NEAR EAST UNIVERSITY
FACULTY OF PHARMACY DEPARTMENT OF PHARMACOGNOSY

NEPHAR 311 PHARMACOGNOSY II Lab.

2015 – 2016 Spring

Prof. Dr. İhsan ÇALIŞ

NEAR EAST UNIVERSITY

FACULTY OF PHARMACY

DEPARTMENT OF PHARMACOGNOSY

The Guide to the Laboratory Practice in Pharmacognosy

NEPHAR 311 PHARMACOGNOSY II Lab.

2015 – 2016 Spring

Prof. Dr. İhsan ÇALIŞ

Student:

Name Surname :

Student Number :

References

Türk Kodeksi (TK 1940)

European Pharmacopoeia 6.0, Vols. 1&2, 2008

The United States Pharmacopeia, 16th Ed, 1985, United States Pharmacopeial Convention, Inc.

J. Bruneton, **PHARMACOGNOSY - Phytochemistry, Medicinal Plants**, 4th Ed., Lavosier, 2009

Egon Stahl & Werner Schild, **Pharmazeutische Biologie 4. Drogenanalyse II: Inhaltsstoffe und Isolierungen**, Gustav Fischer Verlag, Stuttgart, 1981

S. Berger & D. Sicker, **Classics in Spectroscopy – Isolation and Structure Elucidation of Natural Products**, WILEY-VCH Verlag GmBH & Co. KGaA, Weinheim, 2009

H. Wagner, S. Bladt, E.M. Zgainski, **Drogen Analyse – Dünnschicht-chromatographische Analyse von Arzneidrogen**, Springer-Verlag, Berlin, 1983.

Joanne Barnes, Linda A Anderson, J David Philipson, **Herbal Medicines, 3rd Edition,** Pharmaceutical Press, 2007

G. Samuelsson, **Drugs of Natural Origin, A Textbook of pharmacognosy**, Apotekersocieteten, 5th Revised Edition, Kristianstadts Boktryckeri AB, Kristianstadt, Sweden, 2004

R. Hänsel, O. Sticher, **Pharmakognosie – Phytopharmazie**, 8. Auflage, Springer Medizin Verlag, Heidelberg 2007

K. Hostettmann, A. Marston, **Saponins: *Chemistry and Pharmacology of Natural Products***, Cambridge University Press, New York, 1995.

M. Luckner, M. Wichtl, **Digitalis: Geschichte-Biologie-Biochemie-Chemie-Physiologie-Molekularbiologie-Pharmakologie-Medizinische Anwendung**, WVG, Stuttgart, 2000

References for HPTLC

1. CAMAG Instrumental Thin Layer Catalogue- 2013

2. Andok, H.C., Purohit, V.K., **High Performance Thin Layer Chromatography (HPTLC): A Modern Analytical tool for Biological Analysis". Nature and Science, 8, 10, 58-61.** 2010

January 2015, Lefkoşa

Program

NEPHAR 311 Pharmacognosy II – Lab. Calendar

Friday	Group A 09:00 – 10:50	Group B 11:00 – 13:50	Group C 14:00 – 16:50

	DEMO & Experiments
DEMO-1 Week 1	**Isoprenoids: Terpenes, classification , essential oils, iridoids, secoiridoids**

The First Group Circulation Experiments (1 – 3)	
Week 2	**1.1. Quantitative Analysis of Volatile oils (Volumetric) (Thymi Oleum, Kekik) (EU 6)** **1.2. Pharmacopeia Analysis of Oleum Thymi (Thymi aetheroleum)** **1.3. Quantitative analysis of Thymol (TK 1940)**
Week 3	**2.1. Water Determination (EU 6)** **2.2. Isolation of Anethol from the Spirits**
Week 4	**3. Quantitative Analysis of Volatile oil (Gravimetric)** **(Anisi Fructus, Oleum Foeniculi, Anason)**

Week 5	**Quiz for the The First Group Circulation Experiments (1 – 3)**

DEMO-2 Week 6	**The Second Group Circulation Experiments (iv – vi)**

	Midterms

The Second Group Circulation Experiments (iv – vi)	
Week 7	**4. Total Aldehyde Quantitation in citri oleum (Titrimetric)**
Week 8	**5.1. Identification of Triterpene Saponins from *Cylamen* species** **5.2. Pharmacognostical standardization:** Foaming Index
Week 9	**6. Identifications of Cardenolide Glycosides from the leaves of *Nerium oleander* (Nerii folium)**

Week 10	**Quiz for the The Second Group Circulation Experiments (1 – 3)**

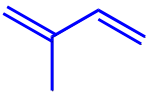
Official Holidays

April 23: National Sovereignty and Children's Day
May 1: Labor and Solidarity Day
May 19: Commemoration of Atatürk, Youth and Sports Day

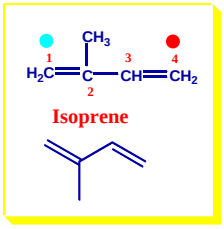
Index

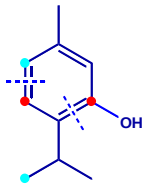
Isoprenoids - TERPENOIDS
ESSENTIAL OILS

Isoprene (short for isoterpene) or 2-methyl-1,3-butadiene is a common organic compound with the formula $CH_2=C(CH_3)CH=CH_2$. It is present under standard conditions as a colorless liquid. It is the monomer of natural rubber and is a precursor to an immense variety of other naturally occurring compounds.

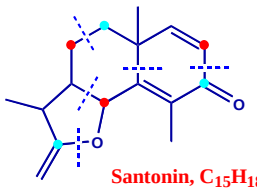
Isoprene (=2-methyl-1,3-butadiene)		$CH_2=C(CH_3)CH=CH_2$

Isoprene is the monomer of natural rubber and is a precursor to an immense variety of other naturally occurring compounds. Isoprenoide, also as Terpene and Terpenoide designation, are natural substances, whose structure develops itself by multiplication of C_5-Isopren units (Isopren = 2-Methyl-1,3-butadien).

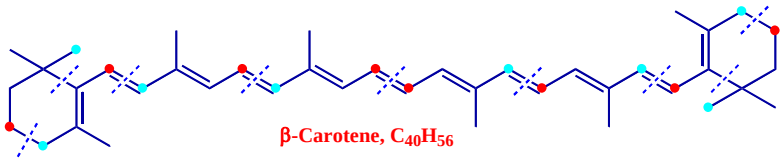




Thymol
$C_{10}H_{14}O$



Santonin, $C_{15}H_{18}O_3$



β-Carotene, $C_{40}H_{56}$

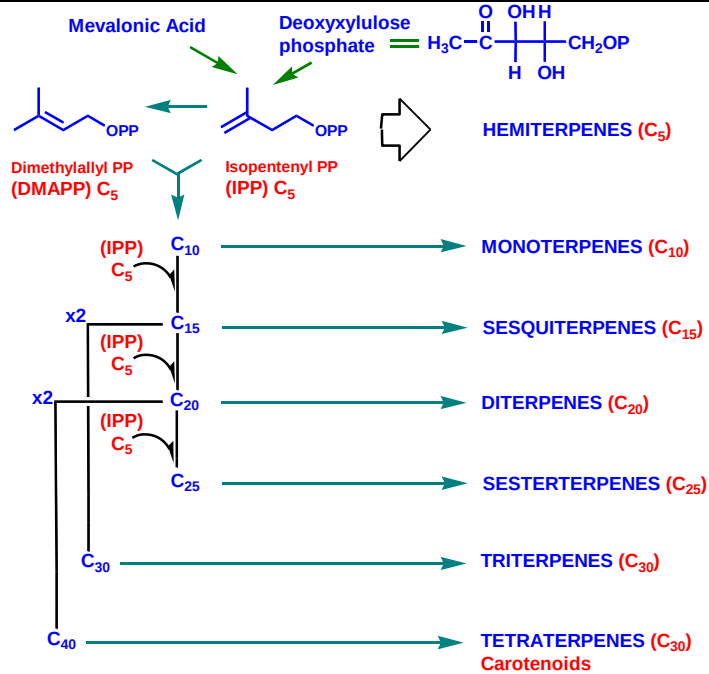
The illustration above brings 3 examples of it.

Thymol is a water vapour-volatile substance and it is an aromatic compound.

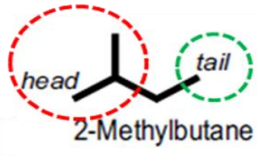
Santonin is color- and odorless, chemically a lacton with the C-skeleton partially hydrogenated naphthalins.

β-Carotene is a red colored material belonging chemically to the hydrocarbones.

The Isoprenoides have different physicochemical characteristics and different chemical structure. In common, the structures of these 3 substances are derived from Isoprene.



<u>Biosynthesis</u>: Depending on the number of 2-methylbutane (isoprene) subunits one differentiates between ***hemi- (C5), mono- (C10), sesqui- (C15), di- (C20), sester- (C25), tri- (C30), tetraterpenes (C40)*** *and* ***polyterpenes (C5)n*** *with n* ***> 8.***



The isopropyl part of 2-methylbutane is defined as the ***head****, and the ethyl residue* as the ***tail****.*

In mono-, sesqui-, di- and sester-terpenes the isoprene units are linked to each other from *head-to-tail;*

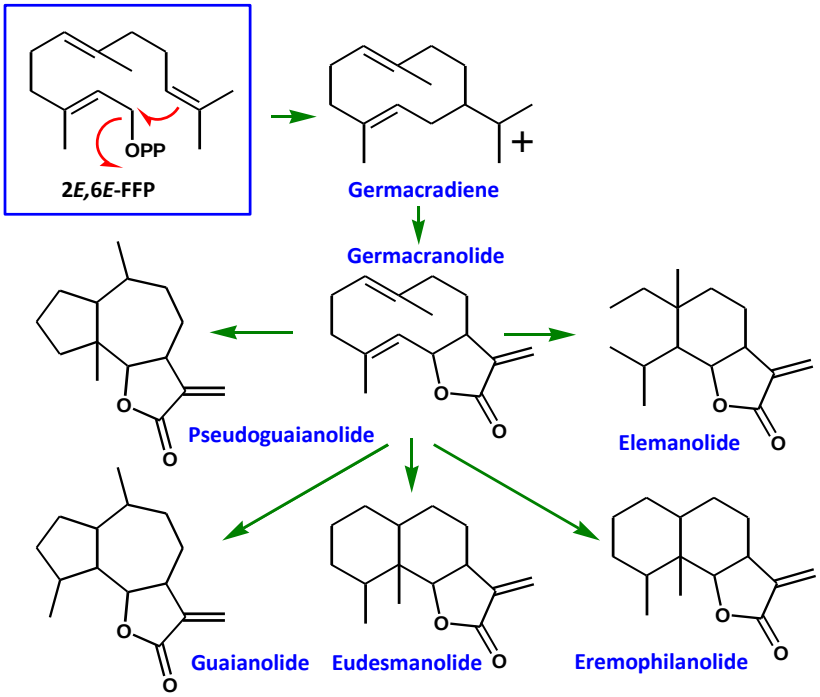
tri- and tetraterpenes contain one tail-to-tail connection in the center.

MONOTERPENES: Many of the compounds belonging to this group are constituents of volatile oils (= essential oils) which are pharmaceuticaly important as means of improving the taste and smell of drugs. Many plant products containing volatile oils of the monoterpene type are used as spices. They are mostly used inperfume industry. Some oxidized monoterpene derivatives (alcohols, aldehydes, ketones, iridoids and secoiridoids) are of medicinal use as rubefacients, sedatives and bitter. Some insecticides like pyrethrins are also monoterpenes.

SESQUITERPENES: These are terpenes containing 15 carbon atoms. There are only a few sesquiterpenes which have medical and pharmaceutical interest.

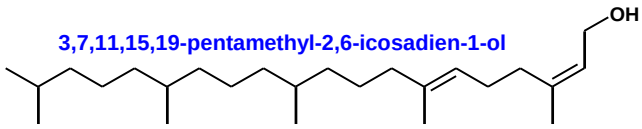
Sesquiterpenoid lactones form a group of substances important by its size. Sesquiterpenoid lactones have a rather scattered botanical distribution. They occur in the **Fungi** and **Bryophytes**, here and there in the Angiosperms (**Apiaceae, Lauraceae, Menispermaceae**), and chiefly in the **Asteraceae**. In the latter, the lactones frequently occur in the glandular trichomes located in the leaves, stems, and inflorescence bracts. The skeleta of sesquiterpenoid lactones vary, but they all arise from the cyclodecadiene-type product of the cyclization of 2*E*,6*E*-farnesyl pyrophosphate.

Although experimental evidence is rare, it is generally accepted that the chief skeletons arise from the cyclization of the **cyclodecadiene**-type cation, *via* the **germacranolides**.



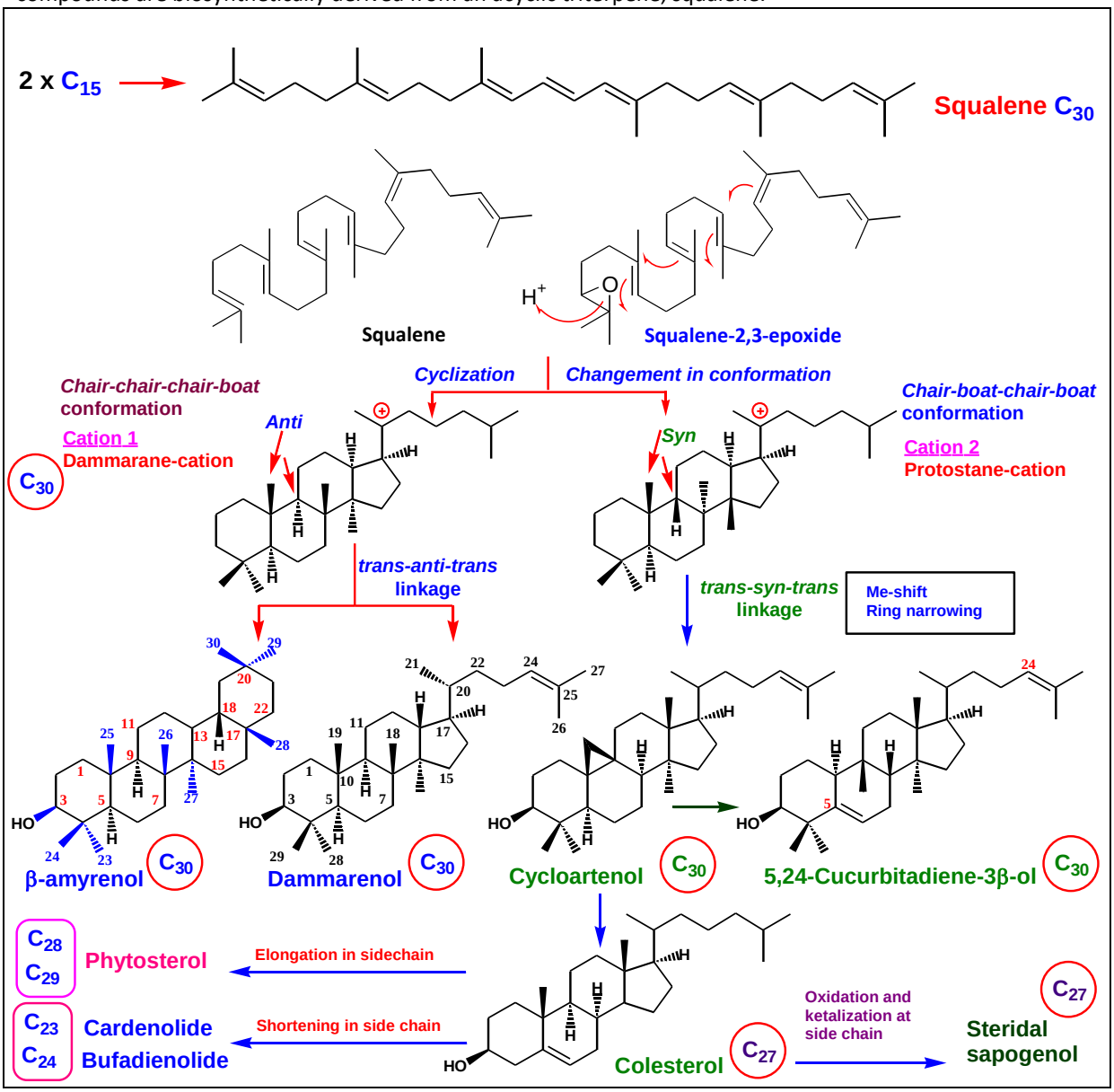
DITERPENES: Many important natural products are diterpenes. The alcohol phytol, which constitutes part of chlorophyll molecule and of the vitamins E and K_1 molecules, is a diterpene with a straight carbon chain. The gibberellins, which are plant growth regulators, and abietic acid, one of the components of colophony, are examples of cyclic diterpenes. Several alkaloids (pseudo alkaloids) contain diterpenoid moieties in their skeletons. Vitamin A is another C20 compound can be regarded as a diterpene, but it is formed by cleavage of tetraterpenes. Forskolin, andromedotoxin (=grayanatoxin I), stevioside, ginkgolides and taxol (= paclitaxel) are diterpenoids which represents pharmacologically active natural products.

SESTERTERPENES: Sesterterpenes rarely occur in higher plants. 3,7,11,15,19-Pentamethylicosa-2,6-dien-1-ol as an example is found in the leaves of potatoes *Solanum tuberosum* (Solanaceae).



About 30 sesterterpenes bridged by furan rings, however, are report occur in various marine sponges. Their structure can also be mono-, bi-, tri-, tetra- and pentacyclic.

TRITERPENES AND STEROIDS: These two groups are treated together because of their biosynthetic origin. There are many triterpenes which are of medicinal interest. Triterpenic and steroidal saponins are the glycosidic compounds. Cycloartenols, qucurbitacins belong to the triterpenes, while phytosterols, cardiac glycosides (cardenolides and bufadienolides) steroidal hormones belong to the steroids. All of these compounds are biosynthetically derived from an acyclic triterpene, squalene.



Biosynthesis of Triterpenes and steroids (R. Hänsel, O. Sticher, 2007)

Figure above shows a brief biosynthetic summary of triterpenes and steroids. They are built up ox six isoprene units and have a common biosynthetic origin in that they are all derived from squalene, presumably via ring opening of squalene-2,3-epoxide (oxidosqualene), followed by a concerted cyclization. While the true triterpenes have 30 carbons, the steroids have only 27 carbons by virtue of the oxidative cleavage of three methyl groups from a C_{30} intermediate. The cyclization of the ***chair-chair-chair-boat*** conformation of squalene-2,3-epoxide yields dammarane-cation (cation 1). Following the rearrangement of the cation 1, either a pentacyclic (e.g. β-amyrenol) or tetracyclic (e.g. dammarenol) is formed. In contrast, cyclization of the ***chair-boat-chair-boat*** conformation of squalene-2,3-epoxide, followed by several rearrengements (protostane-

cation: cation 2), leads to the formation of either cycloartenol or lanosterol (K. Hostettmann&A. Marston, 1995).

Other important classes of secondary metabolites such as phytosterols, cardenolides, cucurbitacins, quassinoids and limonoids are also derived from squalene.

ESSENTIAL OILS (VOLATILE OILS = ESSENCES = HUILE ESSENTIAL)

Essential oils (**EOs**) (Volatile oils = Essences= Huile essential) are products generally of rather complex composition, comprising the volatile principles contained in the plants, and more or less are modified during the preparation process (Bruneton, 1999). To extract officinal volatile principles, there are mainly two methods; steam distillation of oil containing plants or of selected plant parts, and expression. According to the many Pharmacopoeias, EOs are the products obtained from a starting material, either by steam distillation, or by mechanical procedures from the epicarp of *Citrus* fruits, or by simple distillation.

EOs are found as a liquid mixture of compounds including terpenoids (mono- and sesquiterpenes), aromatic compounds (phenylpropanoids, C_6C_3; allyl- and propenylphenols; **anethole**, anisaldehyde, apiole, methylcavicol, **eugenol**, safrole, asarones, cinnamaldehyde), volatile acid and aldehydes , long chain hydrocarbons, compounds arising from terpene degradation, N and S containing heterosides.

Terpen: C,H or C,H,O containing compounds which are accepted to contain two isoprene molecules.

Isoprene: Isopren is molecule which is formulated as C_5H_8 and chemically defined as 2-methyl-1,3-butadien and have not been isolated from the nature.

ISOLATION METHODS :

i. Distillation
ii. Extraction
iii. Mechanical method (Expression)

i. Distillation: Hydrodistillation methods are frequently used as a distillation method.

A. Water distillation
B. Water-Steam distillation
C. Steam distillation

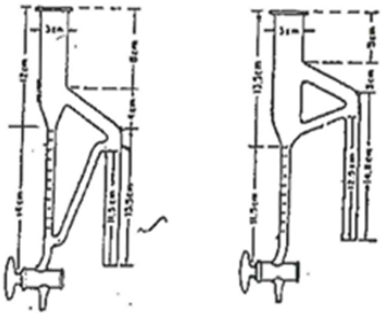
Water distillation is used for the dried and heat resistant (boiling-resistant) compounds containing plant materials. Water-Steam distillation is used for the heat sensitive and deep tissue compounds containing plants. And Steam distillation is used for the fresh and surface-compounds containing plants.

In distillation method, volatile oil is collected in burette of Clevenger equipment in laboratory scale or Florentin Tool in industrial scale. The type of these equipments is decided according to gravity of the volatile oil (heavy from the water or not).

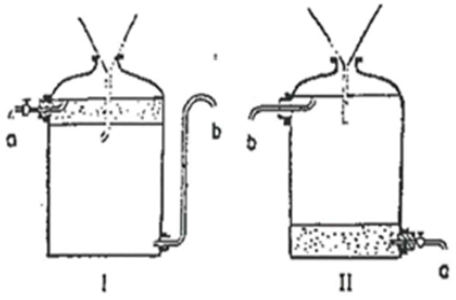
ii. Extraction: Solvent extraction

Most flowers contain too little volatile oil to undergo expression and their chemical components are too delicate and easily denatured by the high heat used in steam distillation. Instead, a solvent such as hexane or supercritical carbon dioxide is used to extract the oils. Extracts from hexane and other hydrophobic solvent are called *concretes*, which is a mixture of essential oil, waxes, resins, and other lipophilic (oil soluble) plant material.

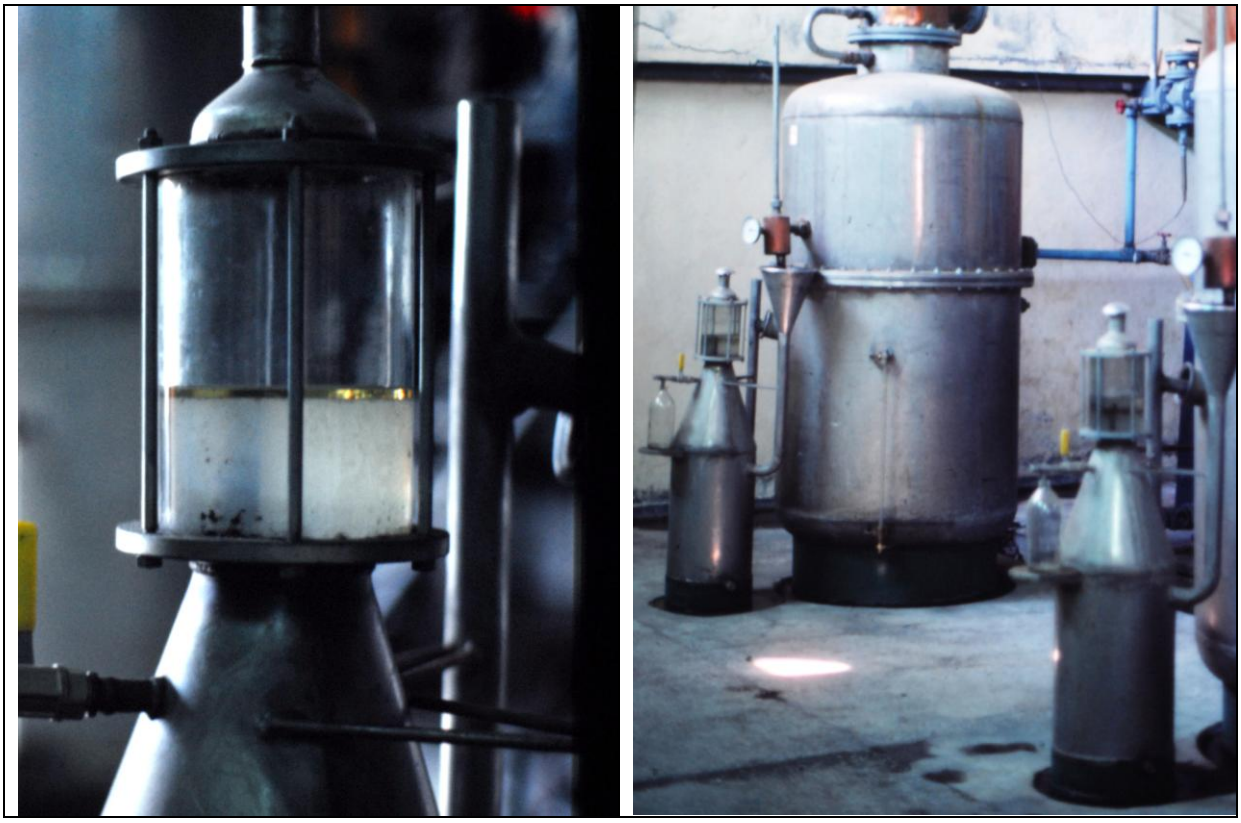
iii. Expression: Most Citrus peel oils are expressed mechanically, or cold pressed. Due to the relatively large quantities of oil in citrus peel and low cost to grow and harvest the raw materials, citrus-fruit oils are cheaper than most other essential oils. Lemon or sweet orange oils that are obtained as by products of the citrus industry are even cheaper



Burette of Clevenger Equipment
1. Light EO from water
2. Heavy EO from water



Florentin Tools (From A.Berk-Baytop)
1. Light from water **2. Heavy from water**
a – Exit of volatile oil
b – Exit of water



Florentin Tools used in Rose Oil production (Isparta). Photo.: Prof. Dr. İ. Çalış

QUANTITATIVE ANALYSIS OF VOLATILE OILS

GRAVIMETRIC METHOD: Included in Turk Codex
VOLUMETRIC METHOD: Included in different Pharmacopeia
MICROSCOBIC METHOD: Special method, usually used for the Labiatae plant.

DIFFERENT ANALYSIS OF VOLATILE OILS

To understand the purity and ingredients of volatile oils some analysis should be performed.

Classical Analysis	**Fractionisation methods:**
Optical rotation	Fractional distillation
Solubility	Derivatisation
Refractive Index etc	

Chromatographic Analysis

TLC	TLC is used for the comparison with the authentic compounds
GC	Adsorban: Silica gel
HPLC	Solvent System: Benzene
	Benzene:$CHCl_3$ (1:1, 3:1)
	Hexane:EtOAc (85:15)
	Benzene:EtOAc (95:5)
	Reagents: Vanilin:H_2SO_4 (1%), Anisaldehit R (1%) or $SbCl_3$
	Phosphomolibdic acid: Terpenes give blue-red or green-grey colors

Two dimensional TLC: This method is used for the separation and determination of compounds which has close Rf value. In this technique, the spotted sample is first developed in one direction; then, after drying, the plate is turned 90^o and developed again, but different solvent may used for the second development.

TAS (Thermomicro Abtrennung nach Stahl): In this method, isolated volatile oil is directly spotted to the TLC plate. 1-25 g plant sample put into the heat resistant glass tool and keep in 200-250 ^oC oven about 30-90 seconds. TLC plate is set to the open edge of the glass tool and periodically moved for the different application spot. After this online application of volatile oil to TLC, plate can be developed in suitable solvent system. This online method gives chance to application to TLC without any degradation of the volatile oil.

a. GC

Gas Chromatography is the partitional chromatography method in which mobile phase is an inert gas and stationary phase is a high boiling liquid.

Application of volatile oil to GC can be done directly or after prefractionation. Generally Flame Ionization Detectors (FID) are used for the detection of the compounds. Structure identification of the determined compounds can be done direct comparison of the compounds with authentic compounds at least two different solvent system.

b. HPLC

This method is also used for the volatile oils although GC is the much frequently used method for the volatile oils. Pressure resistant adsorbents are used for the method and the Silica gel 60 is mostly preferred adsorbent.

Spectral analysis

a. IR
b. Mass Spectrometry
c. NMR

These methods are generally used combination with the Gas Chromatography. Separated compounds with GC is subjected to the spectral analysis to identify their structure. GC/IR and GC/MS are frequently used combination methods.

There are different classical and modern techniques to quantitative analysis volatile oil components.

QUANTITATIVE ANALYSIS METHODS FOR THE VOLATILE OIL COMPONENTS

CLASSICAL METHODS

In this method, volatile oil components grouped according to their structures and quantitative analysis is done on the basis of high percentage structure – Total Alcohol quantitation on the basis of menthol.

In this method, components are isolated from the volatile oils and determined their amount – Sineol quantitation in O. Eucalypti

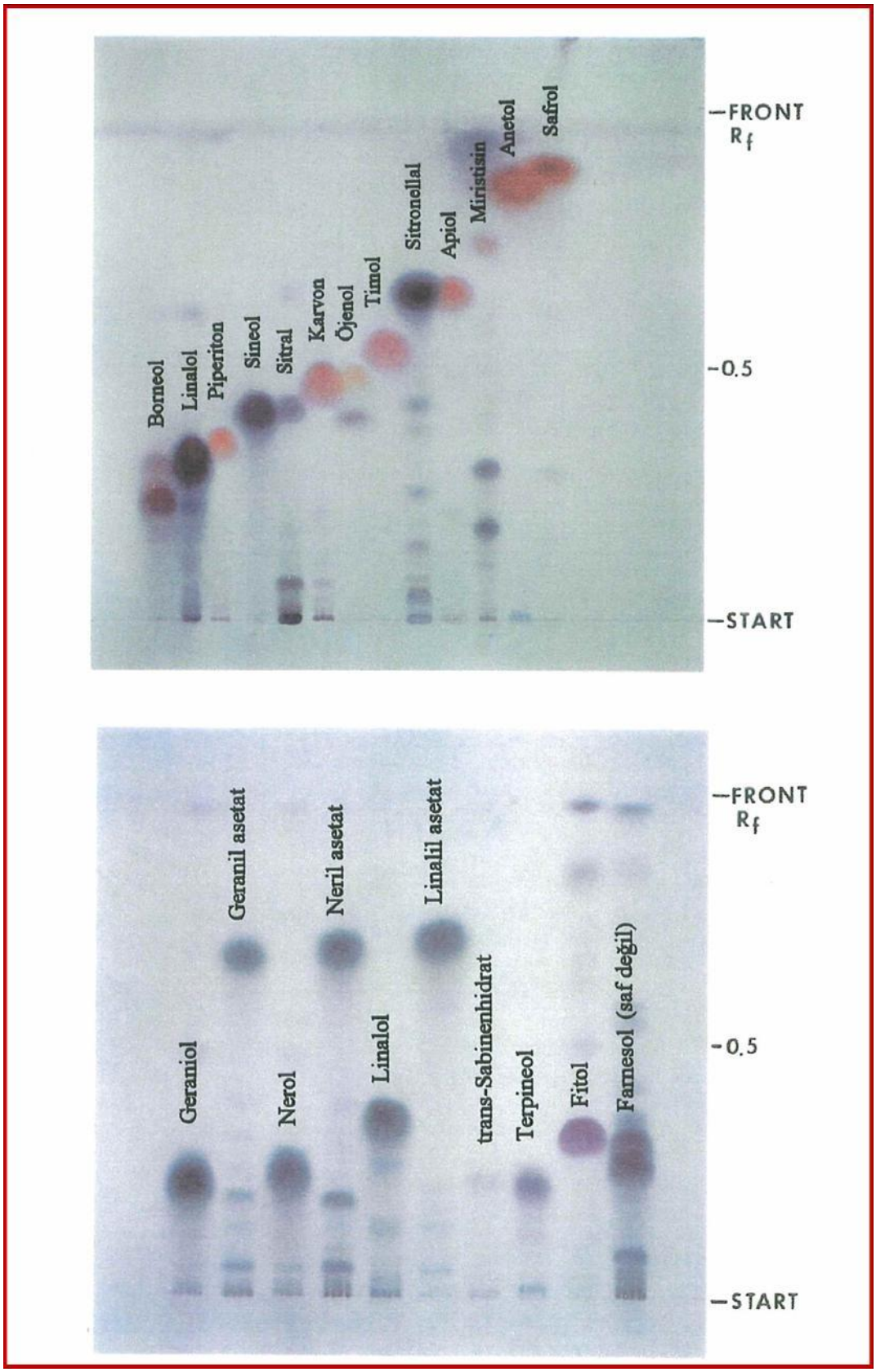
MODERN METHODS

Modern methods mostly include chromatographic methods. Gas Chromatographic methods are the most useful methods among the other chromatographic techniques.

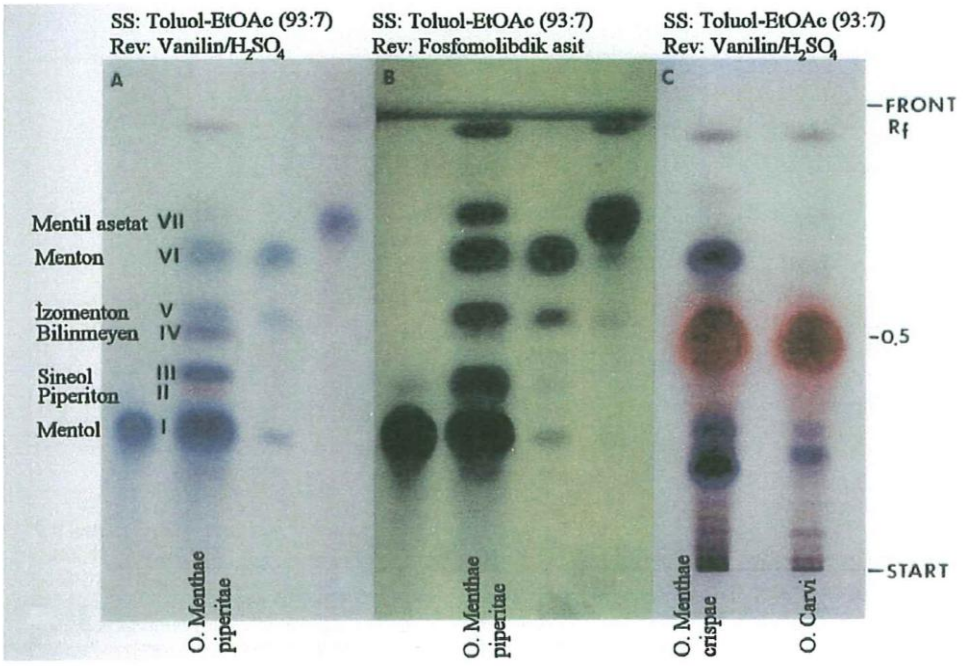
TLC ANALYSIS OF TERPEN AND PHENYLPROPAN STANDART COMPOUNDS

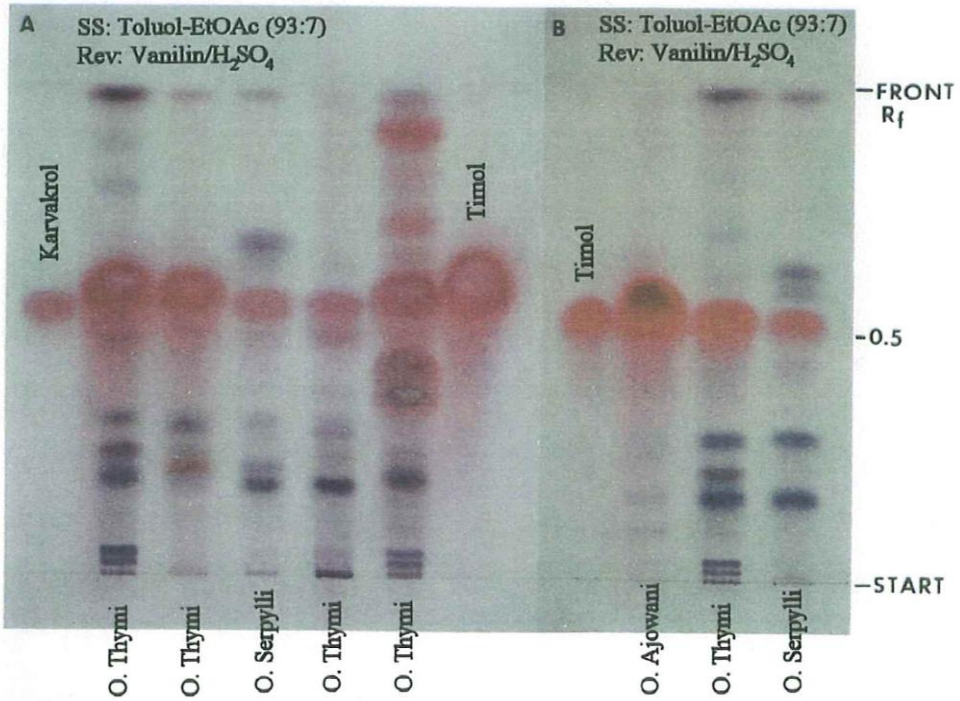
SOLVENT SYSTEM: TOLUEN-ETHYL ACETATE (97:3)

REAGENT (Rev.): VANILIN-H_2SO_4



TLC ANALYSIS OF DIFFERENT VOLATILE OILS





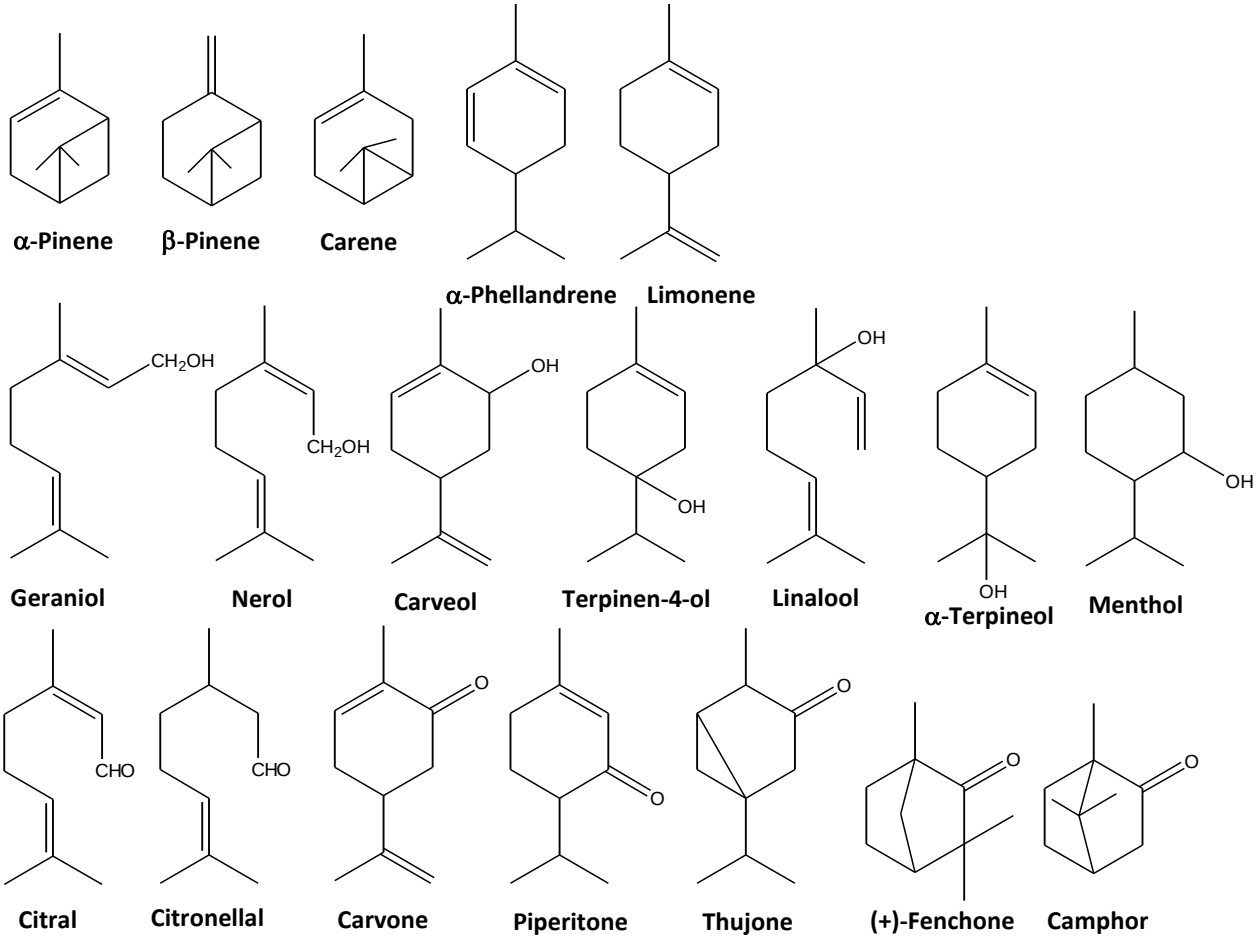
Drugs rich in Essential oils (1/2)

DRUG	*Plant Name*	Family	Main Active Constituents (% in essential oil)
Cinnamomi cortex	***Cinnamomi zeylanicum***	**Lauraceae**	Cinnamoylaldehyde (65-75%), eugenol (4-10%)
Sassafras lignum	***Sassafras albidum***		Safrol (80%), eugenol
Calami Rhizoma	***Acorus calamus***	**Araceae**	α, β, γ-Asarones (50-60%)
Anisi fructus	***Pimpinella anisum***	**Apiaceae**	Anethol (80-90%), methylcavicol, anisealdehyde
Anisi stellati fructus	***Illicium verum***		Anethol (85-90%), safrol, terpineol, phellandren
Foeniculi fructus	***Foeniculum vulgare***		Anethol (50-60%), safrol, methylcavicol, anisealdehyde
Petroselini fructus	***Petroselinum crispum***		Apiol, Myristicin, Allyltetramethoxy-benzol
Carvi fructus	***Carum carvi***		Carvon (50-85%), carveol
Coriandri fructus	***Coriandrum sativum***		Linalool (50-70%), geraniol, borneol, citronellol
Ajowani fructus	***Trachyspermum ammi***		Thymol (35-60%)
Caryophylli flos	***Syzygium aromaticum***	**Myrtaceae**	Eugenol (72-90%), β-caryophyllen (3-12%)
Eucalypti folium	***Eucalyptus globulus***		1,8-cineol (= eucalyptol; 70%),
Myristicae semen	***Myristica fragrans***	**Myristicaceae**	Myristicin (8%), safrol, eugenol, elemicin
Cardomomi fructus	***Elatteria cardamomum***	**Zingiberaceae**	α-Terpinylacetate, 1,8-cineol (50%),
Curcumae rhizomae	***Curcuma xanthorrhiza***		Xanthorrhizol
Matricariae flos	***Chamomilla recutita***	**Asteraceae**	Chamazulen (0-15%), bisabolol (10-25%), bisabololoxids A and B
Cinae flos	***Artemisia cina***		1,8-cineol (80%), α-terpineol, carvacrol
Juniperi fructus	***Juniperus communis***	**Cupressaceae**	Terpinen-4-ol, caryophyllen, camphor
Pinus oleum	***Pinus mugo*** ***Pinus sylvestris***	**Pinaceae**	Bornylacetate, α- and β-phellandrene, α- and β-pinene
Terebinthinae oleum	***Pinus* species**		α- and β-pinene, limonene, phellandrene
Aurantii pericarpium	***Citrus aurantium ssp. amara***	**Rutaceae**	(+)-Limonene
Auranthii flos			Linalylacetate (8-25%), linalool (30%)
Citri pericarpium	***Citrus limon***		(+)-Limonene (90%), citral (3-5%)
Citri pericarpium	***Citrus aurantium ssp. bergamot***		Linalylacetate

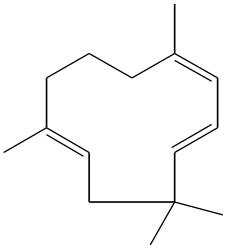
Drugs rich in Essential oils (2/2)

DRUG	*Plant Name*	Family	Main Active Constituents (% in essential oil)
Basilici herba	***Ocimum basilicum***	**Lamiaceae**	Methylcavicol (55%), linalool
Rosmarini folium	***Rosmarinus***	**Lamiaceae**	1,8-cineol (15-30%), Borneol (10-20%)
Lavandulae flos	***Lavandula angustifolia, Lavandula latifolia***	**Lamiacaeae**	Linalyl acetate (30-50%), Linalol (10-15%)
Menthae piperitae folium	***Mentha piperita Mentha pulegium***	**Lamiacaea**	Menthol (50-75%), menthone (10-30%)
Salvia folium	***Salvia officinalis*** *S. officinalis ssp.lavandulifolia**	**Lamiacaea**	Thujone (35-50%), Cineol (14%) *Cineol (30)
Salvia trilobae folium	***Salvia triloba***	**Lamiacaea**	Cineol (60-70%), Thujone (5%)
Thymi herba	***Thymus vulgaris Thymus zygis***	**Lamiacaea**	Thymol and carvacrol (20-60%)
Serpylli herba	***Thymus serpyllum***	**Lamiacae**	Thymol and carvacrol, p-cymol, linalool
Melissa folium	***Melissa officinalis***	**Lamiacaea**	Citronellol (40%), citral (30%)

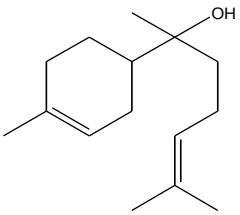
Some non-oxygenated and oxygenated monoterpenes found in the compositon of essential oils.



