University of Szeged

Pharmacognosy practice

for pharmacy students

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Rutae herba
Common rue herb
Ruta graveolens L.
Rutaceae

1. Extraction of Rutae herba with solvents of different polarity

Extraction in a percolator: Add 10 ml of solvent (*n*-hexane, chloroform or 50% aqueous methanol (mixed by yourself)) to 5.0 g of dried Rutae herba in a beaker, cover it and leave soaking for 10 minutes. Fix a percolator hanging over a test tube, and plug its bottom with a small piece of cotton-wool. Pour the wet drug powder inside the percolator, then add more of the same solvent as you used before and collect 20 ml of extract.

Extraction in a Soxhlet extractor: Add 15 ml of methanol to 20.0 g of dried Rutae herba and, after allowing it to stand for 10 min, transfer it into a filter-paper thimble. Put the filled thimble into the extracting part of the Soxhlet extractor and add methanol until it covers the drug. Pour methanol into the distillation flask. The total volume of the methanol used should be 200 ml. Assemble the extractor under the Instructor's guidance, and turn on the heating. The extraction should be continued for 35-40 min after distillation starts.

2. TLC analysis of the extracts:
Sorbent: Silica gel

Spots:

n-Hexane extract of Rutae herba	20 μl
Chloroform extract of Rutae herba	20 μl
50% aqueous methanolic extract of Rutae heba	10 μl
0.1% methanolic solution of rutamarin	10 μl
0.1% methanolic solution of rutarin	10 μl

Solvent systems:
n-hexane – acetone (8:2)
chloroform – methanol (9:1)

ethyl-acetate – formic acid – methanol – water (10:2:2:1)

Detection:
UV_{366} light

Theory 1

Definition of drugs

The term "**drug**" defines a substance of either natural or synthetic origin, which can exert a biological effect and, as such, can be used for therapeutic or other medical purposes.
A **herbal** or **animal drug** is therefore a substance of natural origin conforming to the above definition. It may be an actual therapeutic agent, it may serve surgical or anaesthetic purposes, it may be an additive for pharmaceutical preparations (such as a vehicle, a sweetening, a flavouring or a colouring agent, etc.), or it might also serve diagnostic purposes.
Crude drugs are herbal or animal drugs which consist of natural substances that have undergone no other processes then collection and drying.
The term **natural substance** can refer to a whole plant or plant organ (e.g. leaf, root, etc.), a plant extract, or, in special cases a whole animal or its anatomic parts (e.g. glands).

Processing of drugs

Originating directly from a living organism, drugs typically contain much higher amounts of primary metabolites (i.e. proteins, lipids, nucleic acids, etc.) than secondary ones of therapeutic value. To enrich certain constituents of interest, processing is usually needed, such as

1. comminution: grinding or chopping
2. extraction
3. separation of the constituents

Extraction is of key importance to obtain active principles from drugs (see below, liquid – solid extraction). Extraction removes only those substances which dissolve in the solvent used. The extract is usually a complex mixture of substances.

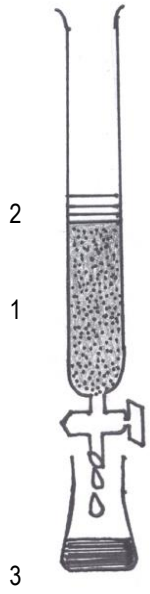
The efficiency of the extraction is determined by the following conditions:

- the particle size (surface area) of the ground drug
- temperature (diffusion increases as the temperature rises; the thermal sensitivity of the compounds of interest must be considered)
- pH value (e.g. in the case of alkaloids)
- solvent polarity (apolar solvents extract mostly apolar components, while polar solvents extract polar components)

Types of extraction:

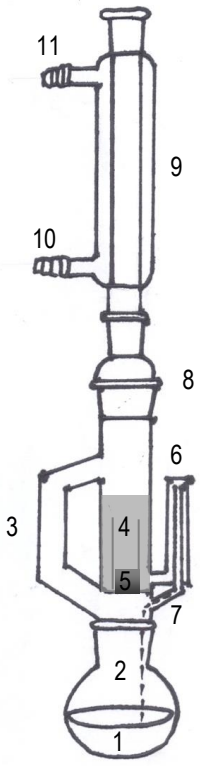
Liquid – solid extraction:
Liquid – solid extraction means extraction from a ground drug into a solvent.

- The drug is able to freely move during the process: infusion, decoction
- The solvent is moving and the drug is steady during the extraction:
  - Percolation: The solvent is passing through a column filled with the drug. This has the advantage that the drug is always in contact with fresh solvent.



1. Grinded drug
2. Solvent
3. Extract

  - Soxhlet extractor: This has the advantage that the temperature is high, the drug is always in contact with fresh solvent, and a relatively small amount of solvent is needed.



1. Stirrer bar/anti-bumping granules
2. Distillation flask (extraction pot) - this should not be overfilled; the volume of solvent in it should be 3 to 4 times the volume of the Soxhlet chamber
3. Distillation path
4. Soxhlet thimble
5. Extraction solid (residue solid)
6. Syphon arm inlet
7. Syphon arm outlet
8. Expansion adapter
9. Condenser
10. Cooling water in
11. Cooling water out

Liquid – liquid extraction:
Liquid – liquid extraction, also known as solvent partitioning, is a method of separating compounds on the basis of their relative solubilities in two different immiscible liquids, usually water (hydrophilic) and an organic (hydrophobic) solvent. It is the extraction of a substance from one liquid phase into another liquid phase. It is commonly performed by using a separating funnel.

Separation of the constituents:

- Centrifugation
- Distillation
- Crystallization
- Filtration
- Chromatography

Chromatography

Definition of chromatography:
Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction (IUPAC, 1993).

Chromatography is a method used for the separation of organic and inorganic compounds. Chromatography can be defined as a science which studies the separation of molecules on the basis of differences in their structures and/or compositions. It was first developed and defined by a Russian botanist Mikhail Tswett in 1903. The word ‘chromatography’ originates from the Greek words for colour "chroma" and write "graphein", chromatography therefore meaning 'to write with colour'.

All chromatographic systems consist of a stationary and a mobile phase. The stationary phase does not move whereas the mobile phase is steadily moving. A sample mixture is placed at the beginning of the stationary phase of a certain length, and the mobile phase is passed through the sample and the stationary phase extracting the constituents of the sample and taking them with it. As a result of the dynamic equilibrium between the two phases, the different compounds migrate at different velocities, depending on their different preferences for the stationary phase and the mobile phase. This eventually results in their separation from each other. Chromatography can be utilized to identify/quantify or isolate constituents of mixtures (analytical or preparative chromatography).

Classification of chromatography

I) Based on the depth of separation
- Column chromatography – the stationary phase is held in a glass column, through which the mobile phase is forced to pass either by pressure or by gravity.

- Planar chromatography – the stationary phase is supported on a flat plate or the interstices of paper and either the capillary force or gravity makes the mobile phase move through the stationary phase.

II) Based on the nature of the mobile phase

- Liquid chromatography (LC)
- Gas chromatography (GC)

III) Based on the mechanism of separation (the nature of the stationary phase)

- Adsorption chromatography: Separation is based mainly on the differences between the **adsorption affinities** of the sample components for the surface of an active solid (e.g. silica gel).
- Partition chromatography: Separation is based mainly on the differences in **solubility** of the sample components in the stationary phase (gas chromatography), or on the differences in solubility in two immiscible solvents (liquid – liquid chromatography).
- Ion-exchange chromatography: Separation is based mainly on the differences in **ion-exchange affinity** of the sample components. The stationary phase is an anionic or cationic ion-exchanger resin. The molecule of interest binds to the column residues and can be eluted by a gradient of a salt (e.g. NaCl – an anionic exchanger) or a buffer (e.g. phosphate, formate or acetate buffers, cationic) solution.
- Gel chromatography (molecular exclusion chromatography): Separation is based mainly on **exclusion effects**, such as the differences in size, shape or charge of the molecules. The term *size-exclusion chromatography* may be used when separation is based on molecular size. The terms *gel fltration* and *gel-permeation chromatography* (GPC) are used to describe this process when the stationary phase is a swollen gel. The liquid or gaseous phase passes through a porous gel, which separates the molecules according to their size. The pores are small enough to exclude the larger molecules. Smaller molecules, however, can enter deeper into the matrix of the gel, and this larger distribution volume leads to their slower migration through the column. Hence, larger molecules pass through the column faster than smaller ones.
- Affinity chromatography: This expression characterizes the particular variant of chromatography in which a **unique biological specificity** of the analyte and ligand interaction is utilized for the separation. For example, an antibody specific to some protein of interest can be immobilized on an otherwise inert stationary phase. When a mixture of proteins is pressed through the system, only the target protein binds to this antibody (and hence to the stationary phase). This protein is later extracted by changing the ionic strength or pH.

IV) Based on the administration of mobile phase

- Capillary action
- Gravity
- Forced
  - Vacuum
  - Pressure
  - High pressure
  - Centrifugal force

Chromatography type	Depth	Mobile phase type	Mechanism	Mobile phase administration
Paper chromatography	planar	liquid	adsorption	capillary action, forced (pressure)
Thin-layer chromatography (TLC)	planar	liquid	adsorption	capillary action, forced (pressure)
Rotation planar chromatography (RPC)	planar	liquid	adsorption	centrifugal force
Column chromatography (CC)	column	liquid	adsorption, ion exchange, etc.	gravity, forced (vacuum, pressure)
Centrifugal partition chromatography (CPC)	column	liquid	partition	forced (pressure, centrifugal force)
Gas chromatography (GC)	column	gas	partition, adsorption	forced (pressure)
High-performance liquid chromatography (HPLC)	column	liquid	many possibilities, depending on the column	forced (high pressure)

Thin-layer chromatography (TLC)

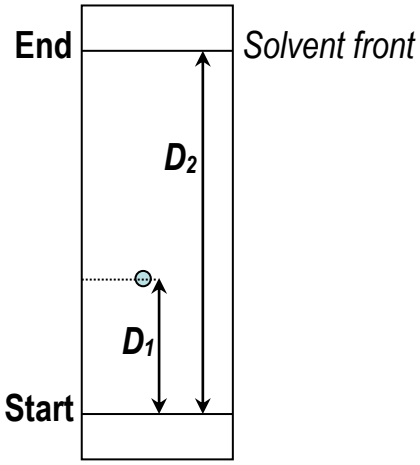
A TLC plate is a sheet of glass, metal or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analysed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate dips into the liquid. This liquid, or the eluent, serves as the mobile phase, slowly migrating up the TLC plate due to capillary action.

When the solvent has reached the top of the plate, the plate is removed from the developing chamber and dried, and the separated components of the mixture are visualized. If the compounds are coloured, visualization is straightforward. Most frequently, the compounds are colourless. The TLC plates used during the practicals contain a fluorescent indicator that fluoresces green under 254 nm light; compounds absorbing at this wavelength appear as dark spots on a green background when the plate is placed under a UV lamp. Other compounds may themselves be fluorescent, providing blue, green, red, etc. spots under UV light of longer wavelength (366 nm). Visualization can be assisted by spraying the TLC plate with an appropriately chosen chemical reagent that provides coloured products when it reacts with certain constituents.

The **retention factor**, R_f, is a simple quantitative indication of how far a particular compound migrates from its starting point in a particular TLC system. The R_f value is a good indicator of whether an unknown compound and a known compound are similar, or even identical. If the R_f value of the unknown compound is close to or the same as the R_f value of the known (reference) compound, then the two compounds are probably similar or identical.
R_f, which can have values in the interval $0 \leq R_f \leq 1$, is defined by

$$\boldsymbol{R_f = D_1 / D_2}$$

where
D_1 is distance that the compound migrats, measured from the centre of its spot to the point where it was originally applied, and
D_2 is the distance of the solvent front from where the sample was applied.



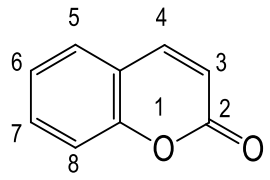
Rutae herba
Common rue herb
Ruta graveolens L.
Rutaceae

This perennial subshrub is native to southern Europe. The herb is rich in volatile oil and also contains other constituents, such as flavonoids (rutin), coumarins (coumarin), furocoumarins (bergapten) and dihydrofurocoumarins (rutamarin and rutarin), and alkaloids (ribalinium).

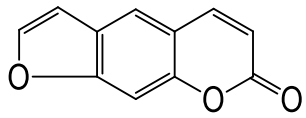
Uses: The drug itself is used as a spasmolytic agent in cases of gastrointestinal cramps, but coumarin is promoted as having beneficial activity against oedema, and its immunostimulant and cytotoxic activities have already led to clinical trials involving patients with advanced cancer. However, one of its metabolites, in rare cases, induces severe hepatonecrosis, which has led to the removal of the drug from the market.

Furocoumarins are phototoxic constituents, and some of them (e.g. bergapten) are used in photochemotherapeutic (PUVA) treatment against psoriasis (administration of phototoxic components followed by UV irradiation).

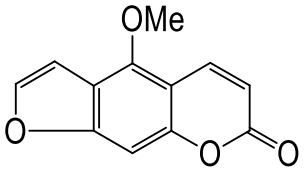
Toxicity: The photosensitizing property of the furocoumarins might result in the solar radiation-induced inflammation of the skin. It occurs after contact with the plant or product followed by exposure to sunlight.



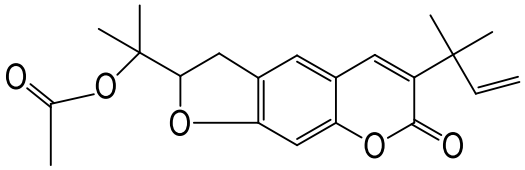
coumarin



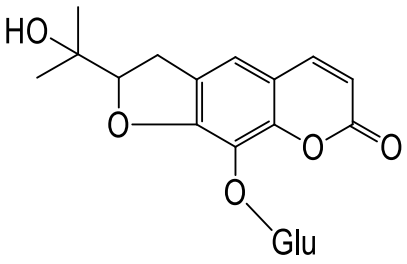
furocoumarin skeleton



bergapten



rutamarin



Glu = glucose

rutarin

Percolation of Rutae herba with solvents of different polarity and TLC analysis of the extracts

The solvents are used in the sequence of increasing polarity, i.e. from the most apolar (i.e. least polar) one to the most polar one: *n*-hexane < chloroform < 50% aqueous methanol. It may be expected that the least polar constituents of the plant will be extracted with *n*-hexane, those of intermediate polarity with chloroform, and the most polar compounds with 50% aqueous methanol.

TLC analysis of the plant extracts reveals their constituents. The surface of the TLC plate is covered with silica gel (representing a highly polar surface, with many –OH groups), which allows the apolar components to migrate further, while the polar components are retained more strongly, with lower *Rf* values. Different solvent systems (apolar: *n*-hexane – acetone; medium polar: chloroform – methanol; and polar: ethyl-acetate – formic acid – methanol – water) will be tested to study how the polarity of the solvent system affects the separation. The detection will be carried out under 366 nm light, where furocoumarin derivatives appear as fluorescent blue or yellow spots.

Test questions 1

Essay questions:

- Define the expressions drug, herbal or animal drug, crude drug, and natural substance.
- Why do we need to process drugs? What are the most important steps for this?
- Define extraction. What conditions influence the efficiency of extraction? What are the main types of extracting methods and their characteristics?
- What kind of methods can be utilized to separate the constituents of an extract?
- Define chromatography and explain how it works in general.
- Classify the chromatographic methods:
    - based on the depth of separation
    - based on the nature of the mobile phase
    - based on the mechanism of separation / the nature of the stationary phase
    - based on the administration of the mobile phase
- Define TLC, explain the mechanism of separation, give the possible visualization methods and describe the retention factor.
- In the TLC of the different extracts of common rue herb, how did we extract the drug? How did we check the composition of the different extracts by means of TLC and visualize them on the developed TLC plate? What could we conclude?

Mechanisms and structures:

- Give the structure of coumarin.
- Give examples of furocoumarins (furocoumarin skeleton, bergapten, rutamarin, and rutarin).

Classification (Latin name of the drug, species, and family) and uses of the following drugs:

- Common rue herb

Practical 2

Basic phytochemical procedures, extraction methods, chromatography, II

1. Separation of the chloroform extract of Rutae herba by column chromatography
2. TLC analysis of the fractions

1. Fractionation by column chromatography (stationary phase: silica gel)

Plug the bottom of the glass column with a small piece of cotton-wool. Suspend 7 g of silica gel with a mixture of *n*-heptane – ethyl acetate (8:2) and pour the suspension into the glass column. Wash the remaining silica gel onto the top of the column with the same solvent. For this purpose, you can use the solvent within the column by opening the tap. **Important**: Never let the top of the column dry. The solvent must always cover it. Once the column has been prepared, let the solvent flow down until its surface just covers the top of the column and then close the tap.

Dissolve the chloroform extract of Rutae herba prepared during the previous week's practical in the mixture of *n*-heptane – ethyl acetate (8:2). Carefully layer 1 ml of this solution (i.e. the sample to be separated) onto the top of the column. Perform the elution with *n*-heptane – ethyl acetate (8:2), (7:3) and (1:1) eluents, and collect 5 fractions (volume: 5 ml) of each.

