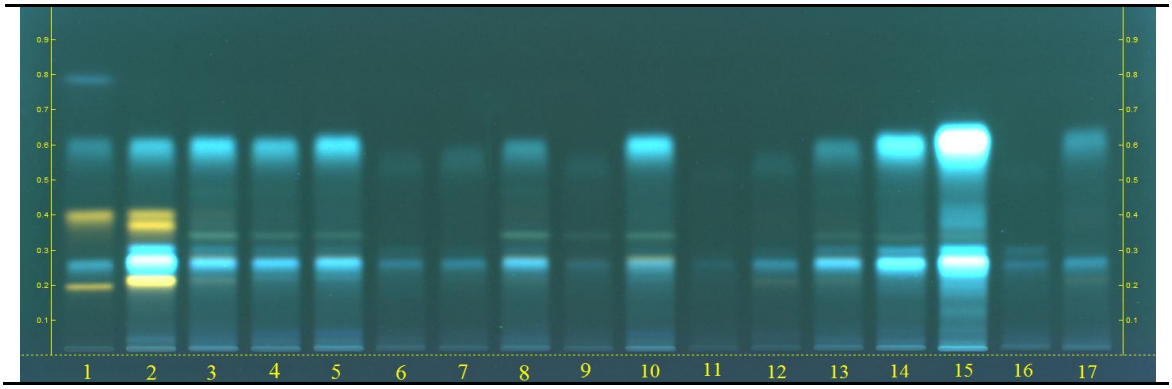


**Figure 40**. Continued.

**Figure 34.**

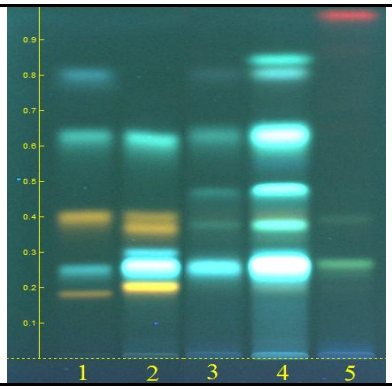



**Figure 36.**

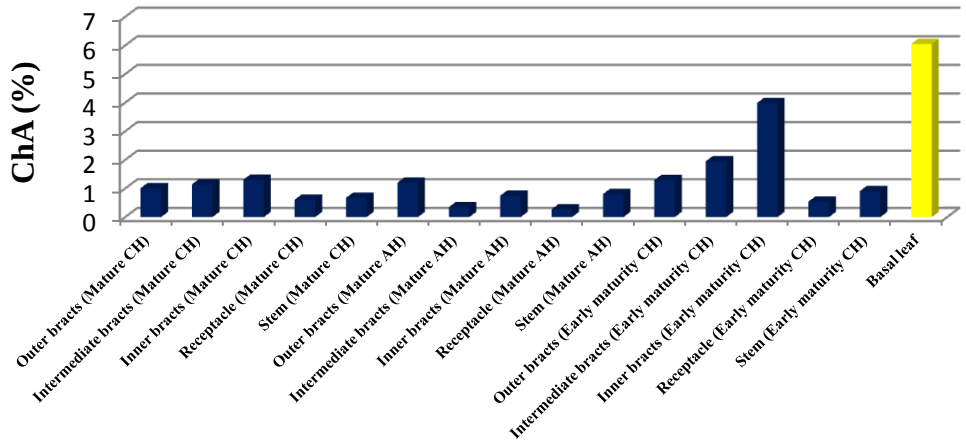
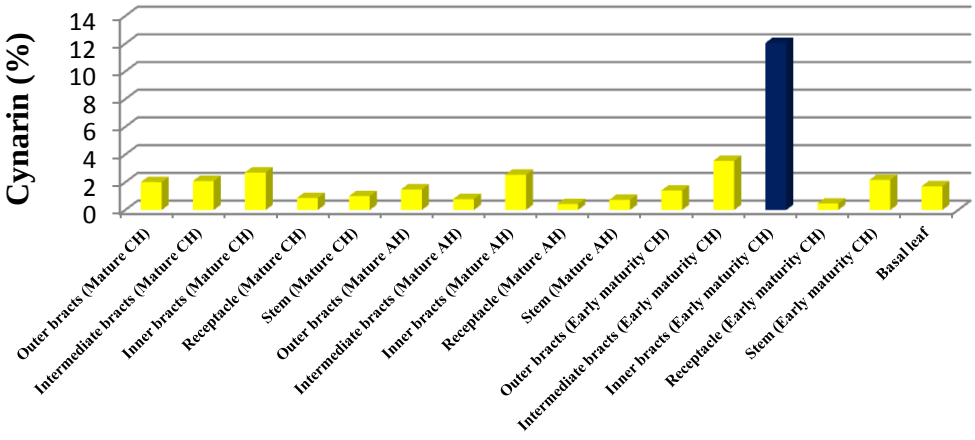



**Figure 37.**



## 6. CONCLUSION

The aim of the present study was to develop and validate qualitative and quantitative HPTLC methods on two medicinal plants from Asteraceae, *M. recutita* and *C. scolymus*, which have widespread applications in phytotherapy.