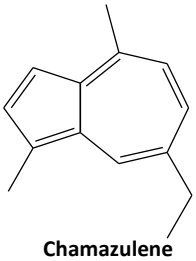
Some sesqui-terpenes found in the compositon of essential oils



α-Caryophyllene

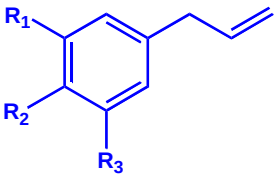


(-)-α-Bisabolol

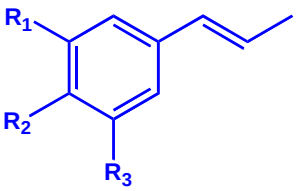


Chamazulene

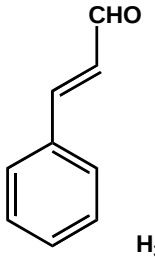
Allyl- and Propenylbenzol (Phenylpropane) Derivatives found in the compositon of essential oils.



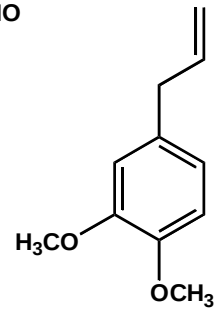
	R_1	R_2	R_3
Myristicin	O – CH_2 - O		OMe
Eugenol	OMe	OH	H
Methyleugenol	OMe	OMe	H
Elemicin	OMe	OMe	OMe



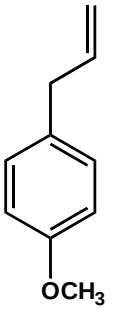
	R_1	R_2	R_3
Isomyristicin	O – CH_2 - O		OMe
Isoeugenol	OMe	OH	H
Methylisoeugenol	OMe	OMe	H
Isoelemicin	Ome	OMe	OMe



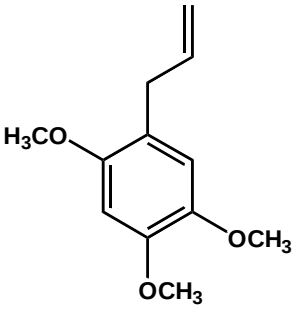
Cinnamic aldehyde



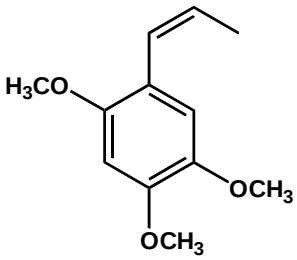
Methyleugenol



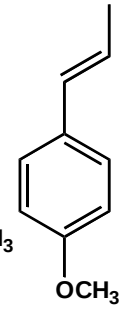
Estragole (=Methylchavicol)



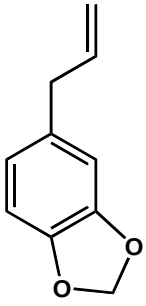
α-Asarone



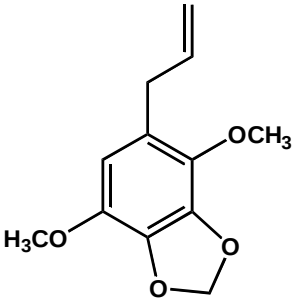
β-Asarone



Trans-Anethole



Safrole

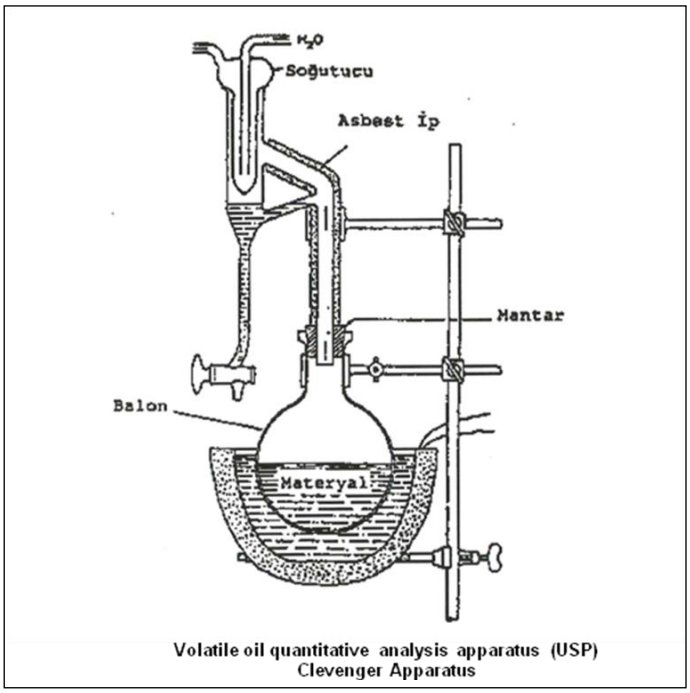


Apiole

1.1. Quantitative Analysis of Volatile oils (Volumetric) (Thymi Oleum, Kekik) (EU 6)

About 50 g of absolute-measured sample is filled to the boiling flask of Clevenger apparatus. Then distilled water is added to the boiling flask and heated. Water vapor and the volatile oil dragged with water vapor are condensed on the cooler and collected in the apparatus' burette. The quantity of volatile oil is read from the burette as ml and recorded. The result should be given as v/w (ml/g).

The acquired volatile oil is transferred to a little Erlenmeyer and dried with anhydrous sodium sulphate (Na_2SO_4). To read the oil output as w/w, the density of volatile oil is determined. For determination of the volatile oil you can use 10 ml or 25 ml specific-gravity bottles (pycnometers)



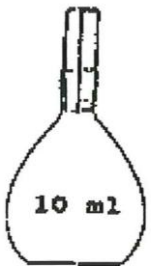
Volatile oil quantitative analysis apparatus (USP)
Clevenger Apparatus

ASSAY PROCESS: 100 g of sample is filled to the boiling flask of Clevenger apparatus, and then 1 liter of distilled water is added. The boiling flask is heated on the gas burner by shaking frequently. Water vapor and the volatile oil dragged with water vapor are condensed on the cooler and collected in the apparatus' burette. The loss of water returns to the boiling flask through curved pipe. The quantity of volatile oil is read from the burette as ml and recorded.

The tap of the apparatus is opened and water from burette is taken till volatile oil level. Then the volatile oil is transferred to a little Erlenmeyer and dried with a tip of a spatula anhydrous sodium sulphate (Na_2SO_4). The clarified volatile oil is transferred to a dry little erlenmeyer and its density is calculated.

10 ml or 25 ml volumed specific-gravity bottle (pycnometer) is filled with water using burette and water volume of pycnometer can be found. Pycnometer's cap is closed properly; its outside is dried and weighed absolutely (m_3). Then 2 ml less than the first volume of water is put to the empty pycnometer and weighed absolutely again (m_1). Then the volatile oil is added carefully to 2 ml water

less picnometer. Pycnometer's cap is closed properly; its outside is dried and weighed absolutely (m_2). The volatile oil's density is calculated with these three weightings.



Pycnometer

$$d = m_2-m_1/m_3-m_1$$

After calculating the volatile oil's density, % quantity of volatile oil can be found as below.

In P g	sample	M g volatile oil
In 100 g	sample	X

X= M. 100/P

Volumetric methods give results within a short time and easier than gravimetric methods. Therefore new pharmacopeias accept the quantitative analysis method of volatile oil with volumetric method.

Ref. **EUROPEAN PHARMACOPOEIA 6.0, Vol. 1, 01/2008, p.251**

DETERMINATION OF ESSENTIAL OILS IN HERBAL DRUGS

The determination of essential oils in herbal drugs is carried out by steam distillation in a special apparatus in the conditions described below. The distillate is collected in the graduated tube, using xylene to take up the essential oil; the aqueous phase is automatically returned to the distillation flask.

Apparatus. The apparatus comprises the following parts :

(a) a suitable round-bottomed flask with a short, ground-glass neck having an internal diameter of about 29 mm at the wide end;

(b) a condenser assembly (see **Figure 1.1**) that closely fits the flask, the different parts being fused into one piece; the glass used has a low coefficient of expansion:

- the stopper **K'** *is* vented and the tube **K** has an orifice of diameter about 1 mm that coincides with the vent; the wide end of the tube **K** is of ground-glass and has an internal diameter of 10 mm;
- a pear-shaped swelling, **J**, of 3 ml capacity;
- the tube **JL** is graduated in 0.01 ml;
- the bulb-shaped swelling **L** has a capacity of about 2 ml;
- **M** is a three-way tap ;
- the junction **B** is at a level 20 mm higher than the uppermost graduation;

(c) a suitable heating device, allowing a fine control;

(d) a vertical support with a horizontal ring covered with insulating material.

(e) ***Method****.* Use a thoroughly cleaned apparatus. Carry out the assay according to the nature of the drug to be examined. Place the prescribed volume of distillation liquid in the flask, add a few pièces of porous porcelain and attach the condenser assembly. Introduce *water R* through the filling funnel **N** until it is at the level **B***.* Remove the stopper **K'** and introduce the prescribed quantity of *xylene R,* using a pipette with its tip at the bottom of the tube **K***.* Replace the stopper **K'** and ensure that the orifice coincides with the vent. Heat the liquid in the flask to boiling and adjust the distillation rate to 2-3 ml/min, unless otherwise prescribed.

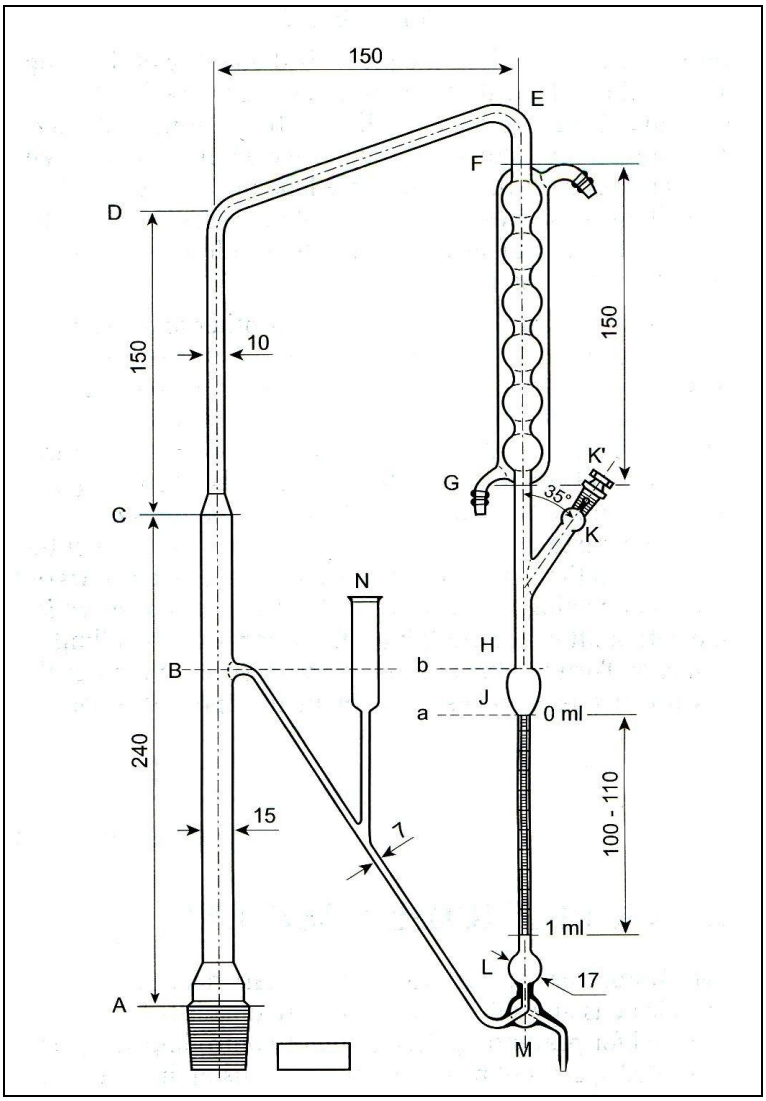
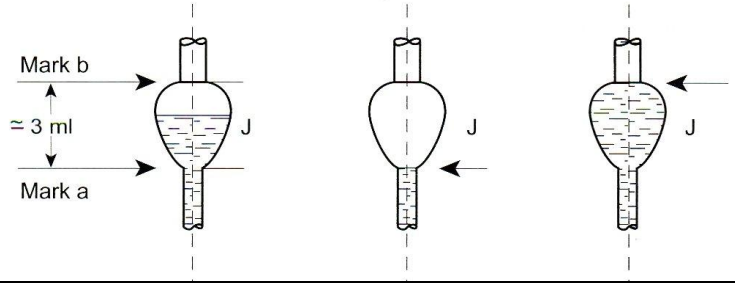


Fig. 1.1. Condenser: Apparatus for the determination of essential oils in herbal drugs (EU 6).
Dimensions in millimeters

Fig. 1.2	

To determine the rate of distillation, during distillation lower the level of the water by means of the three-way tap until the meniscus is at the level of the lower mark (a) (see **Figure 1.2**). Close the tap and measure the time taken for the liquid to reach the upper mark (b). Open the tap and continue the distillation, modifying the heat to regulate the distillation rate. Distil for 30 min. Stop the heating and after at least 10 min read off the volume of xylene in the graduated tube.

Introduce into the flask the prescribed quantity of the drug and continue the distillation as described above for the time and at the rate prescribed. Stop the heating and after 10 min read the volume of liquid collected in the graduated tube and subtract the volume of xylene previously noted. The difference represents the quantity of essential oil in the mass of the drug taken. Calculate the result as millilitres per kilogram of drug.

When the essential oil is to be used for other analytical purposes, the water-free mixture of xylene and essential may be recovered as follows: remove the stopper **K'** and introduce 0.1 ml of a 1 g/l solution of *sodium fluoresceinate R* and 0.5 ml of *water R.* Lower the mixture of xylene and essential oil into the bulb-shaped swelling **L** by means of the three-way tap, allow to stand for 5 min and lower the mixture slowly until it just reaches the level of the tap **M**. Open the tap anti-clockwise so that the water flows out of the connecting tube **BM**. Wash the tube with *acetone R* and with a little *toluene R* introduced through the filling funnel **N**. Turn the tap anti-clockwise in order to recover the mixture of xylene and essential oil in an appropriate flask.

QUESTIONS

1. What is density? Explain.
2. What is volatile oil?
3. Which method is used in TK (Turkish codex) for quantitative analysis of volatile oil?
4. What is refraction index?
5. Draw the head part of apparatus used for volatile oils that are heavy from water.
6. What is the reason of putting 2 ml less water to the pycnometer in the density detection?
7. What are the other methods for measuring density of liquids?

1.2. Pharmacopeia Analysis of Oleum Thymi (Thymi aetheroleum)

Ref. **EUROPEAN PHARMACOPOEIA 6.0, Vol. 1, 01/2008, p.31**

THYME OIL

Thymi aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the fresh flowering aerial parts of *Thymus vulgaris* L., *T. zygis* Loefl. ex L. or a mixture of both species.

CHARACTERS

Appearance: clear, yellow or very dark reddish-brown, mobile liquid with a characteristic, aromatic, spicy odour, reminiscent of thymol.

Solubility: miscible with ethanol and with light petroleum.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography *(2.2.27).*

Test solution. Dissolve 0.2 g of the substance to be examined in *pentane R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 0.15 g of *thymol R,* 25 mg of *α-terpineol R,* 40 pi of *linalol R* and 10 *µl* of *carvacrol R* in *pentane R* and dilute to 10 ml with the same solvent.

Plate*: TLC silica gel plate R.*

Mobile phase*: ethyl acetate R, toluene R* (5:95 *V/V).* ***Application****:* 20 *µl,* as bands.

Development*:* over a path of 15 cm. *Drying:* in air.

Detection*:* spray with *anisaldehyde solution R* and heat the plate at 100-105 °C for 5-10 min while observing. Examine in daylight.

Results*:* see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other bands may be present in the chromatogram obtained with the test solution.

Top of the plate	
Thymol : a brownish-pink zone Carvacrol: a pale violet zone Linalol: a violet zone a-Terpineol : a violet zone	A large violet zone (hydrocarbons) (at the solvent front) A brownish-pink zone (thymol) A pale violet zone (carvacrol) A violet zone (linalol) A violet zone (a-terpineol)
Reference solution	**Test solution**

B. Examine the chromatograms obtained in the test for chromatographic profile.
Results : the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density *(2.2.5):* 0.915 to 0.935.
Refractive index *(2.2.6):* 1.490 to 1.505.

Chromatographic profile

Gas chromatography *(2.2.28) :* use the normalisation procedure.

Test solution. The substance to be examined.
Reference solution. Dissolve 0.15 g of *β-myrcene R*, 0.1 g of *γ-terpinene R,* 0.1 g of *p-cymene R,* 0.1 g of *linalol R,* 0.2 g of *terpinen-4-ol R,* 0.2 g of *thymol R* and 50 mg of *carvacrol R* in 5 ml of *hexane R.*

Column*:*
— ***material****:* fused silica,
— *size: l =* 30 m (a film thickness of 1 μm may be used) to 60 m (a film thickness of 0.2 μm may be used), Ø = 0.25-0.53 mm,
— ***stationary phase*** *: macrogol 20 000 R. Carrier gas: helium for chromatography R. Split ratio:* 1:100.

Temperature:

	Time (min)	*Temperature °C*
Column	0-15 15 - 55	60 60->180
Injection port		200
Detector		220

Detection*:* flame ionisation. *Injection:* 0.2
Elution order*:* order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability*:* reference solution:
— *resolution:* minimum 1.5 between the peaks due to thymol and carvacrol,
— *number of theoretical plates:* minimum 30 000, calculated for the peak due to p-cymene at 80 °C.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution. Disregard the peak due to hexane.
Determine the percentage content of these components. The limits are within the following ranges:
— *P-myrcene:* 1.0 per cent to 3.0 per cent,
— *y-terpinene:* 5.0 per cent to 10.0 per cent,
— *p-cymene:* 15.0 per cent to 28.0 per cent,
— *linalol :* 4.0 per cent to 6.5 per cent,
— *terpinen-4-ol:* 0.2 per cent to 2.5 per cent,
— *thymol:* 36.0 per cent to 55.0 per cent,
— *carvacrol:* 1.0 per cent to 4.0 per cent.

STORAGE
In a well-filled, airtight container, protected from light, at a temperature not exceeding 25 °C.

1.3. Quantitative analysis of Thymol (TK 1940)

Ref. **Türk Kodeksi, 1940**

This assay is performed on the basis of water solubility of phenol's alkaline salts.

Special glass flask called CASSIA flask is used for the determination of phenolic compounds. Cassia flask is 100 – 150 ml volume, neck part is 0-6 or 0-10 ml graduation.

Method

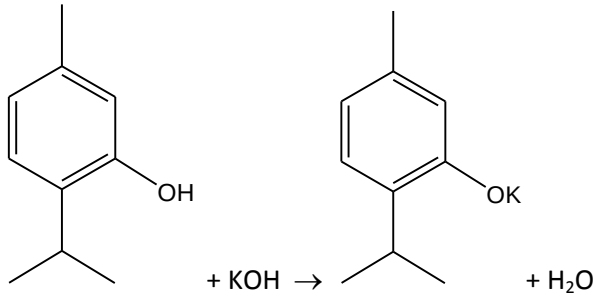
5 ml volatile oil is transferred to the Cassia flask. Solution which contains 35 ml KOH (sud lesivi) + 70 ml water, is added to the volatile oil slowly and mixed. Complete solubility of the volatile oil in the solution should be achieved during this mixing. Solution is added until the top level of the flask and keep for a while. At the end of the experiment insoluble part is read from the neck of the Casia flask (A). When the insoluble volume (A) is subtracted from the volatile oil volume at the beginning of the experiment, this differential volume Gives phenolate formed part of the oil, and the percentage of the phenolic compounds can be calculated as follows:

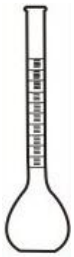
Sample:

A: 4 mL

5 mL – 4 mL = 1 mL (Phenolate formed part of the volatile oil)

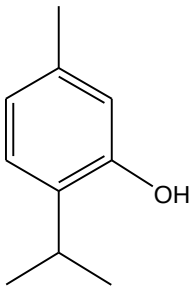
In 5 mL volatile oil 1 mL Phenolic compounds
In 100 mL volatile oil X mL Phenolic compounds
X= % 20 phenolic compounds

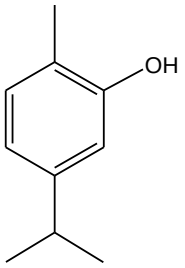




Timol **Fenolat**

Thymus vulgaris is not naturally grows in Turkey. Volatile oils sold by the name of thyme oil are produced from the different *Origanum* species. It is found that Carvacrol content of these oils is more than thymol content.





Thymol (Crystalline) **Carvacrol (Liquide)**

Quantitative analysis of thymol can be done colorimetrically, spectrophotometrically, bromometrically and using Gas Chromatography.

This method is used for the phenolic compounds determination in Caryophylli Oil, Origani Oil, Majoranae Oil. However it should be noted that acids present in these oil can be made salts and these salts can also dissolve in water. This can cause a wrong result for the phenolic contets of the volatile oils.

Thymol Determination on Thymi Oil by TLC

Method: Thin Layer Chromatography
Adsorbent: Silica gel
Solvent system: Petroleum ether : Ethyl Acetate (85:15)
Sample solution : % 4 Thymi Oil in alcohol
Revelatory system: a) Phosphomolibdic acid Reagent
b) Anisaldehyde / H_2SO_4
c) Vanilin / H_2SO_4
d) Antimon III (V) chloride

Questions:

1. Give examples of volatile oils containing phenolic compounds. Is it possible to calculate total phenolic content with the same method?
2. Give the chemical formula of Thymol and Carvacrol and indicate the difference between these two compounds.
3. What kind of compounds is specifically react with phosphomolibdic acid reactive ?
4. What kind of reactives can be used for the determination of thymol and carvacrol ?
5. Compare the registered Thymi Oil and ordinary Thymi Oil which obtained in Turkey and give their chemical contents.

2.1. Water Determination (EU 6)

Ref. **EUROPEAN PHARMACOPOEIA 6.0, Vol. 1, 01/2008, p.31**

DETERMINATION OFWATER BY DISTILLATION

The apparatus (see Figure 2) consists of a glass flask ***(A)*** connected by a tube ***(D)*** to a cylindrical tube ***(B)*** fitted with a graduated receiving tube ***(E)*** and reflux condenser (**C**). The receiving tube ***(E)*** is graduated in 0.1 ml. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the Connecting tube may be insulated.

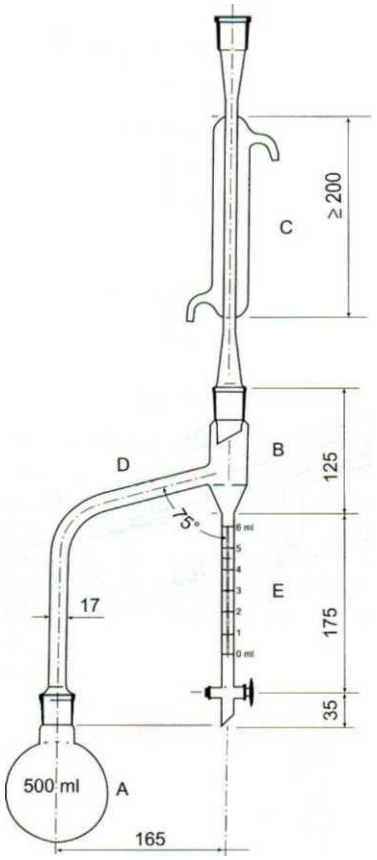


Figure 2. ***Apparatus for the determination of water by distillation***
Dimensions in millimetres

Method. Clean the receiving tube and the condenser of the apparatus, thoroughly rinse with water, and dry.

Introduce 200 ml of *toluene R* and about 2 ml of *water R* into the dry flask. Distil for 2 h, then allow to cool for about 30 min and read the water volume to the nearest 0.05 ml. Place in the flask a quantity of the substance, weighed with an accuracy of 1 per cent, expected to give about 2 ml to 3 ml of water. If the substance has a pasty consistency, weigh it in a boat of metal foil. Add a few pieces of porous material and heat the flask gently for 15 min. When the toluene begins to boil, distil at the rate of about two drops per second until most of the water has distilled over, then increase the rate of distillation to about four drops per second. When the water has all distilled over, rinse the inside of the condenser tube with *toluene R.* Continue the distillation for 5 min, remove the heat, allow the receiving tube to cool to room temperature and dislodge any droplets of water which adhere to the walls of the receiving tube. When the water and toluene have completely separated, read the volume of water and calculate the content present in the substance as millilitre per kilogram, using the formula:

$$\frac{1000\,(n_2 - n_1)}{m}$$

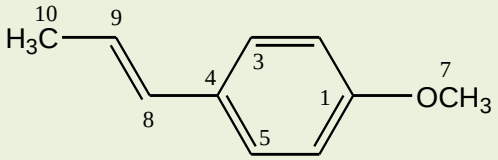
m = the mass in grams of the substance to be examined,

$\boldsymbol{n_1}$ = the number of millilitres of water obtained in the first distillation,

$\boldsymbol{n_2}$ = the total number of millilitres of water obtained in the 2 distillations.

2.2. Anethol isolation from the Oleum Foeniculi
2.2.1. Crystallization
2.2.2. Column Chromatography

Ref. S. Berger & D. Sicker, **Classics in Spectroscopy –** Isolation and Structure Elucidation of Natural Products**,** WILEY-VCH Verlag GmBH & Co. KGaA, Weinheim, 2009



Anethole

*1-Methoxy-4-[(1*E*)-propenyl]benzene*

$C_{10}H_{12}O$, Mol. Wt. 148.20

Colourless crystals, mp 22 °C
Above mp: colourless liquid, bp 234 °C

From anise
Pimpinella anisum L. (Apiaceae) contained as extract in the spirit Rakı and Ouzo

Introduction

Anethole (trans-anethole) is an unsatured aromatic ether with a very aromatic taste occurring in several plants such as anise, star anise and fennel. The correct constitution of anethole was described as early as 1877. However, the very similar taste of licorice, appreciated by many people, is not due to anethole but to another natural sweetener, glycyrrhizic acid, a triterpenoid saponine glycoside, which is 50 times sweeter than sugar. Compounds such as anethole, due to their aromatic flavour, are responsible for the denomination of a class of certain carbocycles as aromatic compounds. Anethole is not only aromatic in its flavour but has a distinctly sweet taste in addition. Dilution experiments showed it to be 13 times sweeter than sugar. For these two reasons, the perception of anethole is a pleasant one on the tongue. Only in large quantities, which are clearly above those taken up by food or beverages, is it slightly toxic (spasmolytic) and irritant.

Anise (or aniseed) is an annual herbaceous plant which grows up to 1 m tall. The small, white flowers are arranged in white umbels and finally produce 3-5 mm long seeds. They arc often used in south and east Asian cooking. The British confectionary *aniseed balls* contain it as an ingredient, and also the Italian cookies *pizelles.* In India, chewing aniseeds is a standard behaviour after a meal, regarded as acting as a mouth freshener and digestive. Aniseed essential oil can be used in aromatherapy against colds and influenza. In former times, the essential oil was even used to treat scabies and lice.

A tasteful compound like this was, of course, not ignored by liqueur manufacturers. Accordingly, their list is long, including e.g. Absinthe (Thujones), Pastis, Sambuca, Rakı, Arak, Mastika and *Ouzo.* Rakı and Ouzo initiates the formation of an oil-in-water emulsion which is opalescent due to the Tyndall effect of the colloid particles (called the louche effect in France). If the Rakı/Ouzo is actually served from a deep freezer, you will find that it contains tiny colourless crystals - these are composed of pure (E)-anethole.

it is of interest that anethole belongs to the groups of chemopreventive agents, i.e. to phytochemicals derived from fruits and vegetables (including also curcumin, eugenol and limonene), which have the ability to suppress the formation of cancer by interfering with several cell-signalling pathways - a matter which is under detailed investigation.

ISOLATION

Based on an idea which arose in one of the authors' minds in a restaurant with a glass of deeply cooled Rakı in hand.

PRINCIPLE

Anethole is a rather nonpolar compound. The solubility of the anethole contained in the Rakı is in a range in which the compound crystallizes out in the deep cold whereas it is still soluble in the beverage at room temperature. This effect can easily be observed where it is served. When Rakı is served as a well-chilled aperitif it appears cloudy due to precipitated anethole crystals. On standing and warming, the cloudiness disappears by dissolution of anethole in the aqueous ethanol. There is no other flavour component in Rakı/Ouzo which undergoes this change. Therefore, it can be used for a simple selective separation of anethole just by filtration of the cold liqueur. However, it should be kept in mind that anethole has a rather low melting point of 22 °C. This has to be taken into consideration during any separation operations. All equipment used for filtration has to be precooled to avoid loss of anethole by liquefaction.

It is not to recommend to cool the liqueur still more than described here because then water ice also begins to crystallize. If you cool several brands of Rakı/Ouzo you will find that the degree of crystallization of anethole is different, which gives a hint about its varying content in the liqueur.

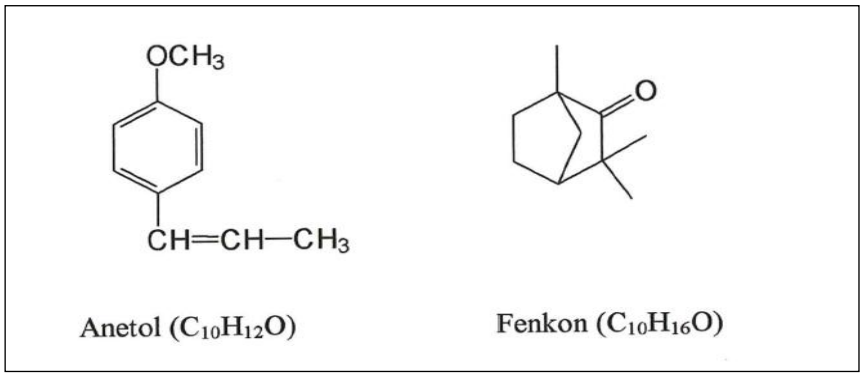
2.2.1. METHOD 1: Crystallization

A 500 mL volume of Rakı/Ouzo is allowed to cool in a deep freezer at -20 °C overnight. The viscosity of the solution increases. Anethole crystallizes in the form of colourless leaflets. A sintered glass filter funnel is precooled in the same freezer and used for the filtration operation. The Ouzo is filtered by suction, which requires 30 min because the glass filter easily tends to become blocked by the anethole crystals. To avoid this, it is to recommended to scrape off the material from the filter surface occasionally by means of a pre-cooled spatula. During filtration, the temperature at the funnel should not rise above -12 °C. Finally, a colourless crystalline mass (300 mg) is scraped out of

the sintered glass filter funnel, put into a glass vial and immediately evacuated with an oil pump at 20 Pa and 15 °C to remove traces of water and ethanol. Colourless crystals of pure anethole (150 mg) remain in the vial, which, depending on the storage temperature, can be kept as a solid or a liquid.

2.2.2. ANETHOL ISOLATION FROM OLEUM FOENICULI BY COLUMN CHROMATOGRAPHY

Oleum Foeniculi is volatile oil , obtained by steam distillation of powdered fructus parts of *Foeniculum vulgare*. It contains 50-60 % anethol and 10-20 % fenkon.



Column Chromatography:

Column:	cooled, 2 x 50 cm
Adsorbent (Stationary Phase):	Silica gel G, 0.063-0.2 mm (50g)
Solvent System (Eluent):	petroleum ether – chloroform (75:25) 100 ml petroleum ether – chloroform (50:50) 50 ml petroleum ether – chloroform (25:75) 50 ml chloroform 100 ml
Application:	0.5 g solution of O. Foeniculi in petroleum ether – chloroform (75:25)
Flow Rate:	2-3 drop/s
Fraction Volume:	~ 10 ml

<u>Control of Fractions by TLC</u> :

Adsorbent	: Silica gel F_{254}
Solvent System	: petroleum ether – chloroform (50:50)
Sample solution	: O.Foeniculi solution in petroleum ether
Standart solution	: Anethol solution in petroleum ether
Revelation	: Vanillin / H_2SO_4

Process of Assay:

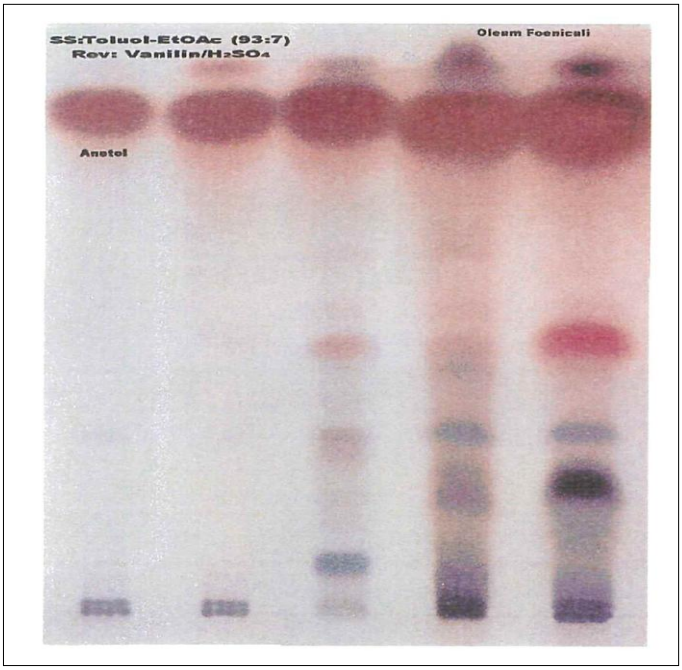
Cooled chromatography column fixed by clamps and water circulation is set up for cooling. A part of cotton is located to the bottom of the column. 100 ml petroleum ether – chloroform (75:25) solvent system is prepared and 50 g silica gel is dissolved in this solvent system, mixed carefully until it become a homogeny mixture and then it is poured into the column. Mobile phase (solvent system)

is passed through the column until the upper level of the adsorbent. 0.5 – 1 ml solution of 0.5 g O.Foeniculi in petroleum ether – chloroform (75:25) is applied on the adsorbent with pasteur pipette. Solvent is started; so sample solution is absorbed on stationary phase (adsorbent) thoroughly. Tap of the column is closed with cotton or glass wool to protect the shape of the upper layer of adsorbent from the velocity of newly added solvent. At this time; 30 test tubes are placed under the column and numerated to collect fractions flowing from the column. Elution is started with 100 ml petroleum ether – chloroform (75:25). Flow rate must be 2-3 drop/s and fraction volume is nearly 10 ml. Fractions , eluted from the column , are controlled using thin layer chromatography (TLC). Elution is continued with in order of solvent systems that indicated above.

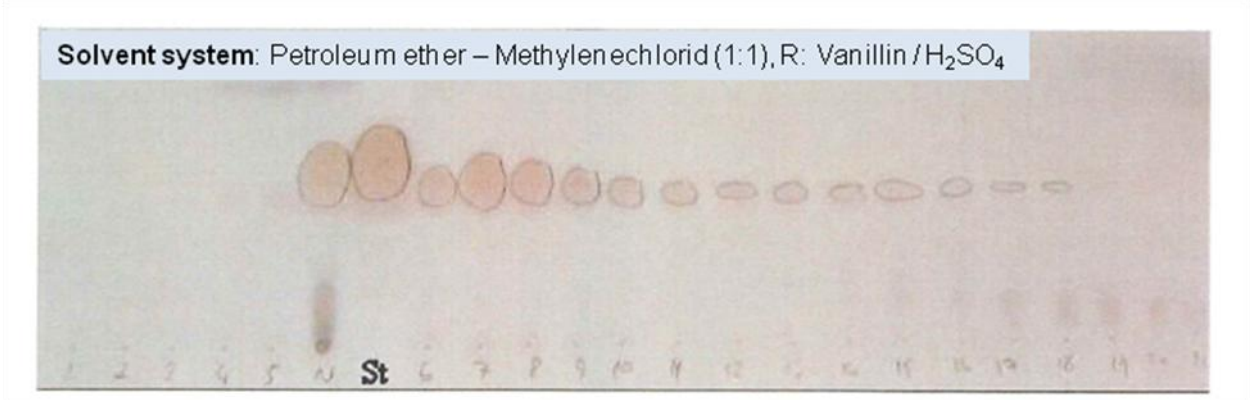
Anethol Identification and Purity Control:

1. **Chromatographic Analysis**
   - TLC, GLC, HPLC
2. **Spectral Analysis**
   - UV, IR, 1D and 2D NMR

ANETHOL IDENTIFICATION IN OLEUM FOENICULI USING TLC ANETHOL

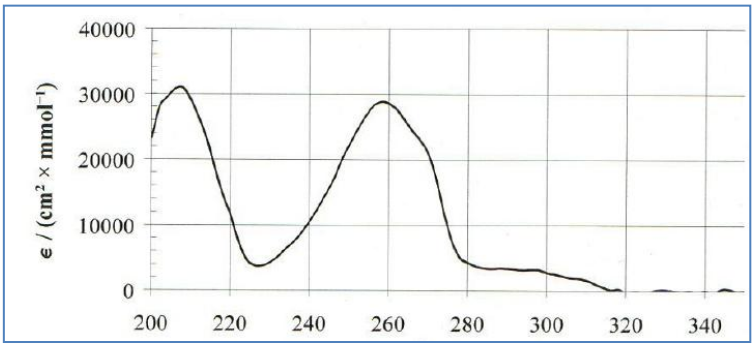


ISOLATION FROM OLEUM FOENICULI BY COLUMN CHROMATOGRAPHY



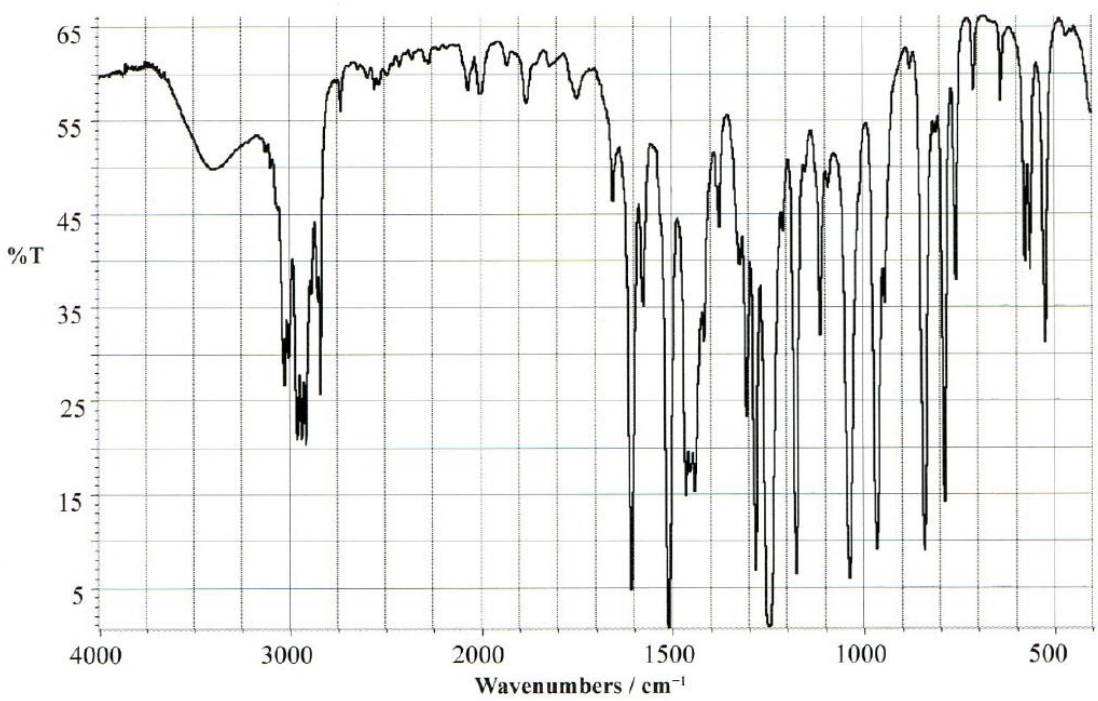
SPECTROSCOPICAL ANALYSIS OF ANETHOLE

UV



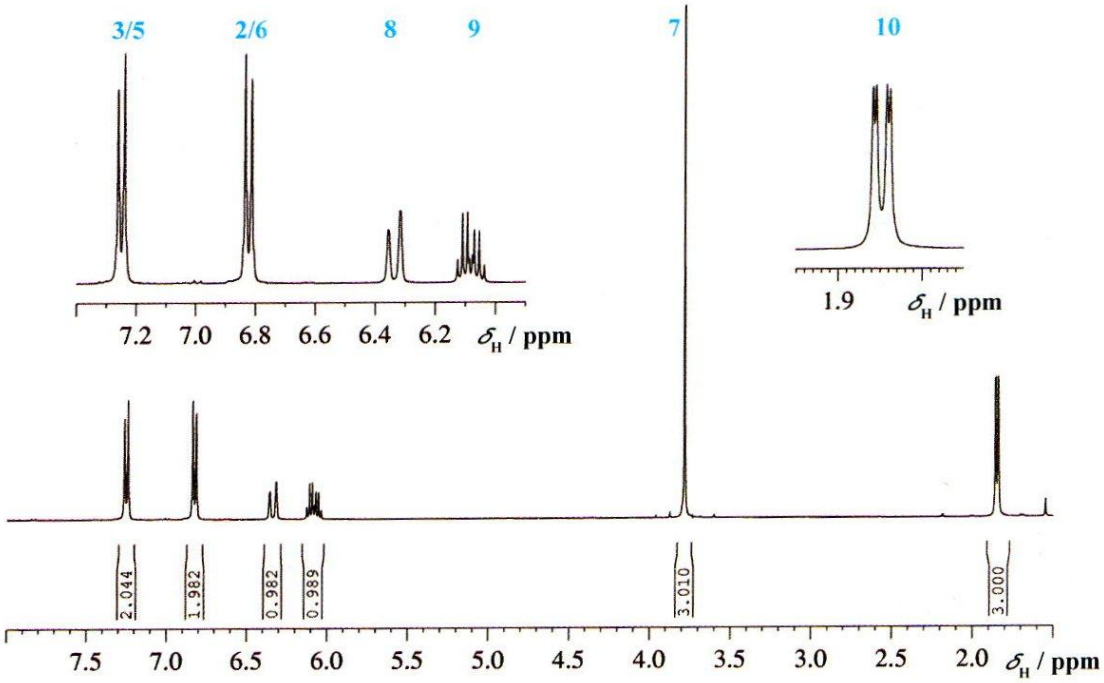
The UV spectrum reveals a typical pattern of an aromatic compound with a main π - π^* transition at 260 nm and a shoulder due to the auxochromic methoxy group reaching to 320 nm. Due to the flexibility of the side chains, there is no vibrational fine structure to be seen.