Elution:

Collect 20 ml to an Erlenmeyer flask first and start the fractioning only after this!

fraction 0: *n*-heptane – ethyl acetate (8:2); 20 ml
fractions 1-5: *n*-heptane – ethyl acetate (8:2); 5 ml each
fractions 6-10: *n*-heptane – ethyl acetate (7:3); 5 ml each
fractions 11-15: *n*-heptane – ethyl acetate (1:1); 5 ml each

2. TLC determination of the fractions

Sorbent: silica gel. Place the TLC plate horizontally.

Spots:

0.1% methanolic solution of rutamarin	10 µl
Original chloroform extract of Rutae herba	10 µl
Fractions eluted from the column	20 µl each
0.1% methanolic solution of bergapten	10 µl

Solvent system:

n-hexane – acetone (7:3)

Detection:

366 nm light

Theory 2

Column chromatography (CC)

In CC, the solid stationary phase is placed in a vertical glass (usually) column and the mobile phase, a liquid, is added to the top so that it flows down through the column either due to gravity or assisted by external pressure. CC is generally used as a purification technique for the isolation of the desired compound(s) from a complex mixture.
The mixture to be the subject of CC is applied to the top of the column. The liquid solvent (the eluent) is passed through the column by gravity or by the application of air pressure. As explained above (see Theory 1), the different components of the mixture bind with different strengths to the stationary phase, and hence their "washing" through the column with the mobile phase results in different migration velocities. If the column is long enough and/or the differences are significant enough, the components of the mixture separate from each other and can be collected one after the other into distinct fractions as the solvent drips from the bottom of the column.
Two main types of CC can be differentiated: normal-phase CC (NP-CC) and reversed-phase CC (RP-CC). Typical adsorbents for NP-CC are silica gel (SiO_2) or alumina (Al_2O_3). These have a polar surface; in these cases less polar mobile phases are used (chloroform, dichloromethane, acetone, etc.) and the elution sequence will be from the leas polar (eluting first) to the most polar compounds (eluting last). In RP-CC, the adsorbent has an apolar surface (e.g. octadecyl silica gel = C_{18}), more polar eluents are used (methanol or water) and the sequence elution of the compounds is the opposite of that in NP-CC (hence the name "reversed-phase").

Separation of the chloroform extract of Rutae herba with CC and TLC analysis of the fractions

The stationary phase of the column is silica gel, which serves as a polar surface for the separation. A gradient elution is to be performed with a stepwise increase of the polarity of the solvent system used: from (8:2) *n*-heptane – ethyl acetate to 1:1. Increase the solvent polarity during the separation will result in a gradual acceleration of the migration of the components of the mixture through the column, allowing the separation of compounds with a broad range of polarity within a reasonable time frame. As above (see NP-CC), the least polar components are expected in fractions 1-5, eluted with *n*-heptane-ethyl acetate 8:2, the intermediate polar components in fractions 6-10, eluted with *n*-heptane – ethylacetate 7:3, and the most polar components in fractions 11-15, eluted with *n*-heptane – ethyl acetate 1:1. It is interesting to examine the relationship between the elution time (i.e. the fraction number) and the R_f values of the individual components: theTLC check of the compositions of the fractions is performed on silica gel, resulting in a certain pattern on the TLC plate.

Test questions 2

Essay questions:

- Define column chromatography. How can it be used to separate the different constituents of a complex mixture such as a plant extract?
- How did we perform CC of thecommon rue herb extract? Describe the detection and visualization of the different compounds obtained from rue extract.

Practical 3

Basic phytochemical procedures, extraction methods, chromatography, III

1. Isolation of rutamarin by preparative TLC
2. Purity examination of the isolated rutamarin by TLC
3. Two-dimensional TLC

1. Isolation of rutamarin by preparative TLC

Rutamarin is isolated out from the previously prepared chloroform extract of the Rutae herba. Load the extract onto the bottom of the TLC plate in the form of a band ca. 7 cm long and ca. 0.5 cm wide. Apply pure rutamarin next to it as a separate spot for reference. Develop the plate in a mixture of *n*-hexane – acetone (8:2). Mark the band whose R_f value and colour are identical with those of rutamarin under 366 nm light and scrape it off together with the silica gel. Put a small amount of cotton-wool into the bottom of an elution tube and put the powder on the top of it. Pour 2 ml of methanol onto it and collect the liquid in a porcelain dish.

2. Purity examination of the isolated component by TLC

Sorbent: Silica gel

Spot:

Isolated rutamarin	40 µl
0.1% methanolic solution of rutamarin	10 µl

Solvent system:

n-hexane – acetone (8:2)

Detection:

366 nm light

3. Two-dimensional TLC

Spot the extract into the left corner of the plate and the samples of the two reference compounds into the right corner. Develop the chromatogram in solvent system A until three-quarters of the plate, and then remove it from the chamber. After drying it, rotate it counterclockwise through 90º. Apply the same two reference compounds as before into the left corner of the plate and develop the chromatogram again until three-quarters of the plate in solvent system B.

Sorbent: Silica gel (use a 20 x 20 cm plate)

Spots:

Chloroform extract of Rutea herba	20 µl
0.1% methanolic solution of rutamarin	10 µl
0.1% methanolic solution of bergapten	10 µl

Solvent system A:
n-Hexane – acetone (8:2)

Solvent system B:
Toluene – ethyl acetate – formic acid (5:4:1)

Detection:
366 nm light

Theory 3

Isolation of rutamarin by preparative TLC

Preparative TLC is a simple isolation method that can be utilized for the purification of small quantities of sample. The fraction containing several components is separated on a TLC plate as discussed above, and the component of interest is removed by scraping it off together with the silica gel. The component can easily be eluted from the silica gel with methanol, and its purity can be rapidly checked with an additional (analytical) TLC (rutamarin should be the only component present in the eluted fraction). The compound is visualized under 366 nm light as above.

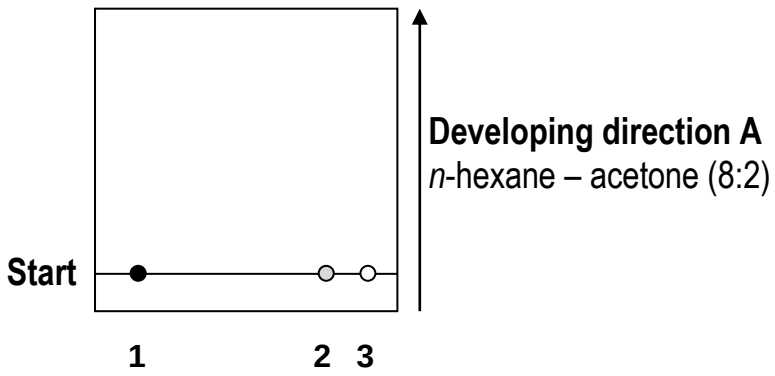
Two-dimensional chromatography

In planar chromatography, two-dimensional chromatography refers to the chromatographic process in which the components are subsequently developed in two directions, in order to gain benefit from the different selectivities of two different solvent systems. R_f is influenced not only by the polarity, but also by various other physicochemical properties of the utilized solvents. Several of these properties of the solvent systems can have different effects on the migration of different compounds. It is therefore frequently observed that two compounds cannot be separated in solvent A, but this is possible in solvent B. Since solvent A might give better separation for other compounds, two-dimensional TLC usually provides a much better resolution than that with either solvent alone: the constituents are scattered around the TLC plate and a better identification of certain components can be achieved.

With this setup, only one sample can be tested at a time, but the number of applied reference compounds can vary.

Procedure:

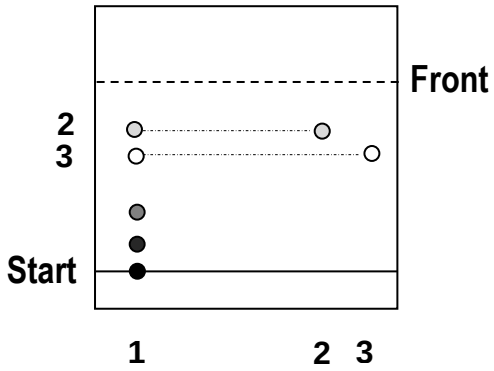
1.



Spot the chloroform extract of Rutae herba (**1**) and the reference compounds (rutamarin, **2**, and bergapten, **3**) onto the TLC plate (test materials should be placed to the right side

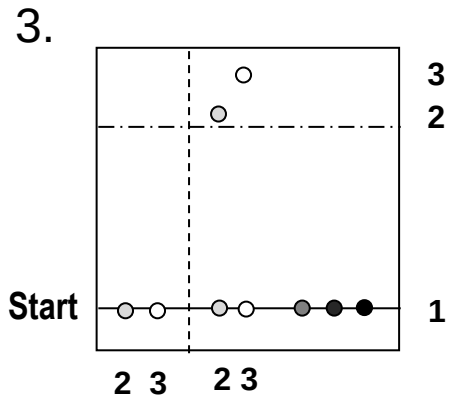
of the plate). Develop the chromatogram in the first direction (direction **A**) in *n*-hexane – acetone 8:2 till the ¾ of the plate.

2.



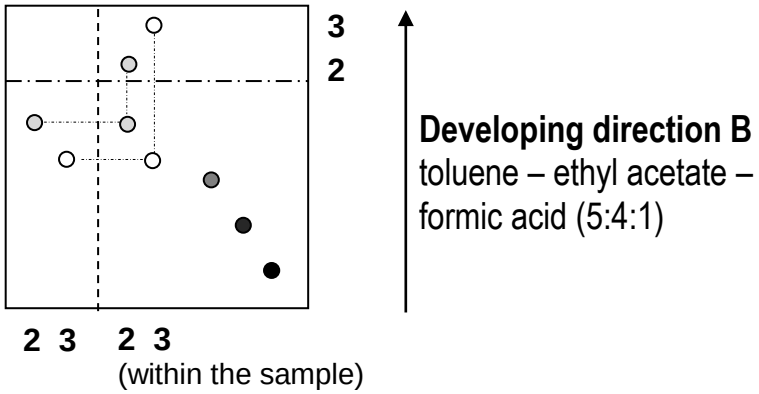
After the development, you can identify the two reference compounds (**2** and **3**) within the extract through their R_f values.

3.



Turn the plate through 90°. Spot the two reference compounds (**2** and **3**) again, but now on the left side of the plate. Develop the chromatogram in the second direction (direction **B**) in toluene – ethyl acetate – formic acid (5:4:1).
After the plate is turned, the chromatogram prepared in direction A becomes a set of starting points of sample components already separated by solvent A.

4.



The two reference compounds (**2** and **3**) can be identified in this direction too: spots of compounds **2** and **3** will provide the coordinates where these two compounds should be found if they are present within the analysed sample. In our case, we can conclude that both compounds are present in the chlorofom extract of Rutae herba.
It can be seen that most of the constituents of the extract (but not all) are scattered around the diagonal of the plate.

Test questions 3

Essay questions:

- What is preparative TLC? How can it be used for isolation? How did we the isolated rutamarin by preparative TLC and how could we check the purity?
- Define 2D TLC, write and draw the procedure of a 2D TLC development, and explain how the components of an extract can be identified.
- What would we observe if we performed a 2D TLC by using the same solvent system twice?

Practical 4

Drugs with carbohydrate content, I

1. Investigation of starch-containing drugs (Maydis amylum, Solani amylum andTritici amylum)
   - Microscopic characteristics
   - General starch tests
   - Tests for impuritites
2. Tests for Gossypii lana
   - Behrens test, Schweitzer test
3. Isolation of polysaccharides from Lini semen and Althaeae radix

Solani amylum	**Tritici amylum**	**Maydis amylum**
Potato starch	Wheat starch	Maize starch
Solanum tuberosum L.	*Triticum aestivum* L.	*Zea mays* L.
Solanaceae	Poaceae	Poaceae

1. Investigation of starch-containing drugs (Maydis amylum, Solani amylum andTritici amylum)

Microscopic characteristics: Place a small amount of starch on a glass slide, add 1 drop of a mixture of glycerol – water (1:1), put a cover slip on the top and examine the characteristics under the microscope.

General starch tests

Test for identification:

Dissolve 0.2 g of starch in 10 ml of hot water, which will give a translucent jelly. Allow it to cool, and then add 1 drop of 0.01 M iodine solution.

- Heat the mixture and allow it to cool down again
- Add 1-2 drops of R-NaOH solution, and then R-HCl solution

Tests for impurities:

- Soluble starch impurity: Shake 1 g of starch with 10 ml of cold water for 5 min. Filter the liquid through double filter paper. Add 1 drop of 0.01 M iodine solution to the filtrate. The liquid turns blue in the presence of soluble starch.
- $CaCO_3$: Put a small amount of $CaCO_3$-containing starch into a porcelain dish. Add 2 drops of R-HCl. You can see/hear the formation of CO_2 bubbles if $CaCO_3$ is present.
- Foreign starch impurity can be recognized by the different shape and size of the starch granules on microscopic investigation.

Gossypii lana
Cotton
Gossypium hirsutum
Malvaceae

2. Tests for Gossypii lana

Identification (Behrens test):
Treat a small sample of cotton-wool with iodine – zinc chloride solution (Behrens reagent) on a glass slide: put the cotton-wool on the slide, cover it with a cover slip and drop the Behrens reagent onto the side of the cover slip. Check the situation under the microscope after 15 minutes.

Schweitzer test:
Put some fibres of cotton-wool on a glass slide and cover them with a cover slip. Add 2 drops of Schweitzer reagent [copper(II) oxide dissolved in concentrated NH_4OH]. Check the situation under the microscope after 15 min.

Lini semen	**Althaeae radix, Althaeae folium**
Linseed	Marshmallow root, leaf
Linum usitatissimum L.	*Althaea officinalis* L.
Linaceae	Malvaceae

3. Isolation of polysaccharide from Lini semen and Althaeae radix

Lini semen
Add 20 ml of distilled water to 5 g of whole linseeds in a flask and place the flask on a water bath for 30 min. To avoid the evaporation of the water, cover the flask with a glass funnel. After the contents have cooled down, filter the extract through 3 layers of gauze. Add cold methanol to the filtrate (3 times the volume of the filtrate). The polysaccharide will precipitate. Filter it off on paper and wash the precipitate with 2 x 5 ml of acetone.

Althaeae radix
Add 50 ml of distilled water to 5 g of Althaeae radix in a flask and place the flask on a water bath for 30 min. To avoid the evaporation of the water, cover the flask with a glass funnel. After the contents have cooled down, filter the extract through 5 layers of gauze. Add cold methanol to the filtrate (double the volume of the filtrate). The polysaccharide will precipitate. Filter it off on paper and wash the precipitate with 2 x 5 ml of acetone.

Theory 4

Carbohydrates

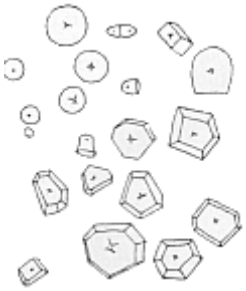
Carbohydrates are the products of the primary metabolism in plants. They contain only carbon, hydrogen and oxygen, the last two elements usually being present in the same proportions as in water. They are among the most abundant constituents of plants. Many carbohydrates, such as the sugars and starches, are important food reserves for plants and foodstuffs for mammals. Cellulose and other polysaccharides are constituents of the cell wall of plants. Sugars can be bound to a wide variety of other compounds, forming glycosides.

Monosaccharides
These sugars contain 3-9 carbon atoms but those with 5 (pentoses) or 6 (hexoses) occur the most frequently. Those containing an aldehyde group (such as glucose) are aldo sugars, while those with a keto group (such as fructose) are ketoses. Through oxidation of the terminal –OH group to –COOH, uronic acids are produced (such as glucuronic acid from glucose, or galacturonic acid from galactose).

Oligosaccharides
These are derived from 2-10 monosaccharide units. The most important representatives of this group are disaccharides, such as sucrose (glucose + fructose), maltose (glucose + glucose) or lactose (glucose + galactose); and trisaccharides, such as gentianose and raffinose.

Polysaccharides
Polysaccharides are formed by from ten to thousands of interconnected monosaccharide units. The most important representatives of this group are starch, cellulose, gums and mucilages.

Maydis amylum
Maize starch
Zea mays L.
Poaceae



The drug is obtained from the grains of *Zea mays*. Starch is a fine white powder. The grains are polygonal, rounded or spherical 10 to 30 µm in size. The hilum is a central triangular or 2 to 5 stellate cleft. No striation is present.

Uses: A disintegrating agent in tablet formulation, the base of dusting powders.

Tritici amylum
Wheat starch
Triticum aestivum L.
Poaceae

Wheat starch is obtained from the grains of *Triticum aestivum*. It is a very fine slippery white powder. The larger particles are lenticular and the smaller ones are globular. The particles are 30-45 µm in size. The hilum is a central point, or seldom a cleft. The striations are concentric, but not well-defined.

Uses: A disintegrating agent in tablet formulation, the base of dusting powders.