The results of experiments during method development for *M. recutita* flower extract indicated that A7G was well separated from the other constituents on pre-conditioned silica gel 60 $NH_2$ $F_{254}$s HPTLC plate using a developing solvent system of ethyl acetate-formic acid-acetic acid-water (30:1.5:1.5:3, v/v/v/v). The developed method was further validated for qualitative and quantitative analyses of A7G in *M. recutita* flower extract. Then, the method was successfully applied for analyzing A7G content in extracts of cultivar and wild *M. recutita* flowers, several Asteraceae members having *M. recutita*-like flowers and also commercial *M. recutita* products. The method was further interpreted for discriminating the genuine specimen, *M. recutita*, from other Asteraceae species and also adulterated products sold as *M. recutita* on market. Consequently, HPTLC densitograms of two nature-picking *M. recutita* and different brands of *M. recutita* tea bags (M1-5) were matched with the reference *M. recutita*. The content of A7G was found as 3.29 mg/g in cultivar *M. recutita* flower extract, up to 0.96 mg/g in wild *M. recutita* and in several *M. recutita*-like flower extracts and in between 0.43 and 0.80 mg/g in commercial *M. recutita* products. Moreover, *M. recutita* products mostly sold in spice shops were found to be adulterated with that of the *M. recutita*-like materials and A7G was either not detected or its content was below the LOQ.

In *C. scolymus* leaf extract, ChA and L7G were separated on pre-developed silica gel 60 $F_{254}$ HPTLC plate by methanol-dichlorometane (1:1, v/v) containing 0.5% TEA using a developing solvent system of ethyl acetate-formic acid-acetic acid-water (35:2:2:5, v/v/v/v). In addition, HPTLC-$DPPH^{\cdot}$ assay on *C. scolymus* leaf extract resulted that ChA and cynarin significantly contribute to antioxidant activity *C. scolymus* leaf extract. However, due to instability of phenolic compounds on the plate during chromatography, an alternative technique HPLC was used to quantify ChA and cynarin contents. Results have shown that the highest ChA content was determined as 6.05% in *C. scolymus* leaf extract whereas the highest cynarin concentration was found as 12.06% in extract of *C. scolymus* inner bracts at the early stage of maturity.

Therefore, usage of *C. scolymus* leaf as well as *C. scolymus* other plant parts particularly inner bracts would be highly beneficial. Moreover, it was found that *C. scolymus* leaf extract had a unique chemical fingerprinting profile versus *C. scolymus* bract, receptacle and stem extracts. The qualitative and quantitative analyses on *C. scolymus* food supplements showed that ChA contents of C1 and C2 but not C3 met the criterion stated in the Ph. Eur, however their fingerprinting profiles were different than the profile of the *C. scolymus* leaf extract.

It is well-known that quality assurance of plant materials has a direct impact on the safety and efficacy. Herbal drugs should fulfill high demands concerning quality parameters before marketing. Therefore, the proposed HPTLC methods in the present study may be a guide for further studies.

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# 8. CURRICULUM VITAE

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**Publication**

Guzelmeric E, Vovk I, Yesilada E. Development and validation of an HPTLC method for apigenin 7-*O*-glucoside in chamomile flowers and its application for fingerprint discrimination of chamomile-like materials. *J Pharm Biomed Anal*. 2015; 107: 108-118.

**Conference Proceedings**

Guzelmeric E., Ristivojević P., Vovk I., Milojković-Opsenica D., Yesilada E. 'Quality evaluation of marketed chamomile tea products by a validated HPTLC method combined with multivariate analysis', *Poster Presentation*, 21$^{th}$ International Symposium on Separation Sciences, Ljubljana, Slovenia, 2015.

Guzelmeric E., Vovk I., Yesilada E. 'Quantification of major antioxidants determined by HPTLC-DPPH$^{\cdot}$ method in *Cynara scolymus* L. and its products', *Poster Presentation*, 11$^{th}$ International Symposium on Pharmaceutical Sciences, Ankara, Turkey, 2015.

**Award**

Scholarship provided by The Scientific and Technological Research Council of Turkey (TUBITAK) during the Ph.D. program (2211-Yurtiçi Doktora Burs Programı).