IR



The IR spectrum shows CH valence bands for both sp^3 and sp^2 units. The aromatic ring is revealed by the overtone vibrations between 2100 and 1700 cm"^{1}. The sharp C=C vibration at 1600 cm-^{1} and the strong band at 840 cm^{-1} indicate the pora-substituted benzene ring. In the fingerprint region, one finds at 1250 cm-^{1} the C-O-C vibration of an aromatic ether.

^{1}H-NMR



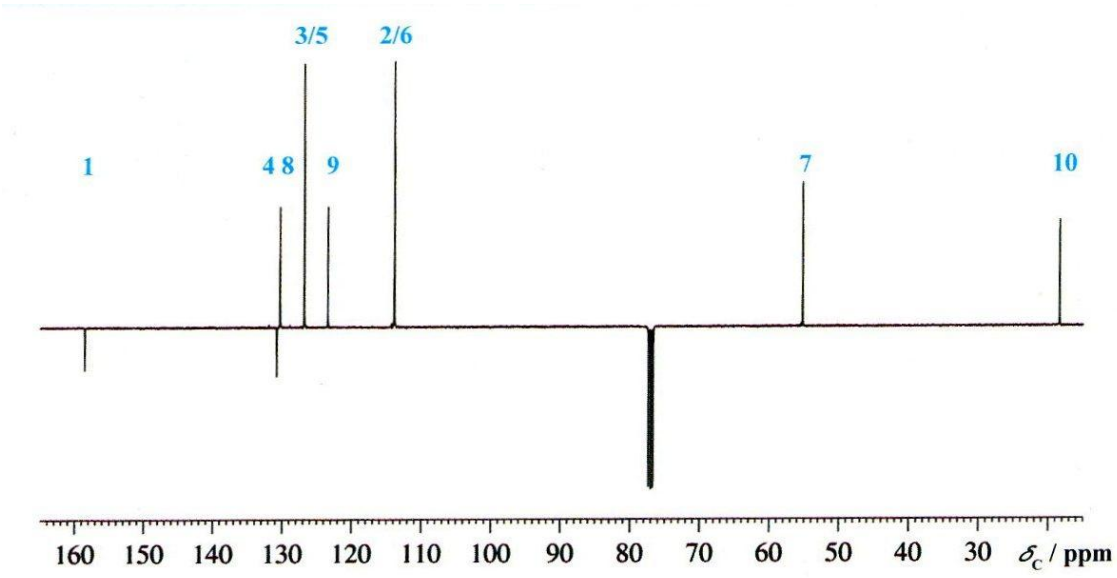
^{1}H NMR Spectrum of Anethole (400 MHz, in $CDCl_3$)

In the ^{1}H NMR spectrum, we first find the typical AA'XX' pattern of a para-substituted benzene ring. The chemical shift of the two protons at 6.8 ppm indicates an oxygen substituent due the shielding effect of the mesomeric contribution of the free electron pair. The next pattern centred at 6.25 ppm is typical of a /rara-double bond with a spin coupling constant of 15.7 Hz and an additional spin coupling to an attached methyl group. The methyl group signal itself nicely reveals a ^{3}J of 6.5 Hz and a ^{4}J (allylic spin coupling) of 1.6 Hz.

^{13}C Signals δ / ppm	Type of Carbon	Assignment	Proton Signals δ / ppm, J / Hz
158.6	C_q	C-1	
130.8	C_q	C-4	
130.3	CH	C-8	6.34, ^{3}J(H-8,H-9) 15.7
126.9	CH	C-3/5	7.25
123.4	CH	C-9	6.08
113.9	CH	C-2/6	6.82
55.2	CH_3	C-7	3.78
18.4	CH_3	C-10	1.84, ^{3}J(H-10,H-9) 6.5, ^{4}J(H-10,H-8) 1.6

^{1}H and ^{13}C NMR Data for Anethole

^{13}C-NMR



^{13}C NMR Spectrum of Anethole (100 MHz, in $CDCl_3$)

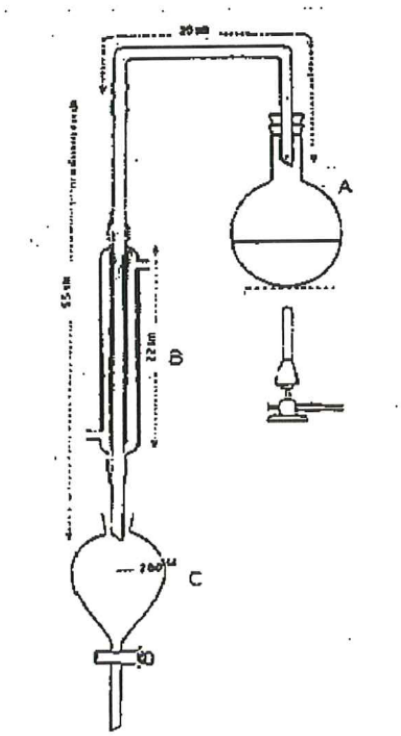
The ^{13}C NMR spectrum reveals a typical pattern of a para-substituted aromatic compound and the assignment for the oxygen-substituted carbon atom C-l at about 160 ppm is obvious. The only difificulty is the relative assignment of C-8 and C-9, but this easily follows by inspection of the HSQC spectrum, since in the proton spectrum the corresponding ^{1}H signals can be distuingished due to the coupling with the methyl group.

3. Quantitative Analysis of Volatile oil (Gravimetric) (Anisi Fructus, Oleum Foeniculi, Anason)

Ref. This method is taken from Deutsche (German) Pharmacopoeia and TK (Turkish Codex) 1948.

Absolutely weighed sample is distilled with 300 ml water on the equipment prepared according to TK, until 200 ml distillate is gained. 60 g NaCl is added to distillate and shaked until it dissolved. By this way, essential oil is easily separated from water which is saturated by NaCl. Essential oil is taken to organic solvent extracting aqueous phase by an organic solvent which has low boiling point such as pentane and hekzane. Organic phase is evaporated in a flask of which tare is determined by bringing to constant weight. Obtained essential oil is brought to constant weight by drying in oven at 50 ºC. Quantity of essential oil is calculated as w/w. Disadvantages of this method :

- Quantitation by this method takes along time. A lot of equipments are used.
- Because of a lot of equipments, amount of essential oil can be lost.
- Due to the distillation and extraction, if the extraction is not successfully performed, yield will be poor.
- Essential oil can not be used after these processes.



Equipment which is used in quantitation of essential oil by Gravimetric method
(TK) (A. Berk- Baytop)
A- Distillation Flask (1 lt); B- Condenser; C- Separating funnel (300 ml)

Experimental Process: Sample is powdered and weighed absolutely about 10 g. It is transferred to a 1 l flask, added 300 ml water, put a boiling stone for boiling properly. Flask is bonded to a smooth cooler by a glass pipe which is flexed birectangular. It's heated on an amiant fiber by strong fire. Distillation is performed properly. Distillate is collected into 250 ml erlenmeyer. When 150 ml distillate is collected, fire is removed for a while. When boiling of the water is stopped, sample which is adhered to wall of the flask is collected to the water by rotating the flask carefully. After that, distillation is started again and continued up to 200 ml line in erlenmeyer. (By the way, if there is a flur on the wall of the cooler due to accumulation of essential oil, cooling is not stopped until the flur is broken). Distillation is stoped when 200 ml of distillate is obtained and 60 g NaCl is added to erlenmeyer and dissolved. Aqueous phase is extracted twice by organic solvent. After that, it is dried by anhydrous Na_2SO_4. It is transferred to a wide mouth flask (100 ml) of which tare is determined. Organic phase is evaporated by an evaporator without excessive heat. Flask is brought to constant weight by a drying oven at 50 ºC and weighed. Increasing of the flask weight gives the quantity of essential oil. By this way, percentage of the essential oil amount is calculated as w/w.

QUESTIONS

Quantitation of Essential oil (Gravimetric)

1-Which Pharmacopoeia is gravimetric method involved in Turkish Codex taken from?

2-What are the disadvantages of gravimetric method?

3-What is the reason of adding NaCl to distillate? Why is 60 g NaCl added?

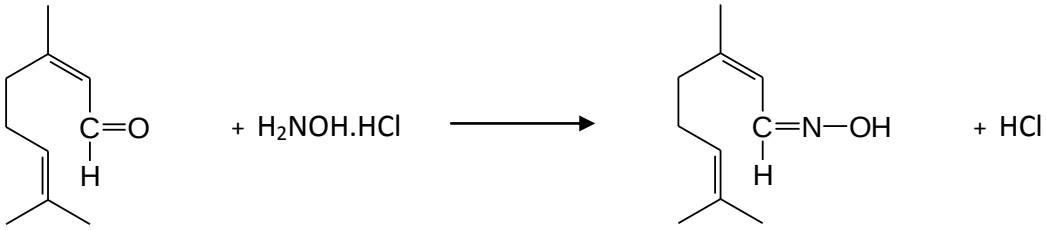
4-Why is essential oil extracted by pentane?

4. Total Aldehyde Quantitation (Titrimetric)

QUANTITATIVE ANALYSIS OF TOTAL ALDEHYDE CONTENT IN LEMON OIL

This method based on occurance of oxime by reaction of aldehydes such as citral, cinnamyl aldehyde, acetaldehyde and cetones such as carvon, menton, pulegon with hydroxylamine hydrocloride ($H_2NOH.HCl$). Formaldehyde can not be determined by this method.

Aldoxime Reaction



Citral Aldoxime

$$HCl + KOH \longrightarrow KCl + H_2O$$

- Put 5 ml of Lemon oil into the tared erlenmayer flask. *****
- Weigh the erlenmayer flask again and calculate the sample's weight.
- Add 1 drop of methyl orange into the erlenmayer flask (methyl orange gives pink color at acidic medium and yellow at basic medium)
- Add 8 – 10 ml of 0.5 N hydroxylamine hydrochloride into the erlenmayer flask. *****
- Titrate the mixture with 0,5 N KOH which is prepared in 60% alcohol until the alcoholic layer's color turns to yellow.
- Record the quantity of 0,5 N KOH that is used during the titration.
- Calculate the percentage of total aldehyde in the oil.

*** Apply the sample onto the TLC plate.**

TLC

Adsorbent: Silica gel

Mobile phase: Toluene – EtOAc (95:5)

Standard: Citral solution (1% in MeOH)

Detection: Vanillin / H_2SO_4 or Anisaldehyde solution R. and heat in the oven for 2 min at 105 ^{0}C

Calculation:

1) 1N	1000 ml KOH	56.1 g
0.5 N	1000 ml KOH	28.05 g
0.5 N	1 ml KOH	0.02805 g
0.5 N	$(a \times f_{KOH})$ ml KOH	**X**

$X = a \times f_{KOH} \times 0.02805$ g

56.1 g KOH is equivalent to	36.5 g HCl
$a \times f_{KOH} \times 0.02805$ g KOH	**A**

$$A = \frac{a \times f_{KOH} \times 0.02805 \times 36.5}{56.1}$$ Equivalent to g **HCl**

152 g citral	36.5 g HCl
B g	A g

$$B = \frac{A \times 152}{36.5}$$ g **Citral**

T g essential oil	contain B g citral
100 g	X

$$X = \frac{100 \times B}{T}$$

2) 1N	1000 ml KOH	152 g citral
0.5N	1 ml KOH	152x0.5/1000
0.5 N	$(a \times f_{KOH})$ ml KOH	X

$X = a \times f_{KOH} \times 152 \times 0.5/1000$ g citral equivalenT

T g essential oil	contain Z g citral
100 g	X

$$X = \frac{100 \times Z}{T}$$

STEROIDS: SAPONINS and CARDIOACTIVE GLYCOSIDES

SAPONINS (= SAPONOSIDES)

K. Hostettmann, A. Marston, **Saponins:** ***Chemistry and Pharmacology of Natural Products***, Cambridge University Press, New York, 1995; R. Hänsel, O. Sticher, **Pharmakognosie – Phytopharmazie**, 8. Auflage, Springer Medizin Verlag, Heidelberg 2007; O. Zerbe, S. Jurt, ***Applied NMR Spectroscopy for Chemists and Life Sciences***, Wiley-VCH, Weinheim, 2014.

An important aspect of the modern use of plant extracts as pharmaceutical preparations is the characterization and determination of the individual active constituents.

In the case for saponin preparations, sophisticated techniques are used for the isolation, structure elucidation and analysis of their component triterpene and steroid glycosides. For biological activity testing, it is also necessary to isolate pure compounds in sufficient quantity and purity. As many foodstuffs contain saponins, their isolation and characterization is vital in order to investigate their biological activities and possible toxic effects.

Techniques of isolation and structure elucidation of saponins (steroid glycosides, steroid alkaloid glycosides) rely on the same or similar methods of chromatography and spectroscopic/chemical analysis (e.g- MS, ^{13}C-NMR, acid hydrolysis, enzymatic hydrolysis, alditol acetate formation, etc.).

Isolation Methods: The isolation of pure saponins requires one or more chromatographic separation steps in order to remove other polar constituents of alcoholic or aqueous plants extracts. A variety of separation techniques are used for these purposes. Open column chromatograpy, flash chromatography, vacuum liquid chromatography (LC), low- and medium-pressure LC, high-performance LC, Countercurrent chromatography (CC) (Droplett CC: DCCC; Rotation Locular CC: RLCC; Centrifugal partition C: CPC). Silica gel, reversed phase silicagel (RP-Silica gel C_8 and C_{18}), dextran supports (Sephadex LH-20), polymers (Polyamide, Diaion HP-20, Amberlite XAD-2) are mostly used stationary phases used in liquid-solide chromatographic techniques. For liquid-liqid chromatographic techniques based on partition such as countercurrent chromatography, DCCC or RLCC, the immiscible solvent systems are used.

Analysis and quantitative determination

Different methods have been employed for the qualitative and quantitative determination of saponins: haemolysis, piscicidal activity, gravimetry, spectrophotometry, TLC, GC, HPLC, etc. Determinations based on classical properties of saponins (haemolysis, surface activity, fish toxicity) have largely been replaced by photometric methods such as densitometry, colorimetry of derivatives and, more recently, by GC and HPLC.

The quantitative analysis of *Ginseng radix* in the *Pharmacopoea Helvetica VII,* for example, relies on reaction with glacial acetic acid/sulphuric acid and spectrophotometry at 520 nm of the red colour formed.

The β-aescine component of horse chestnut (*Aesculus hippocastanum,* Hippocastanaceae) saponin can be spectrophotometrically determined after treatment with a mixture of iron(III) chloride, acetic acid and sulphuric acid.

Spectrophotometric methods are very sensitive but not suitable for estimating saponins in crude plant extracts since the reactions are not specific and coloured products may form with compounds which accompany the saponins, such as phytosterols and flavonoids. Another problem, common to much of the analytical work on saponins, is their incomplete extraction from the vegetable material.

A very simple but inadequate test is based, of course, on the foam-forming properties of saponins

Colour reactions

(1) ***Aromatic aldehydes.*** Anisaldehyde, vanillin and other aromatic aldehydes in strong mineral acid (for example, sulphuric, phosphoric, perchloric acids) give coloured products with aglycones. The absorption maxima of these entities lie between 510 and 620 nm. A dehydration reaction probably occurs, forming unsaturated methylene groups which give coloured condensation products with the aldehydes. These reactions are, however, not very specific and a number of other classes of substance can react. With vanillin-sulphuric acid, spirostan saponins give two visible absorptions, one of them located around the 455 to 460 nm region; triterpene saponins with a C-23 hydroxyl group have a peak located between 460 and 485 nm

(2) ***Liebermann-Burchard test.*** Unsaturated and hydroxylated triterpenes and steroids give a red, blue or green coloration with acetic anhydride and sulphuric acid. Since terpenoid saponins tend to produce a pink or purple shade and steroid saponins a blue-green coloration, differentiation of the two classes is possible.

(3) **Cerium(IV) sulphate or iron(III) salts** and inorganic acids, such as sulphuric acid. *This gives a violet-red coloration of the solution.*

(4) ***A 30% solution of antimony(III) chloride in acetic anhydride- acetic acid.*** This reagent gives colour reactions with hydroxytriterpenes and hydroxysteroids.

(5) ***Antimony(III) chloride in nitrobenzene -methanol.*** The differentiation of 5,6-dehydro-derivatives of steroid glycosides (diosgenin and solasodine glycosides) and 5α- or 5β-H-derivatives (e.g. tomatine) is accomplished with antimony(III) chloride in nitrobenzene-methanol: the 5,6-dehydro-derivatives give a red colour at room temperature.

(6) ***Ehrlich reagent.*** Furostanol derivatives give a red coloration with Ehrlich reagent (1 g p-dimethylaminobenzaldehyde + 50 ml 36% HC1 + 50 ml ethanol; spray and heat) and a yellow colour with anisaldehyde.

(7) ***Carbazole.*** The presence of uronic acids can be established by reaction with carbazole, in the presence of borate and concentrated sulphuric acid.

Several of the reagents used for colour reactions are similarly employed in TLC detection and for the spectrophotometric and colorimetric determination of saponins.

Haemolysis

The ability of saponins to rupture erythrocytes has been used for decades as a detection and quantification method. This procedure has been improved and modified several times, but the most frequently measured parameter is the change in absorbance of the supernatant of an erythrocyte suspension after haemolysis by a saponin or by a saponin-containing mixture. Various amounts of the saponin-containing material are mixed with a suspension of washed erythrocytes in isotonic buffer at pH 7.4. After 24 h, the mixture is centrifuged and haemolysis is indicated by the presence of haemoglobin (red) in the supernatant.

The *European Pharmacopoeia* uses as a unit the quantity in ml of ox blood (diluted 1:50) which is totally haemolysed by 1 g of test substance. As a standard, the saponin mixture from the roots of *Gypsophila paniculata* L. (Caryophyllaceae) has by definition an activity of 30000. The haemolytic index (HI) is calculated as follows:

$$HI \text{ (haemolytic index)} = 30\,000 \times a/b$$

where *a* is the quantity of standard saponin (g) required for complete hydrolysis and *b* is the quantity of substance (g) required for complete hydrolysis.

Haemolytic methods have the disadvantage that they rely on the complete absence of other surface-active compounds which may also be haemolytic. Furthermore, there is often no correlation between the haemolytic activity and the pharmacological activity of the plant drug. Numerous 'typical' saponins, such as glycyrrhizin or sarsaparilloside, show very weak or no haemolytic activity. This is because haemolytic activity depends greatly on the arrangement of the sugar moieties and also on the presence of polar substituents (carboxyl or hydroxyl groups) on the aglycone, especially rings D and E.

Haemolytic property of saponins is also used for identification saponin compounds in a mixture analysed by TLC.

1. TLC
2. Blood-Agar
   (Erythrocyte suspension)
3. Evaluation:
   After 2 hr zone of haemolysis

The foam value (Fr. Ph., 10th Ed., V.4.A):

The foam value in other words: "the dilution of a decoction of the drog which, in the prescribed conditions, produces a lasting foam". In practice, this value is determined on a decoction obtained by prolonged boiling (30 minutes) of 1 g of drug in 100 mL of water. A series of calibrated tubes contains increasing dilutions of this decoction.

The tubes are agitated: the foam value is the drug dilution in the tube that gives 1 cm of foam after 15 minutes at rest.

Thin Layer Chromatography (TLC) of Saponins

The qualitative analysis of saponins by TLC is of great importance for all aspects of saponin investigations. TLC plates (usually silica gel) can handle both pure saponins and crude extracts, are inexpensive, rapid to use and require no specialized equipment. A number of visualization reagents are available for spraying onto the plates (see Table *below*). Methods of preparation of the most common reagents are as follows:

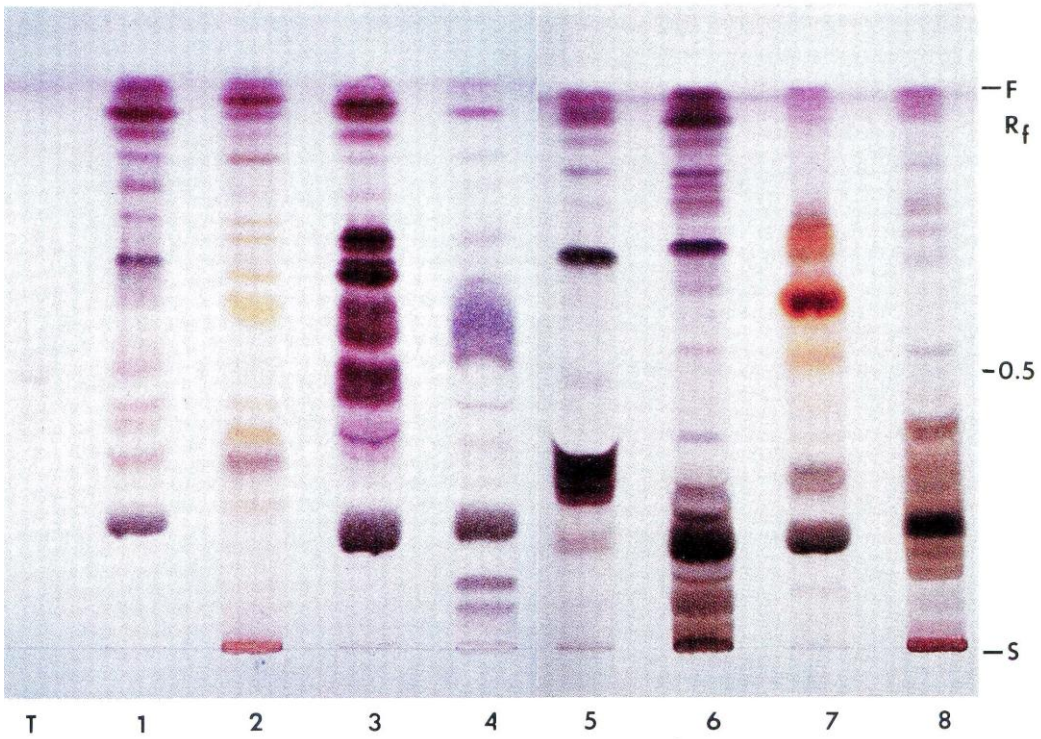
- **Vanillin-sulphuric acid (Godin reagent)**. A 1% solution of vanillin in ethanol is mixed in a 1:1 ratio with a 3% solution of perchloric acid in water and sprayed onto the TLC plate. This is followed by a 10% solution of sulphuric acid in ethanol and heating at 110 °C.
- **Liebermann-Burchard reagent.** Concentrated sulphuric acid (1 ml) is mixed with acetic anhydride (20 ml) and chloroform (50 ml). Heating at 85-90 °C gives the required coloration on the TLC plate. *The resulting colors differ depending on whether the aglycone is a triterpene (pink to red) or a steroid (blue-green).*
- **Antimony-(III)-chloride**. The TLC plate is sprayed with a 10% solution of antimony chloride in chloroform and heated to 100 °C.
- **Anisaldehyde-sulphuric acid.** Anisaldehyde (0.5 ml) is mixed with glacial acetic acid (10 ml), methanol (85 ml) and concentrated sulphuric acid (5 ml). This solution is sprayed onto the TLC plate, which is then heated at 100 °C.

Visualization reagents for the TLC of triterpene saponins

Reagent
Vanillin-sulphuric acid
Vanillin-phosphoric acid
Liebermann-Burchard (acetic anhydride-sulphuric acid)
1% Cerium sulphate in 10% sulphuric acid
10% Sulphuric acid in ethanol
50% Sulphuric acid
p-Anisaldehyde-sulphuric acid
Komarowsky (p-hydroxy benzaldehyde sulphuric acid)
Antimony(III) chloride
Blood
Water

Solvent systems used in TLC of saponins

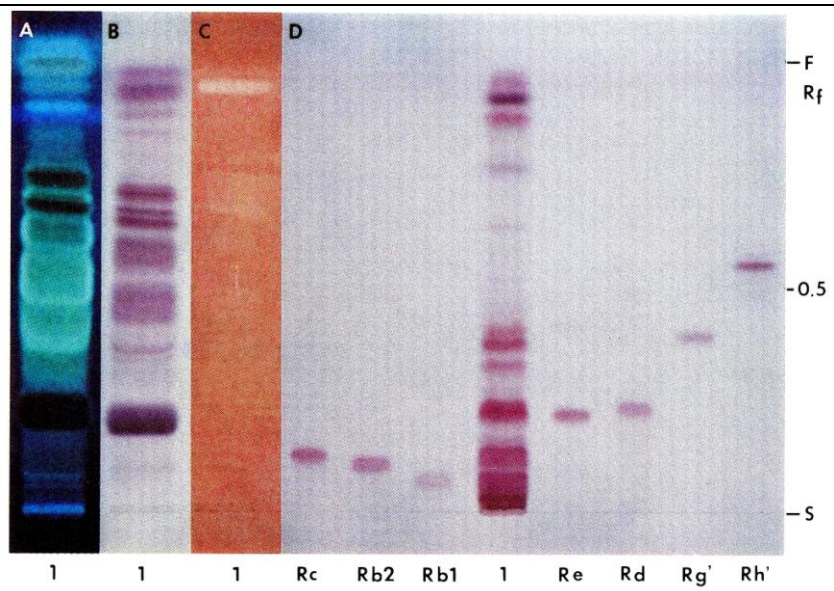
The most frequently used solvent for TLC	chloroform-methanol-water (65:35:10) lower phase, (61:32:7), (80:20:2)
The most popular system	n-butanol-ethanol-ammonia (7:2:5)
Especially for glycosides containing uronic acid residues;	n-butanol-acetic acid-water (4:1:5; upper layer)
For polar saponins	chloroform-methanol-acetic acid- water (60:32:12:8)
Systems employed for the TLC of glycoalkaloids	ethyl acetate- pyridine-water (30:10:30; upper phase).
For ginseng saponins	the upper phase of the solvent system n-butanol-ethyl acetate-water (100:25:50)



TLC of Saponin Drugs (H. Wagner *et al.,* 1983)

Solvent system:	Chloroform – MeOH – H_2O (64:50:10)
Reagent:	Vanillin – H_2SO_4 (1 and 5% in EtOH, resp.) 110°C, 5 – 10 min
T:	**Saponin mixture from Senegae radix**

1: **Senegae radix**	5: **Primula radix**
2: **Sarsaparillae radix**	6: **Saponariae radix**
3: **Ginseng radix**	7: **Liquiritae radix**
4: **Hippocastani semen**	8: **Quillajae cortex**



Solvent system:
Chloroform – MeOH – H_2O
(64:50:10)
Reagents:
A: Antimony-III-chloride,
UV-365nm

A: Antimony-III-chloride,
Day light

C: Blood Agar

D: Vanillin-Phophoric acid
(1% Vanillin solution in 50%
phosphoric acid)
110°C, 5 – 10 min

TLC of Ginseng radix (H. Wagner *et al.,* 1983)

The List of the Saponin Drugs

	Drugs	*Plant name*	Family	Constituents Saponins
TRITERPENIC SAPONINS	Ginseng radix	*Panax ginseng*	Araliacaea	Dammarane-type glycosides **Ginsenosides Rx** **x= o, a, b_1, b_2, c, d, e, f, g_1, h**
	Elutherocci radix	*Acanthopanax senticosus*	Araliaceae	Oleanolic acid glycosides **Elutherosides I - M**
	Hedera folium	*Hedera helix*	Araliacaea	Oleanane-type glycosides **Hederacosides B and C** **α- and β-hederins**
	Hippocastani semen	*Aesculus hippocastanum*	Hippocastanaceae	Oleanane-type glycosides **β-Aescin**
	Liquiritae radix	*Glycyrrhiza glabra*	Fabaceae (Leguminosae)	Oleanane-type glycosides **Glycyrrhizin**
	Primula radix	*Priuma veris, P. elatior, P. longipes, P. megaseifolia*	Primulaceae	13β,28-epoxy-Oleanane-type glycosides **Primulasaponin, Primacrosaponin**
	Cyclamen tuber	*Cyclamen species*	Myrsinacaea (formerly Primulaceae)	13β,28-epoxy-Oleanane-type glycosides **Cyclamin, deglucocyclamin**
	Quillajae cortex	*Quillaja saponaria*	Rosaceae	Oleanane-type glycosides **Quilajic acid**
	Saponaria rubrae radix	*Saponaria officinalis*	Caryophyllacaea	Oleanane-type glycosides **Saponosides A - D**
	Saponaria albae radix	*Gypsophila* spec.	Caryophyllaceae	Oleanane-type glycosides **Gypsoside A**
	Senega radix	*Polygala senega*	Polygalaceae	Oleanane-type glycosides **Senegin**
	Centella asiatica herba	*Centella asiatica*	Apiacaea (Umbelliferae)	Ursane-type glycosides **Asiaticoside**
	Thea semen	*Thea sinensis (Camellia sinensis)*	Theaceae	Oleanane-type glycosides **Theasaponins**
STEROIDAL SAPONINS	Avena sativae herba	*Avena sativa*	Poaceae	Spirofuran (Nuatigenin)-type steroidal saponins **Avenacosides A and B**
	Veronica herba	*Veronica spec.*	Plantaginaceae (formerly Scrophulariaceae)	Spirofuran (Nuatigenin)-type steroidal saponins **multifidoside**
	Sarsaparillae radix	*Smilax* spec.	Liliacaea (Alliacaea)	Spirostanol&furostanol-type steroidal saponins **Parillin, sarsaparilloside**
	Ruscus acuelati rhizome	*Ruscus aculeatus*	Alliacaea	Spirostanol&furostanol-type steroidal saponins **Ruscoside**
	Dioscorea rhizome	*Dioscorea* species	Dioscoreaceae	Spirostanol-type glycosides **Dioscin (Diosgenin glys.)**

STRUCTURE ELUCIDATION

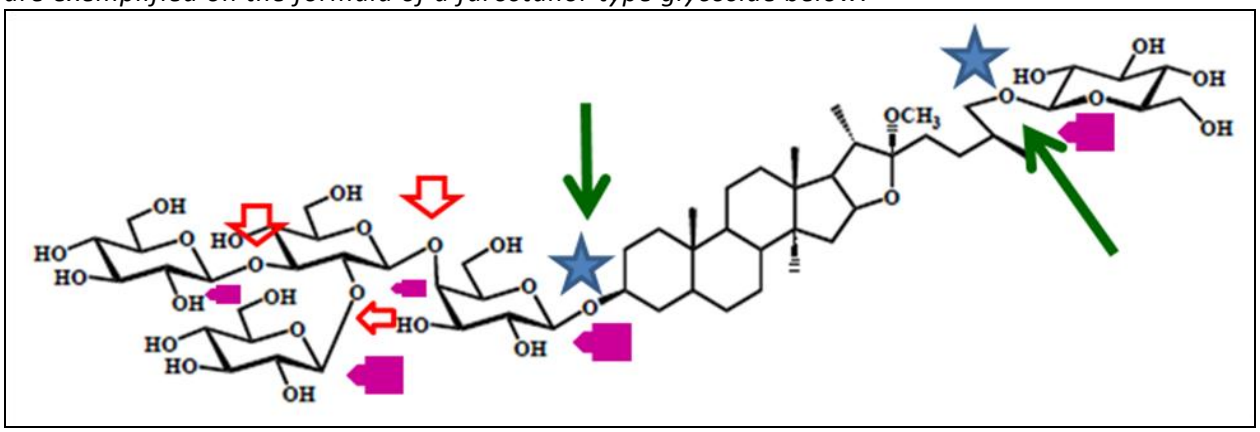
Methods of structure elucidation

Several spectroscopic and spectrometric instruments exist for the process of structure determination of natural products. The combination of physical data e.g. UV- spectroscopy, optical rotation, MS-spectrometry and NMR-spectroscopy, together with information of chemical methods is used to determine the structure of compounds.

Basic problems to be solved in the structure elucidation of glycosides, emphasizing on saponins and cardiac glycosides.

The structure of the genuine aglycone	
The composition and sequence of the component monosaccharides in the carbohydrate moiety , the site of the glycosidations on the agglycone unit.	★
The site(s) of interglycosidic linkage(s)	⇩
The anomeric configuration of each sugar unit (α- or β-anomer)	⬟
The location(s) of the sugar chain(s) on the aglycone.	→
The ring size of sugar units (pyranose or furanose forms)	

The problems to be solved in the structure elucidation of a saponin glycoside indicated above are exemplified on the formula of a furostanol-type glycoside below.



As it is well known, the chemical nature of compounds derived from squalene, steroids and triterpenes, are fairly diverse (see p. 3). In the absence of modern spectroscopic techniques, the identification and structure determination of these molecules were very complicated. NMR spectroscopy has developed very successfully from its early beginnings in the 1940s. Widespread use of NMR started in the 1960s. The progress of modern chemistry became possible with the advent of powerful analytical instrumental methods, with NMR spectroscopy playing a very important role among them.

Considering the importance of NMR in many branches of chemistry, basic NMR knowledge is taught in the chemistry curriculum, and is often often done in combination with other spectroscopic techniques such as IR, UV, Mass (MS) or X-ray.

These Techniques used in Structure Elucidation and the aim of their uses are summarized in Table 3.1.

Table 3.1. Spectroscopical and Chemical Techniques used in Structure Elucidation.

Isolate (Natural Compounds)		Spectroscopical & Chemical Techniques	Answers
	↓	^{1}H-NMR	Number of hydrogens
		^{13}C-NMR	Number of carbons
		DEPT	Determination of carbon types **Quaternary, tertiary, secondary or primary**
		Mass (MS)	Molecular weight
		UV	Chromophores
		IR	Functional groups **(CH, OH, C=O, Ester, usw)**
	1.	**MOLECULAR FORMULA; Double bond equivalent functionalities**	
	↓	HSQC	Heteronuclear one-bond correlation (**C-H**)
		DQF-COSY	Vicinal protons (C-**H**-C-**H**) and geminal protons (C**H$_2$**)
		TOCSY	Protons in a spin system
		HMBC	Heteronuclear long-range multiple bond correlation (**C**-C-C-**H**), (**C**-C-**H**)
		INADEQUATE	**C-C** coupling
	2.	**PLANAR STRUCTURE**	
	↓	X-Ray	**Relative/absolute sterochemistry**
		ROESY,	
		NOESY	
		$[\alpha]_D$	
		Derivatization	
	3.	**COMPLETE STRUCTURE WITH RELATIVE/ABSOLUTE STEREOCHEMISTRY**	

Among the chemical and spectroscopical methods, NMR experiments are the most useful measurements in helping us to establish the chemical nature of compounds.

In the following pages, a group of NMR experiments performed on the structure elucidation of saponin isolated as one of the main saponin, deglucocyclamin a 13β,28-epoxy-oleanan-type saponin from *Cyclamen* species (Figures 3.1 – 3.7).