Solani amylum
Potato starch
Solanum tuberosum L.
Solanaceae

The drug is obtained from the tuber of *Solanum tuberosum*. Potato starch is a fine white powder consisting mostly of simple particles that are irregularly ovoid or spherical. The size is around 45-150 µm and the hilum in the form of a point is excentric. The excentric striations are well marked.

Uses: A disintegrating agent in tablet formulation, the base of dusting powders.

Starch is generally a mixture of two structurally different polysaccharides. One of the components is amylose (present as 25% of the starch), a linear molecule composed of 250-300 D-glucopyranose units uniformly linked by α-1,4-glucosidic bonds, which cause the molecule to adopt a helix-like shape. The other component is amylopectin (present as 75% of the starch), which consists of 1000 or more glucose units, most of them also connected by α-1,4-glucosidic bonds, but a number of α-1,6 linkages also occurr at branch points. Because of these structural differences, amylose is more soluble in water than amylopectin.

General starch tests:
Starch is insoluble in cold water, but forms a colloidal solution in boiling water. This solution forms a transparent jelly on cooling. The starch solution is coloured deep-blue by solution of iodine. The colour disappears upon heating or following the addition of NaOH, but reappears when the solution is cooled down or neutralized by the addition of HCl.

Gossypii lana
Cotton
Gossypium hirsutum
Malvaceae

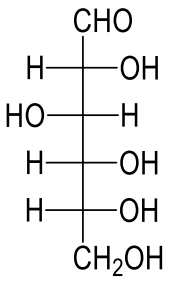
Cotton-wool is prepared from the epidermal trichomes of different Gossypium species. The trichomes should be freed from mechanical contamination, defatted, bleached and combed. Cotton is a white, almost odourless mass, consisting of almost 100% cellulose. Cellulose is built up from around 3000 D-glucopyranose units linked by β-1,4-glucosidic bonds (in contrast with starch), which confers a linear shape to the molecule.

Use: As a wound dressing.

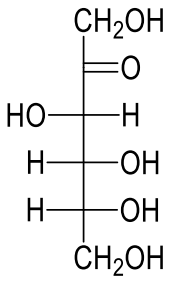
Tests for Gossypii lana

Identification (Behrens test)
See method in Practical 4. After 15 min, the cotton fibres turn blue.

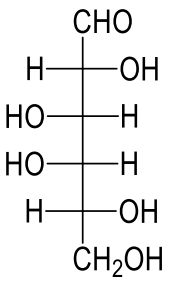
Schweitzer test:
See method in Practicle 4. After 15 min, the cotton fibres start to swell, with the appearance of pearl-like formations, and they eventually dissolve.



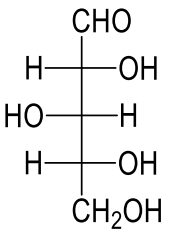
D-glucose



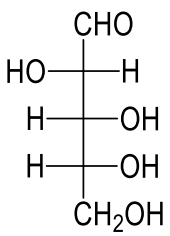
D-fructose



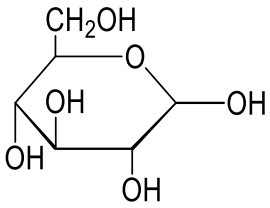
D-galactose



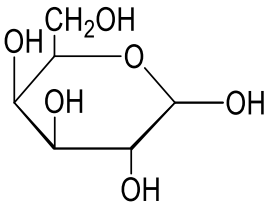
D-xylose



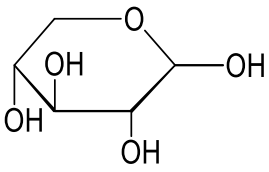
D-arabinose



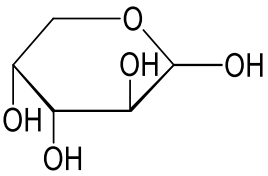
D-glucose



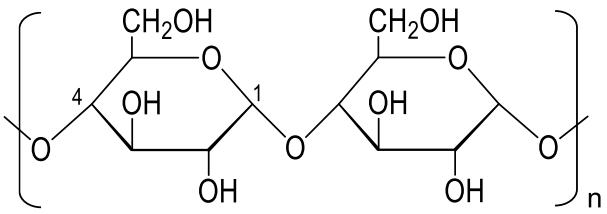
D-galactose



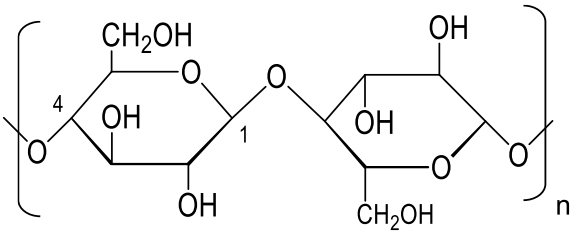
D-xylose



D-arabinose



amylose

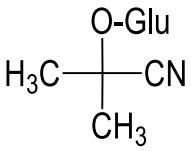


cellulose

Lini semen
Linseed
Linum usitatissimum L.
Linaceae

The seeds are ovate, flattened and around 4-6 mm long and 2-2.5 mm broad with brown testa. The drug is odourless and has a mucilaginous taste. The seed contains mucilage (20%) and fixed oil (30-40%). When the polysaccharide is hydrolyzed, rhamnose, galactose, glucose, xylose, arabinose, fucose and galacturonic acid monosaccharide units can be identified. The drug also contains 0.1-1.5% linamarin, a toxic cyanoglycoside and the enzyme linamarase.

Uses: As a mild laxative because of its polysaccharide content.



Glu = glucose

linamarin

Althaeae radix, Althaeae folium
Marshmallow root, leaf
Althaea officinalis L.
Malvaceae

Root
The drug is 4-10 cm long and 1-2 cm in diameter. It is whitish and fibrous, with a sweet and mucilaginous taste. It contains 25-35% mucilage, starch and sugars. After hydrolysis of its polysaccharide content, glucose, galactose, arabinose, rhamnose and galacturonic acid can be identified.

Leaf
This contains polysaccharide and mucilage.

Uses: Both drugs are used as demulcents; they have anti-inflammatory and immunostimulant effects.

Isolation of polysaccharides from Lini semen and Althaeae radix
The hydrophilic polysaccharides can easily be isolated with hot water. After filtration of the extract, added methanol precipitates the polysaccharides. After filtration and washing, dry the precipitate and keep it for the next practical.

Test questions 4

Essay questions:

- Define carbohydrates.
- Define monosaccharides, oligosaccharides and polysaccharides.
- Starches: describe the general properties and constitution of starches. Give the general starch tests for identification and purity.
- Tests for cotton: Behrens and Schweitzer tests.
- How did we isolate polysaccharides from linseed and marshmallow root?

Mechanisms and structures:

- Give examples of monosaccharides (glucose, fructose, galactose, xylose and arabinose).
- Give examples of polysaccharides (amylose and cellulose).

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Potato starch, wheat starch, maize starch, cotton, linseed, marshmallow root and leaf

Practical 5

Drugs with carbohydrate content, II

1. Hydrolysis and TLC analysis of the monosaccharides obtained from the polysaccharides of Lini semen and Althaeae radix
2. Acaciae gummi and Tragacantha: tests for identification and purity
3. Determination of the swelling value of Agar

1. Hydrolysis and TLC analysis of the monosaccharides obtained from the polysaccharides of Lini semen and Althaeae radix

Hydrolysis

Hydrolyse 50 mg of dried polysaccharide with 5 ml of 1 N H_2SO_4 in a flask on a water bath for 1 hour. To avoid the evaporation of the water, cover the flask with a glass funnel. After the mixture has cooled down, add 10 ml of distilled water and neutralize the mixture with $BaCO_3$ bubble formation can no longer be observed. Filter the mixture through paper into a porcelain dish and evaporate off the liquid on the water bath. After the dish has cooled down, dissolve the dry residue in 1 ml of water.

TLC determination

Sorbent: Silica gel

Spots:

Hydrolysed polysaccharide from Lini semen	5 µl
Hydrolysed polysaccharide from Althaeae radix	5 µl
0.1% aqueous glucose solution + 0.1% aqueous xylose solution	10 µl each
0.1% aqueous galactose solution + 0.1% aqueous arabinose solution	10 µl each

Solvent system:

Chloroform – methanol – water (64:50:10)

Detection:

Spray the TLC plate with thymol – sulfuric acid reagent and heat it at 120 °C for 5 min.

Acaciae gummi
Acacia gum
Acacia senegal Wild.
Mimosaceae

Tragacantha
Tragacanth gum
Astragalus gummifer Labill.
Fabaceae

2. Acaciae gummi and Tragacantha: tests for identification and purity

Acaciae gummi

Tests for identification:
Use 10% solution of acacia gum.

1. Polysaccharide:
   - Add 1 ml of 90% methanol to 1 ml of acacia gum solution
   - Add 10 ml of water to the above-prepared solution
2. Arabic acid:
   - Add 10 ml of 90% methanol previously acidified with a few drops of R-acetic acid to 5 ml of acacia gum solution
3. Calcium:
   - Filter the solution prepared in point 2 through a filter paper
   - Add 1 ml of R-ammonium oxalate solution
   - Add R-HCl to the precipitated solution
4. Arabin:
   - Add 2 drops of basic lead acetate (Pb-acetate) solution to 2 ml of acacia gum solution
5. Peroxidase:
   - Add 5 drops of diluted H_2O_2 and 5 drops of 1% alcoholic benzidine solution to 5 ml of Acacia gum solution
6. Starch:
   - Add 1 drop of 0.01 M iodine solution to 5 ml of acacia gum solution

Tragacantha

Tests for identification:
Use 0.5% tragacanth gum solution.

1. Polysaccharide:
   - Add 5 ml of basic Pb acetate solution to 10 ml of tragacanth gum solution
2. Starch:
   - Add 1-2 drops of 0.01 M iodine solution to 5 ml of tragacanth gum solution

Write down your observations.

Agar
Agar
Gelidium sp.
Rhodophyceae

3. Determination of the swelling value of agar

The test is performed in a 25 ml glass-stoppered volumetric cylinder with 0.2 ml gradations ranging from 0 to 25 ml, which has a height of 10-12 cm. Moisten 1 g of drug with 1 ml of methanol within the cylinder and add 25 ml of distilled water. Shake the fluid thoroughly, holding the stopper firmly. Agitate the mixture every 10 min for half an hour and then leave it to swell for 1 h at room temperature. Read the final volume of the drug in ml together with any adhering mucilage.

Theory 5

Hydrolysis and TLC analysis of the monosaccharides obtained from the polysaccharides of Lini semen and Althaeae radix

The glycosidic bonds linking the monosaccharide units together to form polysaccharides can be broken by hydrolysis with 1 N H_2SO_4 and heating. After the reaction has finished, only the monosaccharide units are detectable, in relative amounts corresponding to the original ratio within the polysaccharide. With the applied reference compounds, these components can be identified on the TLC plate. Spraying the chromatogram with thymol – sulfuric acid reagent (ethanolic solution of 0.5% thymol containing 5% sulfuric acid) is a typical method for the TLC detection of monosaccharides. After spraying, the TLC plates are heated for 5 min at 120 °C in the heating chamber. Sugars appear as pink spots.

Other polysaccharides

Gums are natural plant hydrocolloids which may be classified as anionic or non-ionic polysaccharides or their salts. They are translucent, amorphous substances which are frequently produced by higher plants either spontaneously or (with a protective function) after injury. The compositions of gums are typically heterogeneous. Following hydrolysis, arabinose, galactose, glucose, mannose, xylose and various uronic acids may be detected. Gums in the plants may form salts with Ca^{2+}, Mg^{2+} and other cations.

Mucilages are also heteropolysaccharides; they are produced and stored in higher plants and can be obtained by extraction with cold or hot water.

Acaciae gummi

Acacia gum
Acacia senegal Wild.
Mimosaceae

Acacia gum is a dried gum obtained from the stem and branches of *Acacia senegal*. *A. senegal* is a tree about 6 m high that is abundant in Sudan and Central and West Africa (it is also known as Hashab or Werek). The drug forms rounded or ovoid tears up to about 3 cm in diameter or in angular fragments. The outer surface bears numerous fine cracks, which are formed during the 'ripening' period and make the tears opaque. The gum is white or very pale-yellow. The tears break rapidly with a somewhat glassy fracture and much of the drug consists of small pieces. It is odourless and has a bland and mucilaginous taste. The drug contains 80-90% of a polysaccharide called arabin, which is the Ca, Mg, K salt of arabic acid. Arabic acid is built up from glucose, galactose, rhamnose and arabinose. Acacia gum also contains oxidases and peroxidases, which must be removed before medical use. The gum is used as an ingredient in medications

and these enzymes may oxidize certain active molecules, which can influence their effectiveness. The gum does not contain starch.

Uses: As a general stabilizer in emulsions, and a demulcent. It is widely used in the food stuff, drinks and other industries.

Tests for identification

For polysaccharide: the addition of ethanol results in a whitish precipitate, which is dissolves on dilution it with water.

For arabic acid: on the addition of acidic methanol, a whitish precipitate is formed.

For calcium: when the solution obtained in the testing for arabic acid (see above) is filtered, Ca^{2+} will be present in the filtrate. The addition of NH_4 oxalate leads to a precipitate of Ca oxalate, which is soluble in HCl.

For arabin: the addition of Pb acetate results in the formation of a whitish precipitate.

Peroxidases: the addition of H_2O_2 and benzidine results in a blue colour if peroxidases are present.

Starch: a general starch test with iodine does not give a positive result.

Tragacantha

Tragacanth gum

Astragalus gummifer Labill.

Fabaceae

Astragalus species are thorny shrubs found in the mountainous districts of Anatolia, Syria, Iraq and Iran. Tragacanth gum is the dried gum exudate obtained from incision in the bark of *Astragalus* species. The drug consists of white, horn-like, sometimes translucent and slightly yellowish plates, incurved in a crest or crescent shape. The pulverized drug is pale-yellow, with a mucilaginous taste. The gum is composed of a water-soluble fraction known as tragacanthin and a water-insoluble fraction known as bassorin. After hydrolysis, galactose, arabinose and xylose can be identified. The drug contains a small amount of starch.

Uses: as a suspending agent for insoluble powders, and as a binding agent in pills and tablets.

Tests for identification

For polysaccharide: the addition of Pb acetate results in formation of a whitish precipitate.

For starch: a general starch test with iodine gives a positive result: blue spots appear in the solution.

Agar
Agar
Gelidium sp.
Rhodophyceae

Agar is the dried colloidal substance obtained by concentrating the decoction of various red algae, and especially *Gelidium* species. Agar is known in two forms, one consisting of yellowish translucent stripes, and the other of coarse powder or flakes. The drug is greyish-white or brownish-yellow and has no taste or odour. It consists of polysaccharides, agarose and agaropectine. Agarose consists of galactose and 3,6-anyhydrogalactose. The agaropectine contains uronic acids besides galactose.

Uses: for the preparation of microbiological culturing media, as an emulsifying agent and in the treatment of chronic constipation.

Definition of swelling value
Swelling value is defined as the volume of 1 g of air-dried drug in water or in another liquid specified by monographs, after it has been left to swell at room temperature for 4 h. It is expressed in ml.

Determination of swelling value (Ph. Eur.):
Moisten the prescribed quantity of air-dried drug comminuted to the fineness specified by the monographs with the specified moistening liquid in a 25 ml glass-stoppered volumetric cylinder with 0.2 ml gradations ranging from 0 to 25 ml and a height of 10-12 cm. Shake the drug thoroughly with 25 ml of the specified liquid. Agitate the mixture every 10 min for 1 hour and then leave it for 4 h at room temperature. Read off the volume of the drug in ml together with any adhering mucilage. Calculate the mean value of at least two parallel tests related to 1 g of dried drug.

Test questions 5

Essay questions:

- How did we hydrolyse the polysaccharides obtained from linseed and marshmallow root? How could we detect the monosaccharide units with TLC? What was the visualization technique?
- Define gums and mucilages.
- Define monosaccharides, oligosaccharides and polysaccharides.
- Give the general tests for the identification of acacia gum.
- Give the general tests for the identification of tragacanth gum. How can you distinguish between these two gums?
- Define swelling value.
- Explain the method for determination of the swelling value according to Ph. Eur.

Classification (Latin name of the drug, species, and family) and uses of the following drugs:

- acacia gum, tragacanth gum, agar

Practical 6

Drugs with fixed oil content
1. Comparison of fixed oils derived from different drugs with TLC
2. Test for rancidity
3. Detection of vitamin A from cod liver oil

Drugs with organic acid content
4. Detection of vitamin C from Rosae pseudo-fructus

Helianthi annui oleum raffinatum
Sunflower seed oil
Helianthus annuus L.
Asteraceae

Lini oleum virginale
Virgin linseed oil
Linum usitatissimum L.
Linaceae

Ricini oleum virginale
Virgin castor oil
Ricinus communis L.
Euphorbiaceae

Jecoris oleum
Cod liver oil
Gadus morrhua
Gadidae

1. Comparison of fixed oils derived from different drugs with TLC

TLC determination
Sorbent: Silica gel

Spots:

Chloroform solution of sunflower seed oil	10 µl
Chloroform solution of linseed oil	10 µl
Chloroform solution of castor oil	10 µl

Solvent system:
Petroleum ether – ether – acetic acid (90:10:0.8)

Detection:
Place the TLC plate in a chamber saturated with iodine vapour for 2 min. Then remove it and after a few minutes spray it with starch solution.

2. Test for rancidity
Add 2 ml of cc. HCl to 2 ml of oil in a test tube. Plug the tube with a piece of cotton-wool moistened with a 0.1% ether solution of phloroglucinol. Warm the test tube by hand or on a water bath.

3. Detection of vitamin A in cod liver oil

Add 1 drop of Jecoris oleum to a $SbCl_3$ crystal in a porcelain dish.

Write down your observation.

Rosae pseudo-fructus
Rose hip
Rosa canina L.
Rosaceae

4. Detection of vitamin C from Rosae pseudo-fructus
Extraction: Add 8 ml of 40 ºC water to 2 g of ground drug and extract it for 30 min. Filter the mixture throuhg cotton-wool.

- Add 100 mg of $NaHCO_3$ and 20 mg of $FeSO_4$ to 2 ml of filtrate (a colour will appear). Then add cc. HCl.
- Add 1 ml of Fehling I and 1 ml of Fehling II solution to 2 ml of filtrate. Warm the mixture on the water bath.

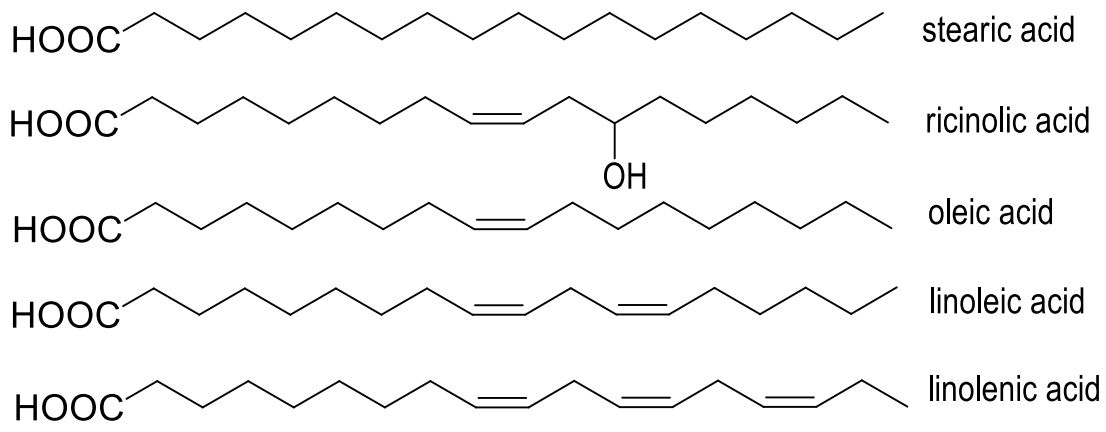
Write down your observation.

Theory 6

Lipids are natural substances, esters of fatty acids and alcohols or polyols. They may be structural elements of cells such as the phospho- and glycolipids of the membranes, while others serve coating (barrier) purposes, such as waxes or cutins, and there are lipids whose primary role is energy storage, such as fixed oils. **Fixed oils** are solid, semi-solid or liquid components built up as esters of alcohols and long-chain (C_{10}-C_{20}) aliphatic or cyclic, saturated or unsaturated fatty acids.
The main difference between waxes and fixed oils is in the nature of the present in their constitution. In fixed oils, the alcohol is always glycerol (a triol, i.e. an alcohol with three –OH groups; fixed oils are also referred to as triacylglycerols) In waxes, the alcohol is a longer-chain monohydric alcohol.

Chemical properties of fixed oils:

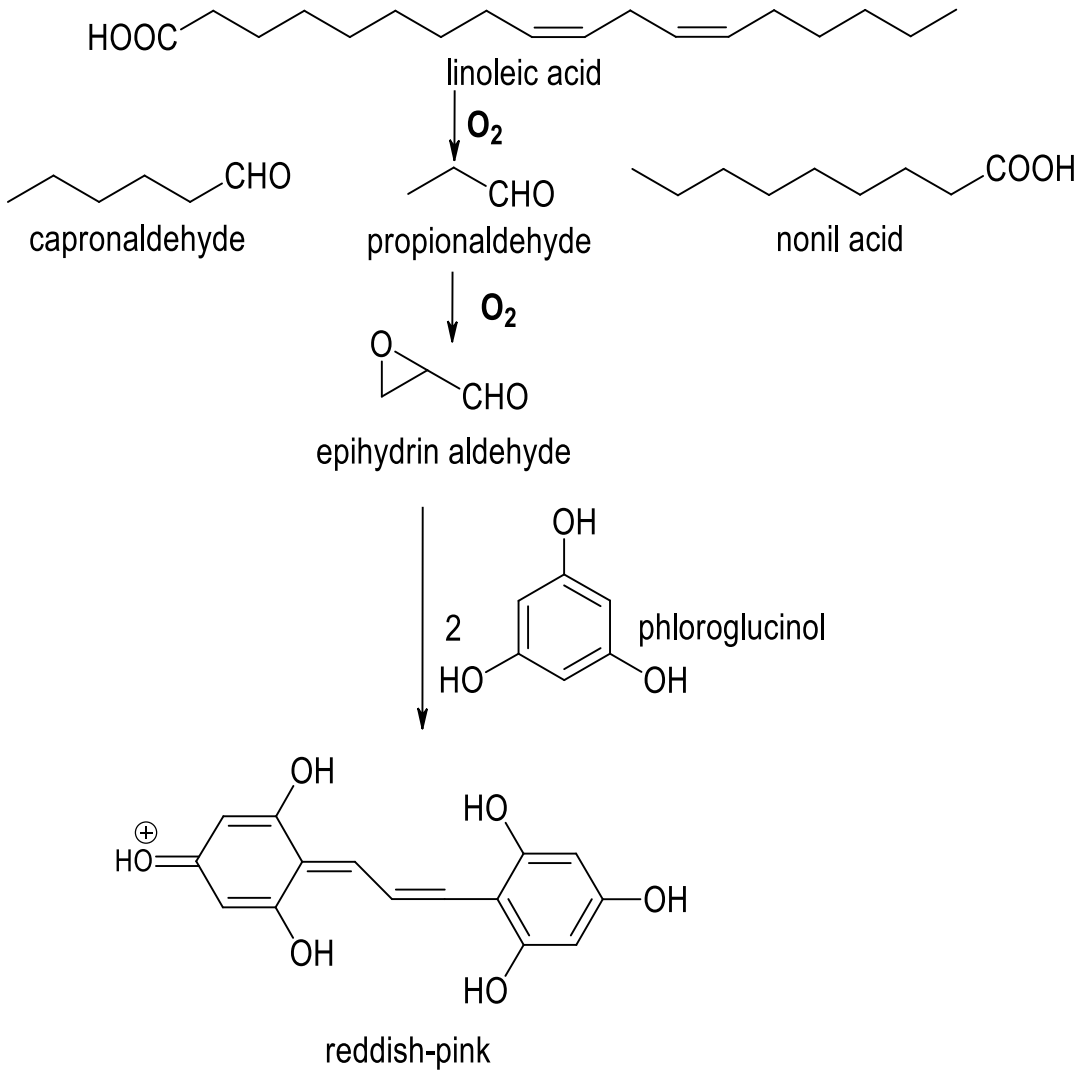
- They are soluble in organic solvents, including acetone
- Triacylglycerols containing unsaturated fatty acid(s) can become rancid: when exposed to air for some time, they develop an unpleasant smell. This is linked to oxidation resulting in peroxides, which may polymerize and change the properties of the original oils. Rancid oils must not be used for medical purposes.
- At room temperature, the consistency of fixed oils depends on the fatty acid composition.
    - Triglycerides of unsaturated fatty acids are liquid
    - Triglycerides of mainly saturated fatty acids are solid. Waxes are always solid.



TLC comparison of fixed oils derived from different drugs
With this TLC, the differences in the constituents of the fixed oils of sunflower seed, linseed and castor bean can be observed. The method is suitable for the detection of compounds containing several double bonds. Solid iodine is placed in a glass chamber, which becomes saturated with iodine vapour. When the previously developed TLC plates

are placed into this chamber, iodine will bind to the double bonds in the different components. After removal of the plate, it is allowed to dry for some minutes in order to allow the excess iodine to desorb from the silica surface. Spraying the plate with starch solution gives a blue colour reaction (general starch reaction with iodine), and the separated fixed oil components appear as blue spots.
Note: as we can visualize double bonds this way, colour intensity of each spot will refer to the degree of unsaturation of that particular compound.

Test for rancidity
See method in practical 6. In the presence of rancid oil, the cotton-wool turns reddish-pink.



Helianthi annui oleum raffinatum
Sunflower seed oil
Helianthus annuus L.
Asteraceae

The drug is the refined fixed oil obtained by cold expression from the seeds of the annual plant *Helianthus annuus.* The plant is native to North America, but is widely cultivated worldwide. The plant can reach 3 m in height, with a head 30 cm in diameter. The ray florets are vivid-yellow in colour. The oil content of the seed (35-50%) consists of 35% linoleic acid, 10% linolenic acid and 39% oleic acid esters.

Uses: Used in ointment, and oily injections as a vehicle.

Lini oleum virginale
Virgin linseed oil
Linum usitatissimum L.
Linaceae

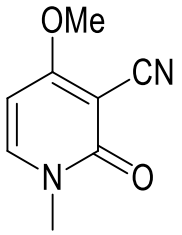
The drug is the fixed oil obtained by cold expression from the seeds of *Linum usitatissimum*. It is a yellowish-brown drying oil (upon exposure to air, it gradually thickens and forms a hard varnish) with a characteristic odour and bland taste. The seed contains 30-40% fixed oil mostly in the form of unsaturated linoleic acid (16-25%), linolenic acid (40-62%), oleic acid (14-16%) and saturated stearic and palmitic acid (10-15%) glycerol esters.

Uses: It is used in the preparation of liniments for the treatment of eczema and psoriasis.

Ricini oleum virginale
Virgin castor oil
Ricinus communis L.
Euphorbiaceae

The drug is the fixed oil obtained by cold expression from the seeds of *Ricinus communis*. Castor is a herbaceous plant, which is annual or perennial depending on the climatic conditions. It is native to India, but is widely cultivated worldwide. The seeds contain 45-55% fixed oil, mostly built up from ricinoleic acid glycerol ester (80%). The seed also contains highly toxic ricins (toxic proteins) and ricinin (a toxic alkaloid), which is not present in the oil. Consequently the castor seed itself is highly toxic.

Uses: It is used as a purgative, but in most of the countries it is restricted to hospital use for administration after food poisoning or as preliminary treatment before intestinal examinations.

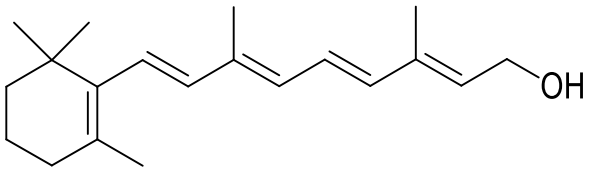


ricinin

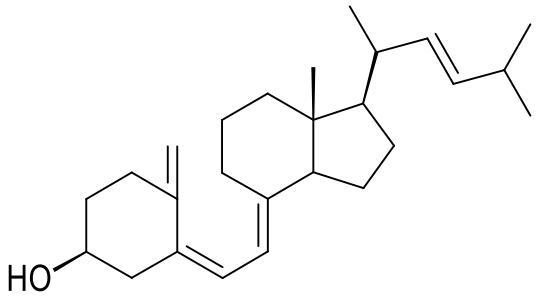
Jecoris oleum
Cod liver oil
Gadus morrhua
Gadidae

The drug is the fixed oil prepared from the fresh liver of *Gadus morrhua,* freed from the solid particles by cooling and filtering. The oil is a pale-yellow, viscous liquid with a characteristic fish odour and taste. The oil contains 85% oleic acid, palmitoleic acid and gadoleic acid (unsaturated fatty acids) triglycerides and 15% palmitic acid and myristic acid (saturated) triglycerides. It contains 180-750 µg/g of vitamin A and 1.5-6.25 µg/g of vitamin D_3.

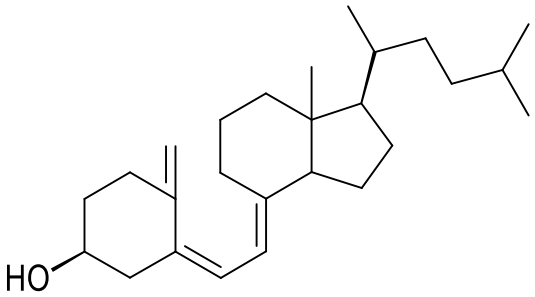
Uses: It is used in cases of vitamin A and D insufficiency and as a constituent of creams and ointments.



vitamin A



vitamin D_2



vitamin D_3

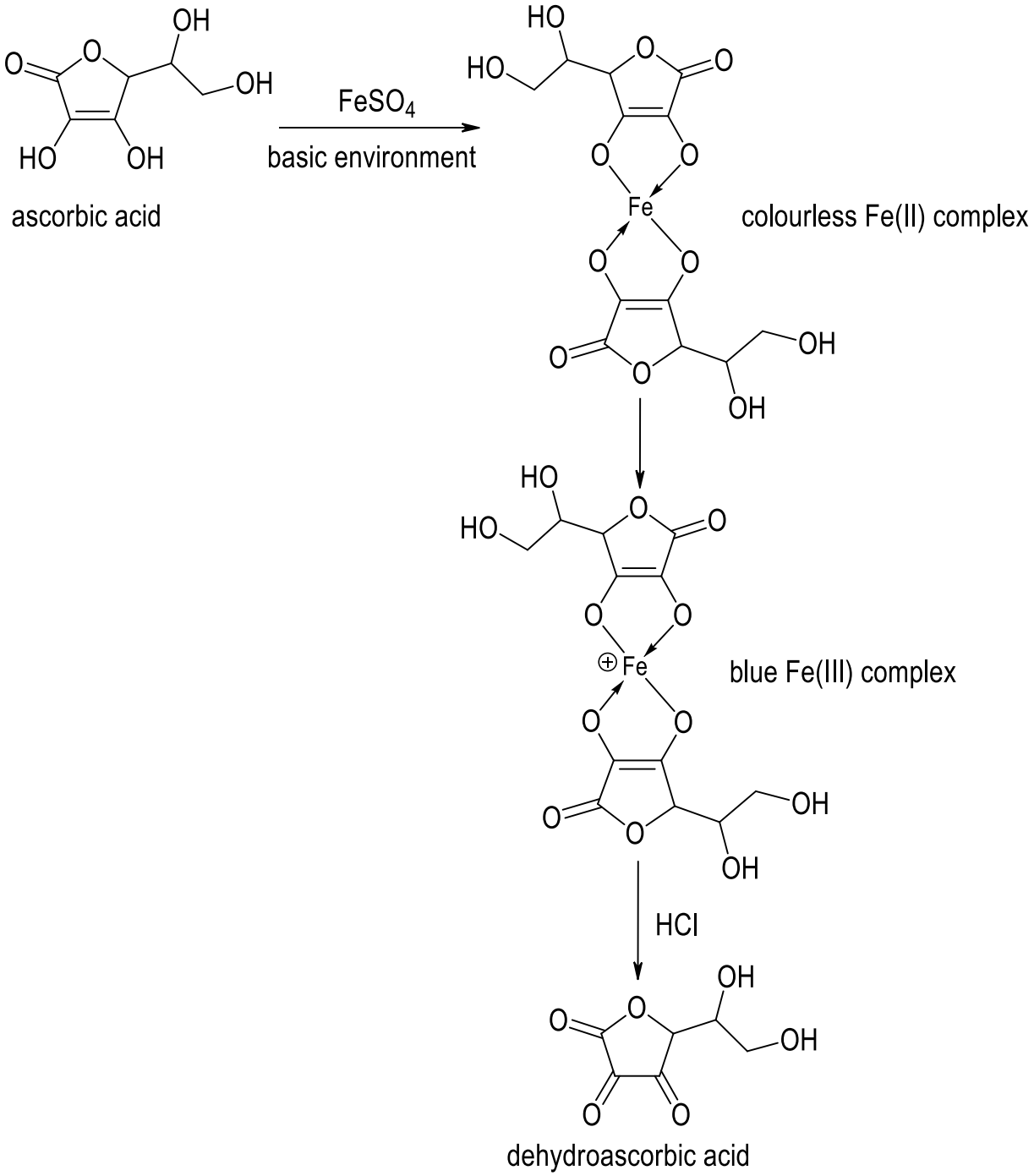
Detection of vitamin A from cod liver oil
See method in Practical 6. As a result, a greyish-violet reaction appears. The mechanism behind this phenomenon is that the electrophilic carbon atoms in the polarized double bonds of vitamin A react with the free electron pairs of $SbCl_3$.