Fig. 3.1. The ^{1}H-NMR Spectrum of Deglucocyclamin (300 MHz, CD_3OD)
Fig. 3.2.a. The ^{13}C-NMR Spectrum of Deglucocyclamin (75.5 MHz, CD_3OD)
Fig. 3.2.b. DEPT Spectrum of Deglucocyclamin
Fig. 3.3. The ^{1}H, ^{1}H- Homonuclear Correlated Spectrum (COSY) of Deglucocyclamin
Fig. 3.4. The ^{1}H, ^{1}H- Homonuclear Total Correlated Spectrum (TOCSY) of Deglucocyclamin
Fig. 3.5. The ^{1}H, ^{13}C- Heteronuclear Correlated Spectrum (HSQC) of Deglucocyclamin
Fig. 3.6. The ^{1}H, ^{13}C- Heteronuclear Multiple Bond Correlation Spectrum (HMBC) of Deglucocyclamin
Fig. 3.7. FAB-MS and The ^{1}H, ^{1}H- Homonuclear Correlated Spectrum (COSY) of Deglucocyclamin dodecaacetate

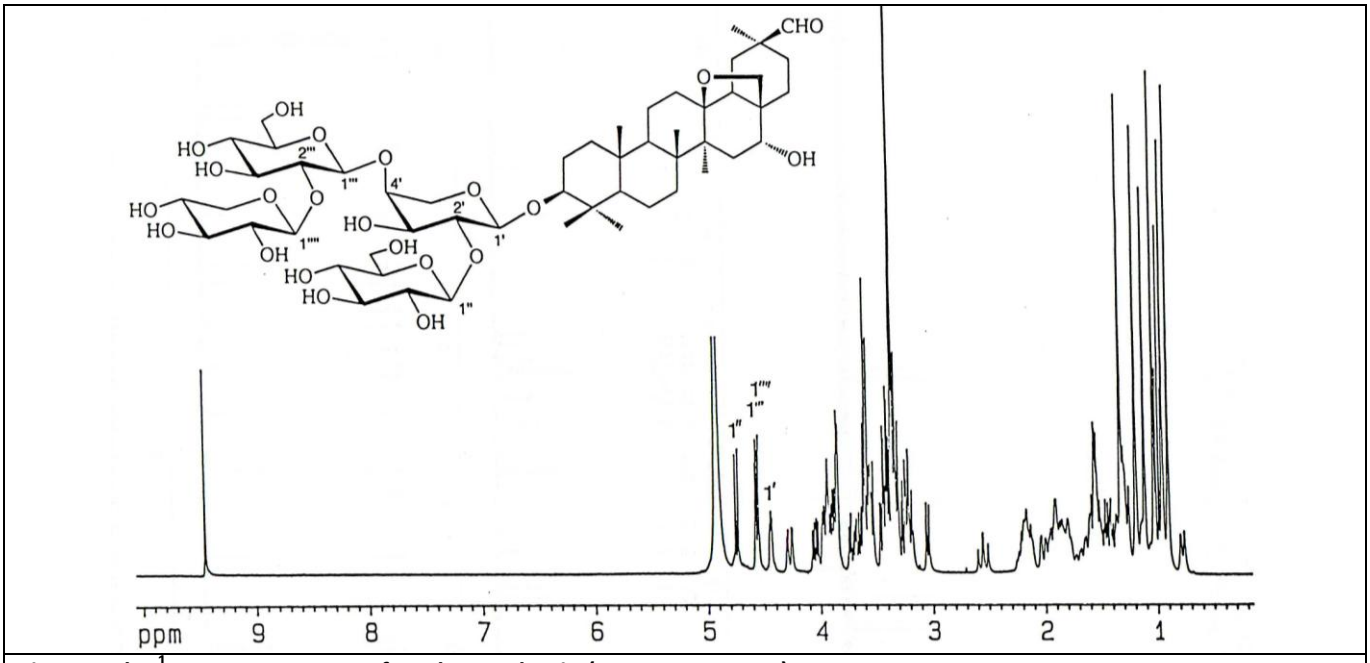


Fig. 3.1. The ^{1}H-NMR Spectrum of Deglucocyclamin (300 MHz, CD_3OD)

Fig. 3.2.b. DEPT Spectrum of Deglucocyclamin

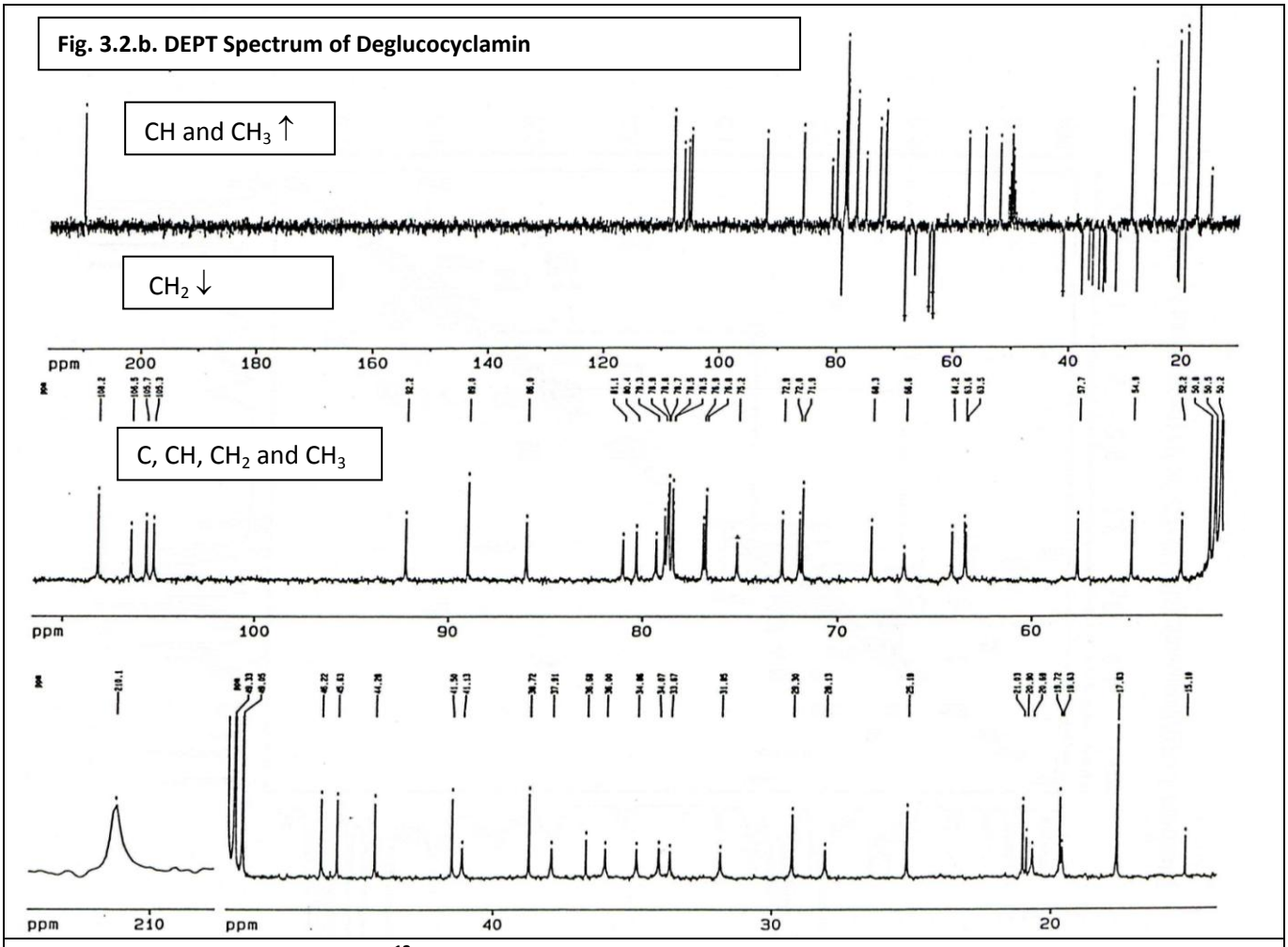


Fig. 3.2.a. The ^{13}C-NMR Spectrum of Deglucocyclamin (75.5 MHz, CD_3OD)

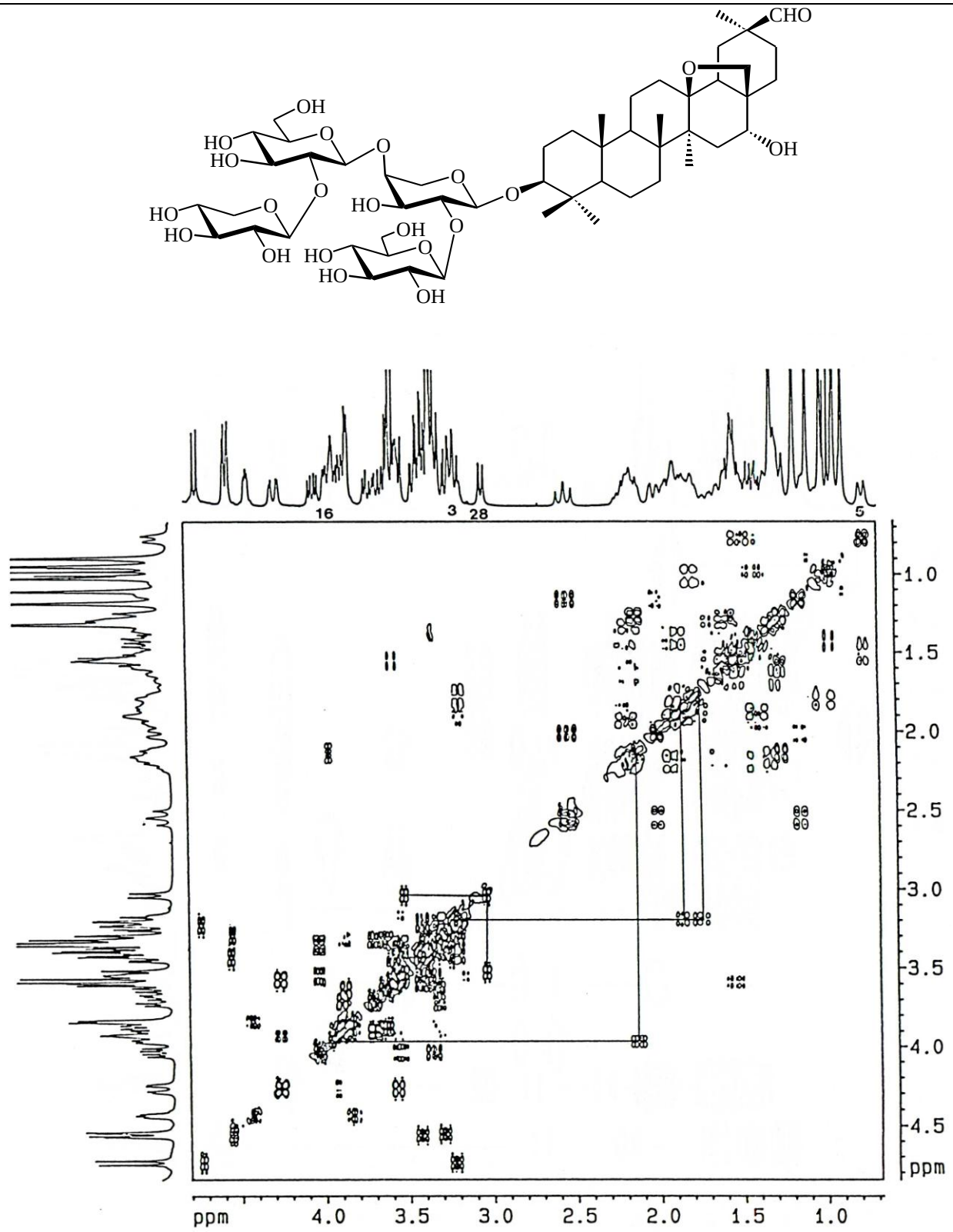


Fig. 3.3. The 1H, 1H- Homonuclear Correlated Spectrum (COSY) of Deglucocyclamin

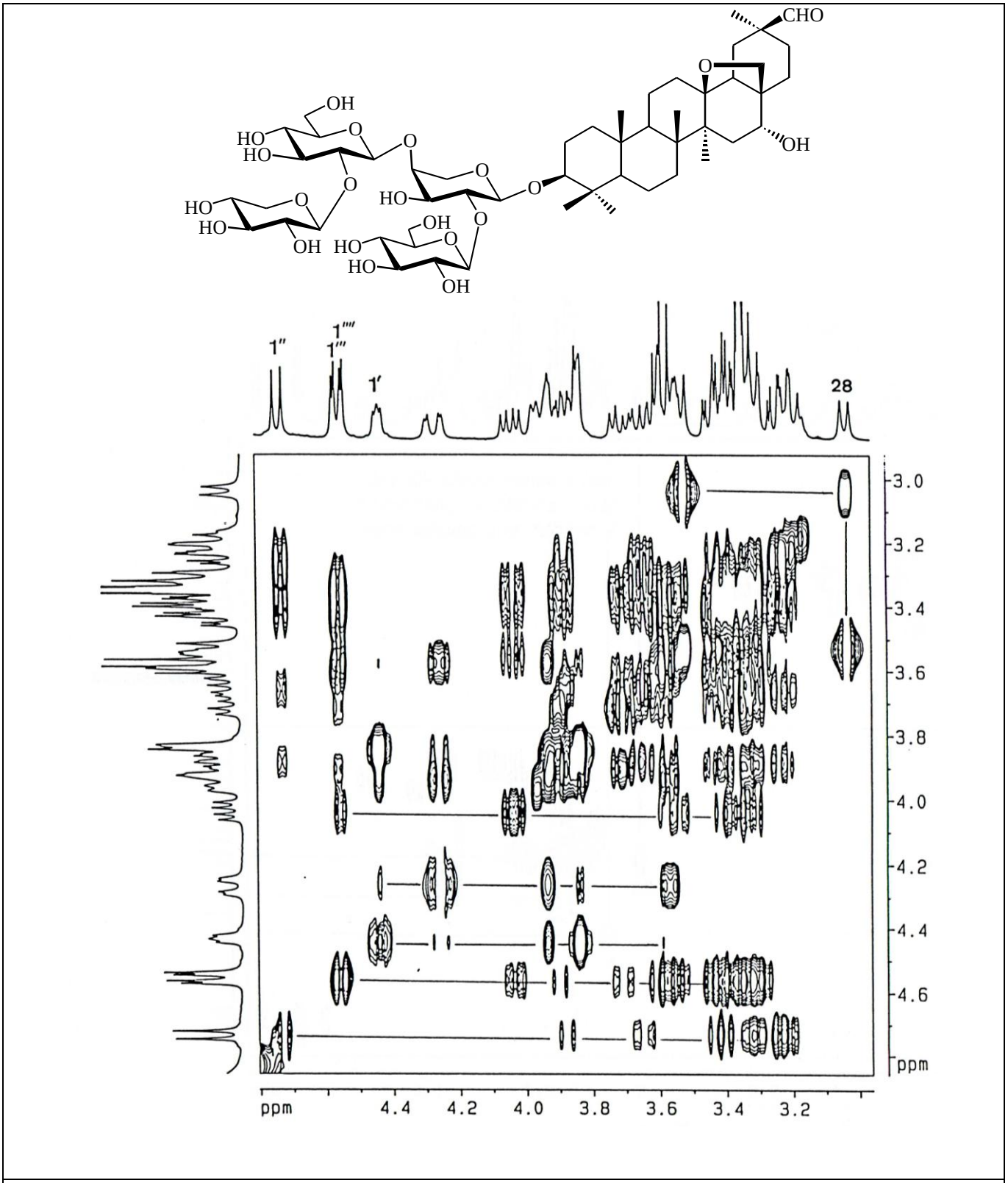


Fig. 3.4. The 1H, 1H- Homonuclear Total Correlated Spectrum (TOCSY) of Deglucocyclamin

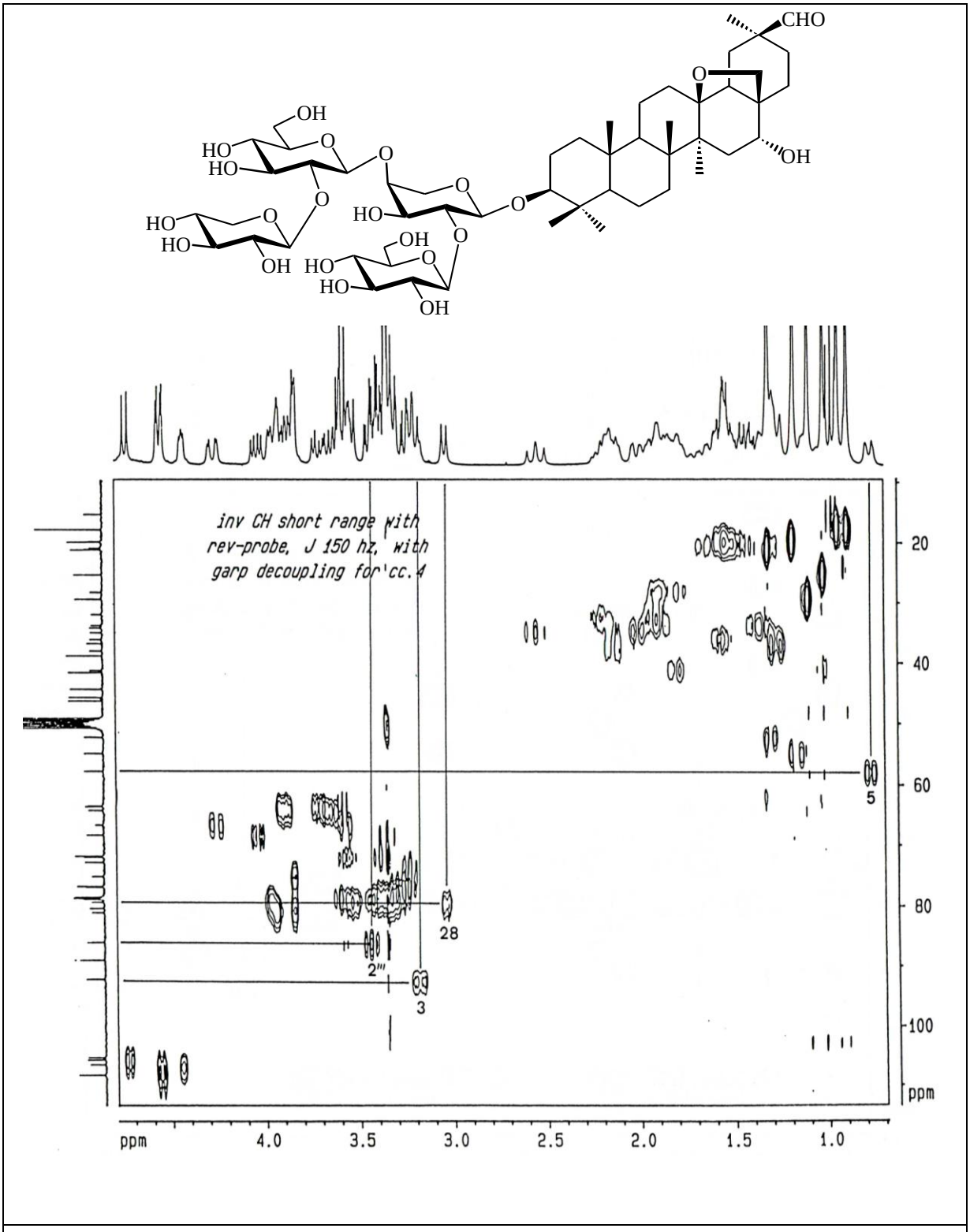


Fig. 3.5. The ^{1}H, ^{13}C- Heteronuclear Correlated Spectrum (HSQC) of Deglucocyclamin

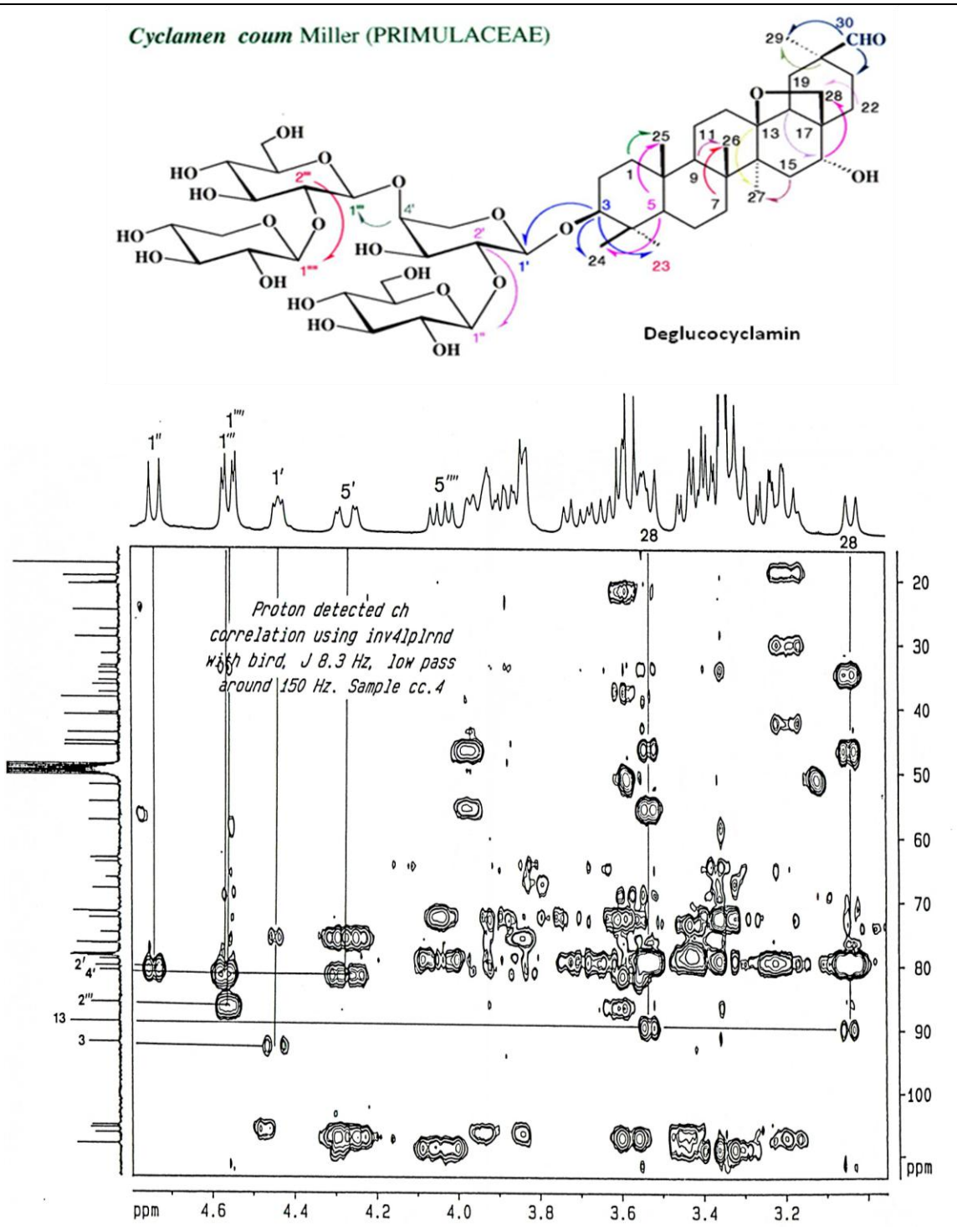


Fig. 3.6. The ^{1}H, ^{13}C- Heteronuclear Multiple Bond Correlation Spectrum (HMBC) of Deglucocyclamin

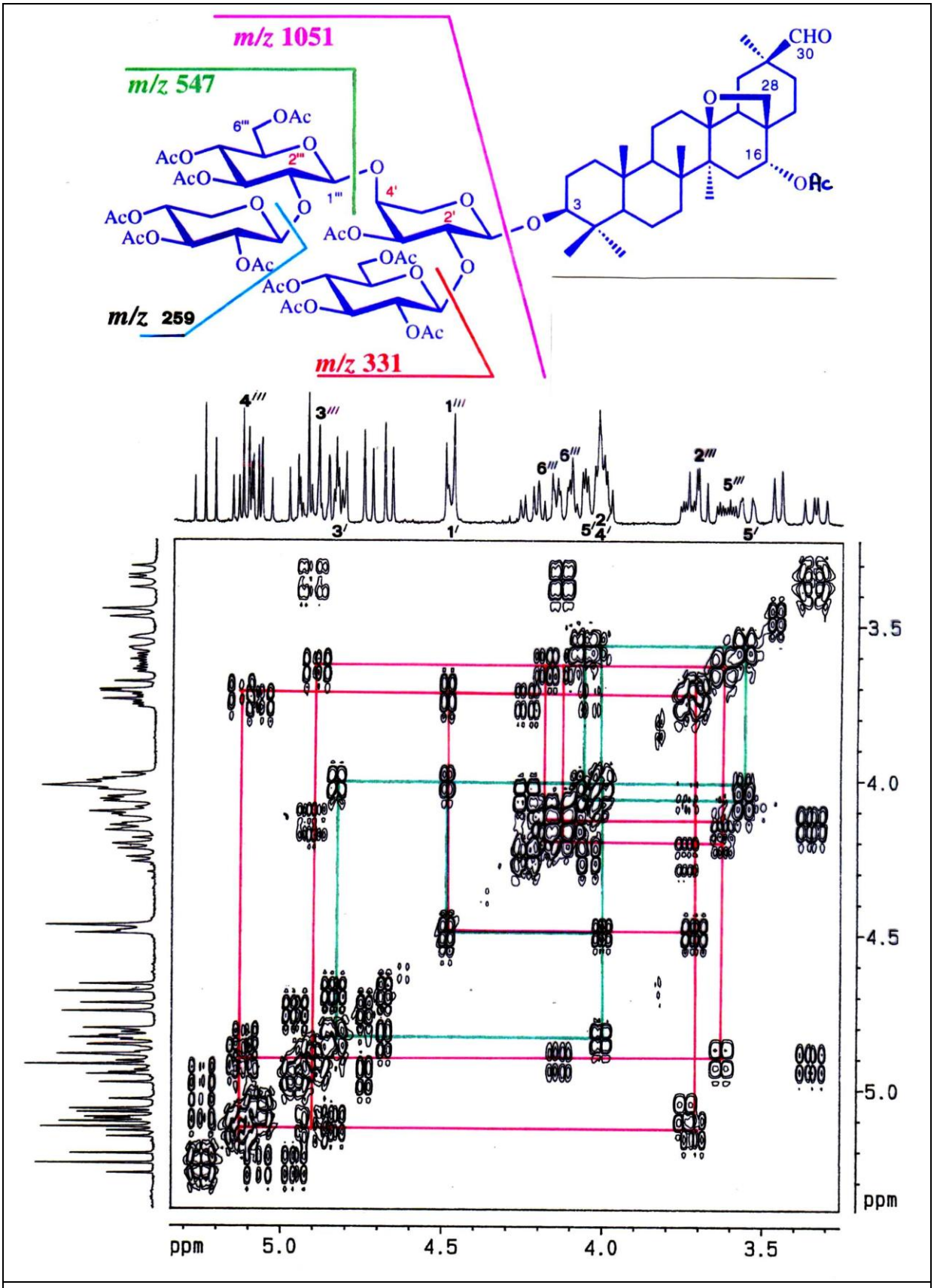
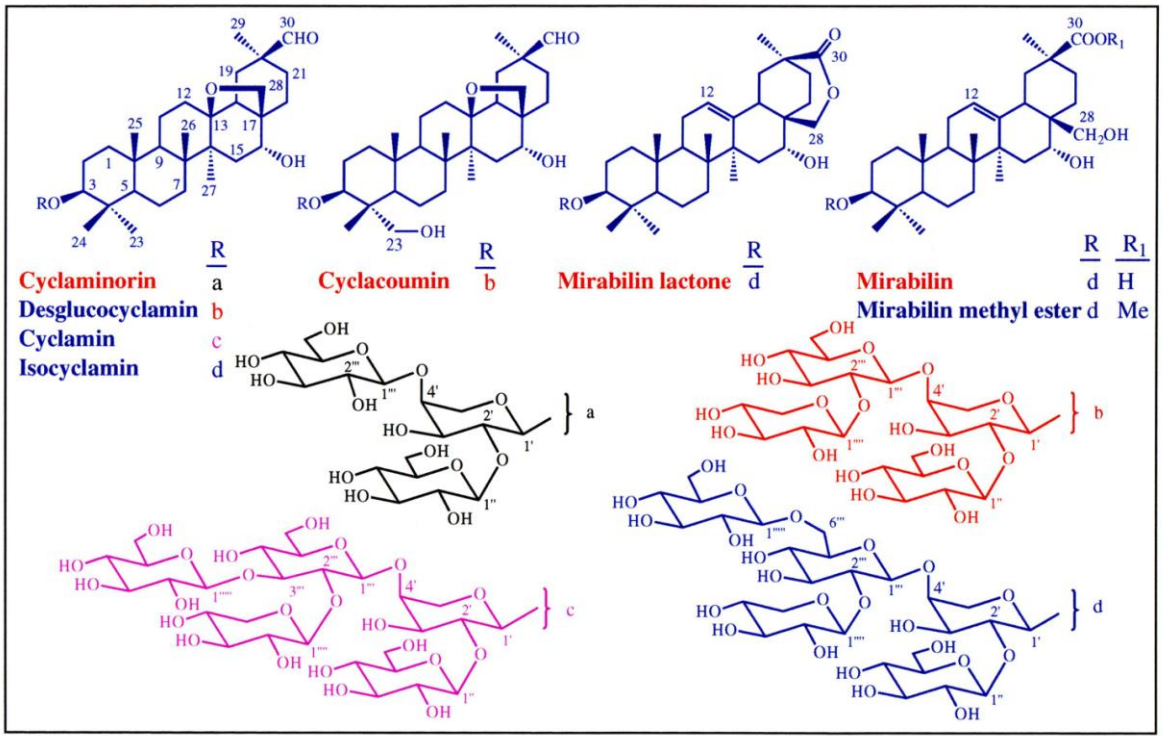


Fig. 3.7. FAB-MS and
The ^{1}H, ^{1}H- Homonuclear Correlated Spectrum (COSY) of Deglucocyclamin dodecaacetate

Table 3.2. ^{13}C-NMR Spectral Data for the Cyclaminorin **(1),** Deglucocyclamin **(2),** Deglucocyclamin dodecaacetate **(2a),** Cyclacoumin **(3)** and Mirabilin lactone **(4)** (**1 – 4** in CD_3OD, **2a** in $CDCl_3$**).**



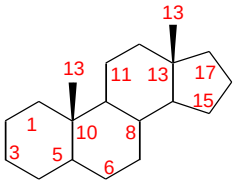
Sapogenol moieties						Sugar moieties					
C No	**1**	**2**	**2a**	**3**	**4**	**C No**	**1**	**2**	**2a**	**3**	**4**
1	40.2 t	40.1 t	39.0 t	40.0 t	39.8 t	1′	104.1 d	104.2 d	102.7 d	104.4 d	105.6 d
2	27.1 t	27.1 t	25.9 t	26.6 t	27.1 t	2′	78.6 d	80.1 d	75.3 d	80.1 d	79.4 d
3	91.4 d	91.2 d	89.8 d	83.9 d	91.2 d	3′	73.4 d	74.2 d	73.5 d	74.4 d	74.4 d
4	40.5 s	40.5 s	39.3 s	44.3 s	40.1 s	4′	78.4 d	79.3 d	77.0 d	79.8 d	80.1 d
5	56.8 d	56.7 d	55.4 d	48.1 d	57.2 d	5′	64.6 t	65.7 t	62.0 t	64.6 t	66.0 t
6	18.7 t	18.6 t	17.6 t	18.2 t	19.2 t						
7	34.0 t	33.9 t	33.1 t	34.5 t	33.4 t	1″	105.6 d	105.5 d	101.1 d	104.7 d	104.3 d
8	43.4 s	43.3 s	42.2 s	43.3 s	44.0 s	2″	75.8 d	75.9 d	71.7 d	75.9 d	75.9 d
9	51.3 d	51.2 d	50.1 d	51.3 d	48.2 d	3″	77.8 d	77.9 d	72.9 d	77.6 d	78.0 d
10	37.8 s	37.7 s	36.7 s	37.6 s	37.9 s	4″	71.5 d	71.9 d	68.2 d	71.8 d	71.3 d
11	19.8 t	19.7 t	18.6 t	19.8 t	24.5 t	5″	78.0 d	77.8 d	71.6 d	78.1 d	77.6 d
12	35.1 t	35.0 t	34.0 t	33.2 t	125.7 d	6″	63.1 t	63.2 t	62.0 t	63.1 t	62.7 t
13	88.1 s	88.0 s	86.3 s	88.2 s	141.1 s						
14	44.7 s	44.6 s	43.6 s	45.4 s	41.4 s	1‴	104.5 d	104.7 d	102.2 d	104.7 d	105.1 d
15	37.0 t	36.9 t	37.0 t	37.0 t	37.3 t	2‴	75.4 d	85.0 d	77.2 d	85.2 d	85.1 d
16	77.8 d	77.7 d	77.9 d	77.6 d	75.6 d	3‴	77.9 d	77.5 d	74.3 d	77.6 d	77.5 d
17	45.3 s	45.2 s	44.0 s	44.7 s	41.0 s	4‴	71.9 d	71.0 d	68.6 d	71.1 d	72.0 d
18	54.0 d	53.9 d	52.4 d	54.0 d	42.8 d	5‴	78.2 d	77.7 d	71.7 d	77.8 d	77.8 d
19	30.9 t	30.9 t	30.0 t	34.0 t	42.8 t	6‴	62.7 t	62.4 t	62.0 t	62.6 t	70.3 t
20	48.3 s	48.1 s	48.0 s	49.5 s	43.0 s						
21	33.2 t	33.1 t	32.2 t	30.9 t	29.3 t	1⁗		107.2 d	101.0 d	107.4 d	107.3 d
22	32.8 t	32.7 t	31.5 t	32.8 t	22.6 t	2⁗		75.8 d	71.4 d	75.9 d	76.0 d
23	28.4 q	28.3 q	27.7 q	64.7 t	28.4 q	3⁗		77.5 d	72.0 d	77.6 d	78.0 d
24	16.7 q	16.6 q	16.1 q	13.1 q	16.9 q	4⁗		70.8 d	69.3 d	71.0 d	71.0 d
25	16.7 q	16.6 q	16.0 q	17.2 q	16.1 q	5⁗		67.3 t	63.0 t	67.4 t	67.4 t
26	18.8 q	18.7 q	18.1 q	18.8 q	16.4 q						
27	20.1 q	20.0 q	19.6 q	20.1 q	28.8 q	1⁎					104.7 d
28	78.5 t	78.3 t	77.0 t	78.5 t	82.6 t	2⁎					75.1 d
29	24.3 q	24.2 q	23.9 q	24.3 q	27.2 q	3⁎					78.1 d
30	209.2 d	209.1 d	207.1 d	209.2 d	180.5 d	4⁎					71.6 d
						5⁎					77.0 d
						6⁎					63.3 t

*) Multiplicities were based on their DEPT and HMQC

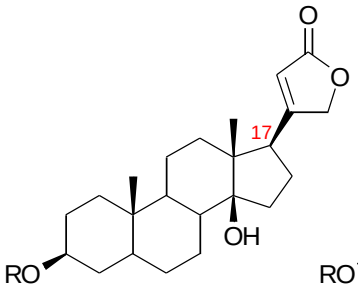
) Additional signals for 2a: 20.7 – 20.5 (each s, CO*C***H_3), 170.6 – 169.4 5 (each s, ***C***OCH_3; totally 12 lines).

CARDIAC GLYCOSIDES (CARDENOLIDES&BUFADIENOLIDES)

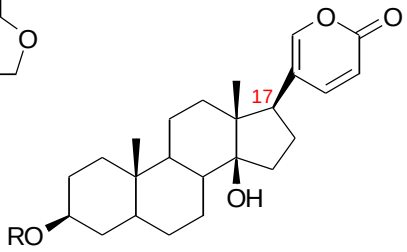
These type of compounds are steroidal glycosides which have a tetracyclic *10,13-dimethylcyclopentano-perhydrophenantren* skeleton linked to a γ-lactone (five-membered) ring (**cardenolide**) or δ-lactone (six-membered) ring (**bufadienolide**) at C-17 in β-position. The glycosidation is occurred at C-3-β-OH. Die structural diversity based on the additional functionalities (OH, C=O etc) at different localities such as C-1, C-6, C-11, C-12, C-14, C-16 and the type and the number of sugars in the sugar chain. Desoxysugars (2-deoxy- or 2,6-dideoxy-hexose derivatives and their C-3-O-methyl derivatives are characteristic features of these glycosides.



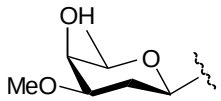
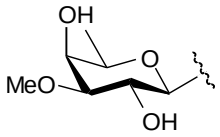
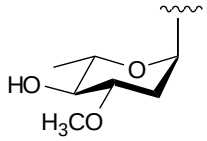
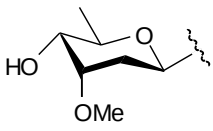
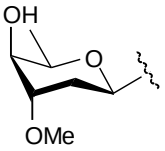
10,13-dimethylcyclopentano-perhydrophenantren



Cardenolide



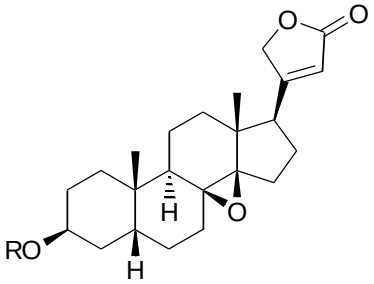
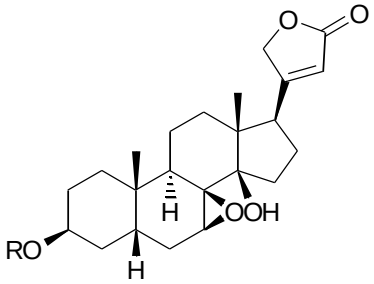
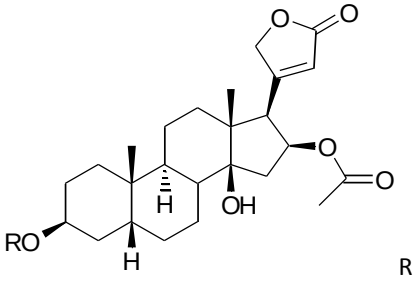
Bufadienolide

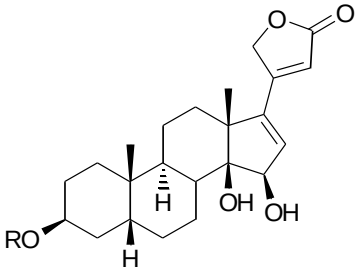
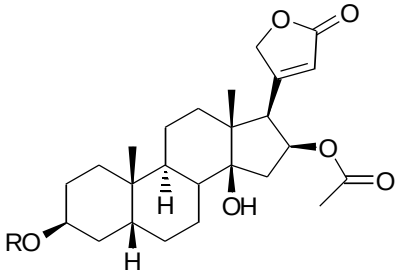
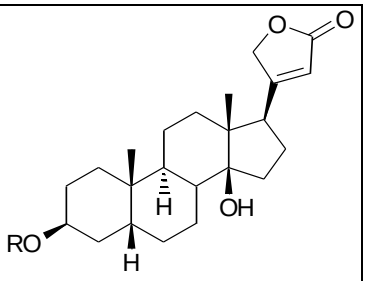
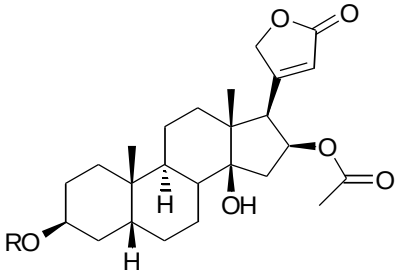
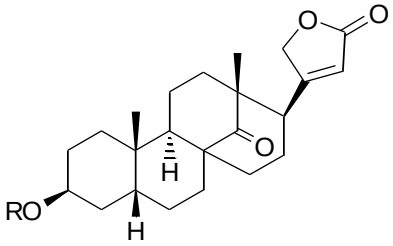
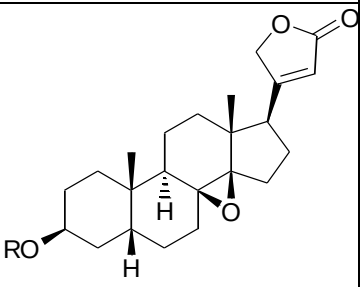
				
D-Diginose	**D-Digitalose**	**L-Oleandrose**	**D-Cymarose**	**D-Sarmentose**

Desoxysugars sugars observed as carbohydrate moiety of cardiac glycosides

CARDENOLIDE GLYCOSIDES from *Nerium oleander* L. (Apocynaceae)

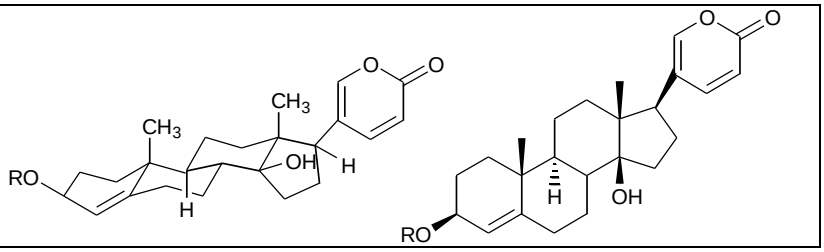
Example Structures for Cardenolide-type Aglycones and their Glycosides from *Nerium oleander* and other *Nerium* species.

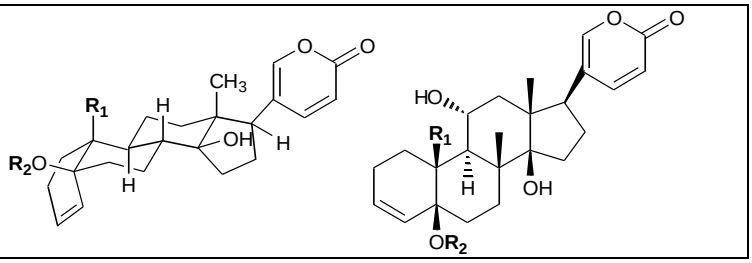
		
R= H, R= D- Digitalosyl*	R= H, R= D-Diginosyl*	= H, ***Oleandrigenin*** R= D- Digitalosyl*
Cardenolide B-1*	**Cardenolide B-2***	**Neritaloside***

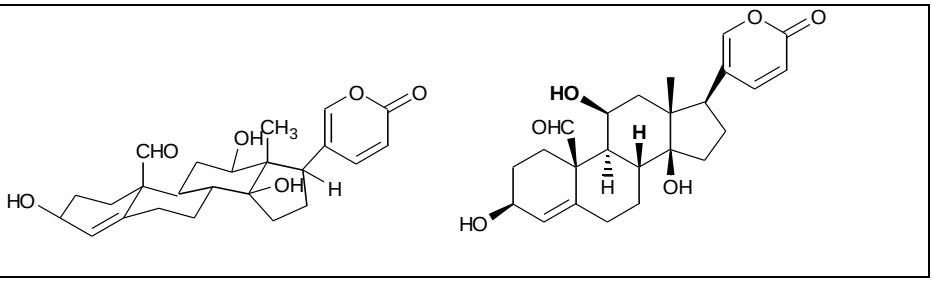
 R= H, R= D- Glucosyl* **Nerianthin***	 R= H, ***Oleandrigenin*** R= D- Diginosyl* **Odoroside A***	 R= H, ***Digitoxigenin*** R= D- Digitalosyl* **Odoroside H***
 R= H, ***Oleandrigenin*** R= D-Oleandrosyl* **Oleandrin***	 R= H, ***Oleagenin*** R= D-Diginosyl* **Oleaside***	 R= H, ***Adynerigenin*** R= D- Diginosyl* **Adynerin***

BUFADIENOLIDE GLYCOSIDES from *Urginea maritima* L. (Alliaceae)

The structures of some bufadienolides and their glycosides from *Urginea maritima* and other *Urginea* species.

Bufadienolides	**R**	
Scillarenin	H	
Proscillaridin A	Rha	
Scillarenin *3-O-β*-D-glucoside	Glu	
Scillaren A	Rha-Glu	

Bufatrienolides	**R_1**	**R_2**	
Scilliglaugenin	CHO	H	
Scilliglaucoside	CHO	Glu	

Bufatrienolides	
12*β*-Hydroxy-scilliglaucin	

A. Characterization

The concentration of cardiac glycosides is often low, and precludes the direct application of the different characterization methods: preparing purified and concentrated extracts is necessary.

The normal technique for the preparation of these extracts is a purification by precipitation with lead acetate. Extraction of the pulverized drug is performed by a mixture of 50% ethanol and lead acetate solution. After boiling, cooling and elimination of the residue by centrifugation, the cardiac glycosides present in the supernatant are extracted with chloroform. The chloroform solution is applied for the characterization reactions and chromatographic analyses.

> **Method for Extraction**: 1 g powdered drog (plant material) is extracted with a mixture of 20 ml 50% ethanol (EtOH) and 10 ml 10% Lead-(II)-acetate solutions under reflux. After cooling, the extract is filtered. The clear filtrate is extracted three times with 15 ml dichloromethane (DCM). After each extraction, lower phases (DCM) are combined and dried by filtration over anhydr-sodiumsulphate. The filtrate is evaporated to dryness amd the residue is dissolved with a mixture of 1 ml DCM and EtOH (1:1).

COLOUR REACTIONS: They can be due to sugars or to the aglycones. The color reactions capture characteristic features, this (1) the Butenolidring in the aglycone part in the case of cardenolide type aglycones like cardiac glycosides from *Digitalis* species and (2) the typical 2,6-Didesoxyzucker.

COLOR REACTIONS OF THE SUGARS

The only color reactions of the sugars that are of interest are those specific to 2,6-dideoxyhexoses. The reaction most frequently carried out is that using xanthydrol, also known as the **Pesez** reaction: addition of xanthydrol to a glycoside solution in concentrated acetic acid, heating in a water bath, and development of a red color.

The Keller-Kiliani: Addition of concentrated **sulfuric acid** containing traces of **ferric salts** to a glycoside solution in **concentrated acetic acid** also containing ferric salts; **a reddish-brown ring** develops and the acetic acid solution slowly turns blue-green.

> Glycosides such as ouabain (whose sugar moiety is rhamnose, i.e., a 6-deoxyhexose) do not produce this reaction, but other glycosides, which comprise 2,6-dideoxyhexoses, but are not cardioactive, do produce a positive reaction: for example digitanol glycosides comprising D-diginose (i.e., 2,6-dideoxy-3-methyl-D-galactose), such as diginoside or digifolein.

COLOR REACTIONS OF THE AGLYCONES

The classic reactions of steroids may be used, but their lack of specificity limits their usefulness.