Rosae pseudo-fructus
Rose hip
Rosa canina L.
Rosaceae

The drug can be collected from various Rosa species. It consists of the ripe and dried receptacle cup (pseudofruits) containing very hard achenes. The plant is a very bushy shrub armed with very strong thorns. The dark-red pseudofruits are 1.5-2.5 cm long and 1-1.2 cm high and contain a number of hard, hairy achenes. The drug is odourless with a slightly sour taste. It contains 0.2-1.5% vitamin C (ascorbic acid), flavonoids, carotinoids (β-carotene and licopine), sugar and mucilage.

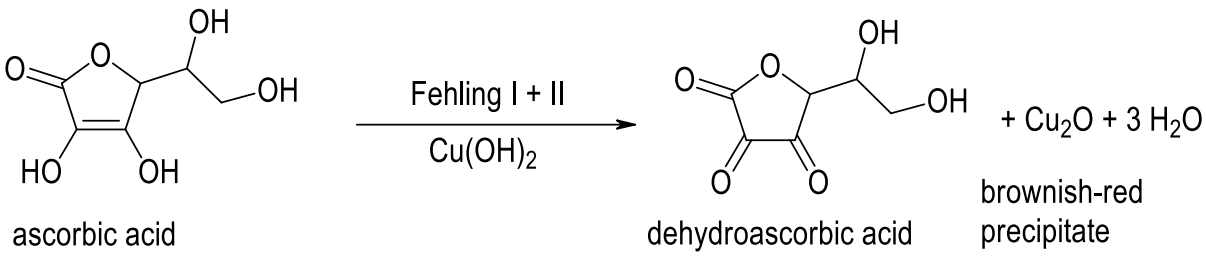
Uses: It is used as a vitamin C supplement in the form of a tea in case of common cold. It is important that vitamin C is not stable at higher temperature so that making tea in the usual way (i.e. by adding boiling water) is not appropriate. For the highest yield of vitamin C, room-temperature water should be used for at least 4 h for the extraction.

Detection of vitamin C from Rosae pseudo-fructus
Through iron complex formation: *See method in Practical 6.* On the addition of $NaHCO_3$ and $FeSO_4$ to the filtrate, a colourless Fe(II) complex is first formed, which is subsequently transformed to a violet Fe(III) complex. On the addition of cc. HCl, it breaks down and the colour disappears.



With Fehling's test: *See method in Practical 6.* Fehling's test is suitable fopr the detection of reducing sugars. Fehling's reagent is a 1:1 mixture of **Fehling I** = hydrated copper(II) sulfate dissolved in distilled water; and **Fehling II** = potassium sodium tartrate and sodium hydroxide in distilled water. Fehling's test makes use of the reactivity of aldehydes with the weak oxidizing agent in alkaline solution. In addition to the Cu^{2+}, Fehling's solution contains tartrate ion as a complexing agent to keep the Cu^{2+} in solution. Without the tartrate ions, copper(II) hydroxide would precipitate from the basic solution. The tartrate ion is unable to form complex with Cu^{+}, so the reduction of Cu^{2+} to Cu^{+} by reducing sugars results in the formation of an orange-red precipitate of Cu_2O

(positive result). Ascorbic acid is able to reduce the Fehling's reagent even without heating, which differs from sugars, revealing the strong reducing capacity of vitamin C.



ascorbic acid dehydroascorbic acid

Test questions 6

Essay questions:

- Define fixed oils. Which group of compounds do they belong to and what are their chemical characteristics?
- How did we compare the different fixed oils with TLC? How did we visualize the TLC plate and what could we conclude?
- How can we detect vitamin A in cod liver oil? Explain the test and write down the result.
- How can we detect vitamin C in rose hip? Explain the two methods (with Fe^{2+} salt and Fehling's reagent) and write down the observations.

Mechanisms and structures:

- Give examples for fatty acids (stearic acid, ricinolic acid, oleic acid, linoleic acid linolenic acid).
- Describe the mechanism and explain the test for rancidity of fixed oils.
- Draw the formulae of ricinin and vitamins A, D_2, D_3 and C.
- Describe and draw the mechanism of the detection of vitamin C with a Fe^{2+} salt.
- Describe and draw the mechanism of the detection of vitamin C with Fehling's reagent.

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Sunflower seed oil, virgin linseed oil, virgin castor oil, cod liver oil and rose hip

Practical 7

Drugs with alkaloid content I

1. General alkaloid tests
2. Drugs with alkaloids derived from ornithine: alkaloids with a tropane skeleton.
   - TLC analysis of Belladonnae folium, Stramonii folium and Hyoscyami folium
   - Vitali test
   - Detection of scopoletin
3. Drugs with alkaloids of phenylalanine origin, I.
   - Detection of emetin: Rubremetin test
   - Detection of cephaelin: Frohde test

1. General alkaloid tests: Use 2-3 ml of quinine hydrochloride solution, and add 1-2 drops of:

- **Mayer reagent**
- **Dragendorff reagent**
- **Wagner reagent**

Write down your observations.

2. Drugs with alkaloids derived from ornithine:

Belladonnae folium	**Stramonii folium**	**Hyoscyami folium**
Belladonna leaf	Stramonium leaf	Hyoscyamus leaf
Atropa belladonna L.	*Datura stramonium* L.	*Hyoscyamus niger* L.
Solanaceae	Solanaceae	Solanaceae
Belladonnae folii extractum siccum normatum *Belladonnae folii tinctura normata* *Belladonnae pulvis normatus*	*Stramonii pulvis normatus*	

TLC analysis of Belladonnae folium, Stramonii folium and Hyoscyami folium

Extraction: Add 10 ml of 0.5% aqueous sulfuric acid to 2 g of drug (**Belladonnae folium, Stramonii folium**) in a flask and warm the flask on a water bath for 10 min. After cooling, filter the mixture through cotton wool into a separating funnel. Adjust the pH of the filtrates to 8-9 with cc. NH_3. Extract it with 2 x 10 ml of chloroform. Combine the organic solvent-containing phases and dry them with anhydrous Na_2SO_4. Pour the liquid into a porcelain dish and evaporate off the chloroform to dryness on the water bath. Dissolve the dry residue in 0.5 ml of methanol for TLC.

In the case of **Hyoscyami folium**, use 4 g of drug and 20 ml of 0.5% sulfuric acid for the extraction. For the liquid–liquid partition, use 2 x 20 ml of chloroform. The pH adjustment protocol is the same as for the other two drugs.

TLC analysis
Sorbent: silica gel

Spots:

Belladonnae folium extract	40 µl
Stramonii folium extract	40 µl
Hyoscyami folium extract	40 µl
Methanolic solution of atropine	20 µl
Methanolic solution of scopolamine	20 µl
Methanolic solution of scopoletin	20 µl

Solvent system:
Dichloromethane – methanol – cc. NH_3 (40:9:1)

Detection:
366 nm light
Spray the TLC plate with Dragendorff reagent.

Vitali test (determination of alkaloids with a tropane skeleton): From the extract that remained after the sample was taken for TLC, evaporate off the methanol and then add a few drops of cc. HNO_3 to the dry residue. Evaporate the mixture to dryness again on the water bath, and then add some KOH granules and a few drops of methanol.

Detection of scopoletin: Add 10 ml of water to 0.2 g of drug and extract the mixture by shaking. Add 10 drops of R-Pb acetate to the aqueous extract, filter it through paper into a separating funnel, and then extract it with 8 ml of chloroform. Add 2 drops of NH_4OH to 3 ml of water in a test tube, add the chloroform phase to it and shake the mixture well. Wait a few minutes, and then check the separated aqueous phase under 366 nm light.

Write down your observations.

3. Drugs with alkaloids derived from phenylalanine, I

Ipecacuanhae radix
Ipecacuanha root
Cephaëlis ipecacuanha
Cephaëlis acuminata
Rubiaceae

Ipecacuanhae extractum fluidum normatum
Ipecacuanhae pulvis normatus
Ipecacuanhae tinctura normata

Detection of emetin and cephaelin

Extraction: Add 10 drops of R-NH_4OH solution to 0.5 g of drug in an Erlenmeyer flask. Extract the mixture with 10 ml of chloroform for 10 min (shake it frequently). Filter the extract through filter-paper and divide it between two porcelain dishes. Evaporate both portions to dryness.

Rubremetin test: Detection of **emetin**. Add a small amount of chlorogen and 2-3 drops of R-HCl to the dry residue in one of the porcelain dishes.

Frohde test: Detection of **cephaelin**. Add a few drops of Frohde reagent (ammonium molybdate dissolved in cc. H_2SO_4) to the dry residue in the other porcelain dish.

Write down your observations.

Theory 7

Alkaloids

Alkaloids are not a homogeneous group of compounds, from either chemical, biochemical or physiological points of view. Their common feature is that they are N - containing secondary plant metabolites. They can be divided into three groups, according to the origin and position of the N atom.

I.) **Typical alkaloids** contain one or more N atom(s) in heterocyclic ring. The N is derived from an amino acid.
II.) **Protoalkaloids** contain one or more N atom(s) in a side-chain. The N is derived from an amino acid.
III.) **Pseudoalkaloids** contain the N atom in the side-chain or in a heterocyclic ring, but it is not derived from an amino acid.

The alkaloids usually have a basic character because of the N atom, which can be present in form of a primary amine (RNH_2), a secondary amine (R_2NH) or a tertiary amine (R_3N) (the R groups may be different). Since the N atom bears a non-bonding pair of electrons, the alkaloids can form cations with aqueous mineral acids (RNH_3^+, $R_2NH_2^+$ and R_3NH^+, respectively), but upon the addition of alkali the cation gives up a hydrogen ion and the free alkaloid base is liberated.

In plants, alkaloids exist either in their **free base** form or as **salts** with organic acid(s). They are usually solids (except for coniine, nicotine and pilocarpine) and most of them are colourless. The free alkaloid bases **differ** greatly in **solubility** from their salts, especially when the salts are formed with mineral acids. While the alkaloid bases are typically soluble in organic, apolar solvents, their mineral salts dissolve in water or polar solvents. This phenomenon can be utilized effectively for the extraction and purification of alkaloids.

Extraction of alkaloids: The method depends on the plant material and the purpose of the investigation.

I.) ***Extraction with organic solvents:*** The first step is to moisten the drug with a base (such as few drops of cc. NH_3). After this step, the alkaloid salts will be liberated as free bases, and in this form they can be extracted with organic solvents (e.g. ether, chloroform or dichloromethane). With this method, free alkaloids can be obtained.
Purification of free alkaloid bases by solvent–solvent extraction: To separate the alkaloids from the other components which were also extracted from the plant material by the organic solvent, we can add acidified water to

the above-mentioned extract, and perform solvent–solvent (organic solvent vs. water) extraction in a separating funnel. With the acid, the free alkaloids will form salts again and, in accordance with their drastically changed solubility, they will be transferred to the aqueous phase during the separation. The solubility of the unwanted non-alkaloidal compounds will not change on the addition of acid, and they will remain in the organic phase.

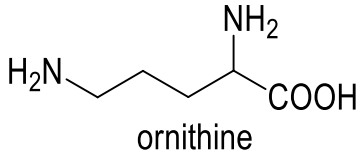
II.) ***Extraction with water or aqueous alcoholic solvents:*** The first step is to extract the drug with dilute acid-containing water or aqueous alcoholic solvents. In this case the alkaloids are isolated in their water-soluble salt form. ***Purification of alkaloid salts by solvent–solvent extraction:*** For a similar phase-transfer purification as above in point I, the acidic aqueous solution of alkaloid salts must now be made alkaline so that the free alkaloid bases can be extracted with an organic solvent. Non-alkaloid compounds will remain in the aqueous phase.

General alkaloid tests

- **Mayer reagent** (an aqueous solution of potassium tetraiodido mercuriate(II)) → yellowish-white precipitate
- **Dragendorff reagent** (potassium tetraiodido bismuthate(III) dissolved in acetic acid) → orange precipitate
- **Wagner reagent** (an aqueous alcoholic solution of iodine + potassium iodide) → a brownish-red precipitate

Drugs with alkaloids derived from ornithine: alkaloids with tropane skeleton

Belladonnae folium
Belladonna leaf, Deadly nightshade leaf
Atropa belladonna L.
Solanaceae



ornithine

The plant is a perennial herb which attains a height of about 1.5 m. The leaves are dull-green or yellowish-green, the upper side being somewhat darker than the lower side. Some of the cells of the spongy parenchyma are filled with microspheroidal crystals of calcium oxalate ("oxalate sand"). The plant contains alkaloids (min. 0.3%) such as atropine [a 1:1 mixture of (-)-S-hyoscyamine and (+)-R-hyoscyamine], scopolamine, coumarins such as scopoletin and flavonoids.

Uses: The alkaloids of the plant have parasympatholytic activity. Atropine is used in medical practice to dilate the pupils before optometric examinations. In cases of hyperacidity, peptic ulcer or before certain surgical interventions, atropine is used to

lower or even block gastric secretion. Because of its spasmolytic properties, it is used in renal or gall bladder spasm and asthma.

Hyoscyami folium
Hyoscyamus leaf, henbane leaf
Hyoscyamus niger L.
Solanaceae

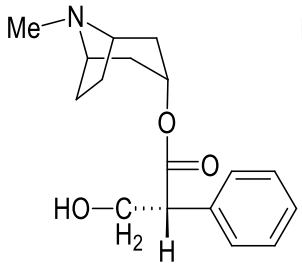
The leaves are sessile, ovate-oblong to triangular-ovate, 10 to 20 cm long and very hairy. The spongy mesophyll contains calcium oxalate crystals, mainly in the shape of single and twin prisms. The drug contains alkaloids (min. 0.05%) such as atropine [a 1:1 mixture of (-)-S-hyoscyamine and (+)-R-hyoscyamine], scopolamine, coumarins such as scopoletin and flavonoids.

Uses: See Belladonna leaf.

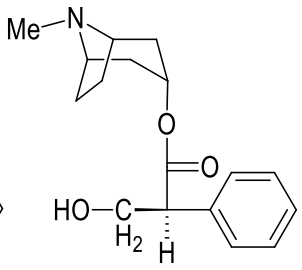
Stramonii folium
Stramonium leaf, thornapple leaf
Datura stramonium L.
Solanaceae

The dried leaves are greyish-green, 8 to 25 cm long and 7 to 15 cm wide. They are short petiolate, ovate or triangular-ovate in shape. Some of the cells of the parenchyma are filled with clusters of calcium oxalate crystals.

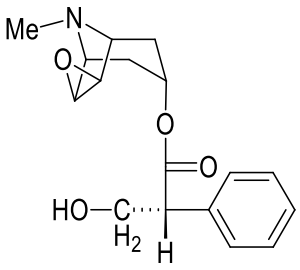
Uses: See Belladonna leaf.



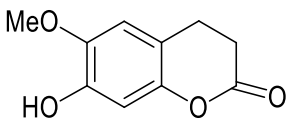
(-)-S-hyoscyamine



(+)-R-hyoscyamine



(-)-S-scopolamine

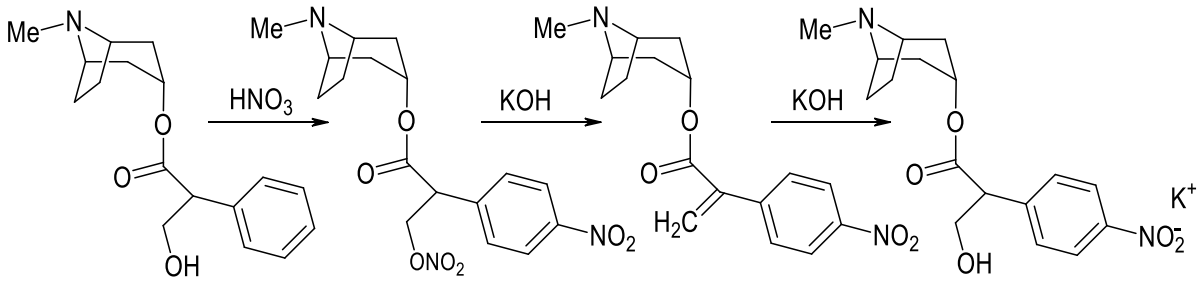


scopoletin

TLC analysis of Belladonnae folium, Stramonii folium, Hyoscyami folium
For the extraction of the alkaloids, the acidified water option was chosen so asto yield the alkaloids in a water-soluble salt form. When solution is made alkaline, the alkaloids are

comverted into into their free base form and can be extracted with chloroform. After TLC development of, scopoletin (a coumarin) exhibits blue fluorescence under 366 nm light, while the alkaloids can be visualized as orange spots after the plate is sprayed with the general Dragendorff alkaloid reagent.