In the case of the cardenolides, it is far more interesting to run specific reactions, linked to the presence of the α,β-unsaturated γ-lactone, such as the **Kedde** reaction or the **Baljet** reaction. For these reactions, an **aromatic nitro derivatives** are used in an alkaline medium (sodium hydroxide). A deeply colorred adduct with the lactone is occurred yielding a fairly stable purplish-red color.

The **Baljet reaction** uses **picric acid** and yields a stable **orangy** color.

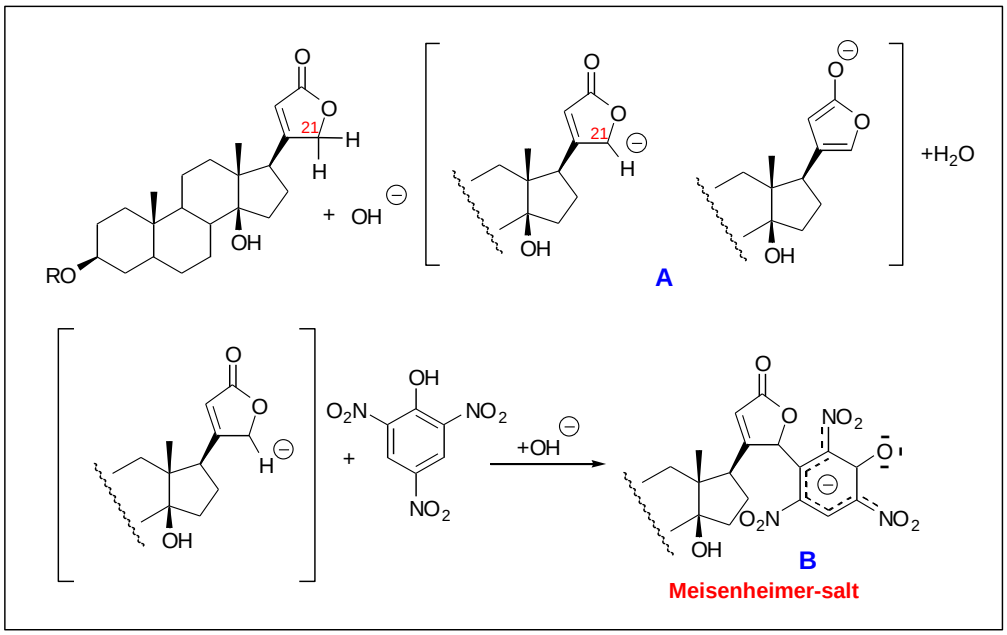
> *These reactions are negative with saponins, and either negative or much weaker with bufadienolides. The digitanol-glycosides sometimes give weakly positive reactions.*

Kedde Reaction: Addition of **3,5-dinitrobenzoic** and NaOH acid to the alcoholic solution of a cardenolide glycoside causes an intense violett colouring, in the presence of digoxin and digitoxin.

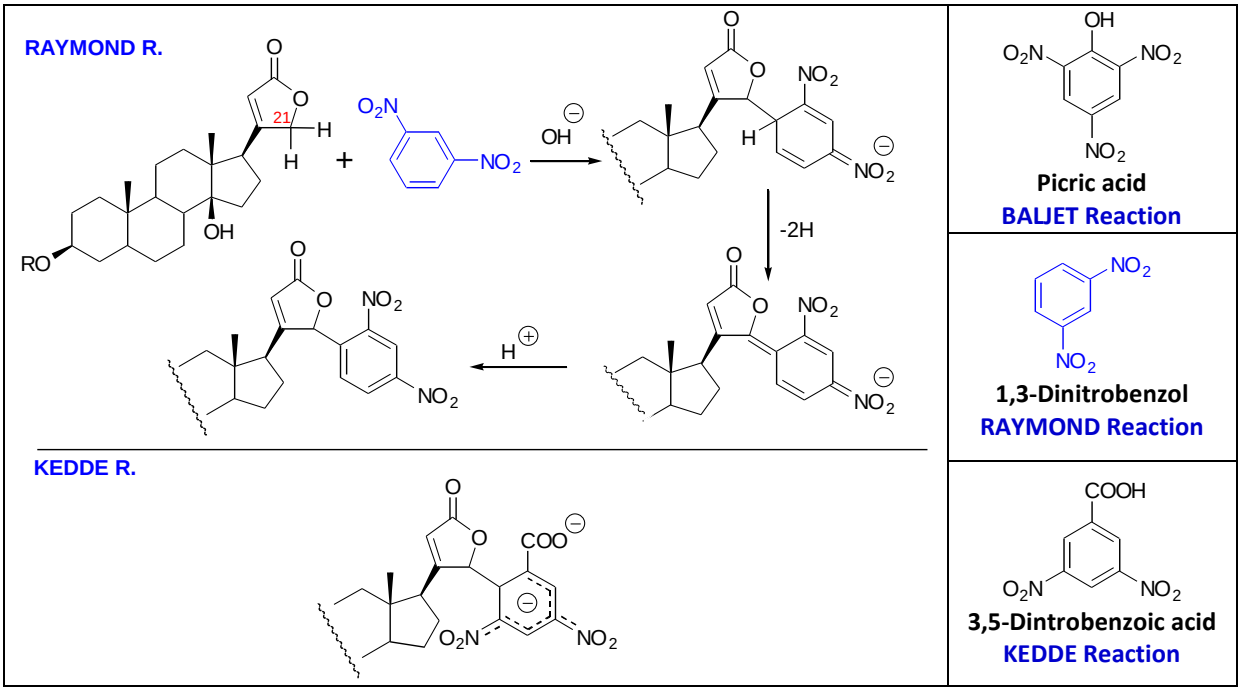
Raymond Reaction: As Kedde reaction, but instead of dinitrobenzoic **1,3-dinitrobenzene** yields blue color in the presence of oubain (g-strophantin).

BALJET Reaction: **Picric acid (= 1,3,5-dinitrophenol)** is used as an aromatic nitro derivative

Detection of Butenolidringes (**BALJET** R.): The most important color reactions, based on the presence of the Butenolid-ringes, are conversions with aromatic nitro compounds, for example the very long known as Baljet reaction. This reaction mechanism, cleared first in 1977, is in the alkaline environment of the activated Methylene-Group (C-21) of the Butenolid-ring easily cleaved a proton. The resulting carbanion (**A**) is added to a **aromatic nitro-derivative** (as **picric acid= 1,3,5-dinitrophenol**) yielding a coloured complex (Meisenheimer-salts) (**B**). In the presence of **picric acid**, an orange-coloured solution is occurred (Absorption max at 494 nm).



Reaction mechanism of **BALJET Reaction** (Aromatic nitro derivative: **picric acid= 1,3,5-dinitrophenol**)



Reaction mechanism of **RAYMOND** and **KEDDE Reactions**
(Aromatic nitro derivatives: **1,3-dinitrobenzene** and **3,5-dinitrobenzoic acid**, respectively)

FLUORESCENCE REACTIONS

Under acidic conditions, cardiac glycosides form fluorescent dehydrated derivatives: 14-dehydro derivatives, and in the case of aglycones substituted at C-16, 14,16-didehydro derivatives. In the latter case, the fluorescence is much more intense, since the resulting trienone has three double bonds conjugated with the carbonyl group. These reactions are mostly useful to visualize chromatograms (TLC).

JENSEN reaction: In practice, the **Jensen reaction** is used by spraying the plates with **trichloroacetic acid** in solution in ethanol. The simultaneous use of an oxidant (**chloramine T**) allows the observation of fluorescent spots of different colors, which facilitates the interpretation of the chromatograms.

TATTJE reaction: **Phosphoric acid** can also be used, alone or mixed with sulfuric acid and ferric chloride: after heating, a red color appears.

> *The reaction is much more sensitive with glycosides comprising an aglycone substituted at C-16 than with those of digitoxin (3,14-dihydroxy-cardenolide glycoside) or digoxin-type (3,12,14-trihydroxy-cardenolide glycoside).*

STRUCTURE ELUCIDATION

NMR Spectra of Cardenolides and Bufadienolides

Cardenolides

^{1}H NMR comparison of the cardenolides

The cardenolides showed complex ^{1}H NMR spectra due to the excessive signal overlap in the 1.0-2.5 ppm chemical shift area. The signals between 4.91-4.92 ppm (*dd*, *J* = 18.2-18.5, 1.5, H_2-21) and 5.90-5.94 ppm (s, H-22) are characteristic signals for the butenolide ring of the cardenolides. The other characteristic signals clearly observed in the spectra are germinal proton of C-3(OH) group (H-3), and the methyl groups located at C-10 and C-13 (Me-18 and Me-19, respectively). H-3 is observed at approximately at 4.00 – 4.10 ppm. Methyl resonances are easily detectable at high field of the spectra as three proton singlet signals (0-8 – 1.20 ppm). The sugar moieties showed shift ranges between 3.28 and 4.74 ppm with a methyl group between 1.21-1.29 ppm (*d, J*= 6.1- 6.7, H-6') for 6-deoxysugars as it can be seen in the ^{1}H-NMR Spectrum of Securigenin 3-β-*O*-[α-allopyranosyl-(1→4)-β-6-deoxyallose]. Anomeric protons are observed at more lower field (ca. between 4.40 – 5.20 ppm) in comparison to those of other sugar signals. Their multiplicities (d or dd) and coupling constants (J values) are indicative for normal series of sugars or 2-deoxy- and 2,6-dideoxy-hexose derivatives.

On the other hand, in some cardenolides methyl (-CH_3) group located at C-18 can be oxidized to -CH_2OH, CHO or COOH. The aldehyde group on C-19 of securigenin 3-β-*O*-[α-allopyranosyl-(1→4)-β-6-deoxyallose] at 9.4 ppm is shifted (**Fig. 3.7**). The 19-hydroxy-sarmentogenin is characterized by an alcohol function at C-19, replacing the aldehyde function of securigenin glycosides. The two doublets were shifted to higher fields and resonated between 3.86 and 3.71 ppm (each *d, J* = 10.8 Hz) (**Fig. 3.8**).

Further characteristic signals for the cardiac glycosides are signals of H-17. Its multiplicities ("*t*" of *dd* or *d*) are based on the lack or presence of the functionalities at C-16. In case of 19-hydroxy-sarmentogenin, H-17 is observed at 2.93 ppm as *t (dd)* with a *J* value (coupling constant) of 6.9-7.1Hz (**Figs. 3.8 & 3.9**).

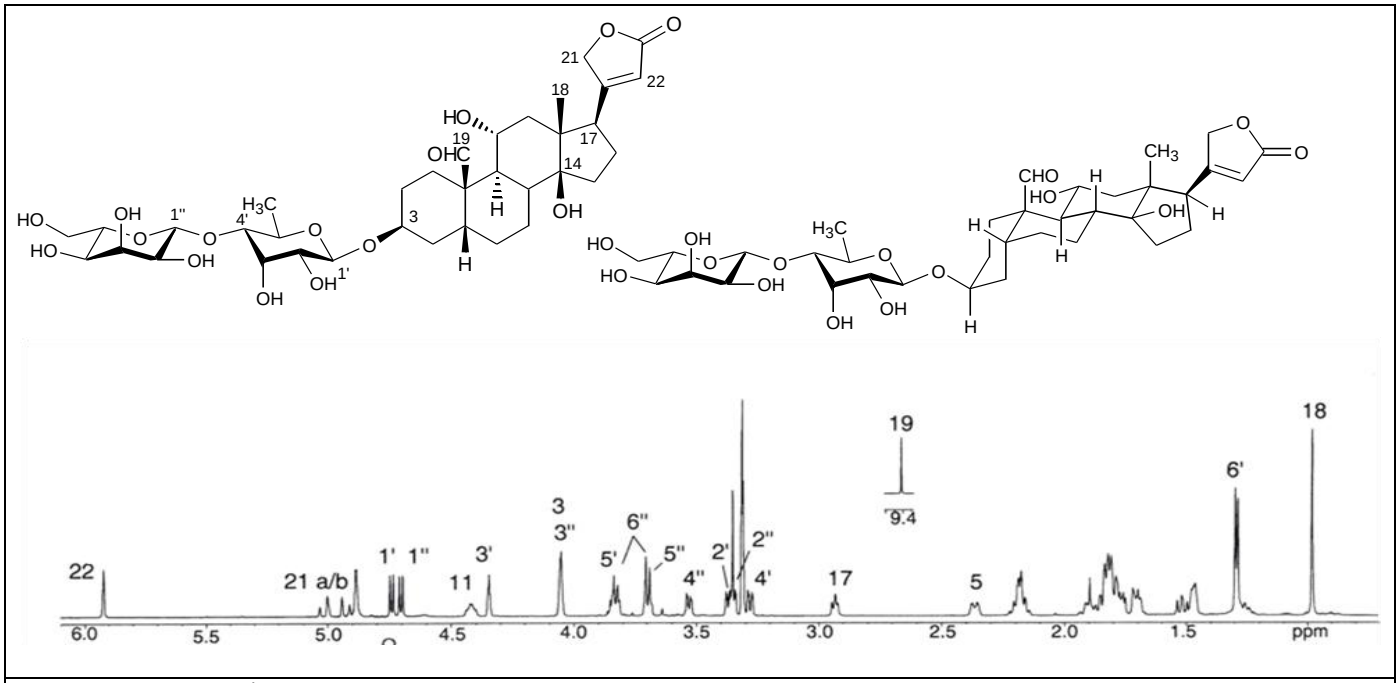


Fig. 3.7. The ^{1}H-NMR Spectrum of **Securigenin 3-β-*O*-[α-allopyranosyl-(1→4)-β-6-deoxyallose]**
(from Diss. ETH No. 13555, Anita ANKLI, 2000)

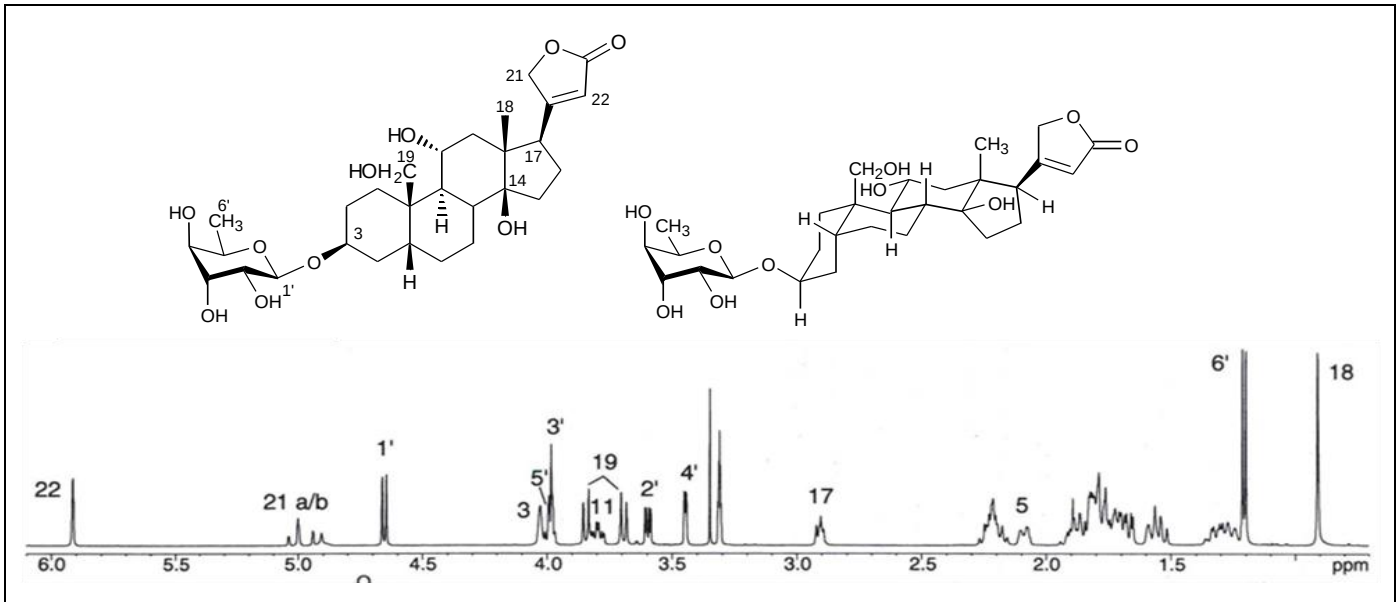


Fig. 3.8. The ^{1}H-NMR Spectrum of **19-hydroxy-sarmentogenin 3-β-*O*-6-deoxygulose**
(from Diss. ETH No. 13555, Anita ANKLI, 2000)

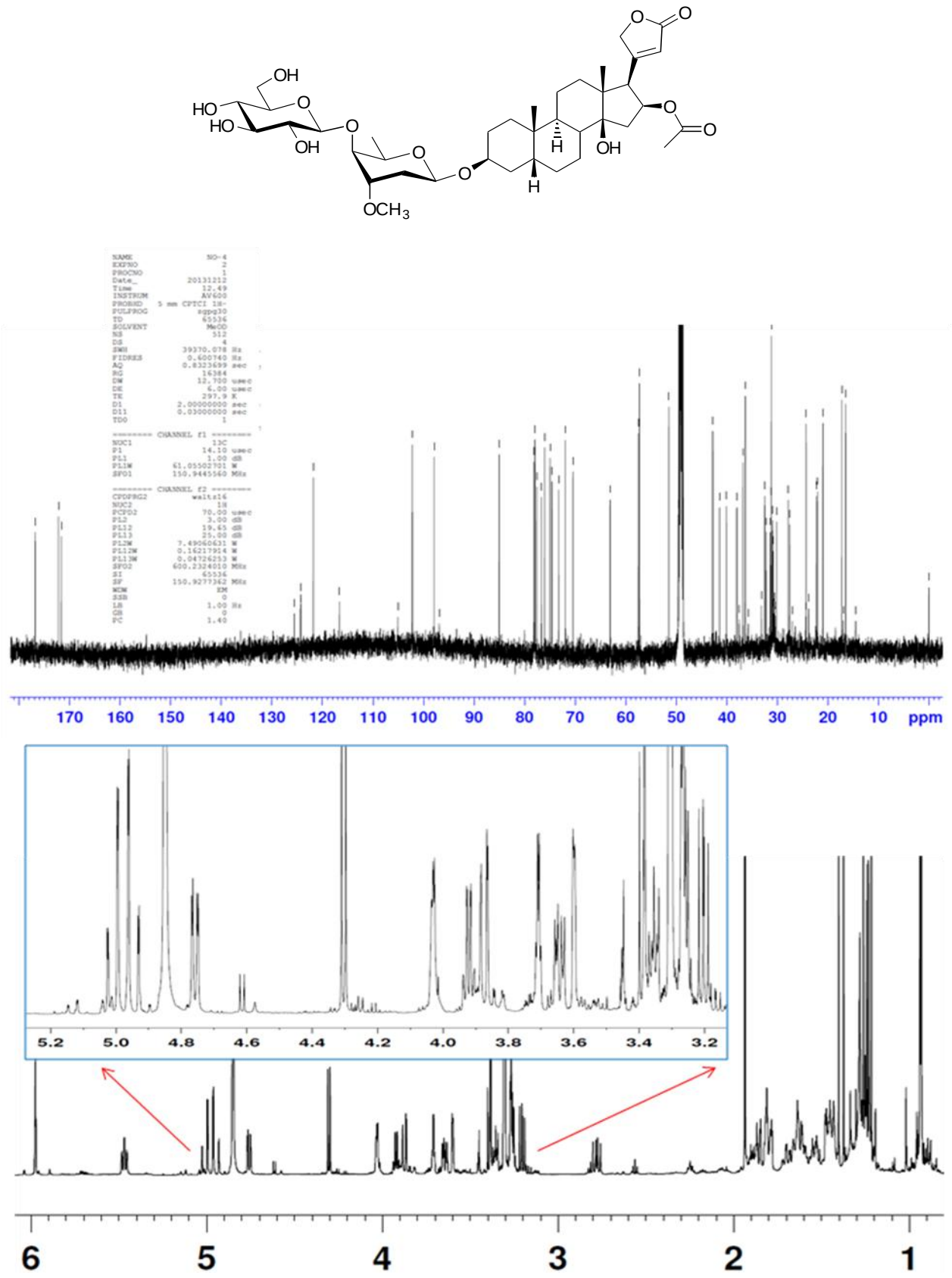


Figure 3.9. The ^{1}H- and ^{13}C-NMR Spectra of Oleandrigenin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-sarmentopyranoside from *Nerium oleander* (^{1}H: 600 MHz; ^{13}C: 150 MHz, MeOD).

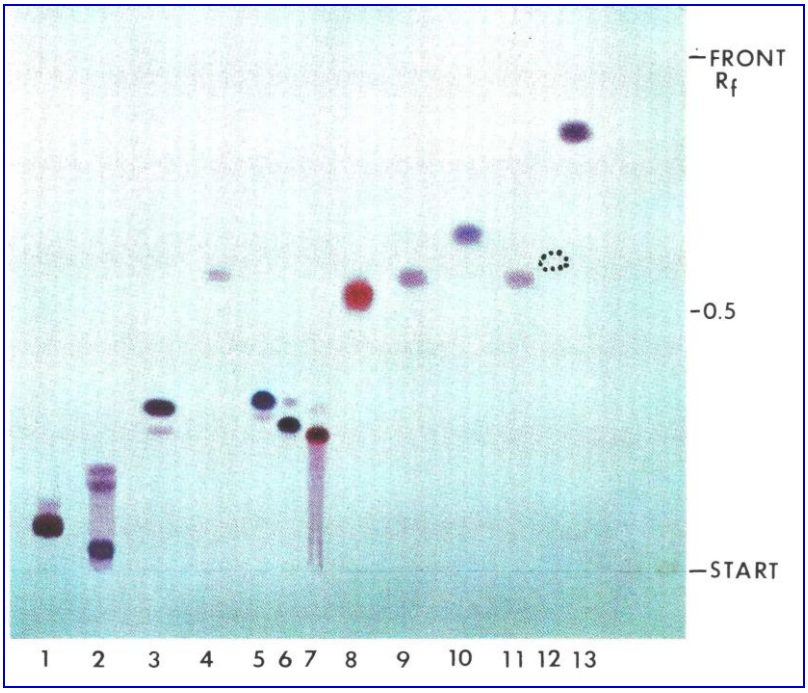
Ref. **Ceyda ERTAYLAN**, NEPHAR 501 GRADUATION PROJECT, NEU, Faculty of Pharmacy, 2013.

Thin Layer Chromatography (TLC) of Cardiac Glycosides (Cardenolides&Bufadienolides)

Method for Extraction: 1 g powdered drog (plant material) is extracted with a mixture of 20 ml 50% ethanol (EtOH) and 10 ml 10% Lead-(II)-acetate solutions under reflux. After cooling, the extract is filtered. The clear filtrate is extracted three times with 15 ml dichloromethane (DCM). After each extraction, lower phases (DCM) are combined and dried by filtration over anhydr-sodiumsulphate. The filtrate is evaporated to dryness and the residue is dissolved with a mixture of 1 ml DCM and EtOH (1:1).

Adsorban : Silica gel 60 F254
Application : 20 µL extraxt
Solvent system : Ethylacetate – Methanol – H_2O (81:11:8)
Detection 1 : KEDDE Reagent (A mixture of solutions A and B is sprayed on TLC).
Solution A: 3% 3,5-Dinitrobenzoic acid;
Solution B: 2N NaOH
Cardenolides exhibit a rose or blue-violet colours.
Bufadienolides do not give any colur.

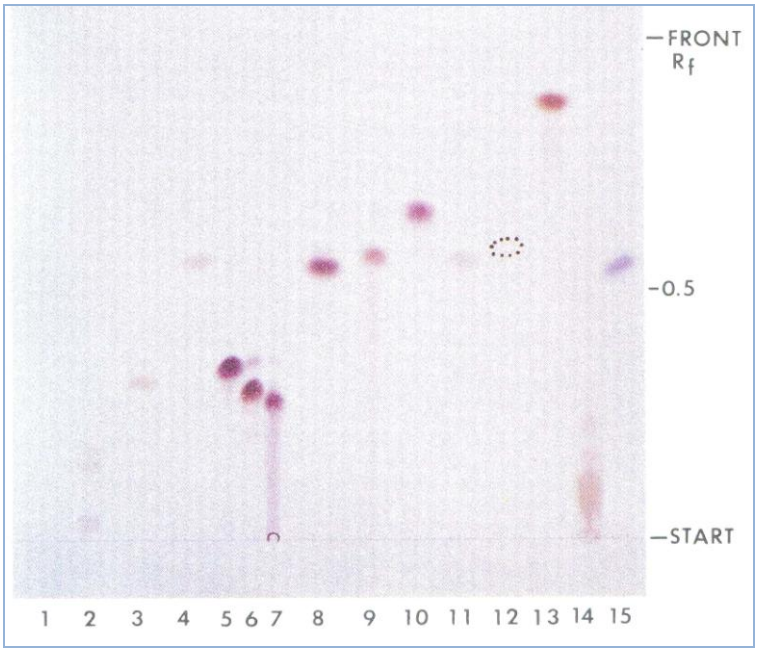
Detection 2 : Cons. H_2SO_4 (After spraying, TLC plate is heated at 100°C for a few minutes.
Cardenolides and ***bufadienolides*** are observed as fluorescens producing blue, brown, green or yellow coloured spots under UV-365 light.



TLC of Cardiac Glycosides (H. Wagner *et al.,* 1983)

Solvent system:	Ethylacetate – MeOH – H_2O (81:11:8)
Reagent:	Kedde Reagent

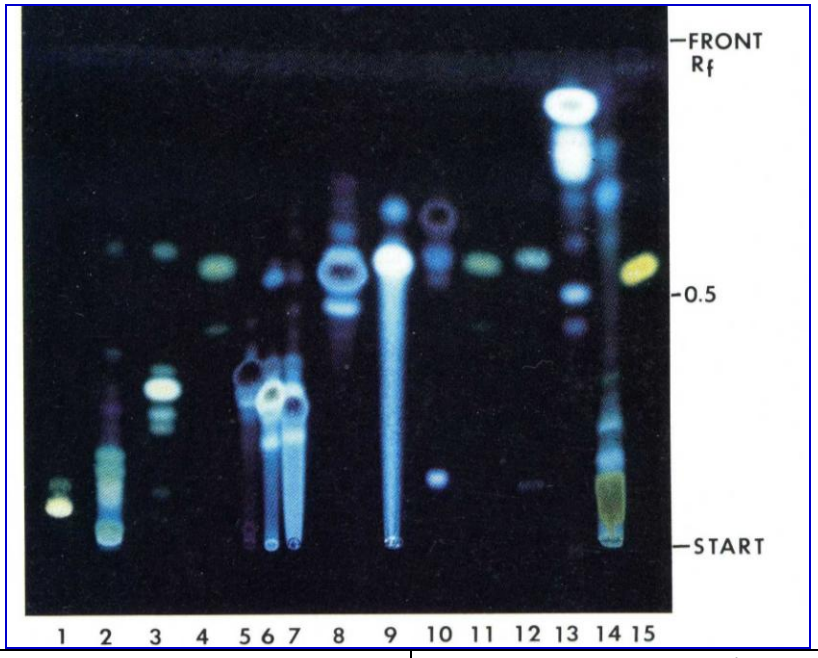
1: **g-Strophanthin**	8: **Digoxin**
2: **k-Strophanthin**	9: **Gitoxin**
3: **Convallotoxin**	10: **Digitoxin**
4: **Cymarin**	11: **Cymarol**
5: **Lanatoside A**	12: **Peruvoside**
6: **Lanatoside B**	13: **Oleandrin**
7: **Lanatoside C**	



TLC of Cardiac Glycosides (H. Wagner *et al.,* 1983)

Solvent system:	Ethylacetate – MeOH – H_2O (81:11:8)
Reagent:	Conc. H_2SO_4, day light

1: **g-Strophanthin**	8: **Digoxin**
2: **k-Strophanthin**	9: **Gitoxin**
3: **Convallotoxin**	10: **Digitoxin**
4: **Cymarin**	11: **Cymarol**
5: **Lanatoside A**	12: **Peruvoside**
6: **Lanatoside B**	13: **Oleandrin**
7: **Lanatoside C**	14: **Hellebrin**
	15: **Proscillaridin**



Solvent system:	Ethylacetate – MeOH – H_2O (81:11:8)
Reagent:	Cons. H_2SO_4 and UV 365 nm

The List of the Cardioactive Glycoside Drugs (Cardiac Drugs)

	Drugs	***Plant name***	**Family**	**Constituents**
CARDENOLIDE-TYPE	Digitalis lanata folium	*Digitalis lanata*	Scrophulariacaea	Lanatosides A - E
	Digitalis purpurea folium	*Digitalis purpurea*	Scrophulariacaea	Purpureaglycosides A – B Digitoxin, Gitoxin, Gitaloxin
	Nerii folium	*Nerium oleander*	Apocynaceae	Oleandrin, Odorosides A, H Oleaside A, E, Adynerin
	Adonidis herba	*Adonis vernalis*	Ranunculaceae	Adonitoxin k-strophanthidin glycoside
	Convallaria herba	*Convallaria maialis*	Alliaceae	Convallotoxin, Convalloside, Glucoconvalloside
	Strophanti grati semen	*Strophanthus gratus*	Apocynacaea	g-strophanthin Sarmentosides A, D, E
	Xysmalobii radix (Uzarae radix)	*Xysmalobium undulatum*	Asclepiadacea	Uzarin, Xysmalomonoglucoside
	Thevetia folium	*Thevetia peruviana*	Apocynacaea	C-nor-D-homo-cardenolides Thevetoside
BUFADIENOLIDE-TYPE	Hellebori radix	*Helloborus niger* *Helloborus odorus* *Helloborus viridis*	Ranunculaceae	Hellebrin Hellebrigenin glycosides
	Scillae bulbus	*Urginea maritima*	Alliacaea	Proscillaridine Scillaren A Glucoscillaren Scilliroside Glucoscilliroside

5.1. Oleanane-type Triterpene Saponins from *Cyclamen* species (Myrsinacaea): Identification and Characterization of Cyclamin

İ. Çalış, A. Yürüker, N. Tanker, Anthony D. Wright, O. Stricher. **Triterpene Saponins from *Cyclamen coum* var. *coum*. Planta Med. 63**, 166 - 170 (1997); İ. Çalış, M. E. Şatana, A. Yürüker, P. Kelican, R. Demirdamar, R. Alaçam, N. Tanker, H. Rüegger, O. Stricher. **Triterpene Saponins from *Cyclamen mirabile* and their Biological Activities. J. Nat. Prod. 60**, 315 - 318 (1997); İ. Çalış&O. Sticher, Triterpene Saponins from Plants of the Flora of Turkey. **Saponins used in Traditional and Modern Medicine**, Plenum Press, New York, 1996.
http://www.cyclamen.org/medicine_set.html

Cyclamen persicum Mill., February 2014, Çayırova, Photo: İhsan Çalış

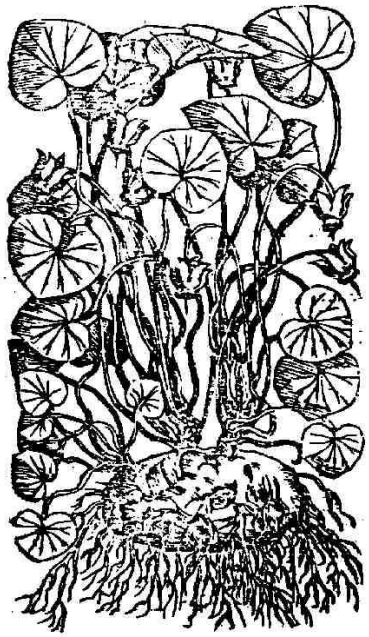
Cyclamen L. is common geophyte cultivated throughout temperate zones and it is a genus of twenty species within the family **Myrcinaceae** (formerly Primulaceae). *Cyclamen* species inhabit a range from eastern and southern France, Switzerland, Italy, Austria, Croatia, Bosnia, Serbia, Albania, Greece, Bulgaria, Turkey, Georgia, the Caucasus, Iraq, Iran, Syria, Lebanon, Israel, diverse parts of North Africa including parts of Tunisia, Algeria and Libya, Northern Somalia, and many Mediterranean islands including the Baleariacs, Corsica, Sardinia, Malta, Sicily, Cyprus, and the Greek islands of the Ioannian Sea, Saronic Islands, Sporades, Cyclades, Aegean Islands, Crete and the Dodecanese.

The genus *Cyclamen* is represented by ten species in Anatolia (Turkey) and tree species in Cyprus. *Cyclamen* species are known as tavşan kulağı (=davşankulağı), dağ menekşesi, domuz ekmeği, deve tabanı and topalak at different regions of Turkey and Cyprus. Several investigations on the different

species of Cyclamen have resulted in the isolation of oleanane-type triterpene saponins as exemplified above. The tubers of *Cyclamen coum* Miller are used in Turkish folkloric medicine against infertility by women as an ovule in their natural form (after removing the outer surface). Several *Cyclamen* species are widely used in traditional folk medicine for their laxative, abortive, purgative, emmenagogue and anti-helmintic properties. Tubers of *Cyclamen* species have been used against worms afflicted to the roots of *Nicotiana* tobaccum in the agriculture.

They are tuberous plants and have no obvious affinity with Primroses, although they do resemble the North American *Dodecatheon* in having reflexed petals. Their habitats range from *Fagus* (Beech) woodland, through scrub and rocky areas, to alpine meadows where they flower in snow meltwater. The genus is notable for the fact that although it is small, there are species which flower in every month of the year.

Cyclamen were put to many medicinal uses during the first few centuries A.D. according to Pedacio (or Padanius) Dioscorides, a Greek military surgeon and naturalist of the first century.



Gerard's illustration: ***Cyclamen hederifolium***

Many centuries later Gerard in his Herbal says - 'it is reported to me by men of good credit, that cyclamen or sow-bread growth upon the mountains of Wales; on the hills of Lincolnshire and in Somerset-Shire. Being beaten and made up into trochisches, or little flat cakes, it is reputed to be a good amorous medicine to make one love, if it be inwardly taken'.

http://www.cyclamen.org/medicine_set.html

In modern herbals, *Cyclamen* are described as a 'Self-esteem builder essence', which allows the person to get in touch with their self-esteem and confidence. A homeopathic tincture is made from fresh tubers and is applied as a liniment externally over the bowels, causing purging. The fresh tubers, bruised, and made into a cataplasm, make a stimulating application to indolent ulcers. An ointment called 'Ointment of Arthainta' was made from the fresh tubers for expelling worms, and was rubbed on the umbilicus of children and on the abdomen of adultsto cause emesis, and in the region over the bladder to increase urinary discharge.

Dermatology

There are a number of references to dermatological problems with *Cyclamen*, but these are rare and no growers known to the author have been effected in any way.

Animals

The popular name 'Sowbread' comes from the fact that the tubers were a source of food for wild boar. There is a report that *Cyclamen* are poisonous to cats and fish.

5.1. Identifications of Saponins from *Cyclamen persicum* Mill.
5.2. Pharmacognostical Standardization: foaming Index

Method for Extraction: 1 g powdered drog (tubers: underground part of the plant) is extracted with a mixture of 5 mL MeOH. After cooling, the extract is filtered and concentrated to 2-3 mL using rotary evaporator. The concentrated extract is directly used for TLC (A) and colour reactions (B).

6.1.1. **Thin Layer Chromatography (TLC)**: For thin layer chromatography, 5 and 10 µL filtrates are applied in the presence of reference saponins, cyclamin, isocyclamin and deglucocyclamin.

Reference saponins: Pure glycosides are solved in MeOH (1mg/1 mL MeOH), 5 µL is applied to TLC.

Thin Layer Chromatography (TLC):

Adsorban : Silica gel 60 F254
Application : 5 - 10 µL extraxt
Solvent system : Dichloromethane – Methanol – H_2O (61:32:7)
Detection : ***Solution A***: 1%Vanillin in MeOH
Solution B: 5% H_2SO_4 in H_2O
Saponins exhibit a red or violet colours.

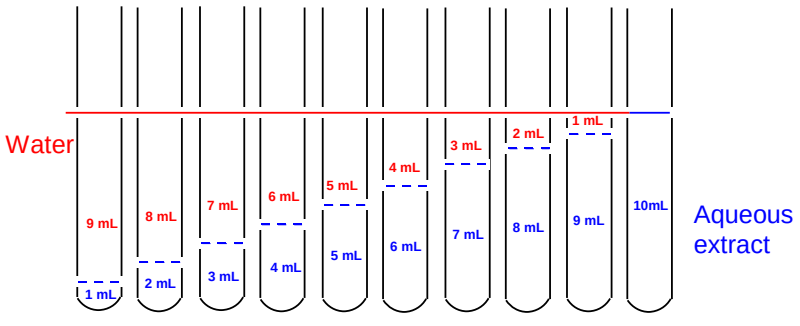
6.1.2. **Colour Reaction**: 0,5 ml concentrated extract is transferred to reagent glass. A few drops of cons. H_2SO_4 are added to the extract.

The colour of solution occurred is recorded.

6.2.1. **The foam value** (Fr. Ph., 10th Ed., V.4.A):

The foam value in other words: "the dilution of a decoction of the drog which, in the prescribed conditions, produces a lasting foam". In practice, this value is determined on a decoction obtained by prolonged boiling (30 minutes) of 1 g of drug in 100 mL of water. A series of calibrated tubes contains increasing dilutions of this decoction.

The tubes are agitated: the foam value is the drug dilution in the tube that gives 1 cm of foam after 15 minutes at rest.

1g Drug; Extraction with water Filtration Complete the volume of the filtrate to 100 mL using a flask.		

6. Cardenolide Glycosides from *Nerium oleander* L. (Apocynaceae)

İ.Çalış, Ceyda ERTAYLAN, Cardioactive Glycosides and Their Anticancer Activities from the Flora of Cyprus, Emphasizing on *Nerium oleander* L., Near East University, Faculty of Pharmacy, Graduation Project, 2013

Nerium oleander L., N. Cyprus, June 2013, Photo: İhsan ÇALIŞ

Introduction

Nerium oleander L. is an evergreen shrub or small tree in the dogbane family Apocynaceae, toxic in all its parts. It is most commonly known as **oleander** and widely cultivated that no precise region of origin has been identified, though southwest Asia has been suggested. This plant is represented in the flora of Turkey as well as Cyprus and is known as "Zakkum, Ağu Çiçeği, Kanağacı, Zıkkım ağacı, Avu, Ayan, Fattak" in Turkish folk medicine. It is commonly known as "kaner" and its various parts are reputed as therapeutic agents in the treatment of swellings, leprosy, and eye and skin diseases. Various parts of the plant are used in traditional medicine because of their antibacterial, anticancer, antileprotic and cardiotonic properties.

Nerium oleander has historically been considered a poisonous plant because some of its compounds may exhibit toxicity, especially to animals, when consumed in high amounts. It is one of the most poisonous of commonly grown garden plants. Among the compounds isolated from all parts of the plant, oleandrin is a cardenolide type cardiac glycoside, which is one of the toxic glycosides of the plant. Odoroside, neritaloside, nerianthin, nerizoside, oleasides A – D, adynerin are some of the

further examples for cardenolide type glycosides. In addition to several cardenolides, *Nerium oleander* contains a number of secondary metabolites such as triterpenes, pregnanes, flavonoids.

The cardiac glycosides are a group of natural natural compounds occurring in a limited number of plant families. Digitoxin and digoxin have been used in the treatment of cardiac diseases for many years, but they have a narrow therapeutic window because of arrythmia and disturbance of atrio-ventricular contraction. Recent studies shows that cardiac glycosides have cytotoxic activity and anticancer effect and are used for treatment of several different cancer types.

Table 3.3. Cardenolide-type Aglycones and their Glycosides from *Nerium oleander* and other *Nerium* species.

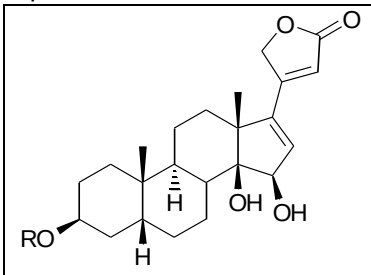
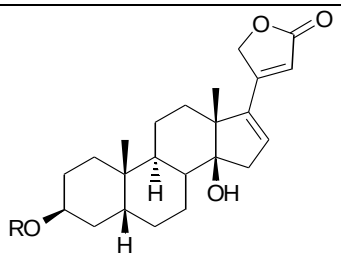
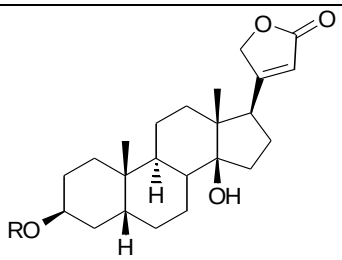
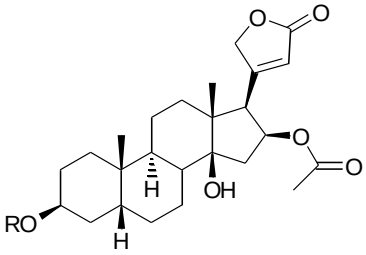
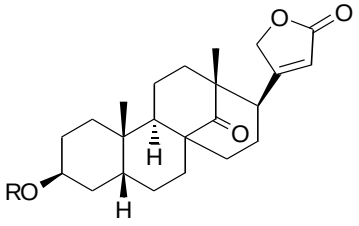
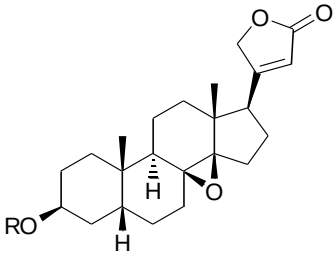
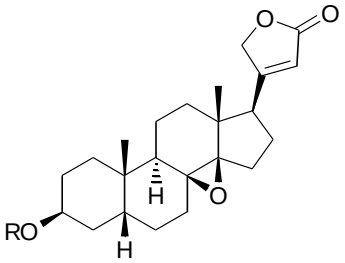
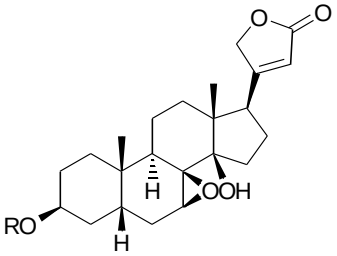
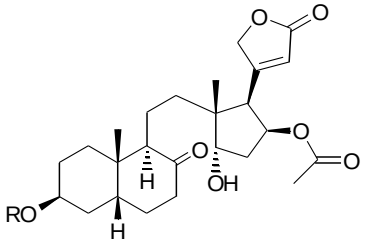
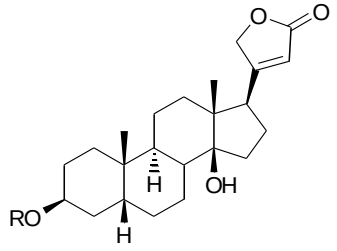
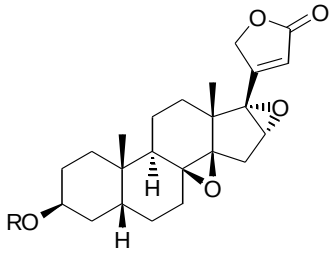
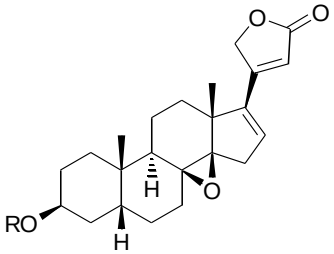
 R= H	 R= H	 R= H ***Digitoxigenin***
R= D- Glucosyl **Nerianthin**	R= 2-*O*-methyl-D-Digitalosyl **Nerizoside**	R= D- Digitalosyl **Odoroside H**
	R= D-Sarmentosyl **3β-*O*-(β-D-sarmentosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide**	R= Oleandrosyl **3β-*O*-(α-L-oleandrosyl)-Digitoxigenin**
 R= H ***Oleandrigenin***	 R= H, ***Oleagenin*** **(8*R*)-3β-hydroxy-14-oxo-15(14→8)*abeo*-5β- cardenolide**	 R= H ***Adynerigenin***
R= D-Oleandrosyl **Oleandrin**	R= D-Diginosyl **Oleaside A**	R= D-Diginosyl **Adynerin**
R= D-Diginosyl **Odoroside A**	R= D-Digitalose **Oleaside B**	R= D-glucosyl-(1→4)-D-Diginosyl **3β-*O*-(β-D-glucopyranosy-(1→4)-β-D-diginopyranosyl)-8,14-epoxy-5β,14β-cardenolide**
R= D-Sarmentosyl **3β-*O*-(β-D-sarmentosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide**	R= D-glucosyl-(1→4)-D-Diginosyl **Oleaside C**	R= D-glucosyl-(1→4)-D-Digitalosyl **3β-*O*-(β-D-glucopyranosyl-(1→4)-β-D-digitalopyranosyl)-8,14-epoxy-5β,14β-cardenolide**
R= D-Digitalose **Neritaloside**	D-glucosyl-(1→4)-D-Digitalosyl **Oleaside D**	

Table 3.3 (cont.). Cardenolide-type Aglycones and their Glycosides from *Nerium oleander* and other *Nerium* species.

 R= H **3β-hydroxy-8,14-epoxy-card-20(22)-enolide**	 R= H **3β,11β-dihydroxy-7β,8-epoxy-card-20(22)-enolide**	 R= H ***Neriagenin***
R= D-Digitalosyl **Cardenolide B-1**	R= D-Diginosyl **Cardenolide B-2**	R= D-glucosyl-(1→4)-D-Diginosyl **3β-*O*-(β-D-glu-(1→4)- β-D-dig)-14α-hydroxy-8-oxo-8,14-sec-5β,14β-card-20(22)-enolide**
 R=H **Digitoxigenin**	 R=H **3β-hydroxy-8,14;16α,17-diepoxy-5β,14β-card-20(22)-enolide**	 R= H **3βhydroxy-8,14-epoxy-5β,14β-card-16,20(22)-dienolide**
R= Sarmentosyl **3β-*O*-(β-D-sarmentosyl)-digitoxigenin**	R= Diginosyl **3β-*O*-(β-D-diginosyl)-8,14;16α,17-diepoxy-5β,14β-card-20(22)-enolide**	R= Sarmentosyl **3β-*O*-(β-D-sarmentosyl)-8,14-epoxy-5β,14β-card-16,20(22)-dienolide**
		R= Diginosyl
		3β-*O*-(β-D-diginosyl)-8,14-epoxy-5β,14β-card-16,20(22)-dienolide

6. Identifications of Cardenolide Glycosides from *Nerium oleander*

Method for Extraction: 5 g powdered drog (plant material) is extracted with a mixture of 50 ml 50% ethanol (EtOH) and 20 ml 10% Lead-(II)-acetate solutions under reflux. After cooling, the extract is filtered. The clear filtrate is extracted three times with 15 ml dichloromethane (DCM). After each extraction, lower phases (DCM) are combined and dried over anhydr-sodiumsulphate. The filtrate is used for TLC (**A**) and colour reactions (**B**).

A. **Thin Layer Chromatography (TLC)**: For thin layer chromatography, 5, 10 and 20 µL filtrates are applied in the presence of reference cardenolide glycosides

Reference cardenolides: Pure glycosides are solved in MeOH (1mg/1 mL MeOH), 5 µL is applied to TLC.

Thin Layer Chromatography (TLC):

Adsorban : Silica gel 60 F254
Application : 20 µL extract
Solvent system : Ethylacetate – Methanol – H_2O (81:11:8)
Detection 1 : KEDDE Reagent (A mixture of solutions A and B is sprayed on TLC).
Solution A: 3% 3,5-Dinitrobenzoic acid;
Solution B: 2N NaOH
Cardenolides exhibit a rose or blue-violet colours.
Bufadienolides do not give any colour.

Detection 2 **: sprayed with vanillin-H_2S0_4 reagent and heated to 110 °C for 5-10 min,** Evaluation under visible and under UV light 366 nm.

Color reaction on planar chromatography (TLC)

The cardiac glycosides showed characteristic color reactions on TLC after detection with vanillin-H_2S0_4 reagent and heated to 110 °C for 5-10 minutes. The color pattern included greenish to intense blue colors, depending on the substitution pattern of the genin and the sugar moieties. Only cardenolides, which are not oxidized on C-19, respond with a blue color.

B1.Colour Reaction: 0,5 ml filtrate is transferred to reagent glass. Solutions A (**3% 3,5-Dinitrobenzoic acid**) and B of (**2N NaOH**) are added to the filtrate, respectively. The colour occurred in a few seconds is recorded (**KEDDE Reaction**).

Not: Instead of **3,5-dinitrobenzoic acid**, **picric acid** can also be used.

B2. The Keller-Kiliani test for deoxysugars: Glycoside is dissolved in 99% acetic acid (or glycoside solution is diluted with acetic acid). A few drops of 10% ferric(III)chloride solution is added. This mixture is transferred to the surface of **concentrated sulphuric acid (conc. H_2SO_4).** At the junction of the liquids **a reddish-brown coloured ring** is developed which gradually becomes blue-green.

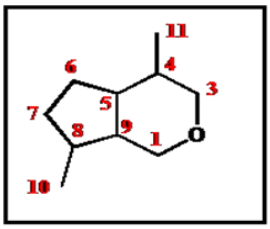
Appendix

MONOTERPENES: IRIDODS & SECOIRIDODS

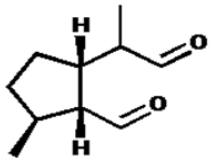
Ref. J.Bruneton, **Pharmacognosy,** Phytochemistry, Medicinal Plants, 4th Ed., Lavosier, 2009

Introduction

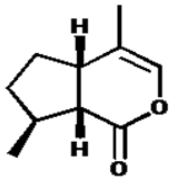
Iridoids in the strict sense of the term are monoterpenes characterized by a cyclopenta[c]pyranoid skeleton, also known as the iridane skeleton (cis-*2*-oxa-bicyclo-[4,3,0]-nonane). In the broad sense, it is acceptable to include in this group the secoiridoids, which arise from the latter by cleavage of the 7,8 bond of the cyclopentane ring. Some authors even limit their definition to the concept of "methylcyclopentane". **Structurally related alkaloids (e.g., skytanthine) are merely extraction artefacts formed by the replacement of the pyran ring oxygen atom by a nitrogen atom.**



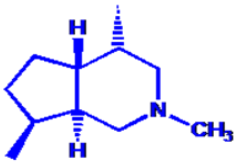
Iridane



Iridodial



Nepetelactone



Skytanthine

The iridoids were named after ants of the Iridomyrmex genus, from which were isolated compounds involved in the defense mechanisms of these insects: iridodial, iridomyrmecin, and related compounds. Such simple structures also exist in plants; for example, nepetalactone from *Nepeta cataria* L. (Lamiaceae), or teucriumlactone C from *Teucrium marum* L. have marked properties (the effects of the former on cats earned it some evocative names: catnip, Katzenmelisse, herbe-aux-chats). Iridoids normally contain ten carbon atoms, may also have more, and present multiple structural variations, ranging from simple functionalization to the formation of polycyclic structures.

Iridomyrmex purpureus

http://de.wikipedia.org/wiki/Iridomyrmex

The group is biosynthetically homogeneous, and is represented, except for the few structures specific to insects, only in the Dicotyledon Angiosperms. They are elaborated preferentially by gamopetalous plants: Dipsacales, Gentianales, Lamiales, Scrophulariales; which makes them interesting chemotaxonomic markers.

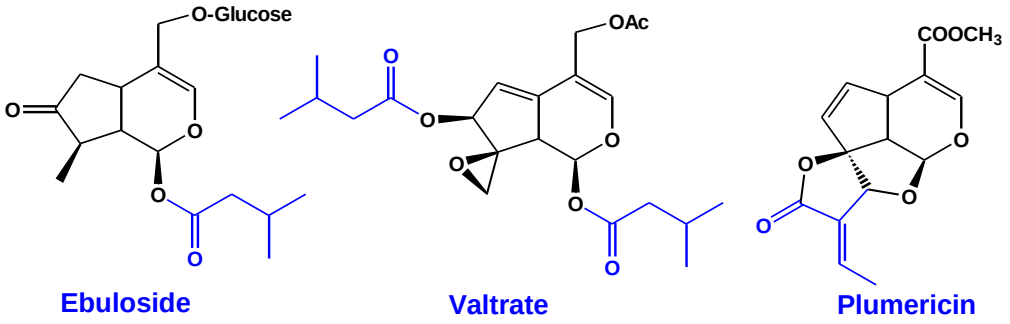
STRUCTURE OF IRIDOIDS

The majority of iridoid glycosides in the broad sense of the term are glucosides, with the glycosidic linkage established between the hydroxyl group on the anomeric carbon of D-glucose and the hydroxyl in the 1-position of the aglycone. Also known are structures in which the glucose is linked to the 11-hydroxymethyl

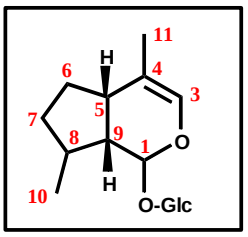
group (e.g., ebuloside in Caprifoliaceae). Non-glycosidic iridoids may be, among other things, alkaloids (skytanthine), polycyclic compounds (plumericin), polyesters (valepotriates), or intramolecular ethers (rehmaglutin B). Non-glycosidic secoiridoids are exceptional (*Syringa* sp., *Olea* sp. [Oleaceae]).

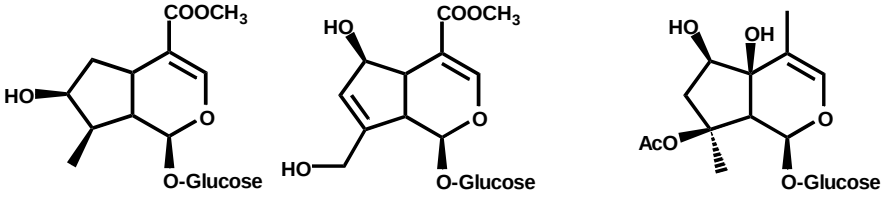
Iridoids generally have ten carbon atoms. When there is a C-11, it is generally part of a carbomethoxyl group (loganin, geniposide) or part of a carboxylic acid group (monotropein); in rarer cases, this group is replaced by a hydroxymethyl group (Valerianaceae, Caprifoliaceae), or by an aldehyde or methyl group (lamioside). In a certain number of cases, the C-ll is absent (aucubin, catalpol, harpagoside). The pyran ring is only exceptionally open (for example, in the case of the gentiobioside of iridodial and of nepetariaside, the precursor of nepetalactone).

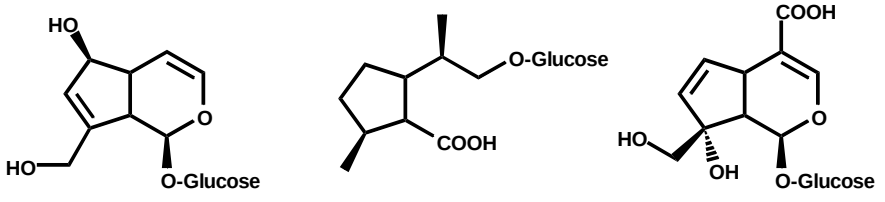
There are multiple structural variations. They have allowed some authors to propose subdivisions within the group. The methyl group which is normally at C-8 can be more or less oxidized: examples include a hydroxymethyl group (aucubin, monotropein) and an epoxide (valtrate); it is rarely absent (deutzioside). There may be an unsaturation at C-7(8) (geniposide, aucubin), which may become a center of oxidation **(catalpol) or hydration (lamioside). Note the possible oxidation of C-6 (aucubin, verbenalin, harpagoside) and the potential unsaturation at C-6(7) (monotropein).**

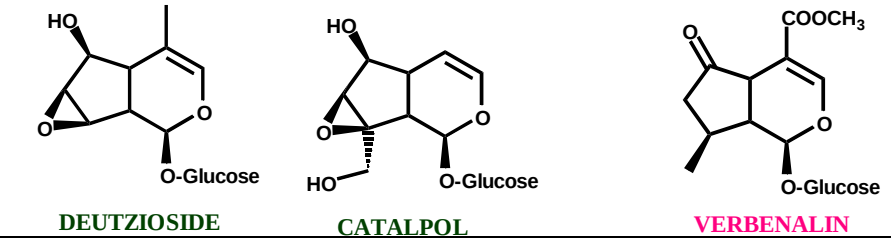


Non-glycosidic Iridoids



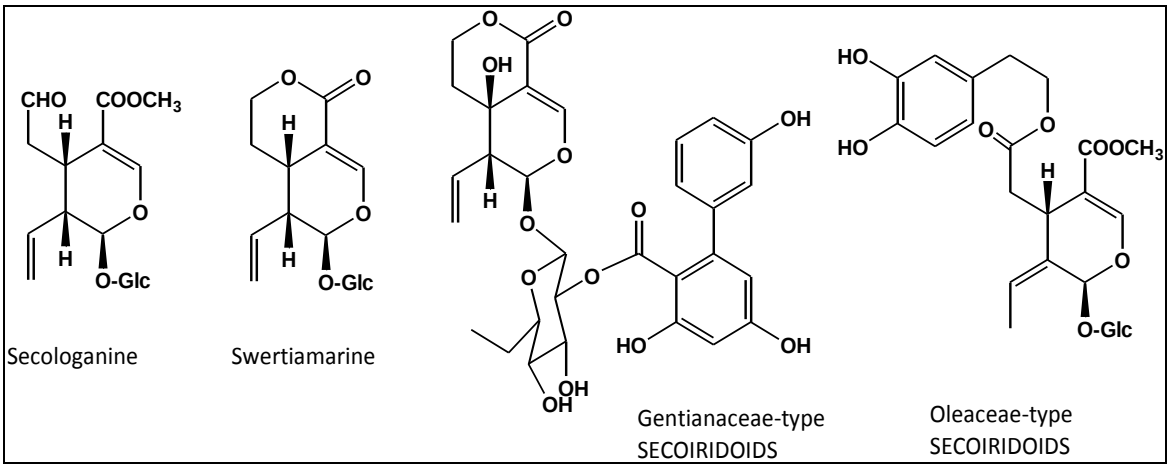






Structural Variations in Iridoid Glycosides

There are several types of secoiridoid-type aglycones. Some of them are shown below.



Secoiridoid-type Aglycones

- Those which, like secologanin, have a vinyl group at C-9. Polyfunctionalization of which permits lactonization (gentiopicrin).
- Those which, like oleoside, have an ethylidene or hydroxyethylidene group at C-9. The carboxyl group may be esterified (oleuropein).
- Those which are amidified by an aromatic amine (alkaloids).

MAIN IRIDOID&SECOIRIDOID CONTAINING DRUGS

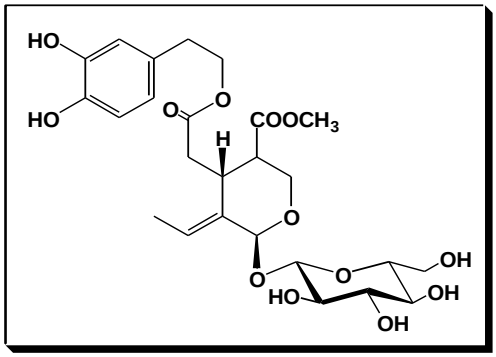
DRUG	*Plant Name*	Family	Active Constituents
Valeriana radix VALERIAN	***Valeriana officinalis***	Valerianaceae	**SECOIRIDOIDS (Valepotriates)**
Harpogophyti radix DEVIL'S CLAW	***Harpagophytum procumbens***	Pedaliaceae	**IRIDOIDS (Harpagide=Harpagoside, isoharpagoside, procumbid)**
Olea folium OLIVE TREE	***Olea europea***	Oleaceae	**SECOIRIDOIDS Oleuropein**
Gentiana radix GENTIAN	***Gentiana lutea***	Gentianacaea	**SECOIRIDOIDS Gentiopicroside Amorapanin, Amoroswerin, Amoragentin**
Centaurii herba	***Centaurium minus***	Gentianacaea	**Swertiamarin, Gentiopicroside**
Scrophularia herba	***Scrophularia nodosa***	Scrophulariacaea	**Aucubin and Catalpol derivatives, harpagoside**
Menyanthidis folium	***Menyanthes trifoliata***	Menyanthaceae	**SECOIRIDOIDS Foliamenthin, Sweroside IRIDOIDS; Loganin**
Plantaginis folium	***Plantago lanceolata***	Plantaginaceae	**IRIDOIDS Aucubin**

Pharmacopeia Analysis
Olive leaf (Olea folium) - Zeytin Ağacı Yaprakları

Ref. **EUROPEAN PHARMACOPOEIA 6.0, Vol. 2, 01/2008, p.2545**

DEFINITION

Dried leaf of *Olea europaea* L.
Content: minimum 5.0 per cent of oleuropein
Mol. Formula: $C_{25}H_{32}O_{13}$
Mol. Weight: 540.52



Oleuropein is a chemical compound found in olive leaf from the olive tree together with other closely related compounds such as 10-hydroxyoleuropein, ligstroside, and 10-hydroxyligstroside. All these compounds are tyrosol esters of elenolic acid that are further hydroxylated and glycosylated. Oleuropein and its metabolite hydroxytyrosol have powerful antioxidant activity both in vivo and in vitro and give extra-virgin olive oil its bitter, pungent taste. Oleuropein preparations have been claimed to strengthen the immune system.

IDENTIFICATION

A. The leaf is simple, thick and coriaceous, lanceolate to obovate, 30-50 mm long and 10-15 mm wide, with a mucronate apex and tapering at the base to a short petiole; the margins are entire and reflexed abaxially. The upper surface is greyish-green, smooth and shiny, thelower surface paler and pubescent, particularly along the midrib and main lateral veins.

B. Reduce to a powder (355) *(2.9.12).* The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R.* The powder shows the following diagnostic characters: fragments of the epidermis in surface view with small, thick-walled polygonal cells and, in the lower epidermis only, small anomocytic stomata *(2.8.3);* fragments of the lamina in sectional view showing a thick cuticle, a palisade composed of 3 layers of cells and a small-celled spongy parenchyma; numerous sclereids, very thick-walled and mostly fibre-like with blunt or, occasionally, forked ends, isolated or associated with the parenchyma of the mesophyll; abundant, very large peltate trichomes, with a central unicellular stalk from which radiate some 10 to 30 thin-walled cells which become free from the adjoining cells at the margin of the shield, given an uneven, jagged appearance.

C. Thin-layer chromatography *(2.2.27).*

Test solution. To 1.0 g of the powdered drug (355) *(2.9.12)* add 10 ml of *methanol R.* Boil under a reflux condenser for 15 min. Cool and filter.

Reference solution: Dissolve 10 mg of *oleuropein R* and 1 mg of *rutin R* in 1 ml of *methanol R.*

Plate: *TLC silica gel plate R.*

Mobile phase*: water R, methanol R, methylene chloride R* (1.5:15:85 *V/V/V).*

Application*:* 10 *µl,* as bands.

Development*:* over a path of 10 cm.

Drying*:* in air.

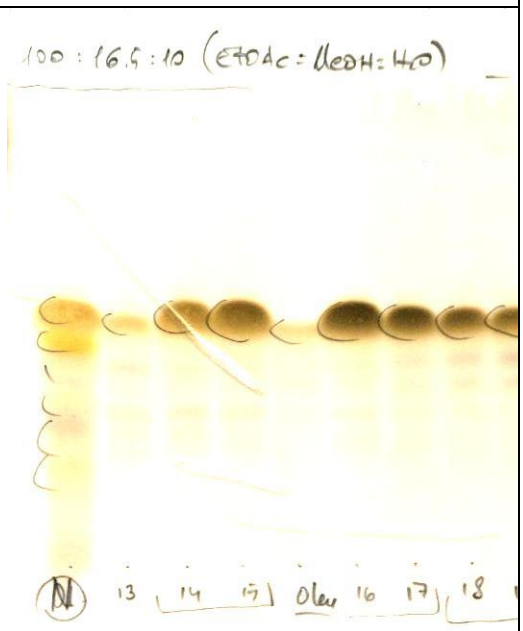
Detection*:* spray with *vanillin reagent R* and heat at 100-105 °C for 5 min; examine in daylight.

Results*:* see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

<table>
<tr><td colspan="2">Top of the plate</td><td></td></tr>
<tr><td rowspan="2">Oleuropein : a brownish-green zone

Rutin: a brownish-yellow zone</td><td>A dark violet-blue zone (solvent front)

A dark violet-blue zone

A brownish-green zone (oleuropein)</td><td rowspan="2"> 
</td></tr>
<tr><td>Reference solution</td><td>Test solution</td></tr>
<tr><td colspan="3">Mobile phase: ETOAc-MeOH-H_2O (100:16.5:10)
Detection: 1% Vanillin/conc. H_2SO_4</td></tr>
</table>

Alternative extraction method: To 1.0 g of the powdered drug add 10 ml of EtOH-H_2O (1:1) mixture. Keep in ultrasonic bath at 50°C for 10 min (Extraction). Cool and filter to another reagent glass. After partition of filtrate with ethyl acetate (EtOAc) (2 mL), wait until the phase separation. Use EtOAc phase (upper phase) for thin layer chromatography (TLC).

Reference solution: Dissolve 10 mg of *oleuropein R* and 1 mg of *rutin R* in 1 ml of *methanol R.*

Plate: *TLC silica gel plate R.*

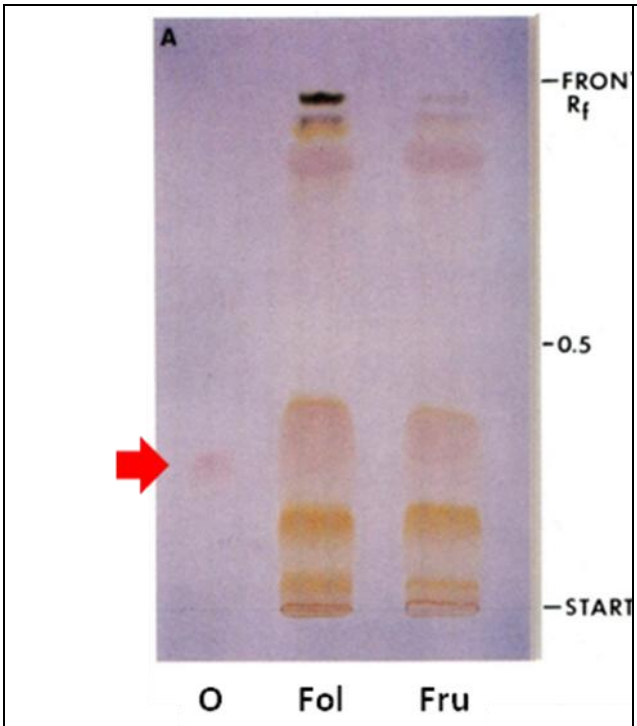
Mobile phase*:* EtOAc-MeOH-H_2O (100:16,5:13,5; *V/V/V).*

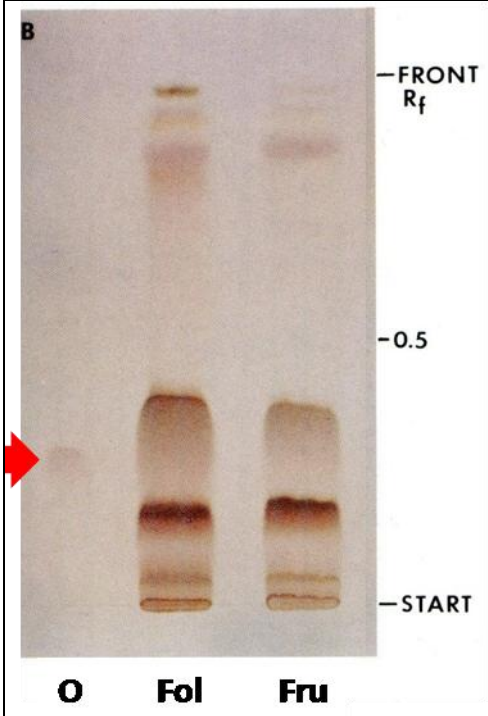
Application*:* 10 *µl,* as bands.

Development*:* over a path of 10 cm.

Drying*:* in air.

Detection*:* spray with *vanillin reagent R* and heat at 100-105 °C for 5 min; examine in daylight

<table>
<tr>
<td> 
</td>
<td>Reference solution:
O: Oleuropein (1 mg / 1 ml of methanol)
Fol: Olea folium
Fru: Olea fructus (fresh)
Plate: TLC silica gel plate.
Mobile phase: Ethylacetate-Dioxan-H_2O
(30:10:0.3 V/V/V).

Application: 10 µl, as bands.
Development: over a path of 10 cm.
Drying: in air.
Detection: Examine in daylight</td>
</tr>
<tr>
<td> 
</td>
<td>Reference solution:
O: Oleuropein (1 mg / 1 ml of methanol)
Fol: Olea folium
Fru: Olea fructus (fresh)
Plate: TLC silica gel plate.
Mobile phase: Ethylacetate-Dioxan-H_2O
(30:10:0.3 V/V/V).

Application: 10 µl, as bands.
Development: over a path of 10 cm.
Drying: in air.
Detection: 10% $FeCl_3$ solution</td>
</tr>
<tr>
<td colspan="2">Ref. H. Wagner, S. Bladt, E.M. Zgainski, Drogen analyse: DC Analyse von Arzneidrogen,</td>
</tr>
</table>

TESTS

Loss on drying *(2.2.32) :* maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) *(2.9.12)* by drying in an oven at 105 °C for 2 h.

Total ash *(2.4.16):* maximum 9.0 per cent.

HPLC
Use of High Performance Liquid Chromatography in Drug Analysis

ASSAY - 1

Ref. **EUROPEAN PHARMACOPOEIA 6.0, Vol. 2, 01/2008, p.2545**

Liquid chromatography *(2.2.29).*

Test solution. In a flask, place 1.000 g of the powdered drug (355) *(2.9.12)* and add 50 ml of *methanol R.* Heat in a water-bath at 60 °C for 30 min with shaking. Allow to cool and filter into a 100 ml volumetric flask. Rinse the flask and the filter with *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of this solution to 20.0 ml with *water R.*

Reference solution. Dissolve 5.0 mg of *oleuropein CRS* in 5.0 ml of *methanol R.* Dilute 1.0 ml of this solution to 25.0 ml with *water R.*

Column*:*

— ***size****: l* = 0.15 m, Ø = 3.9 mm;

— ***stationary phase*** *: octadecylsilyl silica gel for chromatography R* (5 |jm);

— ***temperature****:* 25 °C. *Mobile phase :*

— ***mobile phase A*** *:* dilute 1.0 ml of *glacial acetic acid R* to 100 ml with *water R ;*

— ***mobile phase B*** *: methanol R ;*

Time (min)	**Mobile phase** A **(per cent** *V/V)*	**Mobile phase B (per cent** *V/V)*
0 - 5	85 -> 40	15 60
5 - 12	40 20	60 > 80
12 - 15	20 -> 85	80 > 15

- ***Flow rate****:* 1 ml/min.
- ***Detection****:* spectrophotometer at 254 nm.
- ***Injection*** : 20 *µl.*
- ***Retention time****:* oleuropein = about 9 min.

Calculate the percentage content of oleuropein using the following expression:

$$\% \text{ Oleuropein } P = \frac{A_1 \times m_2 \times p \times 8}{A_2 \times m_1}$$

A_1 = area of the peak due to oleuropein in the chromatogram obtained with the test solution;

A_2 = area of the peak due to oleuropein in the chromatogram obtained with the reference solution;

m_1 = mass of the drug to be examined, in grams;

m_2 = mass of *oleuropein CRS* in the reference solution, in grams;

P = percentage content of oleuropein in *oleuropein CRS*

ASSAY - 2

Ref. **D. Özkum, F. Nuray Yalçın, İ Çalış,** EVALUATION OF THE OLEUROPEİN CONTENT OF OLIVE LEAVES COLLECTED FROM CYPRUS AND BALIKESİR REGION**, BiHAT 2008, Kuşadası**

Olea europaea L. (Oleaceae) is one of the plants grown in Mediterranean countries, both in terms of economic value as well as important plant nutrient. And also among the public the aqueous extract of olive leaf have also great importance in terms of use as constipation, diuretic, arrhythmia and spasm to resolve regulatory. Pharmacological effects of olive leaf have been attributed it contens; polyphenols and secoiridoits. Especially "Oleuropein",secoiridoits content of Olive leaf indicated that increase in the hypotensive effect, and also showed antioxidant and antimicrobial properties.

In this study, amounts of oleuropein content olive leaf collected from the Cyprus-Kyrenia and Balıkesir-Ören region were compared. This analysis was carried out using High Performance Liquid Chromatography (HPLC)-DAD. In this study the oleuropein, used as reference material, was isolated from study materials, the structure determination has been illuminated with the aid of UV, ^{1}H, ^{13}C NMR, and by Mass (ESI-MS) spectroscopy.

MATERIALS AND METHODS:

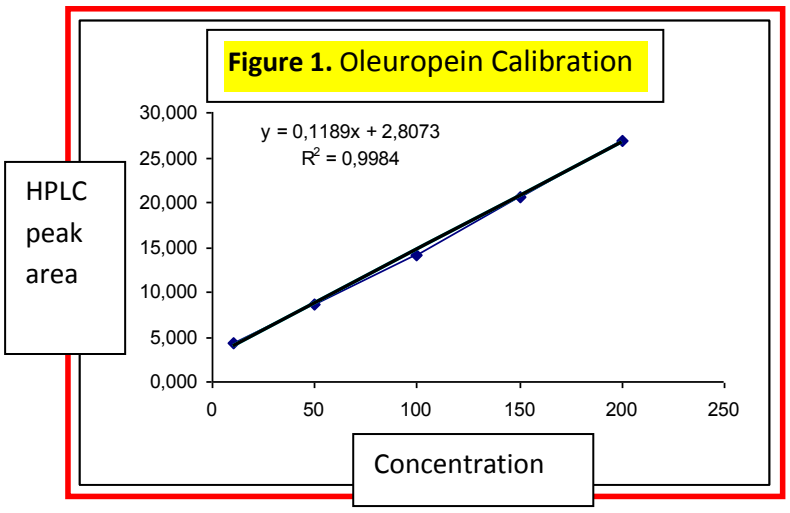
Plant Materials: *Olea europaea* L. (Oleaceae) leaf samples used in the study were collected from Cyprus-Kyrenia and Balıkesir-Ören region, in May 2007.

Properties of HPLC: Dionex P680 HPLC pump, Dionex ASI-100 Automated Sample Injector, Dionex Thermostatted Column Compartment TCC-100, Dionex DAD, ACE 5 C18 column (4.6X250 mm) and HI-5 C18-10C pre-column was used.

Moving phase has been formed of 79% distilled water acidified with 0.1 M ortofosforic acid and 21% acetonitrile again acidified with 0.1 M ortofosforik acid. Izocratic method is used, flow rate is set to 1 ml per minute and injection volume as 20 µl. Quantification of oleuropein made from chromatogram at 233 nm.

5 g of pulver drug of leaf samples is extracted in 30 ml 60% aqueous methanol 15 minutes, 3 times by keeping in ultrasonic bath. Obtained extracts combined and was completed to 100 ml with the same solvan. Then extracts diluted at a rate of 1:5 followed by 1:10 with the mobile phase.

Calibration curve is obtained from the reference substance (oleuropein) prepared with 200, 150, 100, 50 and 10 ppm solution of mobile phase (Figure 1). Both from reference / standard substance and extracts made 5 times applications and the average of these values were taken.



From calibration curve y = 0.1189 x + 2.8073 (R2 = 0.9984) equation were obtained.

RESULTS AND DISCUSSION: The oleuropein content of leaf samples collected from the area Balıkesir-Ören was found to be % 4.41 (a / a). The value obtained was found to be close to previously studies value of olive leaf samples collected from this area.

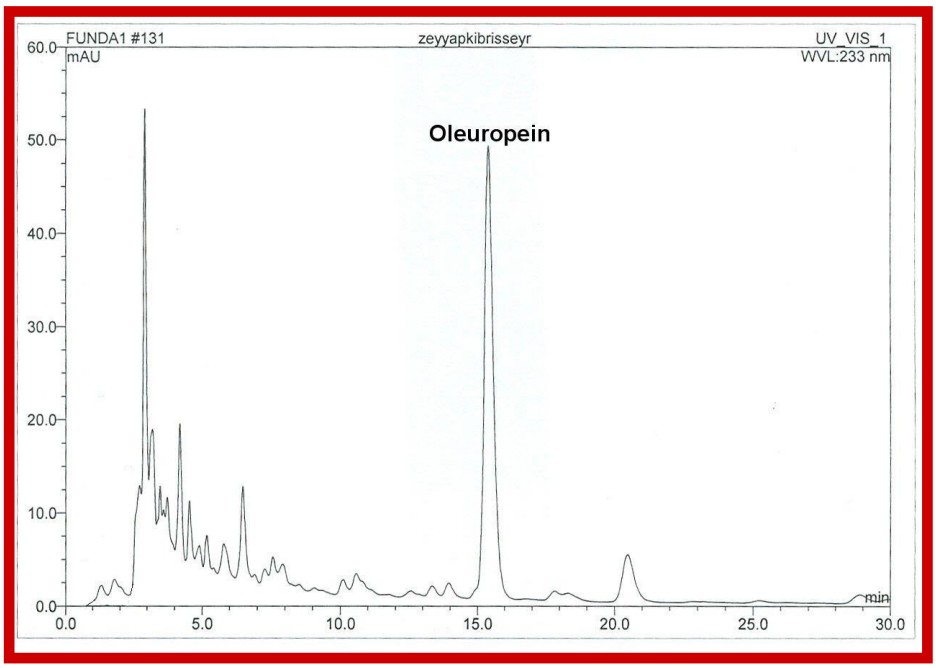
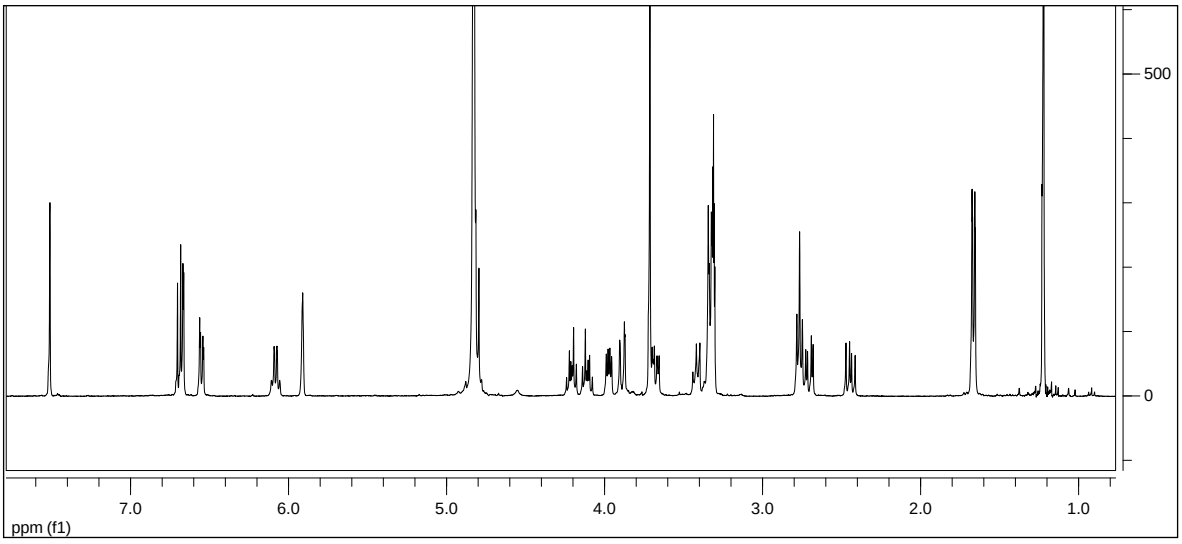


Figure 2. The HPLC of Cyprus-Kyrenia District Sample



^{1}H-NMR Spectrum of Oleuropein (400 MHz, CD_3OD)

The number of terpenic compounds in Oleum Menthae by 2D - TLC

DETERMINATION OF THE NUMBER OF TERPENES IN OLEUM MENTHAE

Method: Two dimensional thin layer chromatography

Adsorbent: Silicagel

Mobile phase: Benzene-Chloroform (75:25) or

Petroleum ether- Ethyl acetate (80:20)

Sample: 4 % O. Menthae in alcohol

Detection: Anisaldehyde solution+ 0.5 ml concentrated H_2SO_4 (It must be fresh)

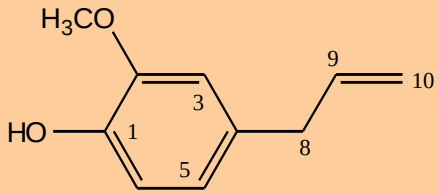
A plates (20x20 cm) 0.30 mm thickness is used. Sample is applied as a spot, which is 2-3 mm diameter, on the diagonal and 3 cm far away from the square. The spot must be dried and then sample is applied to the same spot, two or three times.

Put the plate in the tlc tank and allow the solvent to develop the tlc plate until 2 cm from the top of the plate. Mark the point of start and the front. Keep it until drying. After drying, turn the plate 90° according to the first route of development and put the tlc tank. Develop the plate until 2 cm to top of the plate. Remove the plate and wait for drying.

Spray the reactive solution for visulation and heat in the oven for 5-10 min at 100-110 °C. Mark the spots and copy it to onionskin.

Eugenol from *Syzgium aromaticum*
***Syn.: Eugenia caryophyllata*, *E. Aromaticum* (Myrtaceae)**

Ref. S. Berger & D. Sicker, **Classics in Spectroscopy –** Isolation and Structure Elucidation of Natural Products, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 2009



$C_{10}H_{12}O_2$, Mol. Wt. 164.20
Pale yellow oil
Synonymous names: 4-Allylguaicol, Bioxeda

Syzgium aromaticum
*Syn.***: *Eugenia caryophyllata*, *E. aromaticum***
(Myrtaceae)

Introduction

Eugenol is a long-known natural product occurring in essential oils from clove, cinnamon, piment, oil of bay, laurel, basil and nutmeg. Eugenol is a clear, pale yellow oil with a pleasant scent and (in dilute aqueous solution) a spicy taste. It has a link to the history of organic chemistry. In 1825, Faraday isolated 3 g of a very strange liquid from town gas, in which the C:H ratio was as high as 1:1! (it was the first benzene). In 1865, Kekulé proposed a cyclic formula for it with alternating double bonds, which explained not all but quite a lot of the properties of such compounds with low H content. Soon, a name for the whole class was wanted. Because compounds such as eugenol, vanillin and anisóle showed an aromatic flavour, the term aromatic compounds was used for a class of carbocycles with the feature of a cyclic conjugated system of π-electrons. Later, it was found that they all follow Huckel's ($4n + 2$) rule.

Eugenol belongs to the group of phenylpropanes such as anethol, estragóle and cinnamaldehydc, which are formed via the shikimate pathway and are frequently found in essential oils. Eugenol is used in perfumeries for spicy, clovc-like and oriental-type fragrances. A molecular formula of $C_{10}H_{12}O_2$, leading to a boiling point of 251-254 °C ensures a distinct vapour pressure at ambient temperature necessary for a smell from the physical point of view. Eugenol is slightly soluble in water, a prerequisite for all other uses. It is used as a flavoring in the kitchen, e.g. for aromatized red wine and red cabbage. As with all phenols, eugenol is an antiseptic: think of thymol used in toothpaste and cresols used for disinfection. Similarly, eugenol is a constituent of mouthwash. Due to its local antiseptic and also analgesic properties, it is used in dentistry. Mixed with zinc oxide, it forms a cement used for fillings of the teeth. Finally, eugenol can be used as an insect attractant.

Together with other spice constituents, eugenol is under detailed investigation for its biological effects in the human body. Erlenmeyer was the first to recognize the chemical nature of eugenol: he still called it Eugensäure (= eugenic acid) due to its acidic nature, which he used during purification. As a chemical, eugenol was used to produce vanillin via isoeugenol. It is classified as a hazardous material (classification Xn Harmful). Eugenol shows an allergenic potential and should therefore be handled with care. Avoid skin contact, as with all phenols. Never try it in pure form (it tastes extremely bitter). Overdosing by oral intake causes a range of serious symptoms, e.g. convulsions, dizziness and heartbeat acceleration.

EUGENOL

PRINCIPLE

Eugenol is sparingly soluble in water, but dissolves well in organic solvents such as diethyl ether, petrol ether, chloroform and ethanol. Eugenol as a phenol is OH-acidic (pK_a 10.3) and can be deprotonated by NaOH solution. The ionic phenolate form is soluble in water; this property is used for separation from other organic constituents of the cloves.