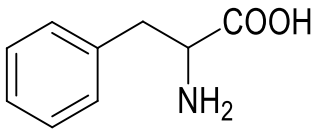
Vitali reaction
See method in practical 7. On the addition of cc. HNO_3 and KOH, a bluish-red colour can be seen around the KOH, according to the following mechanism.



atropine 4-nitroatropine nitrate 4-nitroapoatropine

Drugs with alkaloids derived from phenylalanine, I

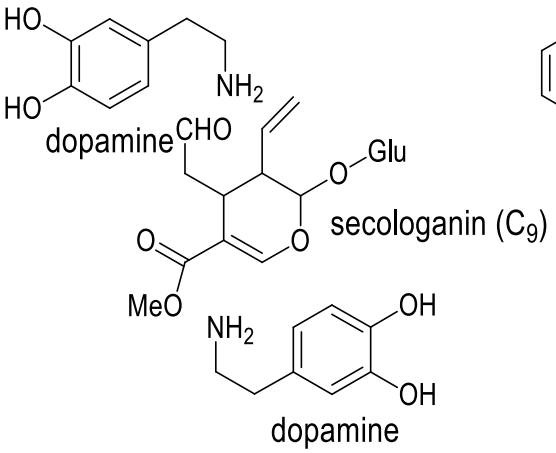
Ipecacuanhae radix
Ipecacuanha root
Cephaëlis ipecacuanha
Cephaëlis acuminata
Rubiaceae

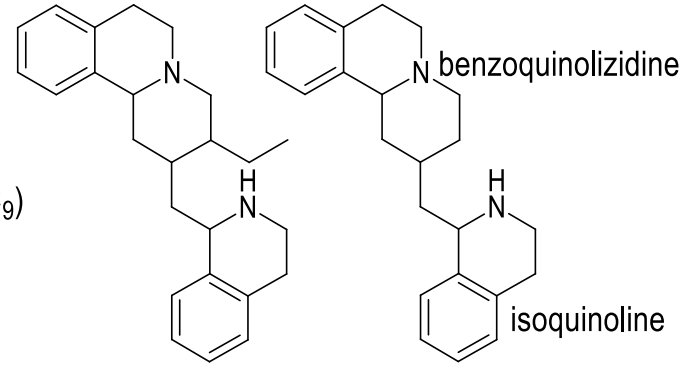


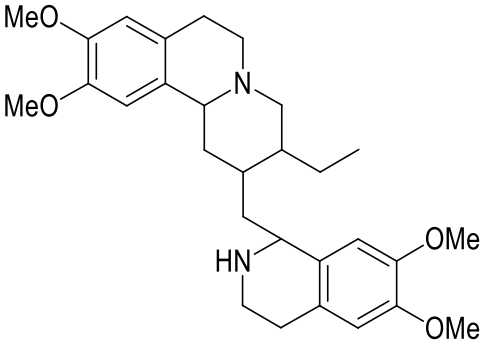
phenylalanine

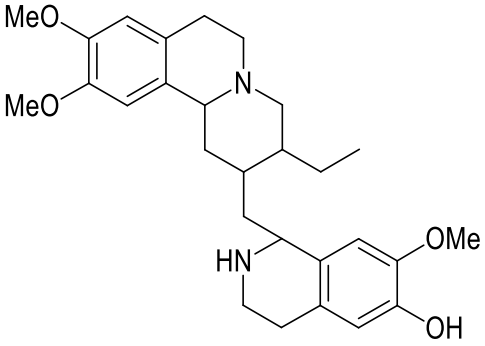
The drug consists of the dried roots and rhizome of *Cephaëlis ipecacuanha* and *Cephaëlis acuminata*. The former is referred to as Brazilian and the latter as Cartagena ipecacuanha. The underground part consists of thin horizontal rhizomes from which the roots originate, pointing downwards. Some of the roots remain thin; others develop an unusually thick bark which is characteristic of the drug. The colour of the outer surface varies from brick-red to dark-brown and depends on the type of soil in which the plant is grown. It has a weak odour and an unpleasant taste. The drug contains 1.8-4% alkaloids (emetin and cephaelin), 40% starch and 3-4% triterpenes.
The alkaloids are biosynthesized from the fusion of two phenylalanine units (dopamine) and a C_9 secoiridoid unit (secologanin).

Uses: Emetin and cephaelin are expectorants when taken in small amounts, but can cause nausea and vomiting in higher doses. Overdosing results in severe unrelievable vomiting and haemorrhagic diarrhoea. Emetin hydrochloride is also used to treat amoebic dysentery.





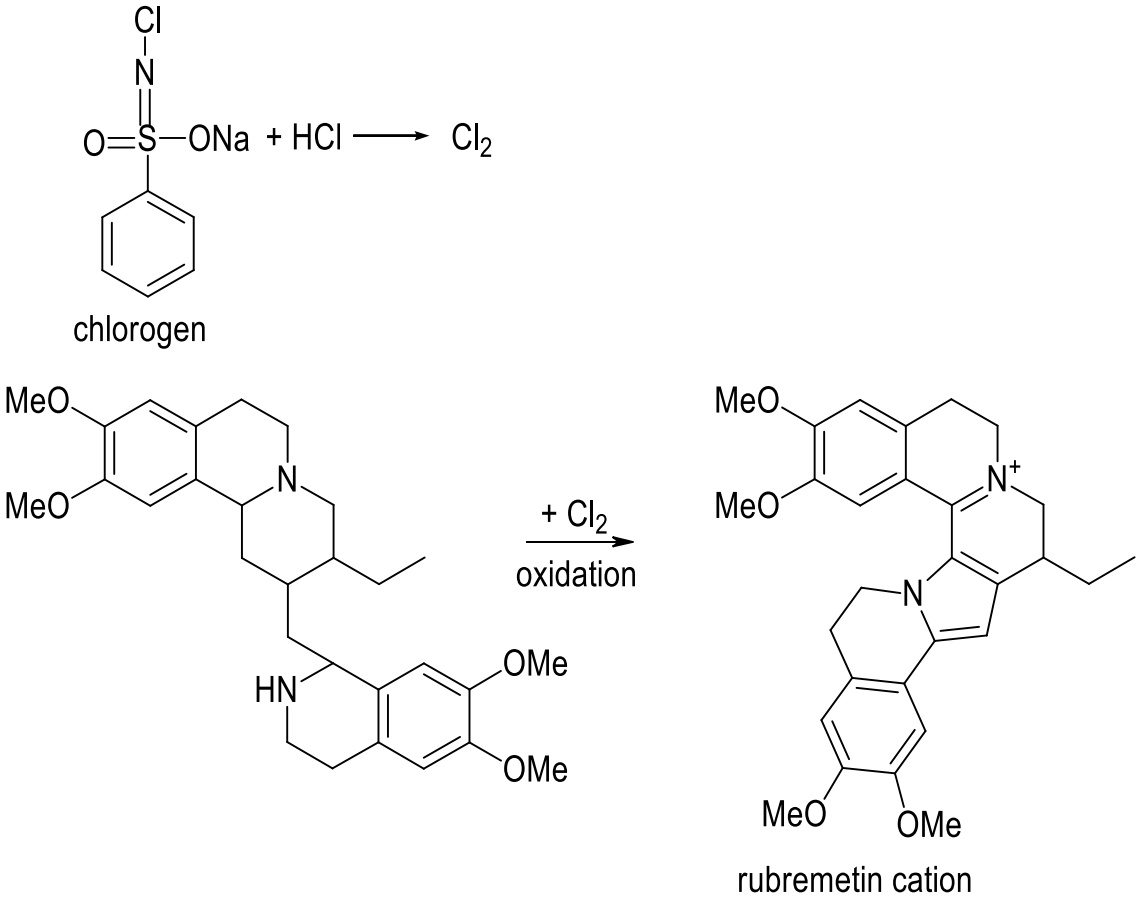




Detection of emetin and cephaelin

Rubremetin test

See method in Practical 7. On the addition of chlorogen and HCl, Cl_2 is formed and oxidizes emetin to an orange-reddish rubremetin cation.



Frohde test

See method in Practical 7. On the addition of Frohde reagent (ammonium molybdate dissolved in cc. H_2SO_4) to the dry residue a blue colour appears.

Test questions 7

Essay questions:

- Define and classify alkaloids. Give their chemical properties.
- Explain the two different options for the extraction of alkaloids from plants, and their subsequent purification by solvent–solvent extraction.
- What are the general tests for alkaloids? What are the reagents used?
- TLC analysis of alkaloids with a tropane skeleton: extraction of the drugs, visualization of the TLC plate and conclusions. Detection of scopoletin (drug, reagent used, and observation).
- Describe the procedure for the detection of cephaelin (Frohde test) starting from the extraction of the drug until the observed result.

Mechanisms and structures:

- Give examples of alkaloids with a tropane skeleton (hyoscyamine and scopolamine). What is atropine?
- Describe and draw the mechanism of the Vitali test. What drug did we use? How did we extract it and what was the result?
- Draw the structure of scopoletin.
- Draw the structures of emetin and cephaelin.
- Describe and draw the mechanism of the detection of emetin (Rubremetin test).

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Belladonna leaf, stramonium leaf, hyoscyamus leaf and ipecacuanha root

Practical 8

Drugs with alkaloid content, II

Drugs with alkaloids derived from phenylalanine, II:

1. Detection of carotenoids from Capsici fructus
2. Marquis test, detection of meconic acid from Opium
3. Separation of morphine from other opium alkaloids
4. Purity test of the isolated morphine by TLC

Capsici fructus
Paprika fruit
Capsicum annuum var *minimum* L.
Solanaceae

1. Detection of carotenoids from Capsici fructus

Extract 0.2 g of drug with 5 ml of petroleum ether at room temperature by shaking. Filter the extract through on paper into a porcelain dish and evaporate it to dryness on a water bath. Add 1-2 drops of 80% H_2SO_4 to the dry residue.

Write down your observation.

Opium crudum
Opium
Papaver somniferum L.
Papaveraceae

Opii pulvis normatus

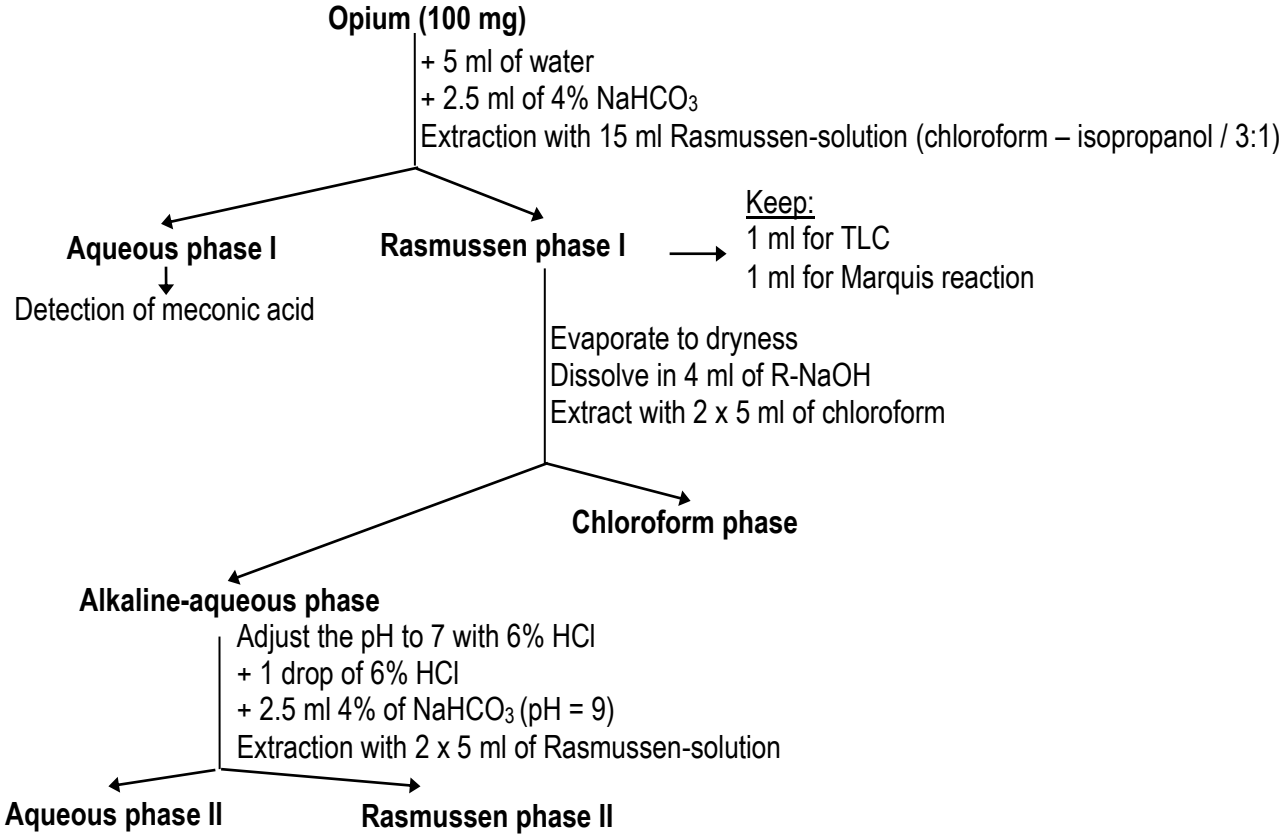
2. Marquis test, detection of meconic acid

Marquis test: Detection of morphine. Evaporate the Rasmussen phase I (see below) to dryness. Add 1-2 drops of Marquis reagent (1 ml of cc. H_2SO_4 + 1 drop of formaldehyde).

Meconic acid: Add first 1-2 drops of R-H_2SO_4 and then 2-3 drops of $FeCl_3$ to the aqueous phase I. (see below).

Write down your observations.

3. Separation of morphine from other opium alkaloids



4. Purity examination of the isolated morphine by TLC

Sorbent: Silica gel

Spots:

Rasmussen phase I.	60 µl
0.1% methanolic solution of papaverine + 0.1% methanolic solution of morphine	10 µl each
Chloroformic phase	60 µl
0.1% methanolic solution of thebaine + 0.1% methanolic solution of codeine	20 µl each
Rasmussen phase II	60 µl

Solvent system:

Ethyl acetate – methanol – cc. NH_3 (85:10:5)

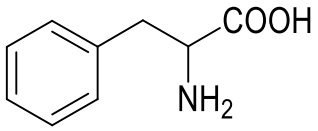
Detection:

Spray the TLC with Dragendorff reagent

Theory 8

Drugs with alkaloids derived from phenylalanine II.

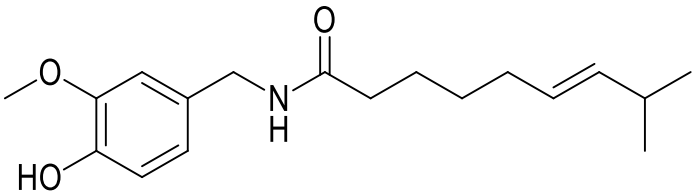
Capsici fructus
Paprika fruit
Capsicum annuum var *minimum* L.
Solanaceae



phenylalanine

The drug consists of the spicy fruit of paprika. It contains 0.1-2% capsaicin and other capsaicinoids, which are protoalkaloids derived from phenylalanine, carotenoids, vitamin C, B1 and B2, flavonoids, sugars, and fixed and volatile oils.

Uses: Paprika is used in great amounts as a spice in the Hungarian kitchen or as a stomachic agent to support digestion. Topically, it is applied as a rubefacient in cases of rheumatic disorders, neuralgias, muscle pain and hair loss. Rubefacients cause the reddening of the skin by dilating the capillaries and increasing the blood supply in the applied surface, which might help reduce inflammation.



capsaicin

Detection of carotenoids
The apolar carotenoids can easily be extracted from the drug with petroleum ether. 80% H_2SO_4 is a strong dehydrating agent that causes the development of a conjugated electron system with a dark-blue colour.

Opium crudum
Opium
Papaver somniferum L.
Papaveraceae

Opium is the dried latex obtained by incision from the unripe capsules of *Papaver somniferum*, an annual plant native to Asia and cultivated widely, from the tropical territories to northern Norway. The unripe capsule contains a considerable amount of latex. To obtain this, small parallel incisions are made in the wall of the capsule. The originally white latex leaks out, after which it rapidly coagulates and turns brown due to

enzymatic oxidation. The partly dried latex is scraped off with a knife or a special scraper from the capsules. The morphine content of the raw opium is about 15%. The pulverized drug is brown, like cinnamon, with a characteristic odour. Opium contains 20-25% total alkaloids, 5-10% natural rubber, 1-4% fixed oil and oxidases. The main alkaloid is morphine, accompanied by codeine, thebaine, papaverine and narcotine, also in high amounts. In opium, alkaloids are present as salts of meconic acid, fumaric acid and lactic acid.

Physiological effects: The effect of opium can be attributed to the synergism and antagonism of the different alkaloids.

Opium: it is an obstipant (against diarrhoea) and a strong painkiller, and it depresses the respiratory centre, increases smooth muscle spasm, causing euphoria.