The ground cloves are first treated in a Soxhlet apparatus with petrol ether to extract all ethereal oil components from the spice. From the extract obtained, all strongly acidic compounds such as carboxylic acids arc removed by a first extraction with saturated $NaHCO_3$, solution, a very weak base which does not deprotonate eugenol. In a second extraction, eugenol is then selectively removed by NaOH extraction as phenolate from the organic into the aqueous phase. Acidification leads to eugenol as a cloudy precipitate in water, which is re-extracted into petrol ether. Final purification is possible by distillation in vacuo or even at ambient pressure, because eugenol has remarkable thermal stability.

METHOD

Crushed cloves (50 g) are placed in the thimble of a Soxhlet apparatus and extracted with 650 mL of petrol ether (boiling range 50-80 °C) for 6 h. The cloudy extract is filtered and the filtrate is reduced on a rotary evaporator to obtain a clear yellow solution of 200 mL volume. Strongly acidic compounds are then removed by shaking with saturated $NaHCO_3$ solution (2 x 50 mL) in a separating funnel. The organic phase is extracted with 5% NaOH (4 x 50 mL) to transfer eugenol into the aqueous phase. To the combined NaOH extracts crushed ice (50 g) is added followed by concentrated HCI (30 mL), which is added slowly with stirring until pH 2 is reached. Test the acidic reaction with pH paper. Acidification is complete if addition of HCI does not cause further precipitation of eugenol as cloudy droplets. Eugenol is re-extracted with petrol ether (4 **x** 50 mL).

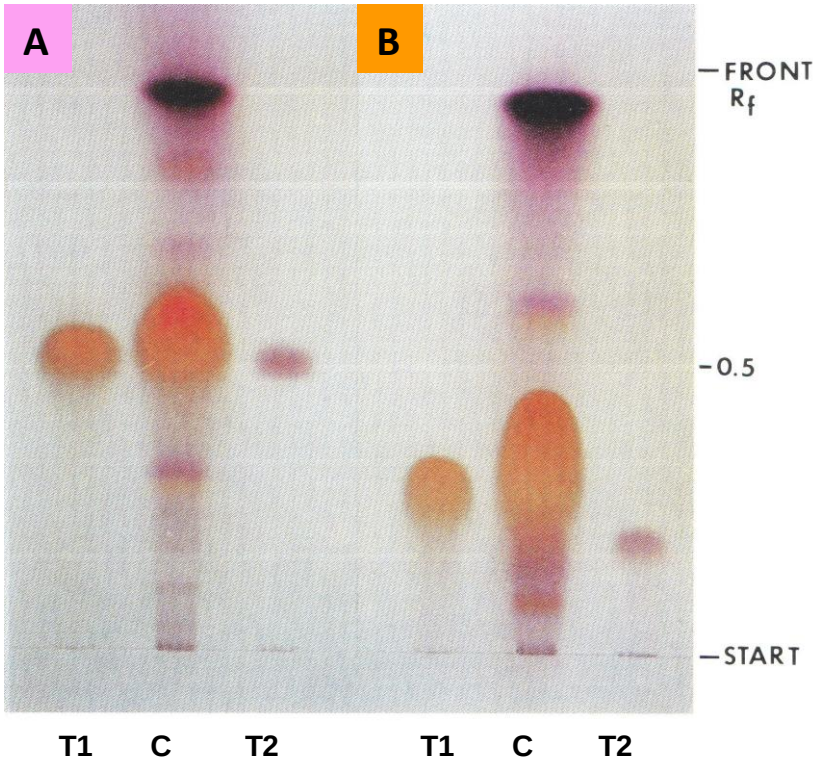
A collection of a raw eugenol samples of a students' laboratory course was collected and distilled in vacuo. This 28 g sample was obtained starting from 400 g of clove (Caryophylli flos).

PURIFICATION

The combined organic extracts are washed with saturated *$NaHCO_3$*, (50 mL) to remove traces of HCI and dried over $MgS0_4$. The sol vent is removed in vacuo. A dark yellow oil of crude eugenol remains: 5.0 g, n_D 1.5385 (20 °C). Distillation in a micro distillation apparatus at ambienl pressure using two heat guns yields 4.0 g of pale yellow eugenol: ***bp*** 251-254 °C, n_D 1.5410 (20 °C). Alternatively, distillation in vacuo is possible: bp 125-126 °C (1.9 kPa). On standing in a deep freezer, eugenol crystallizes (mp -9 °C).

TLC of Caryophylli Flos

Syzgium aromaticum, *Syn.*: ***Eugenia caryophyllata, E. Aromaticum*** **(Myrtaceae)**



Reference solutions:

T1*: Eugenol* (1 mg / 1 ml of methanol)

T2*: Aceteugenol* (1 mg / 1 ml of methanol)

C: Caryophylli Oleum

Plate: *TLC silica gel plate.*

Development: over a path of 10 cm.

Drying: in air.

Detection: 1% Vanillin/H_2SO_4

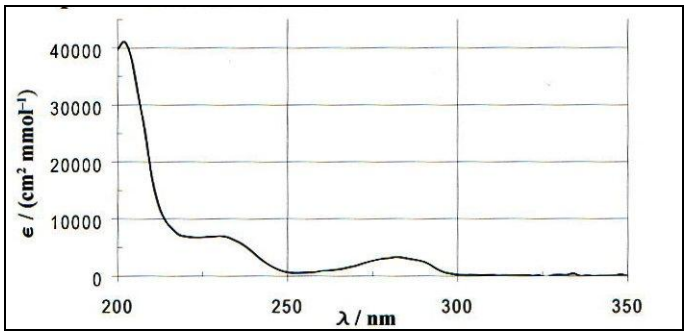
Eugenol: Orange-brown

Humulen&**Caryophyllen**: Red-violet (front)

A: Mobile phase*: Toluol – Ethylacetate (93:7)*

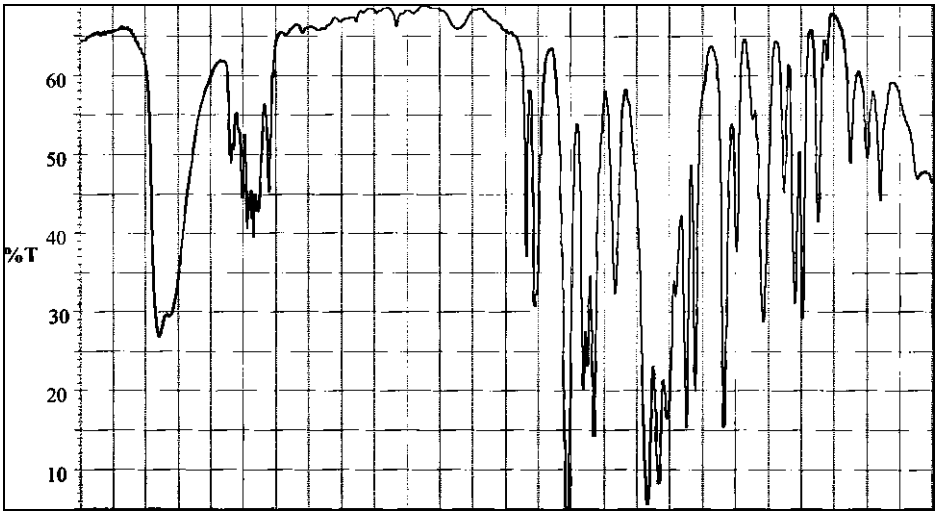
B: Mobile phase*: Toluol*

UV Spectrum



UV spectrum of Eugenol in EtOH

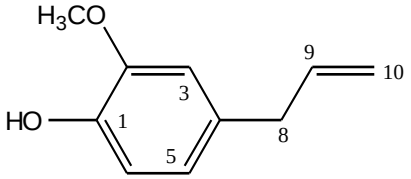
IR spectrum

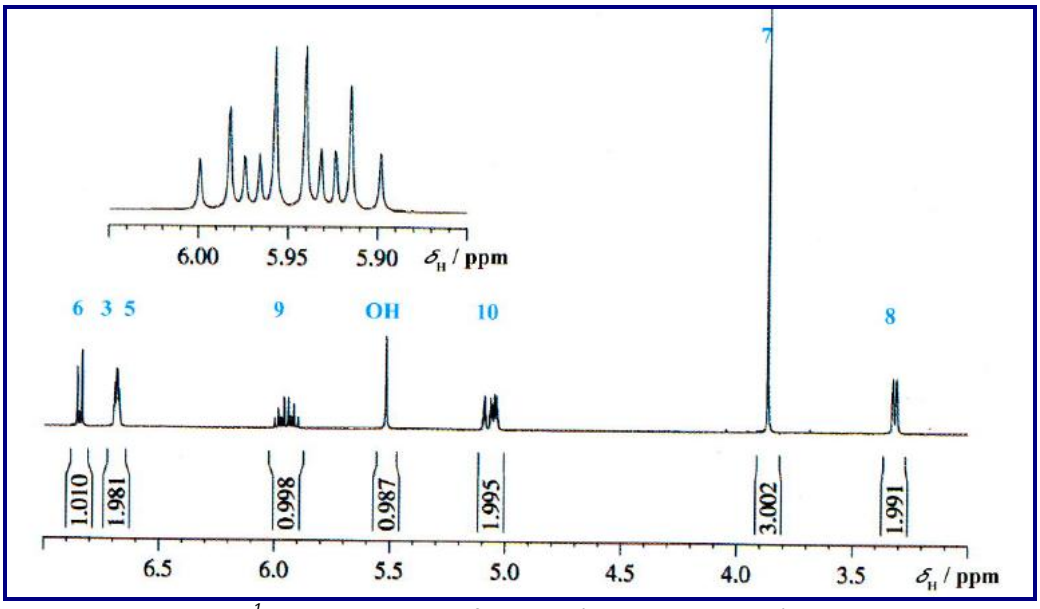


IR spectrum of Eugenol as film

In the IR spectrum, the OH valence vibration at 3250 cm^{-1} dominates the spectrum followed by sp^2- and sp^3 type CH valence vibration bands. The splitting of the two bands in the C=C region at 1620 cm^{-1} is probably due to the presence of aromatic and olefinic double bonds.

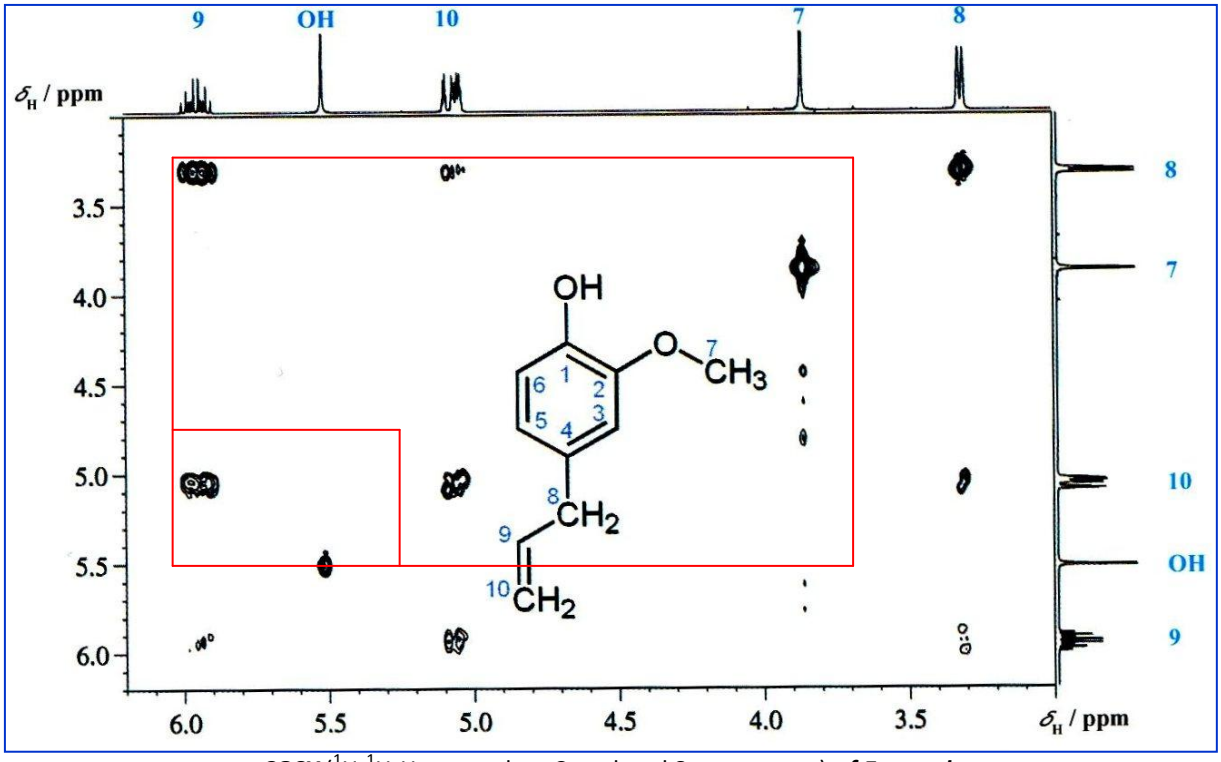
NMR Spectra





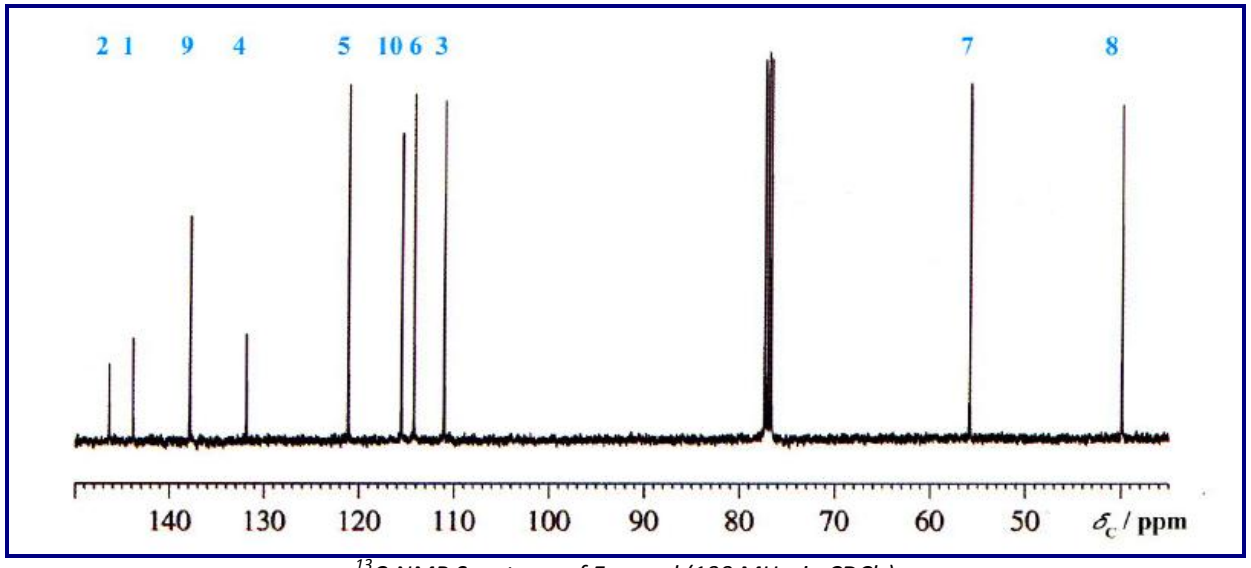
^{1}H-NMR Spectrum of Eugenol (400 MHz, in $CDCl_3$)

In the ^{1}H NMR spectrum, the very typical pattern of a terminal vinyl group at 5.06 ppm is revealed displaying a large **trans-** and a smaller m-olefinic vicinal spin coupling further complicated by allylic spin coupling to the methylene protons at C-8.



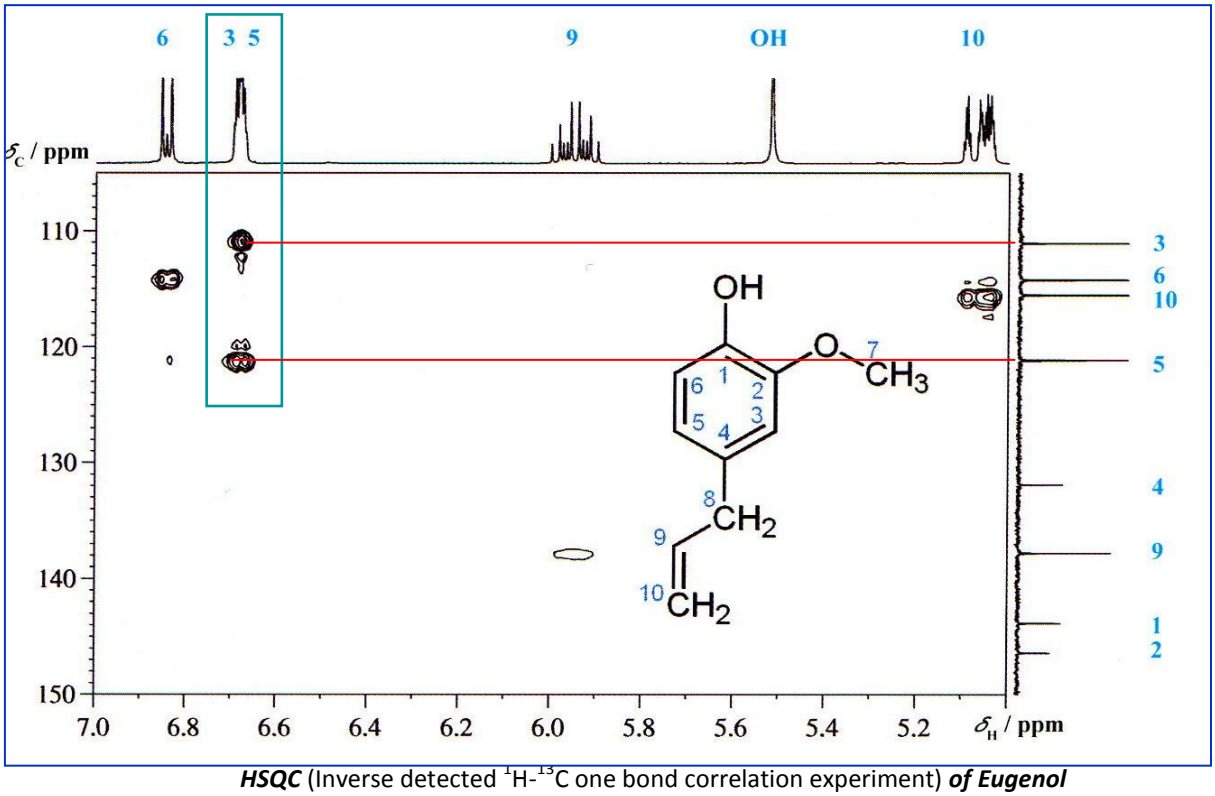
COSY (^{1}H-^{1}H-Homonuclear Correlated Spectroscopy) ***of Eugenol***

Cosy displays the connectivities within the allylic moiety H-8, H-9 and H-10, demonstrating that in a COSY spectrum not only vicinal and geminal but also allylic spin coupling constants yield cross peaks.



^{13}C-NMR Spectrum of Eugenol (100 MHz, in $CDCl_3$)

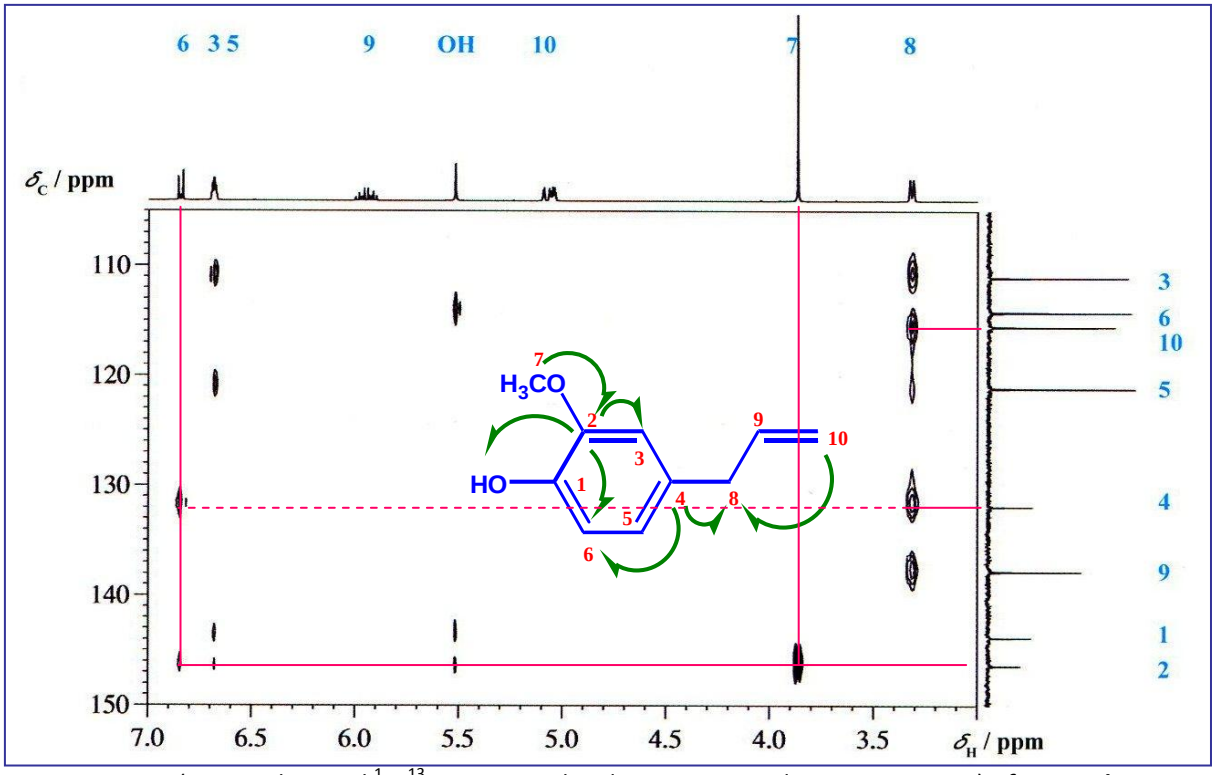
The ^{13}C NMR spectrum displays 10 different signals showing that there is no symmetry in eugenol with its 10 carbon atoms. Whereas the assignment in the aliphatic part is straightforward, the assignment in the olefinic/aromatic part of the spectrum requires the study of the HSQC, HMBC and NOESY spectra.



HSQC (Inverse detected ^{1}H-^{13}C one bond correlation experiment) ***of Eugenol***

HSQC = Heteronuclear Single Quantum Coherence

The HSQC spectrum reveals that two aromatic proton signals overlap each other. However, with this information alone it is not possible to decide which is which carbon and proton signal.



HMBC (Inverse detected ^{1}H-^{13}C-Heteronuclear long-rangecorrelation experiment) ***of Eugenol***

HMBC = Heteronuclear Multiple Bond Correlation

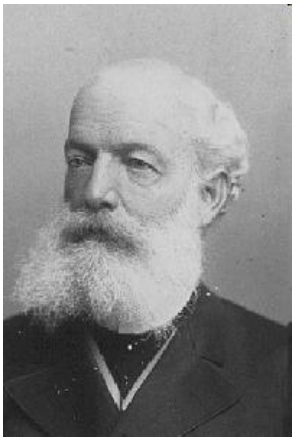
In the HMBC spectrum, it is remarkable that the signal of the phenolic OH proton is rather sharp in $CDCl_3$ solution and even gives useful correlations in the HMBC spectrum. This is probably due to an intramolecular hydrogen bond to the oxygen atom of the methoxy group. The analysis of the HMBC spectrum starts best with the proton signal of the methoxy group, which shows a correlation to the carbon signal at 146.2 ppm and thus assigning this signal to C-2, which in turn fixes the signal at 143.9 ppm to C-1. The chemical shift of the remaining quaternary carbon atom C-4 is therefore at 131.9 ppm, in the HMBC spectrum seen as expected from the protons of methylene group H-8. Since the chemical shifts of C-9 and C-10 could be unequivocally identified in the HSQC-spectrum (137.8 and 115.5 ppm), only three more carbon atoms, C-3, C-5 and C-6, have to be assigned.

^{13}C Signals δ / ppm	Type of Carbon	Assignment	Proton Signals δ / ppm, J / Hz
146.4	C_q	C-2	
143.9	C_q	C-1	
137.8	CH	C-9	5.95, J = 17.0, 9.7, 6.7
131.9	C_q	C-4	
121.2	CH	C-5	6.68
115.5	CH_2	C-10	5.06
114.2	CH	C-6	6.85, J = 8.4
111.1	CH	C-3	6.68
55.8	CH_3	C-7	3.87
39.9	CH_2	C-8	3.32, J = 6.7

NMR Data of Eugenol

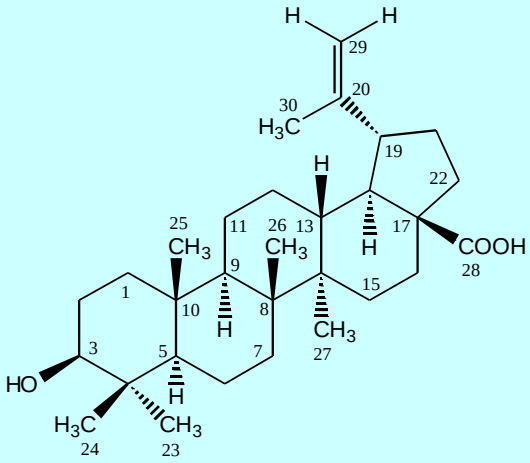
Kekulé, Friedrich August (1829-1896)
Born: Darmstadt (Germany), 1829
Died: Bonn (Germany), 1896

Kekulé, who started studies in architecture, turned himself to chemistry led by Liebig in Giessen (1849-1851). After his doctors degree in Giessen (1852), he traveled through England and France, where he did research under Williamson and Dumas. In 1856 he obtained a professorship at Heidelberg. By that time he announced the tetravalence of carbon (1853). In 1858 he took up a professorship at the University of Ghent (Belgium). In 1865 he introduced the structure of benzene and in 1867 Kekulé moved to Bonn.



Betulinic Acid from *Platanus orientalis* (Çınar)

Ref. S. Berger & D. Sicker, **Classics in Spectroscopy –** Isolation and Structure Elucidation of Natural Products, WILEY-VCH Verlag GmBH & Co. KGaA, Weinheim, 2009



Betulinic Acid
Syn. Names: (+)-Betulinic Acid, β-Betulinic Acid
Lup-20(29)en-oic acid

$C_{30}H_{48}O_3$, Mol. Wt. 456.71
Colorless crystalls, mp 293 – 295 °C

Platanus orientalis
(Platanaceae**)**

Introduction

Platanus orientalis, translated into English as the **Oriental plane**, is a large, deciduous tree of the Platanaceae family, known for its longevity and spreading crown. The species name derives from its historical distribution eastward from the Balkans, where it was recognized in ancient Greek history and literature. Following Greek usage it is called *Platane* or related names in Europe. It was equally as famous to the Iranian-speaking world and from Turkey to India is called *Chenar* or related names, following the Iranian. The native range of the Oriental plane is Eurasia from the Balkans to as far east as at least Iran.

Betulinic acid (source: plane tree bark – **Platanus orientalis cortex**) and his close relative **betulin** (with CH_2OH group instead of COOH) from birch bark (***Betula*** species – Huş ağacı) called birch cork were both named after the birch trees of the genus *Betula* L., The birch bark (Betulae Cortex) consists of two layers, and outer white one and an inner darker one, the real rind. White birch cork contains betulin to about 25% of its dry mass. For a certain reason, betulin is a particular compound in organic chemistry. It is well known that the famous chemist C. W. Scheele was the first to discover a variety of organic compounds from natural sources in the late 18th century, e.g. uric acid, oxalic acid (1776), glycerol (1779), lactic acid (1780) and gallic acid (1786). However, it is not as well known that soon afterwards, betulin was described from birch bark. Betulin was discovered by chance as a sublimate from birch cork and described as a "modified resin in form of a salt". This opinion on betulin was obviously due to the crystalline shape of sublimed betulin which reminded the finder of a crystalline salt. From our contemporary point of view, this betulin can be regarded as the first bioactive principle ever isolated from a plant source. The first detailed investigations of this strange material were

reported only 88 years later. The correct stereochemistry was elucidated in 1953 by chemical means only, i.e. before spectroscopic methods were used for structural assignments.

Betulin is an abundant triterpene diol occurring extremely widely in hundreds of different plant species, e.g. in the European hornbeam *(Carpinus betulus* L.) and the common hazel (*Corylus avellana* L.). The natural purpose for betulin seems to be that it prevents the tree from drying out. Both betulin and betulinic acid belong to the pentacyclic triterpenes with a **lupane skeleton**. For lupane, a procedure of numbering exists which differs from the numeration necessary for the related stem hydrocarbon chrysene. From this point of view, the CA index name **3-hydroxy-3p-lup-20(29)-en-28-oic acid** is a typical example of how a complicated compound can be named shortly in the manner of a semi-trivial nomenclature by using the abbreviated term lupane.

Birch tree products have been used in ethnomedicine for a very long time by different cultures, both in the Indogerman habitat and in the Northern America Indian region. Birch bark was known as a cure for skin diseases at that time. Birch bark powder was also used by the healer Hildegard of Bingen in the 12th century to treat open wounds. Birch trees were worshiped as symbols of life and fertility over the centuries. Even the traditional maypole has its origin in such ancient rituals. However, it is a rare and therefore rather special problem for making dermatologically useful preparations that the hydrophobic betulin is also a lipophobic compound which cannot be integrated easily in creams or unguents. This special behaviour was recently overcome by the development of an oleogel in which a betulin- and betulinic acid- containing birch bark extract is incorporated by means of a vegetable oil and water.

Betulinic acid, has been found to be biologically more active than betulin. Betulinic acid was shown to be a selective inhibitor of human melanoma by inducing apoptosis. Furthermore, it possesses anti-malarial, anti-HIV, antibacterial and anti-inflammatory properties and is active against several other tumour cell lines at a level comparable to drugs for clinical use. Obviously, a principle reason for this is the structural relationship of these natural products to compounds produced naturally in the body, in other words the ability of cell structures to interact with this kind of triterpene skeleton.

In principle, it can be made chemically by oxidation of betulin as a primary alcohol. However, despite the fact that birch bark is very rich in betulin, it is not an easy task to isolate pure betulin. The problem is that betulin is always accompanied by a number of structurally related triterpenoids such as lupeol, erythrodiol and oleanolic acid. It is reported and consistent with our own experience from attempts to isolate betulin in a pure state that crystallization is an insufficient means, and instead elaborate chromatography is required. Based on this finding, methods have been developed for the isolation of pure betulinic acid from the bark of plane trees as the best source. It can be done on a technical scale in procedures without chromatographic steps. In 2006, a Betulin Institute was founded in Darmstadt whose work is dedicated to research on betulin, betulinic acid and their use for pharmaceutical purposes (http://betulininstitut.de/).

ISOLATION

Betulinic acid, due to its steroid-like skeleton a highly hydrophobic carboxylic acid, is isolated from the dried and milled bark of plane trees by Soxhlet extraction with dichloromethane. Purification is possible just by recrystallization. Chromatographic separations are not necessary.

It is advantageous that the bark of a plane tree comes off unassisted each year in autumn at the end of the growth period. A tree of 15 m height drops several kilograms of bark just following its biological rhythm without having a problem. In contrast, greater efforts are necessary to obtain the white bark of birch trees. Moreover, betulinic acid cannot be isolated from it directly, but only the corresponding alcoholic precursor betulin. Furthermore, it is our experience that betulin obtained from birch tree bark by extraction is not nearly as pure as betulinic acid obtained by the method described below. Purification of crude betulin is impossible without chromatography. This clear drawback and the circumstance that plane tree bark is a renewable raw material make it a preferred source for betulinic acid.

METHOD

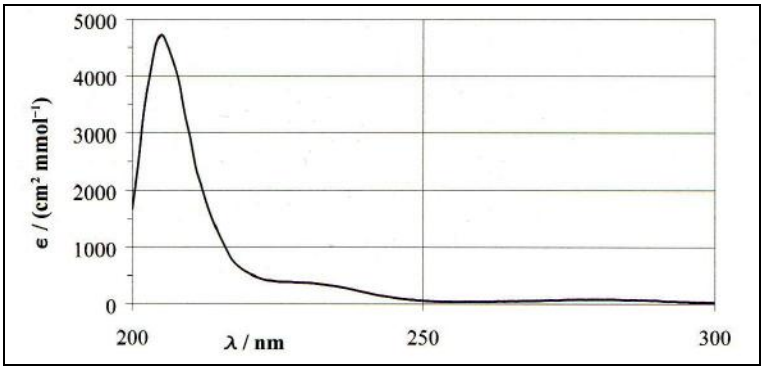
Bark of plane trees is air dried and ground by means of a grinder. The milled bark (70 g) is placed in the thimble of a Soxhlet apparatus and extracted with 700 ml of dichloromethane for 6 h. The yellow extract obtained is filtered to remove a few mechanical impurities coming from the bark.

The filtrate is concentrated to half of its volume and allowed to stand in a stoppered flask in a deep freezer (-18 °C) overnight for crystallization. Filtration yields 1.0 g of yellow crystals of crude betulinic acid (mp 290-293 °C, see text below). On work-up of the mother liquor, another crop of 100 mg of crude product can be obtained.

PURIFICATION

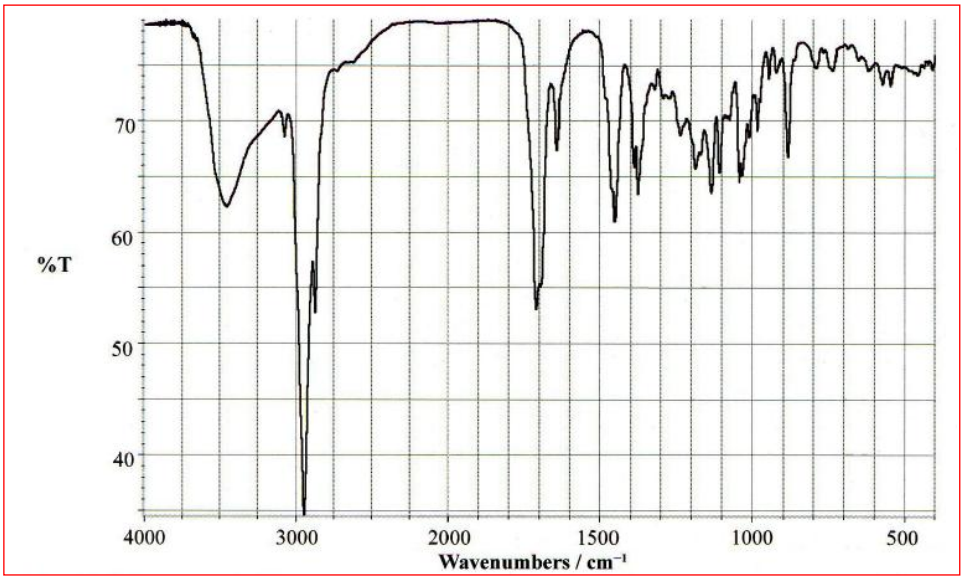
The crude betulinic acid obtained is dissolved in boiling methanol (100 ml) and a small amount of undissolved material removed by filtration. The filtrate is poured into an Erlenmeyer flask, stoppered and allowed to stand in a deep freezer (-18 °C) overnight. Colourless crystals separate and are filtered off, rinsed with 5 mL of ice-cold methanol and dried to yield 600 mg of pure betulinic acid, [a] +11.1° (c 0.00036 g/mL, chloroform).

Melting point: 293-295 °C (with sublimation at normal pressure between two glass plates); 316 °C (without sublimation, sealed in a Fischer cuvette).



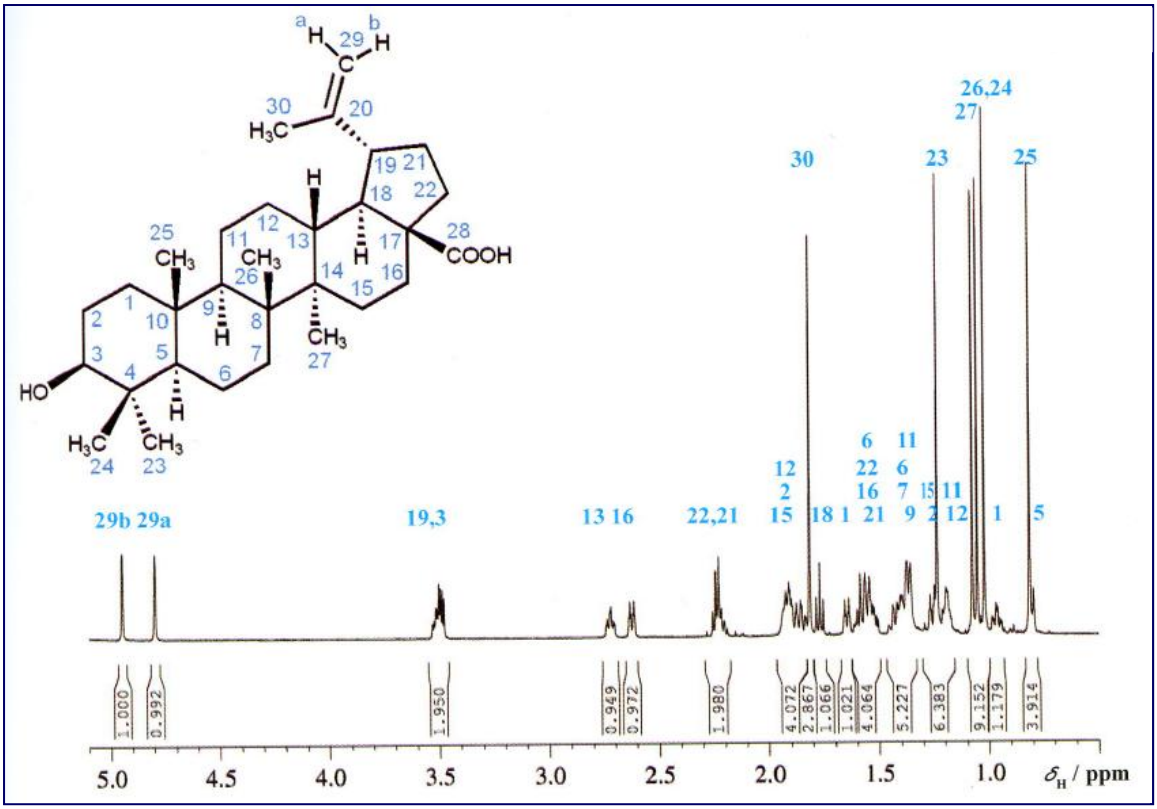
UV Spectrum of Betulinic Acid in EtOH

The two isolated chromophores, the double bond and the carboxyl group, give rise to a UV spectrum with a maximum at 205 nm (ε ~ 5000). A weak and a very weak shoulder can be seen at 240 and 280 nm.



IR Spectrum of Betulinic Acid in KBr

In the IR spectrum, the OH valence vibration of the carboxylic acid, the sp^2 and sp^3 CH valence vibrations, the C=0 double bond and the C=C double bond can be clearly identified. Note the typical wave-number of about 3100 cm"[1] for the terminal $=CH_2$ group of C-29.



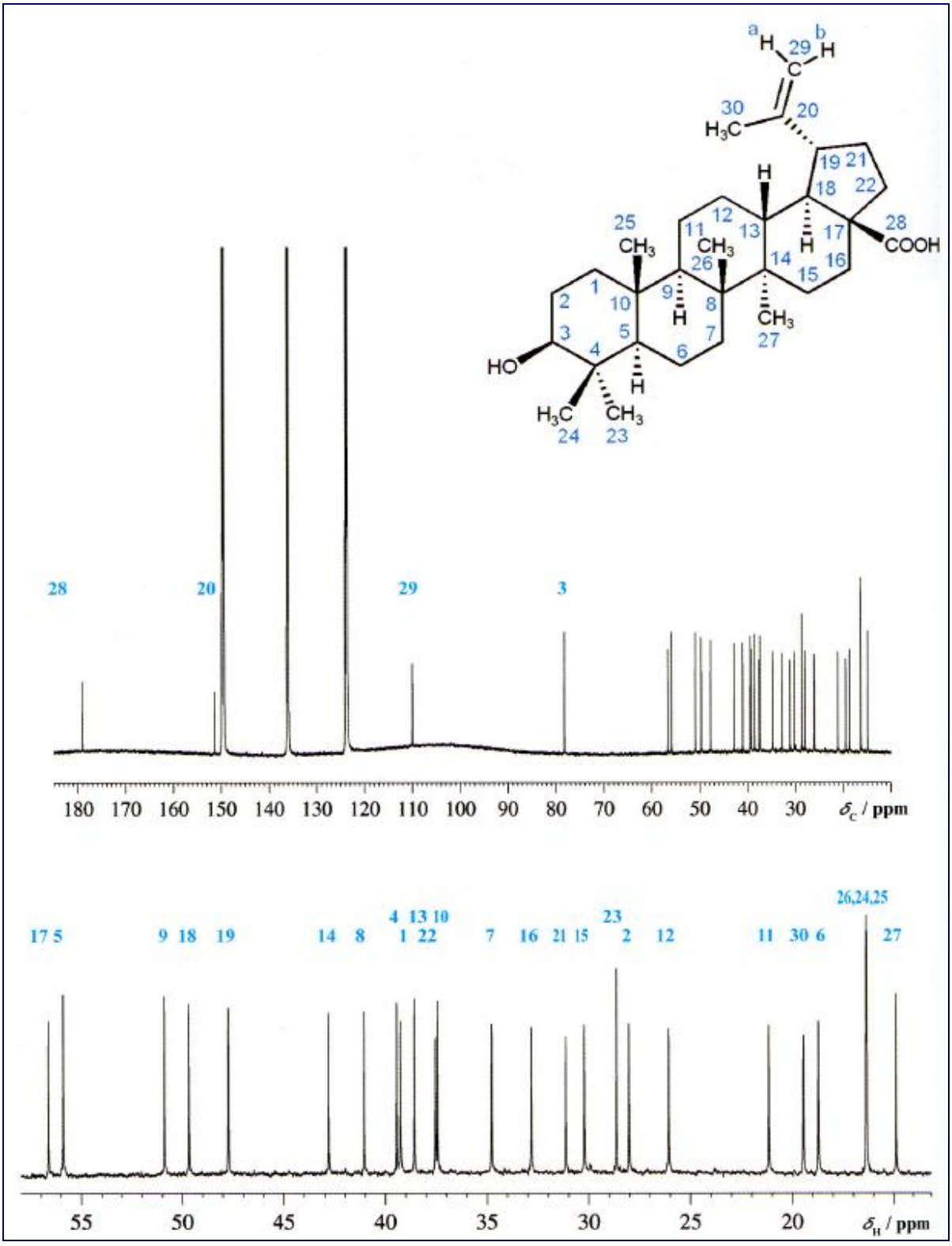
^{1}H – NMR of Betulinic Acid (700 MHz, in Pyridin-d_5)

Betulinic acid, as a triterpene, consists of 30 carbon atoms and is only sparingly soluble in the usual NMR solvents. The spectra shown here were therefore recorded in pyridine-d_5 and it is advisable to use an NMR spectrometer with the highest available field strength due to the complexity of the spectra; here a 700 MHz spectrometer was applied.

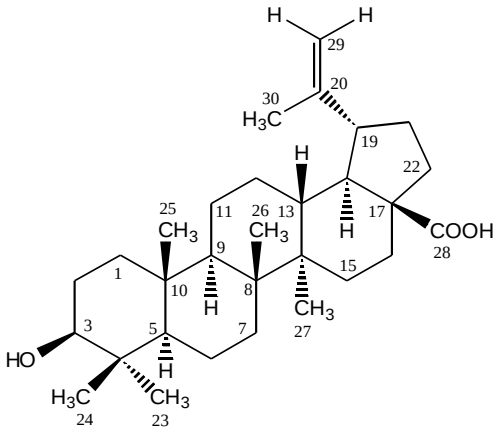
For the assignment strategy, it is best to start with the vinyl group C-20/C-29, since here the chemical shifts are obvious, and then try to get into the inner part of the molecule as far as possible. In a second step, one starts from C-3, because also here the assignments are easy. Hopefully, one arrives from these two ends of the molecule at a correct assignment also for the inner part.

The two olefinic protons H-29a and H-29b resonate at δ_H = 4.80 and 4.95 ppm and their relative assignment can be made from the NOESY spectrum due to the vicinity of H-29a to the methyl group C-30. The next signal in the proton spectrum at 3.5 ppm integrates for two protons, which from their chemical shifts are assigned to H-19 and H-3. The remaining aliphatic protons can not be assigned at this stage; only the signal of the methyl group C-30 attached to a sp^2 centre can be safely identified from its chemical shift of 1.82 ppm.

Similarly to the proton spectrum, only four signals in the ^{13}C NMR spectrum can be easily assigned from their typical chemical shifts, C-28 at 179.1, C-20 at 151.3, C-29 at 111.0 and C-3 at 78.2 ppm. The forest of signals between 60 and 10 ppm has to await the discussion of the HSQC and HMBC spectra.



^{13}C – NMR of Betulinic Acid (175 MHz, in Pyridin-d_5)



^{13}C Signals δ / ppm	Type of Carbon	Assignment	^{1}H Signals δ / ppm, J / Hz
179.1	C_q	C-28	
151.3	C_q	C-20	
110.0	CH_2	C-29	4.95/4.80
78.2	CH	C-3	3.49
56.6	C_q	C-17	
55.9	CH	C-5	0.80
50.9	CH	C-9	1.36
49.7	CH	C-18	1.77
47.7	CH	C-19	3.52
42.8	C_q	C-14	
41.1	C_q	C-8	
39.4	C_q	C-4	
39.3	CH_2	C-1	1.65/0.96
38.6	CH	C-13	2.72
37.5	CH_2	C-22	2.24/1.59
37.4	C_q	C-10	
34.8	CH_2	C-7	1.42/1.37
32.8	CH_2	C-16	2.62/1.56
31.1	CH_2	C-21	2.22/1.52
30.2	CH_2	C-15	1.85/1.26
28.6	CH_3	C-23	1.235
28.0	CH_2	C-2	1.89
26.1	CH_2	C-12	1.93/1.20
21.0	CH_2	C-11	1.40/1.19
19.4	CH_3	C-30	1.82
18.7	CH_2	C-6	1.54/1.37
16.38	CH_3	C-26	1.05
16.36	CH_3	C-24	1.02
16.32	CH_3	C-25	0.80
14.9	CH_3	C-27	1.08

NMR Data for Betulinic Acid

TLC

Ref. T Galgon, D Höke and B Dräger, **Identification and Quantification of Betulinic Acid,** *Phytochem. Anal.* **10**, 187–190, (1999)

Extraction

Dried and powered tissue (1 g) was repeatedly extracted with methanol until complete extraction was achieved as determined by thin layer chromatography (TLC). The combined extracts were evaporated to yield a final concentration equivalent to 1 g bark/10 mL methanol. This extract was used for TLC and/or gas chromatographic (GC) analysis.

TLC

The stationary phase: Silica gel 60 F254 (0.2 mm thick layer) on aluminium foil with fluorescence indicator (Merck).

Aliquots (20–50 mL) of extract were applied to each lane.

Solvent systems:

Extracts were separated by developing twice in different solvent systems:

(i) dichloromethane:petroleum ether (1.2:1, v/v); and

(ii) dichloromethane:methanol: acetic acid (500:20:1, v/v/v).

Between the first and the second development the layer was dried in a cold air stream.

Detection:

i. Anisaldehyde reagent (0.5 Ml anisaldehyde mixed with 10 mL acetic acid and 85 mL methanol followed by the addition of 5 mL concentrated (96%) sulphuric acid), or

ii. Acetic anhydride reagent (acetic anhydride mixed with sulphuric acid (9:1, v/v)).

HPTLC

Use of High Performance Thin Layer Chromatography in Drug Analysis

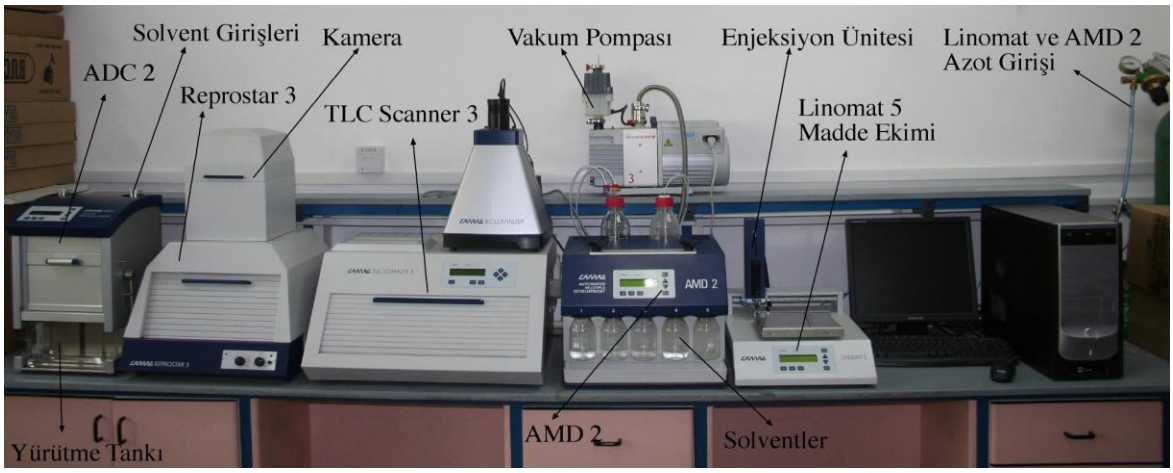
HPTLC – High Performance Thin Layer Chromatography

The high performance version of TLC "HPTLC" is a planar chromatography technique which involves instrumental features in regards to the common TLC. Unlike conventional TLC, HPTLC employs automated application of the sample on the TLC plate, development and spectral identification of the separated substances. Automation of the sample application provides better shaped bands and improves the separation of the substances in the sample. Automation of the sample application to the TLC plate enable analyst to have control on application speed, band length, band concentration and the exact position of the band on the plate. The development of TLC plates in HPTLC also provides beneficial features to the analyst. In the development of TLC plates analyst have control on automated drying of the solvent on applied bands, dipping the TLC into the development mobile phase, tank saturation time, plate saturation time, relative humidity, exact position of the solvent front and drying of the developed plate from the mobile phase. HPTLC also provides a spectral investigation of the TLC plate after development by the use of a TLC-Scanner. TLC-Scanner provides a spectral analysis of the TLC plate in the UV-Vis. range of the electromagnetic radiation. Spectral investigation provides UV-Vis. Spectrum of the seperated substances as well as precise exact R_f values of the substances.

Overall automation of TLC steps improves precision, repeatability and reproducibility of the TLC analysis. Additionally the problems associated with the high concentration of sample application, development process (smile effect) and unconsistent R_f values are avoided with HPTLC.

In addition to chromatogram detection/evaluation under visible or ultraviolet light, pre- and post-chromatographic derivatization is also possible in HPTLC. There are practically unlimited variety of reagents can be used in derivitization process. Unlike with column chromatography, the user has a complete overview of the chromatogram as all fractions remain stored on the plate and any substances remaining in the start position are detectable. It is another advantage of planar chromatography that sample preparation can often be simplified, due to the one-time use of the stationary phase. Standardized HPTLC analysis methods – qualitative or quantitative – can be validated. Automation of TLC also enables hypenation with other spectral techniques such as TLC-MS which enables analyst to have information on the molecular weight and structural components of the separated substances. TLC-MS online coupling, the comparatively new hyphenation of HPTLC and mass spectrometry, has the potential to become an indispensable technique for many analytical laboratories.

Figure 1. A HPTLC system including TLC applicator, TLC devoloper, TLC Scanner, TLC Visualizer and data controller.



HPTLC Analysis could be employed in many different fields of applications. These applications include analysis in pharmaceutical evaluations, herbals, foods, feed stuff, clinical evaluations, industrial evaluations, cosmetics, environmental evaluations.

Table 1. Applications of HPTLC analysis in different fields

Field of Application	Type of Analysis
Pharmaceutical applications	• Quality control • Content Uniformity Test (CUT) • Identity- and purity checks • Stability tests, etc.
Herbals	• Identification • Stability tests • Detection of adulteration • Assay of marker compounds, etc.
Food and feed stuff	• Quality control • Additives (e. g. vitamins) • Pesticides • Stability tests (expiration), etc.
Clinical applications	• Lipids • Metabolism studies • Drug screening • Doping control, etc.
Industrial applications	• Process development and optimization • Process monitoring • Cleaning validation, etc
Cosmetics	• Identity of raw material • Preservatives, coloring materials, etc. • Screening for illegal ingredients, etc.
Environment	• Water • Soil • Residue analysis, etc.

“HPTLC Analysis” An overview

A HPTLC analysis involves four important steps these are:

1. Application of the sample to the TLC plate (TLC Applicator);

2. Development of the TLC plate (TLC developer);

3. Spectral Analysis of the TLC plate (TLC scanner);

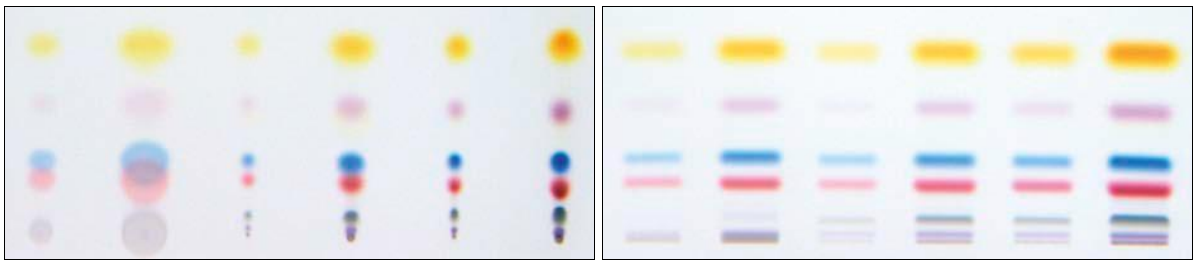
4. Visualization of the TLC plate (TLC visulizer).

TLC Applicator:

Sample application is the first step in the workflow of planar chromatography. This step affects the quality of the result at the end of the process significantly. Therefore the choice of the application technique and the device depend on the requirements of precision, sample volumes, number of analyses and the desired grade of automation. There are two types of sample application on TLC plates are possible these are:

1. Contact transfer: Sample is applied to the TLC by the help of a capillary either automatically or manually. Capillary filled with sample is touched on the application position on the TLC plate. Sample application volume range with contact transfer method is between 0.1–5 µL. This method is the conventional method of application sample to the TLC but it could also be employed in the automated systems. The disadvantages of this method involves the risqué of damaging the TLC plate in application process in manual applications, only application as spots are convenient and requires use of new capillary in each application. Spot wise sample application using a fixed volume capillary is the simplest way of sample application in TLC. Sample volumes of 0.5 to 5 µL can be applied as spots onto conventional layers without intermediate drying, on HPTLC layers it is up to 1 µL per spot. It is recommended to guide the capillary by means of an automated device.

Figure 2. Spot application (contact transfer) and rectangular band application (spray on).



2. Spray on techniques: Sample is sprayed onto the TLC plate by the help of special injector and spraying device. Sample application volume range with spray on method is between 0.5 to > 50 µL. This method could only be applied by using automated TLC systems. The main disadvantage of this method is it is limited only to automated systems. This method is appropriate for applying the

sample as either as spots or rectangular bands. Spraying-on samples as narrow bands allows the application of significantly larger volumes. Starting zones in the form of narrow bands ensure the best resolution that can be achieved with the chromatographic system selected. Very large sample volumes or samples with a high matrix content can be sprayed-on in the form of rectangles which, prior to chromatography, are focused into narrow bands by a short development step with a solvent of high elution strength.

TLC Developer:

In normal TLC development procedure after the sample is applied on the TLC plate it is dipped into the mobile phase system which moves upwards on the plate by the help of capillary action. Prior to dipping process analyst could employ tank saturation period by the help of a filter paper impregnated with mobile phase or with an known amount of excess mobile phase in the twin trough development chambers. These special development chambers contains double compartment for mobile phase. One of the compartments is used for development solvent and the second compartment is used for mobile phase saturation of the tank. In the regular developing tanks without compartments mobile phase saturation could be employed by using saturation pads or filter paper impregnated with the mobile phase.

TLC/HPTLC differs from all other chromatographic techniques in the fact that in addition to stationary and mobile phases, a gas phase is present, which can significantly influence the result of the separation. The gas phase consists of atmospheric gases and the vapors of the mobile phase components. The components of the gas surrounding the plate in the chamber interact with the silica gel. The following considerations primarily concern the separations where silica gel is used as a stationary phase and in the process usually described as adsorption chromatography.

Figure 3. The processes that occur in the development chamber.

Processes that occur in the development chamber

1. Between the components of the developing solvent and its vapor, an equilibrium will be established gradually. This process is called chamber saturation. Depending on the vapor pressure of the solvent components the composition of the gas phase can differ from that of the developing solvent.

2. The part of the layer which is already wetted with mobile phase contributes to the formation of the equilibrium with the gas phase.

3. While still dry, the stationary phase adsorbs molecules from the gas phase. Thereby polar components will be preferentially withdrawn from the gas phase and loaded onto the surface of the stationary phase. Allowing the plate to interact with the gas phase prior to starting chromatographic development is called layer preconditioning, which is not possible with all types of developing chambers. Lining the chamber with filter paper soaked with developing solvent supports this process. In case that preconditioning is not desired, a counter glass plate arranged a few mm apart suppresses it. This is called sandwich configuration.

4. During solvent migration, the components of the mobile phase may be separated by the stationary phase under certain conditions, causing the formation of secondary fronts, which is usually not desired

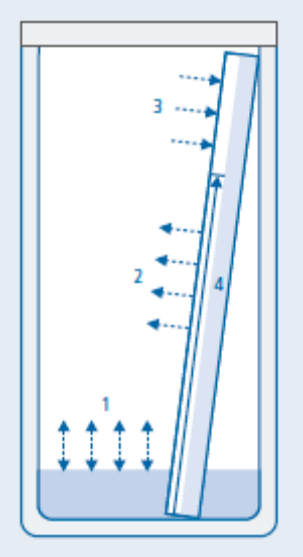
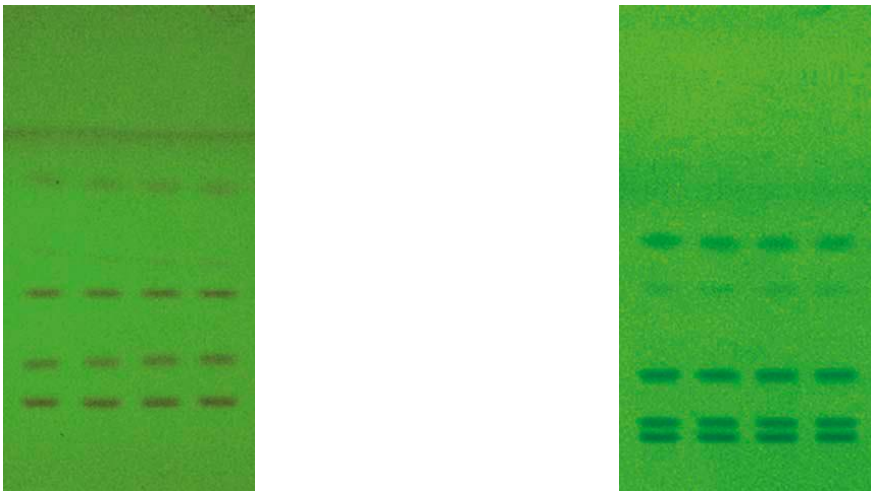


Figure 4. Effect of chamber saturation in development.

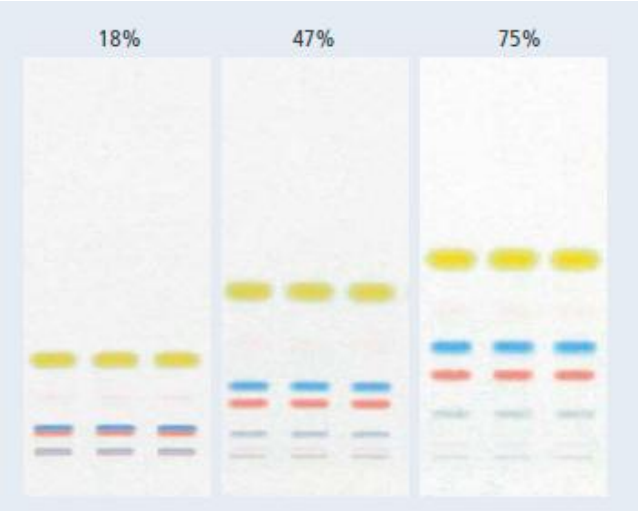


Development without chamber saturation. **Development with chamber saturation**

As explained above the interaction with the gases in the development chamber have effect on the separation therefore relative humidity in the chamber also has a dramatic effect on the separation. In regular TLC development the amount of relative humidity in the development chamber could not be controlled. Therefore depending on the weather conditions and humidity in the laboratory environment different results could be obtained from the development process. This effects the R_f values of the separated substances considerably.

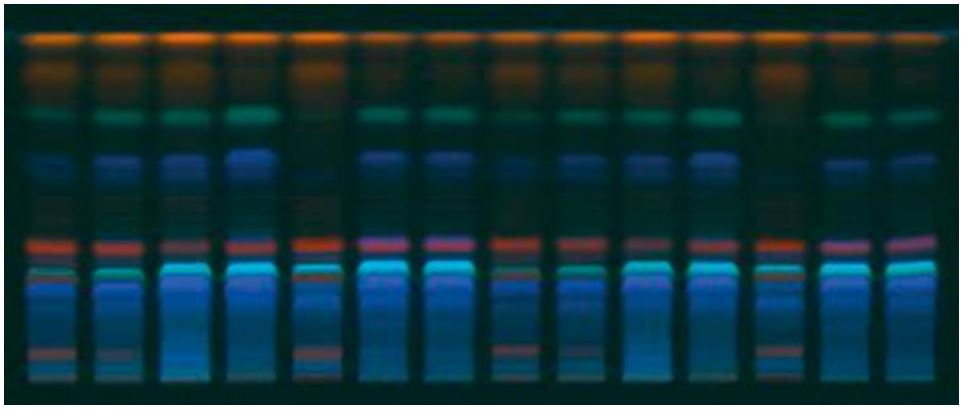
Figure 5. The effect of relative humidity on the R_f values of the separated components.

Influence of relative humidity ("activity of the layer") with the same solvent migration distance. (Same mobile phase and solvent front is applied to separation of the same sample separated at different relative percent humidity values)



In TLC developer systems two types of separation process could be employed regarding the instrument. These separation processes include isocratic elution and gradient elution similar to the applications used in column chromatography. In isocratic system only one type of mobile phase is used during the separation process however in gradient elution different types of mobile phases could be used during the separation process. Gradient elution and isocratic elution requires different kinds of instruments. In gradient elution the TLC plate is developed repeatedly in the same direction. In each successive run extends over a longer solvent migration distance than the one before. Between each run the mobile phase is completely removed from the developing chamber and the TLC plate is dried under vacuum. In each successive run uses a solvent of lower elution strength than that of the one used before. Therefore a stepwise elution gradient is formed. This procedure produces extremely narrow bands (typical peak width : 1 mm).

Figure 6. Separation of various *Rhubarb* sample extracts using gradient elution.



Detection: UV 366 nm; Gradient in 10 steps: Methanol – dichloromethane from 40:60 to 10:90 in 9 steps over 40 mm developing distance followed by one step methanol – dichloromethane 10:90 over 70 mm.

TLC Scanner:

In classical densitometry the tracks of the chromatogram are scanned with monochromatic light in the form of a slit selectable in length and width. Usually the spectral range of these scanners are 190–900 nm. Reflected light could be measured either in the absorbance or in the fluorescence mode. From the acquired data quantitative results can be computed with high precision and spectral selectivity. In scanning process blank reading is taken below the application position where the TLC plate is free from the sample. The scanner scans the whole plate according to the sample application position used in TLC applicator and the solvent front position used in TLC developer. TLC Scanner takes readings from the plate at different steps which means densitometry measurement is taken at regular intervals from the first reading position. If the distance between the data reading steps are shortened the resolution of the analysis increases. Usually TLC-Scanners provide a data resolution range of 25 – 200 µm/step. UV-Vis. spectrum of the separated bands could also be obtained using the TLC scanner. In this case scanner makes measurement in all of the wavelengths for each spot.

TLC Visualizer:

The main purpose of TLC visualizers is to provide photographic records of the TLC analyses, however they can also be used to screen biological activity, track chemical changes in substances and for quantification. The simple TLC visualizer contains a camera that could take pictures of the TLC plate under different light conditions (white light, 254 nm and 366 nm). However other types of cameras which could take series of photographs in a given time period (video scan) or in other applications such as biological activity screening photographs could be taken in certain conditions (at different temperatures) are also used.

TLC or HPTLC Plates?

In HPTLC instrumentation both TLC and HPTLC plates could be used for different purposes. The difference between the TLC and HPTLC plate comes from the type of silica gel used in preparing the plates and the preparation of the plates itself. The main difference of HPTLC plate is that it is produced with smaller adsorbent particles with more uniform shape. The second difference is that HPTLC plates are produced with a smaller and more uniform layer of the adsorbent. Third and the most important difference of HPTLC plates is that they are produced with a vast array of adsorbent types where as TLC plates are produced only with couple of adsorbents.

Table 2. Differences between TLC and HPTLC plates

	Conventional TLC Plate	**HPTLC Plate**
Support Material	Glass or Aluminum Sheets	Glass or Aluminum Sheets
Possible Type of Adsorbent	Silica gel, Alumina, Kieselguhr	RP-18, RP-8, Cellulose, Diol, CN, NH_2, Silica Gel, Alumina
Thickness of Adsorbent Layer	250 µm or >250 µm	100 µm
Particle Size and type of Adsorbent	Big Mesh Size – Nonuniform	Small Mesh Size – Uniform
Efficiency	Low	High (Due to small particle size !)
Sample Spotting	Spray on and Contact transfer.	Spray on and Contact transfer.
	Not Suitable for contact transfer with instrument !	Suitable for all kinds of instrumental spotting
Mobile Phase	Requires high amounts of M.P.	Requires low amounts of M.P.
Sample Capacity	High	Low

Use of HPTLC in Pharmacognosy and Natural Products Chemistry

HPTLC is a powerfull and cost effective technique that is widely used in phytochemical analysis, isolation and identification studies in natural product studies and in biological activity screening studies related with natural products chemistry. HPTLC provides quantification and qualification of compounds of interest in extracts obtained from natural drugs. Qualification could be made by using R_f and spectral similarity (UV-Vis. Or MS) of compounds. Additionally bio assays could be intercorporated with HPTLC to provide fast and convenient way to screen extracts, fractions or pure compounds. A list of commonly used biological activity assays done with HPTLC were given below.

Table 3. Examples of HPTLC bio-autography techniques.

Method	Bio-assay	Application Method	Evaluation
HPTLC-DPPH	DPPH Free Radical Scavenging Activity	Extract, fraction or substance of interest is applied on TLC and than TLC is dipped into DPPH free radical solution. Than plate is incubated in dark for radical scavenging reaction to take place.	Substances that can donate hydrogen to DPPH change the color of the DPPH solution from purple to yellow.
HPTLC-DPPH*	DPPH Free Radical Scavenging Activity	Extract, fraction or substance of interest is mixed with DPPH solution. Samples are incubate at dark for reaction to take place. All of the samples are applied on TLC plate and densitometric measurement is done with a TLC scanner at 517 nm.	Substances that can donate hydrogen to DPPH decrease the absorbance at 517 nm.
HPTLC-Cytotoxicity	*Vibrio fischeri* cytotoxicty test.	*Vibrio fischeri* medium and culture is prepared at dark. This marine microorganism have the bioluminiscence property in dark. The extract, fraction or substance of interest is applied on the TLC plate. The plate is dipped into *V. fischeri* culture and after incubation period it is photographed with a bioluminizer.	Substance that are toxic to *V. fischeri* produce black spots on the photograph, non-toxic substance on the other hand appear as white illuminated spots.
HPTLC-Antimicrobial & Antifungal Activity	Antibacterial, Antifungal activity.	Medium and culture of the microrganism of interest is prepared. The extract, fraction or substance of interest is applied on the TLC plate. The plate is dipped into microorganism culture and after incubation period it is photographed with TLC visualizer.	Substance that are toxic to microorganism produce inhibition zones which can be identified with color differences and their size could be measured from the photographs. Non toxic substances produce smaller or no inhibition zones.

HPTLC Identification of Hawthorn leaves & flowers (*Crataegus sp.*)

Sample:

In a test tube 1.0 g powdered drug is heated with 10 mL methanol for five minutes in a water bath at 65°C. After cooling to room temperature, the mixture is filtered and diluted to 10 mL with methanol. This is the test solution.

Standards (optional):

1 mg caffeic acid, 2 mg chlorogenic acid, 5 mg hyperoside, 5 mg rutin, 5 mg vitexin, and 5 mg vitexin-2"-O-rhamnoside are dissolved in 20 mL methanol each.

Derivatization reagent:

Natural Product reagent: 1.0 g diphenylborinic acid aminoethylester is dissolved in 200 mL ethyl acetate.

Chromatographic conditions:

Stationary phase:

HPTLC plates silica gel 60 F254 (Merck), 10x10 cm or 20x10 cm.

Mobile Phase:

Ethyl acetate, methanol, water, formic acid (50:2:3:6)

Sample application:

2 µL each of test solution and standard are applied as 8 mm bands, min. 2 mm apart, 8 mm from lower edge of plate.

Development:

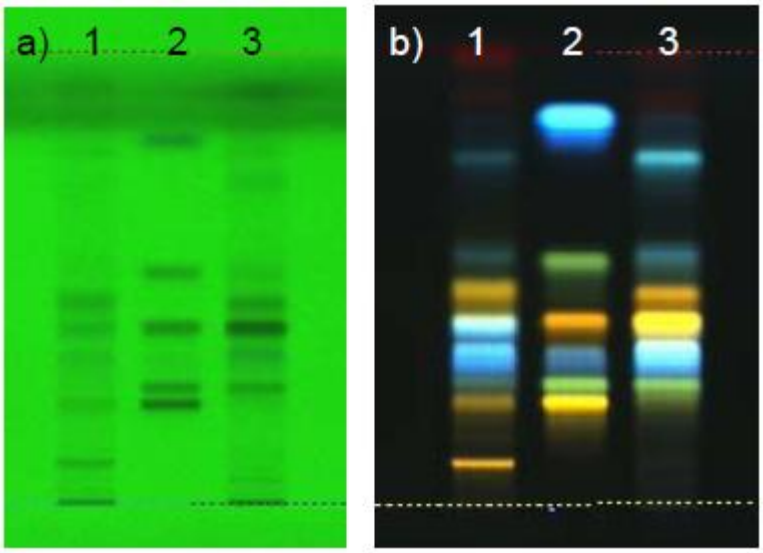
10x10 cm or 20x10 cm Twin Trough Chamber, saturated for 20 min (filter paper), 5 mL (respectively 10 mL) developing solvent per trough, developing distance 70 mm from lower edge of plate. The plate is then dried with a hair dryer (warm air) for 5 min.

Detection:

a) UV 254 nm

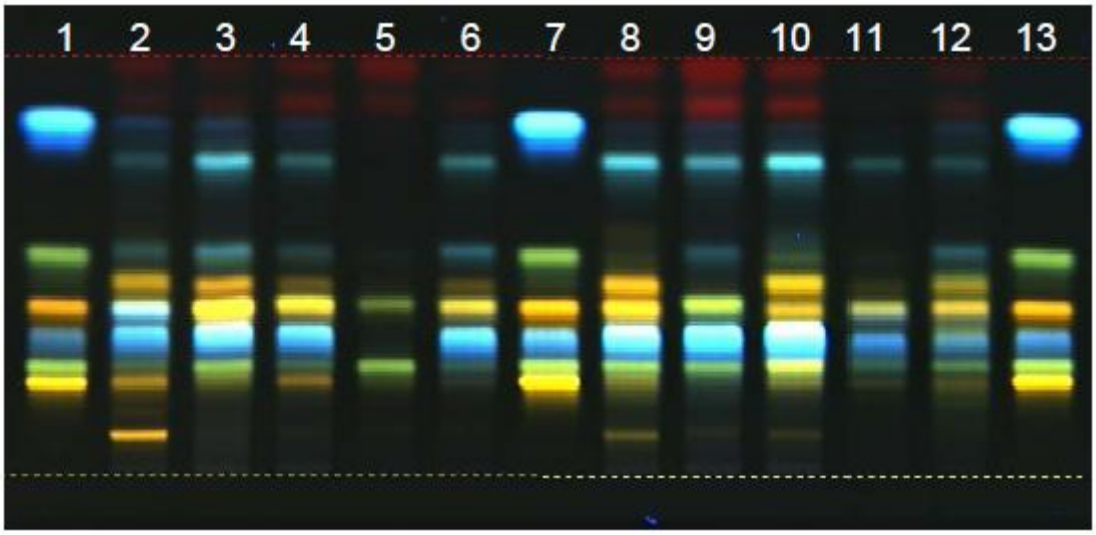
b) Natural product reagent: heat the plate at 100°C for 5 min. Using the CAMAG immersion device, the still hot plate is dipped in Natural product reagent (speed 1, time 0), then dried in stream of cold air. Examination under UV 366 nm.

Results:



HPTLC of *Crataegus,* a) UV 254 nm, b) NP Reagent
1: *Crataegus laevigata*

2: Standards (increasing Rf):
rutin, vitexin-2”-O-rhamnoside, chlorogenic acid, hyperoside, vitexin, caffeic acid
3: Crataegus monogyna



HPTLC of various *Crataegus* species, NP Reagent

1, 7, 13: Standards (with increasing Rf): **rutin**, **vitexin-2”-*O*-rhamnoside**, **chlorogenic acid**, **hyperoside**, **vitexin**, **caffeic acid**
2, 8, 9: *Crataegus laevigata*
3-6: *Crataegus monogyna* (sample on track 5 is fermented)
10: *Crataegus* x *macrocarpa*
11: *Crataegus azarolus ssp. azarolus*
12: *Crataegus pentagyna*

References

1. CAMAG Instrumental Thin Layer Catalogue- 2013

2. Andok, H.C., Purohit, V.K., 2010, “High Performance Thin Layer Chromatography (HPTLC): A Modern Analytical tool for Biological Analysis”. Nature and Science, 8, 10, 58-61.

3. Shikov, A.N., Pozharitskaya, O.N., Ivanova, S.A., Makarov, V.G., 2007. Separation and evaluation of free radical-scavenging activity of phenol components of *Emblica officinalis* extract by using an HPTLC-DPPH method. J. Sep. Sci. 30, 1250-1254.

4. Polatoglu, K., Demirci, F., Demirci, B., Gören, N., Başer, K.H.C., 2010, Antibacterial Activity and the Variation of *Tanacetum parthenium* (L.) Schultz Bip. Essential Oils from Turkey. J. Oleo Sci. 59, 4, 177-184.

5. Verbitski, S.M., Gourdin, G.T., Ikenouye, L.M., McChesney, J.D., 2008. Detection of *Actaea racemosa* adulteration by thin-layer chromatography combined thin-layer chromatography bioluminescence. J. AOAC Int. 91, 268-275.

6. Choma, I.M., Grzelak, E.M., 2011. Bioautography detection in thin-layer chromatography. Journal of Chromatography A. 13; 1218(19), 2684-2691.

Crateagus, Hawthorn, Alıç

Crataegus laevigata (Poir.) DC. (Rosaceae)
Crataegus monogyna Jacq.
Synonym(s) C. oxyacanthoides, C. oxyacantha, Crataegus oxyacantha.
Part(s) Used: *Fruit, leaf, flower*

Ref. Joanne Barnes, Linda A Anderson, J David Philipson, **Herbal Medicines, 3rd Edition,** Pharmaceutical Press, 2007

Pharmaceutical Importance

Hawthorn is characterised by its phenolic constituents, in particular the flavonoid components to which many of the pharmacological properties associated with hawthorn have been attributed. The phytochemistry of hawthorn fruit and hawthorn leaf with flower are, in general, qualitatively similar, although separate monographs for the fruit (berries) and leaf with flowers appear in the British Pharmacopoeia and European Pharmacopoeia. Pharmacological actions support some of the traditional uses of hawthorn, although clinical evidence is limited to preparations containing hawthorn leaf with flower.

Constituents

Amines

Phenylethylamine, *O*-methoxyphenethylamine and tyramine.

Flavonoids

Up to 1%. Flavonol (e.g. kaempferol, quercetin) and flavone (e.g. apigenin, luteolin) derivatives, rutin, hyperoside, vitexin glycosides, orientin glycosides. The fruits contain relatively more hyperoside and the leaves relatively more vitexin glycosides.

Tannins

Proanthocyanins (catechin-type oligomers).

Other constituents

Cyanogenetic glycosides and saponins.

Quality of plant material and commercial products

As with other herbal medicinal products, there can be variation in the qualitative and quantitative composition of hawthorn crude plant material and commercial preparations of hawthorn fruit and/or leaf with flower. According to the British and European Pharmacopoeias, hawthorn leaf with flower consists of the whole or cut, dried flower-bearing tops of *C. laevigata* or *C. monogyna* (or rarely certain other *Crataegus* species), and contains not less than 1.5% of flavonoids, expressed as hyperoside and calculated with reference to the dried drug.

Aqueous-alcoholic (40–70% ethanol or methanol) extracts of hawthorn leaf with flower obtained from several different manufacturers were reported to have a similar profile of constituents (procyanidins, flavonoids, total vitexin and total phenols) both qualitatively and quantitatively, whereas an aqueous extract was stated to have a lower concentration of procyanidins, flavonoids and total phenols, and a similar total vitexin content. However, no statistical analyses were described to support these findings and there were in fact also substantial quantitative variations between aqueous-alcoholic extracts in content of procyanidins (8.7–13.5%), flavonoids calculated as hyperoside (1.1–3.4%), vitexin-2''-*O*-rhamnoside and hyperoside (5.7–7.9%), total vitexins (4.1–4.9%) and total phenols (19.5–25.6%); values for these constituents for the aqueous extract were 8.0%, 0.7%, 5.6%, 4.1% and 14.0%, respectively. Further work showed that all the extracts had a relaxant effect on norepinephrine (noradrenaline)-induced contractions of guineapig aortic rings *in vitro*, achieving relaxations of 29–44% of baseline values. EC_{50} (effective concentration) values were between 4.2–9.8 mg/mL for the aqueous-alcoholic extracts and 22.4 mg/mL for the aqueous extract, although maximum effects were similar for all extracts. The positive control milrinone had a significantly greater effect than any of the extracts (EC_{50} 1.3 mg/mL). Basic statistical analyses indicated that there were no statistically significant differences between the aqueous-alcoholic extracts in terms of their relaxant effects, indicating pharmacological equivalence. However, more sophisticated analysis allowing for multiple analyses indicated that the effects of the 40% ethanol and 55% methanol extracts differed significantly from those of the other aqueous-alcoholic extracts. These findings provide some support for the equivalence of 40–70% ethanol or methanol extracts of hawthorn leaf with flower, although this would be strengthened by more consistent results in further studies. The equivalence of preparations of hawthorn fruit also requires investigation.

Food Use

Hawthorn is not commonly used in foods. It is listed by the Council of Europe as a natural source of food flavouring (category N2). This category indicates that hawthorn can be added to foodstuffs in small quantities, with a possible limitation of an active principle (as yet unspecified) in the final product. In Cyprus, the fruits of hawthorn are used in the preparation of jam.

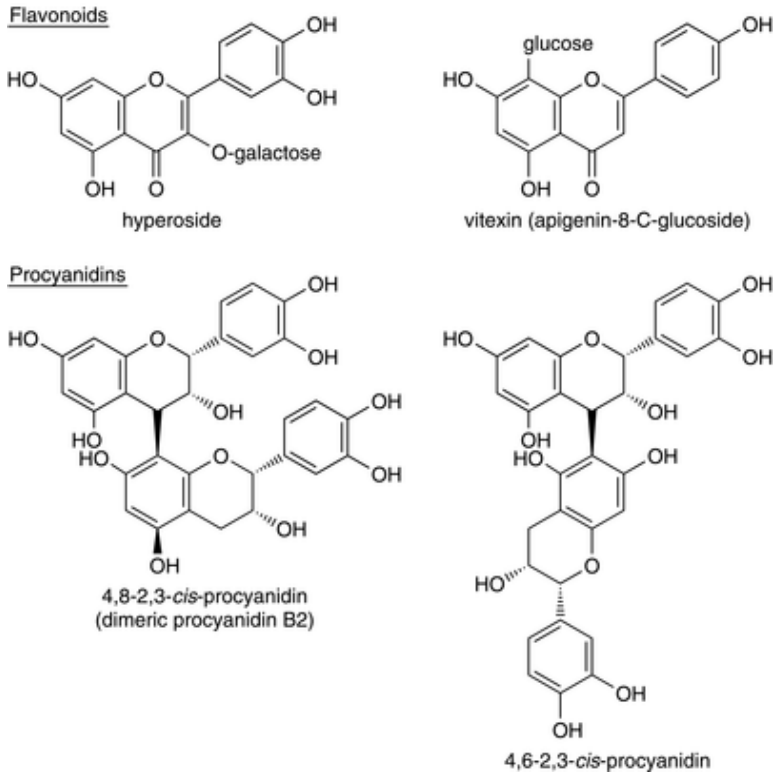
Herbal Use

Hawthorn fruit is stated to possess cardiotonic, coronary vasodilator and hypotensive properties. Traditionally, it has been used for cardiac failure, myocardial weakness, paroxysmal tachycardia, hypertension, arteriosclerosis and Buerger's disease. The German Commission E did not approve therapeutic use of the fruit. Modern interest in hawthorn is focused on the use of hawthorn leaf with flower in reduced cardiac performance.

Dosage

Dosages for oral administration (adults) for traditional uses recommended in older standard herbal reference texts are given below.

Dried fruit: 0.3–1.0 g as an infusion three times daily.
Liquid extract: 0.5–1.0 mL (1 : 1 in 25% alcohol) three times daily.
Tincture: 1–2 mL (1 : 5 in 45% alcohol) three times daily.



Selected constituents of hawthorn.

Leaf with flower

For the treatment of decreasing cardiac efficiency corresponding to functional stage II of the New York Heart Association 160–900 mg aqueous-alcoholic extract (ethanol 45% v/v or methanol 70% v/v; drug to extract ratio 4–7 : 1, with defined flavonoid and/or procyanidin content corresponding to 3.5–19.8 mg flavonoids, calculated as hyperoside, and 30–168.7 mg procyanidins, calculated as epicatechin) daily, in two to three divided doses.

Clinical trials of hawthorn leaf with flower in patients with different stages of heart failure (according to the NYHA classification) mainly have assessed the effects of a standardised extract (WS-1442, containing 18.8% oligomeric procyanidins) administered orally at doses ranging from 160–1800 mg daily for up to 16 weeks.

Contra-indications, Warnings

In view of the nature of the actions documented for hawthorn, there is a view that preparations of hawthorn berries and leaves with flowers are not suitable for self-medication.

Drug interactions

In view of the documented pharmacological actions of hawthorn, the potential for preparations of hawthorn to interfere with other medicines administered concurrently, particularly those with similar or opposing effects, including antihypertensive, antihypotensive, and inotropic agents, should be considered.

In an open-label, randomised, crossover study involving healthy volunteers who received digoxin 0.25 mg for ten days and digoxin 0.25 mg in addition to an extract of hawthorn leaves and flowers (WS-1442) 450 mg twice daily (equivalent to 168.6 mg oligomeric procyanidins) for three weeks with a three-week wash-out period, there were no statistically significant differences between digoxin alone and digoxin plus hawthorn extract in any of the measured pharmacokinetic parameters. Digoxin concentrations were lower in the digoxin plus hawthorn group and, although this did not reach statistical significance (p = 0.054), it is possible that the study did not have sufficient statistical power to detect a difference. Furthermore, the study involved healthy volunteers, and the effects of co-administration of digoxin and hawthorn extract in patients with heart failure requires investigation.

Pregnancy and lactation

Certain hawthorn extracts exhibit activity on uterine tissue (reductions in tone and motility) *in vitro* pharmacological activities described for hawthorn, together with the lack of information on the use of hawthorn during pregnancy and breastfeeding, preparations containing hawthorn fruit or hawthorn and *in vivo* (animals). The clinical relevance of these findings is not known. In view of the these and other leaf and flower are contra-indicated during these periods.

REPORT FORM

The Name of Test/Assay	
The Name of Plant Material	
Glass Material used in the work	
Chemicals	
Solvents	
Reagents (Name & composition)	
Equipment	

Complete the table given above! Write down your study briefly step by step including with your own observations!

Results & Discussion