Morphine: painkiller, sedative, causing euphoria, depresses the respiratory center, suppresses cough and causes spastic obstipation

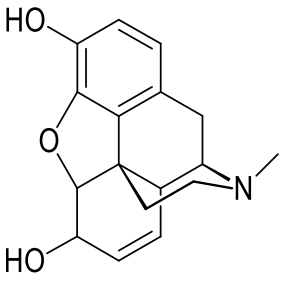
Codeine: it is a painkiller and suppresses cough

Papaverine: it is a spasmolytic and a smooth muscle relaxant, and dilates the arterioles

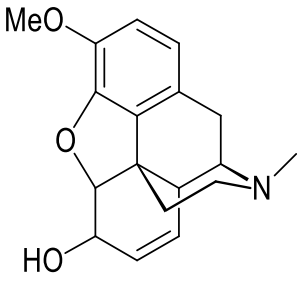
Narcotine: it stimulates the respiratory centre

Thebaine: it is a weak painkiller, and causes smooth muscle spasm

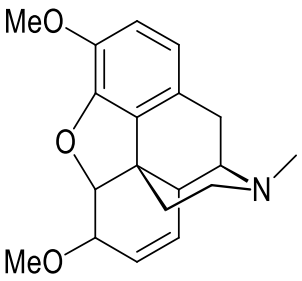
Abuses: it is used as a narcotic drug in the form of a tea, or an injection, or by inhalation of its fumes. The drug causes strong physical and emotional addiction.



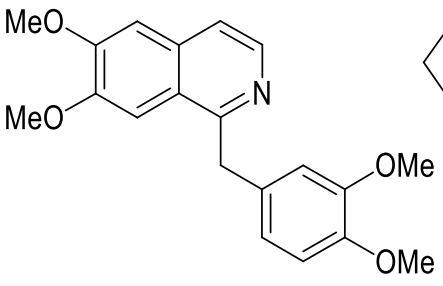
morphine



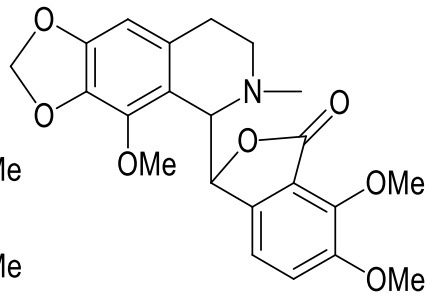
codeine



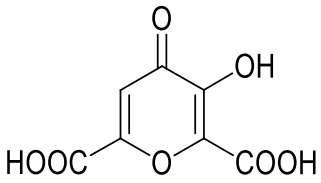
thebaine



papaverine



narcotine



meconic acid

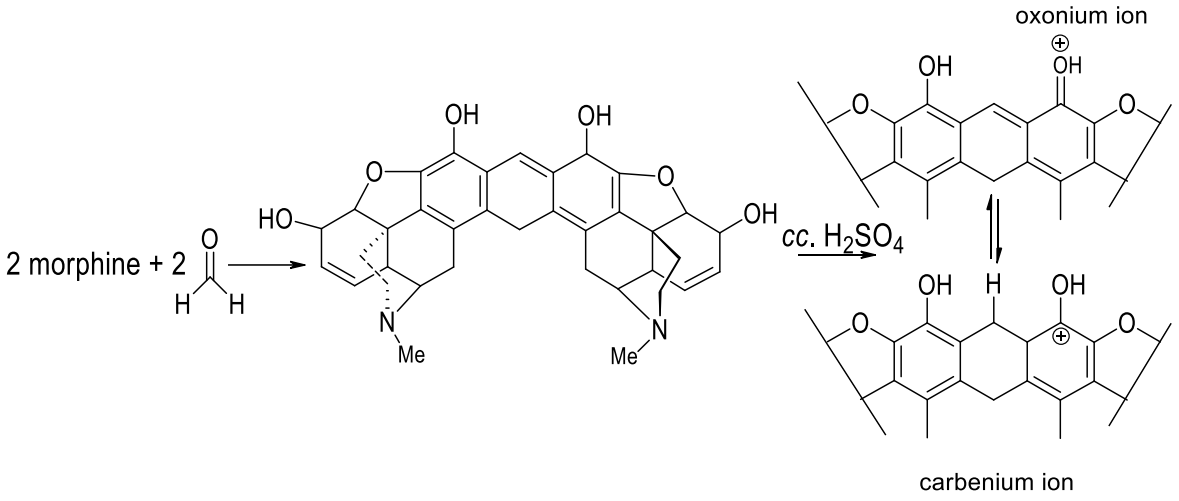
Separation of morphine from other opium alkaloids

The alkaloids of opium are mainly present in the drug in their salt form, in which most of them can be dissolved in water. On the addition of $NaHCO_3$, the pH becomes alkaline which converts all alkaloids (including morphine) to their base form, and they can be

extracted with organic solvents (Rasmussen solution). After this step, the Rasmussen I phase contains all of the alkaloids. Due to the free phenolic –OH group of morphine, it will become a sodium phenolate salt (which is water-soluble) upon the addition of the strong base NaOH to the dry residue of Rasmussen phase I. Other opium alkaloids do not contain a phenolic –OH group, and they therefore cannot form salts with NaOH, and will remain in their free base form. After the extraction of this solution with chloroform, the chloroform phase contains all of the alkaloids except for the salt of morphine, which remains in the aqueous phase. When the pH of this aqueous phase is adjusted to 9, which is the isoelectric point of morphine, its water solubility will decrease considerably so that it can be extracted with organic solvents. The Rasmussen phase II should contain only morphine. By means of TLC analysis, the purity of the isolated morphine can be checked. The TLC plate is visualized by spraying it with the general Dragendorff alkaloid reagent.

Marquis test

See method in Practical 8. First a reddish, and then a violet colour will appear. Two molecules of morphine condense with two molecules of formaldehyde to form a mesomeric system according to the following mechanism.



first reddish, then a violet mesomeric system

Detection of meconic acid

See method in Practical 8. In this acidic environment, a red Fe^{3+} complex forms with meconic acid.

Test questions 8

Essay questions:

- Describe and explain the detection of carotenoids.
- Explain how we separated morphine from the other opium alkaloids? What is the difference in their chemical structures that allows this separation? How did we check its purity by TLC?
- Describe the pharmacological effects of opium alkaloids.
- Describe how we detected meconic acid (starting from the extraction until the observed result).

Mechanisms and structures:

- Draw the structure of capsaicin.
- Give examples of opium alkaloids (morphine, codeine, thebaine, papaverine and narcotine).
- Describe and draw the reaction mechanism of the Marquis test. How did we extract the drug and what could we observe?
- Draw the structure of meconic acid.

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Paprika fruit and opium

Practical 9

Drugs with alkaloid content, III
Drugs with alkaloids derived from tryptophan, I:
1. Cinchonae cortex: thalleioquin reaction and Grahe test
2. Quantitative determination of Cinchonae cortex

Cinchonae cortex
Cinchona bark
Cinchona pubescens Vahl. (*Cinchona succirubra* Pavon)
Rubiaceae

1. Cinchonae cortex: thalleioquin reaction and Grahe test

Thalleioquin test: Use the dichloromethane phase prepared for the quantitative determination (see below, point 2). Measure 5 ml of into a porcelain dish and put the dish on the water bath to evaporate. Dissolve the dry residue in 3 ml of 50% methanol and pour the solution into a reaction tube. Add a few drops of bromine water until the precipitate that forms dissolves. Add 2-3 drops of R-NH_4OH solution.

Grahe test: Put 0.05 g of the drug into a dry reaction tube and heat it with a Bunsen burner until purple fumes form and condense on the upper part of the tube. Take the tube out of the flame, wait until it cools down, and then dissolve the sublimate in 2 ml of methanol. Put 15 ml of water into another test tube, and add 2 drops of R-sulfuric acid and 1-2 drops of the solution of the sublimate. Check it under 366 nm light.

Write down your observations.

2. Quantitative examination of Cinchonae cortex

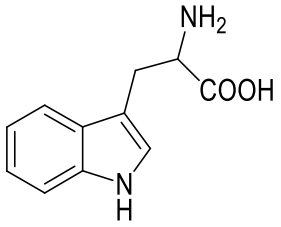
Measure 0.500 g of powdered drug and write down the precisely measured mass (**m**). Add 20 ml of 6.25% formic acid solution and warm the mixture on the water bath for 30 min. After it has cooled down, add exactly 50.00 ml of dichloromethane from a burette and 7.00 ml of 40% NaOH solution. Plug the flask with its stopper and shake it well for 10 min. Then add 2 g of tragacanth gum and continue to it until the emulsion clarifies. Filter the liquid through filter paper and measure precisely 10.00 ml of it into a volumetric flask (*the thalleioquin test is to be performed on the remainder*). Pour the measured 10 ml into a round-bottomed flask, wash the volumetric flask twice with 5 ml portions of dichloromethane and add these too to the round-bottomed flask. Evaporate the mixture to dryness under vacuum. Dissolve the dry residue in 10 ml of methanol and pour the solution into a 25 ml volumetric flask. Wash out the round-bottomed flask twice with 5 ml

of methanol and pour the washings too into the volumetric flask. Fill the flask up to the mark with methanol. Pipette 1 ml of this solution into a dry 10 ml volumetric flask, make the volume up to the mark with methanol and measure the absorbance (***A***) with an UV-spectrophotometer at 282 nm with methanol as reference. Calculate the alkaloid content (**%**) of the drug by using the following formula (*m* = measured drug mass):

$$\% = \frac{A}{0.436} \times 0.00406 \times 12.5 \times \frac{100}{m}$$

Theory 9

Drugs with alkaloids derived from tryptophan, I

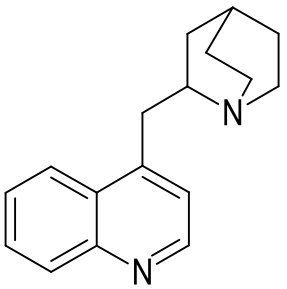


tryptophan

Cinchonae cortex
Cinchona bark
Cinchona pubescens Vahl. (*Cinchona succirubra* Pavon)
Rubiaceae

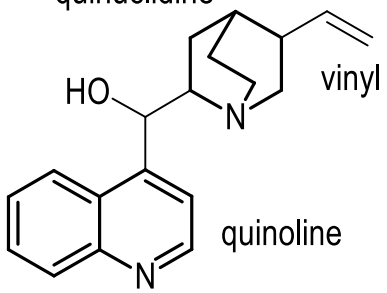
The drug consists of the bark of various species, races and hybrids of the Cinchona genus. The tree is native to the countries located close to the equator in South America (Venezuela, Columbia, Ecuador, Peru and Bolivia) and can be found at an altitude of 1600-3400 m in the Andes. It is cultivated in Indonesia, Malaysia and Zaire. The drug is about 30 cm long and 4-6 mm thick. The outer part is greyish or brownish-grey, and the inner part is deep reddish-brown. The colour of the pulverized bark depends on the species and can range from reddish-brown to yellow. It has a slight odour, tastes bitter and is a strong astringent. The drug contains 5-15% alkaloid salts, of which 30-60% is quinine-type alkaloids (quinine, quinidine, cinchonine and cinchonidine). It also contains a high amount of catechin-type tannins (3-5%).

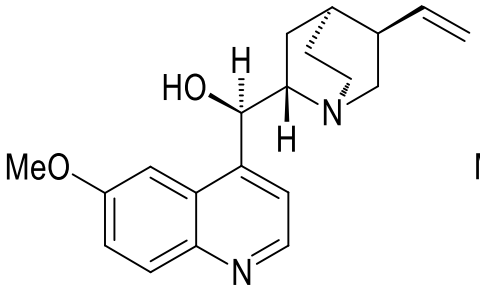
Uses: Quinine is used against malaria fever, and it has an oxytocin-like effect. Quinidine has an antiarrhythmic effect; it decreases the frequency of the stimulus generation and it can therefore be used to treat heart rhythm problems. In high dilution, quinine and quinidine are also used as bitter tonics and stomachics because of their strong bitter taste.



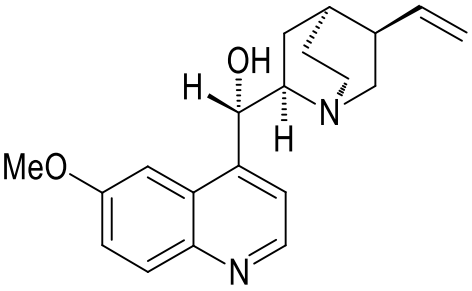
ruban skeleton

quinuclidine

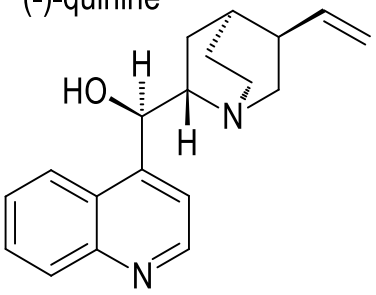




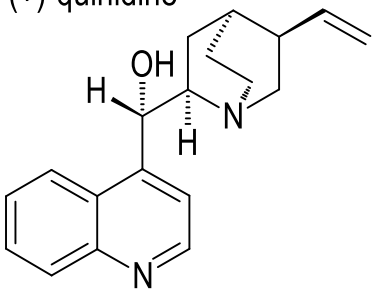
(-)-quinine



(+)-quinidine



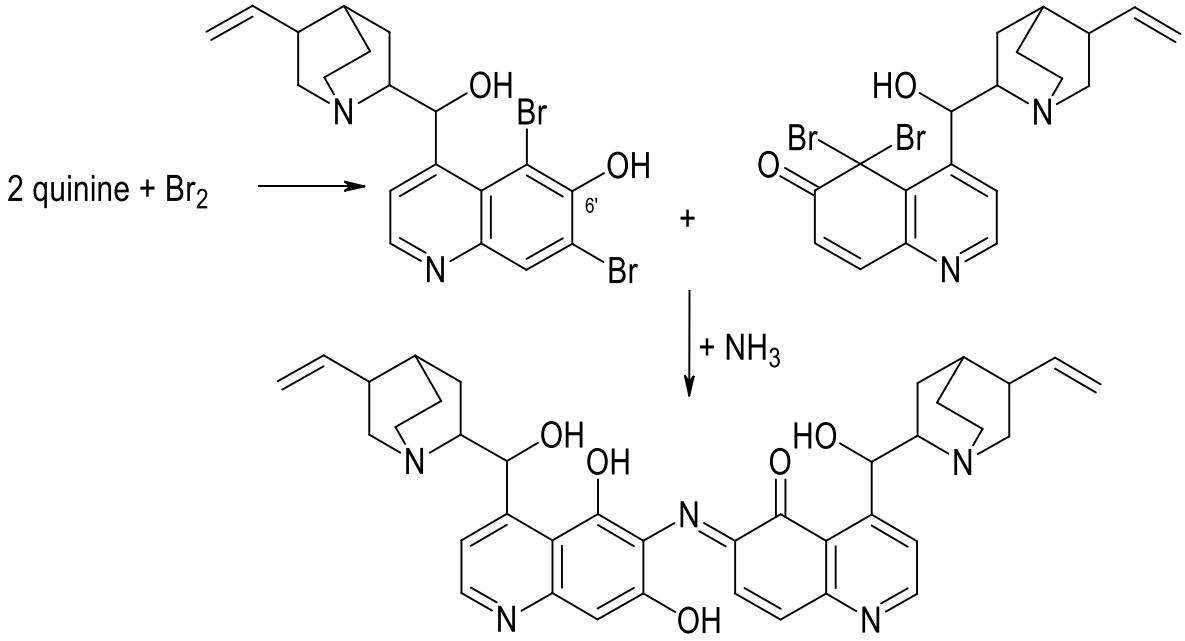
(-)-cinchonidine



(+)-cinchonine

Thalleioquin test (quinine and quinidine)

See method in Practical 9. This reaction can be used for the detection of quinine or quinidine, which possess an oxygen function at position 6'. After extraction of the drug with acidic water, alkali solution is added to the aqueous extract so that the alkaloids are converted into their free base form and can be extracted with an organic solvent. On the addition of bromine water, electrophilic aromatic substitution takes place and a precipitate forms. On the addition of NH_3, two bromine-substituted quinine molecules can condense and develop a mesomeric system with a characteristic green colour.



thalleioquin (green)

Grahe test (quinine and quinidine)

See method in Practical 9. Quinine and quinidine sublime as purple fumes when heated, and condense on the upper, relatively cold part of the test tube. When the sublimate is dissolved in methanol, the solution is diluted with water and diluted acidis added, a blue fluorescence can be observed under 366 nm light. Aqueous solutions of oxyacidic salts of quinine and quinidine display a blue fluorescence.

Quantitative determination of Cinchonae cortex

Extraction of the drug is carried out with acid-containing water so that the alkaloids are obtained in salt form. On the addition of alkali, the alkaloids are converted to their free base form and become soluble in organic solvents. During vigorous shaking, an emulsion is formed, but in response to the addition of tragacanth gum, which absorbs the water, this emulsion is broken down and the organic phase clarifies. The alkaloid content of the drug is determined by spectrophotometry, and is expressed as a percentage of quinine equivalents by using the following formula, where A = measured absorbance, and m = exactly measured mass of the drug.

$$\% = \frac{A}{A_0} \times m_0 \times 12.5 \times \frac{100}{m}$$

A_0 = 0.436
m_0 = 0.00406
Dilution factor= 12.5
Where A_0 is the absorbance of a solution containing quantity m_0 of quinine (0.00406 g).

Test questions 9

Essay questions:

- Explain how we performed the quantitative determination of cinchona bark. Start with the preparation of solutions and describe how we calculated the alkaloid content of the drug.
- Describe how we performed the Grahe test and what we observed.

Mechanisms and structures:

- Draw the ruban skeleton.
- Give examples of alkaloids from cinchona bark (quinine, quinidine, cinchonine and cinchonidine).
- Describe and draw the mechanism of the thalleioquin test reaction. How did we extract the drug and what was the observation?

Classification (Latin name of the drug, species and family) and uses of the following drug:

- Cinchona bark

Practical 10

Drugs with alkaloid content, IV
Drugs with alkaloids derived from tryptophane, II:
1. Strychni semen: detection of strychnine, brucine and loganin
2. Secale cornutum
   - Separation and TLC analysis of water-soluble and peptide alkaloids
   - van Urk reaction and detection of anthraquinones (sclereritrine)

Strychni semen
Nux vomica seed
Strychnos nux vomica L.
Loganiaceae

1. Strychni semen: test for strychnine, brucine and loganin

Extraction: Add 20 ml of water to 0.3 g of drug and boil the mixture for 10 min on a water bath. After cooling the mixture, filter it through cotton wool into a separating funnel. Extract it with 2 x 5 ml of chloroform. Combine the organic phases and dry the liquid with anhydrous Na_2SO_4. Divide the liquid between two porcelain dishes and evaporate both portions to dryness on a water bath.

Test for strychnine: Add a few $K_2Cr_2O_7$ (K dichromate) crystals and a few drops of 80% sulfuric acid to one of the porcelain dishes.

Test for brucine: Add a few drops of cc. HNO_3 to the other porcelain dish.

Test for loganin: Take 1-2 ml from the aqueous phase and add a few drops of R-H_2SO_4 to it. Evaporate the liquid to dryness on the water bath.

Write down your observations.

Secale cornutum
Ergot, secale
Claviceps purpurea
Clavicipitaceae

3. Secale cornutum

Separation and TLC analysis of water-soluble and peptide alkaloids

Extraction and separation: Add a few drops of cc. NH_3 solution to 1 g of drug, and then extract it with 10 ml of ethyl acetate by shaking the mixture for 10 min. Filter it through

cotton-wool into a separating funnel and extract the liquid with 10 ml of 1% tartaric acid solution. Adjust the pH of the aqueous phase to 5 with R-NaOH solution, and then extract it with 2 x 10 ml of chloroform (**chloroform phase I**). Adjust the pH of the aqueous phase to 8 with R-NaOH and extract it with 10 ml of chloroform (**chloroform phase II**). Evaporate the chloroform phases to dryness and dissolve each of the dry residues in 1 ml of methanol.

TLC determination
Sorbent: Silica gel

Spots:

Chloroform phase I.	20 µl	
Chloroform phase II.	40 µl	
0.1% methanolic ergometrin solution		20 µl
0.1% methanolic ergotamine solution		20 µl

Solvent system:
Toluene – dichloromethane − ethanol 28.5:57:14.5

Detection:
Spray the TLC with van Urk reagent, and then heat it for 5 min on 105 °C.

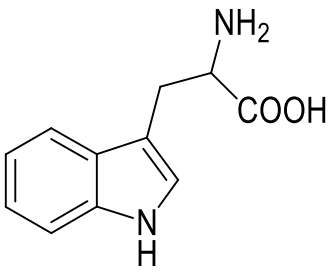
van Urk reaction - detection of ergot alkaloids: After applying it to the TLC plate, evaporate the remaining portion of the **chloroform phase I** to dryness on a water bath. Add 3-5 drops of van Urk reagent (*p*-dimethylaminobenzaldehyde) and 2-3 drops of H_2O_2 to the dry residue.

Detection of anthraquinones (sclereritrine): Add 5 drops of 20% sulfuric acid to 0.2 g of drug, and then extract it with 5 ml of ethyl acetate by shaking it for 2 min. Filter it into a reaction tube and add 2-3 ml of saturated $NaHCO_3$ solution. Shake the mixture, and then add 1 ml of R-H_2SO_4 solution to it.

Write down your observations.

Theory 10

Drugs with alkaloids derived from tryptophan, II



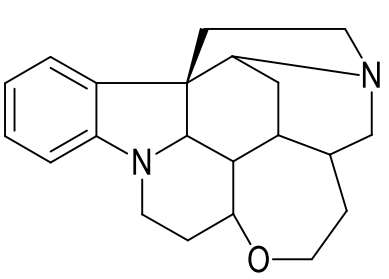
tryptophan

Strychni semen
Nux vomica seed
Strychnos nux vomica L.
Loganiaceae

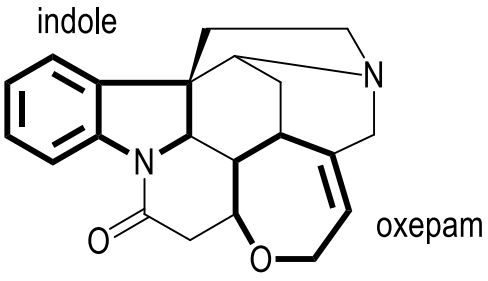
The drug consists of the dried seeds of *Strychnos nux vomica,* a tree with a height of around 25 m. The plant is native to India and Sri Lanka. The fruit of the plant is an orange berry 4-6 cm in diameter that contains 4-6 greenish-grey or grey seeds. The seeds are extremely hard, disk-shaped, 10 to 30 mm in diameter, and 4-6 mm in height. The surface of the seed is covered with silky hairs. It contains 2-3.5% alkaloids (strychnine and brucine), mostly in the form of salts with chlorogenic acid, 1-2% iridoid glycosides (loganin), fixed oil and proteins.

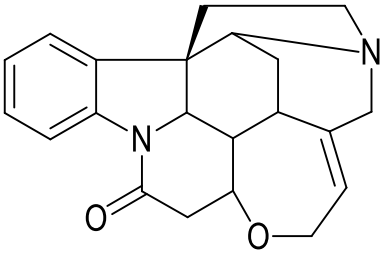
Uses: Strychnine can be used as a tonisant and circulatory stimulant in cases of severe weakness and recovalescence after a long illness or surgery. Since strychnine and brucine have an intensive bitter taste, they are also used as amara. Bitter drugs increase the excretion of the digestive juices in a reflex manner.

Toxicity: The mechanism of action leading to strychnine toxicity is well understood. This compound is a competitive antagonist of the inhibitory neurotransmitter glycine on the receptors in the spinal cord, brain stem and higher centres. Its effect is manifested in increased neuronal activity and excitability, resulting in increased activity of the skeletal muscles. The classical features of strychnine poisoning occur from 15 to 30 min after ingestion and include heightened awareness, muscular spasms, twitches and hypersensitivity to stimuli. Its ingestion in large amounts can cause these features to progress to painful generalized convulsions, during and after which the patient retains full consciousness. The cause of death is usually respiratory arrest secondary to respiratory muscle spasms, although a prolonged muscular spasm can lead to hyperthermia, rhabdomyolysis and associated renal failure due to myoglobinuria. The lethal dose for adults is 30-100 mg.

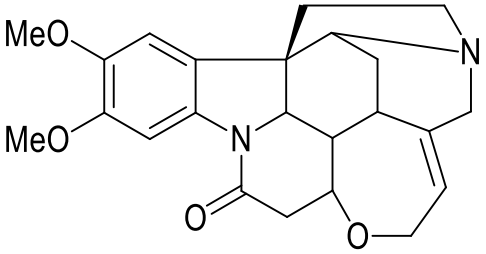


strychnane skeleton

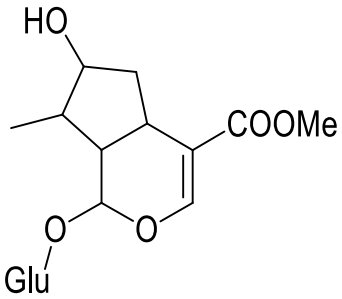




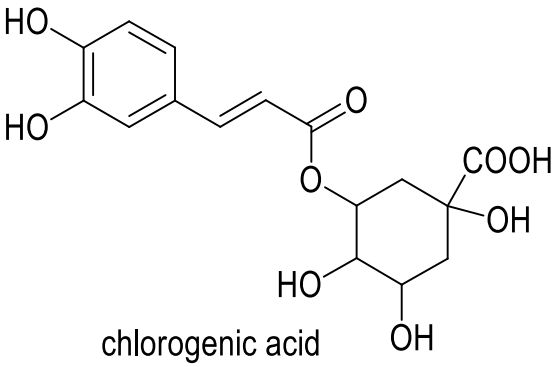
strychnine



brucine



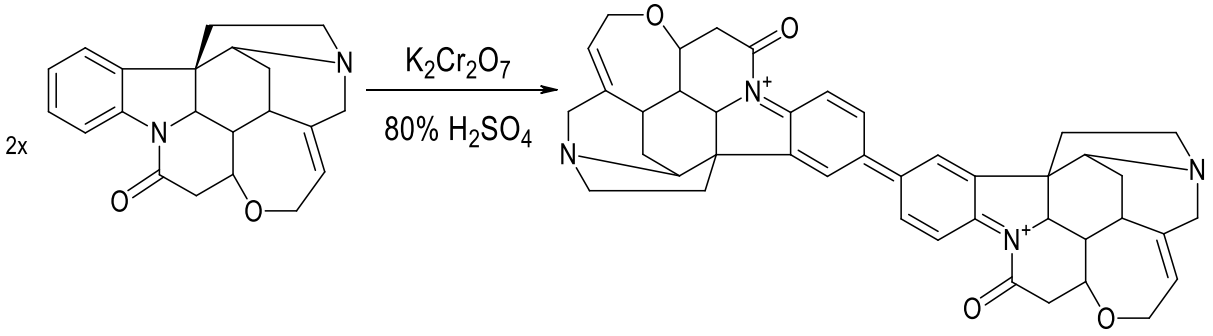
loganin



chlorogenic acid

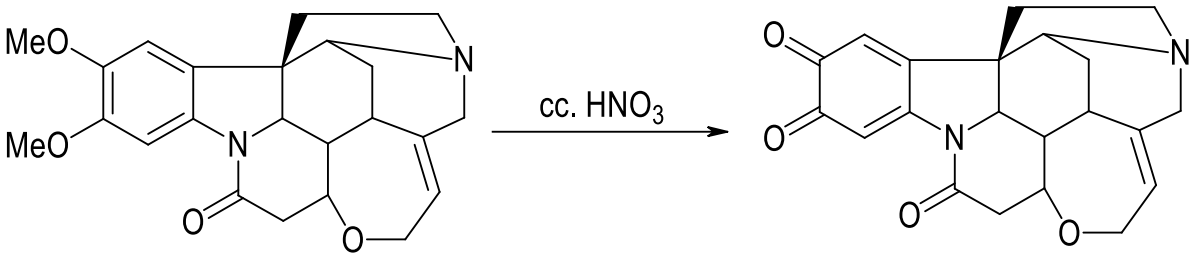
Test for strychnine

See method in Practical 10. A greyish-violet colour will appear. The mechanism involves the condensation of two strychnine molecules to give a dimer with quinoidal structure.



Test for brucine

See method in Practical 10. A blood-red colour appears as a result of the conjugated double bond−keto function system.



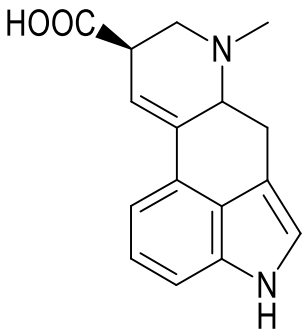
Test for loganin
See method in Practical 10. As a result, the dry residue will have a purplish-black colour, which will be lighter after the addition of a small amount of water, but the dark colour will return if the liquid is evaporated again.

Secale cornutum
Ergot, secale
Claviceps purpurea
Clavicipitaceae

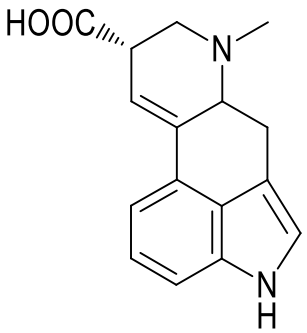
Ergot is the dried sclerotium of a fungus, *Claviceps purpurea*, which inhabits the ovary of rye. Thanks to modern farming methods, the natural occurrence of ergot has greatly decreased, and the drug is now obtained by cultivation. Each sclerotium is about 1-4 cm long and 2-7 mm wide, and is usually slightly curved. It is dark-purple or even black. It contains 0.2-1% indole alkaloids with an ergoline skeleton, 30% fixed oil, carbohydrates, biogenic amines (histamine and acetylcholine), anthraquinone derivatives (sclereritrine) and other colouring substances.

Classification of the alkaloids with an ergoline skeleton:

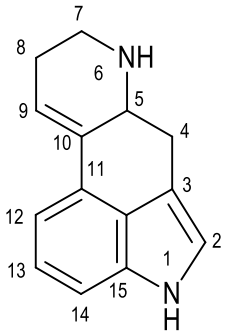
1. Clavines
2. Lysergic acid amides
    a. Water-soluble alkaloids (ergometrine)
    b. Peptide alkaloids (non-water-soluble)
        i. Ergotamine group (ergotamine)
        ii. Ergotoxin group (ergocristine, α- and β-ergocryptine and ergocornine)



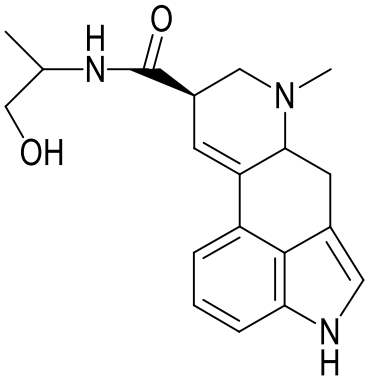
lysergic acid



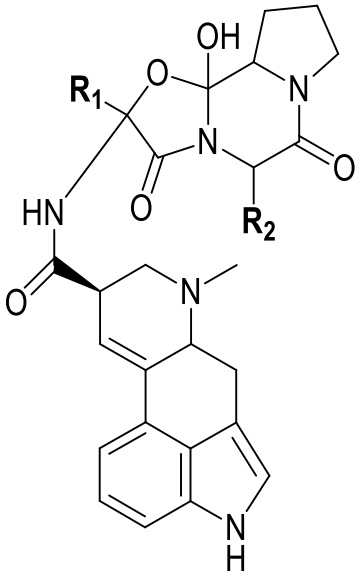
isolysergic acid



ergoline



ergometrine



	R1	R2
ergotamine	—Me	
ergocristine		
alpha-ergocryptine		
beta-ergocryptine		
ergocornine		

Uses: Little is known about the physiological effects of the clavines; this group is of low pharmacological importance. Ergometrine causes strong and long-lasting contractions of the uterus, which makes it unsuitable for the induction of delivery (for which oxytocin can be used), but it can be utilized as a styptic to stop the post-delivery bleeding. The peptide alkaloids have a blood vessel-constricting effect; they therefore increase the blood pressure and decrease the pulsation of the arteries. These derivatives are used in anti-migraine products. Synthetically modified 9,10-dihydro derivatives are strong α-

parasympatholytics, causing dilatation of the vessels and resulting in decrease of the blood pressure.

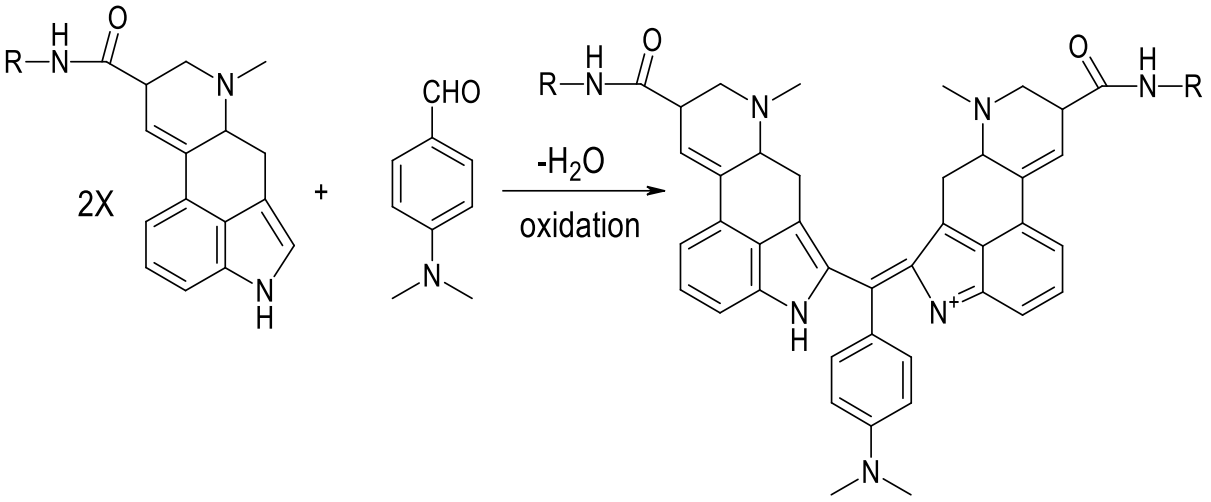
Toxicity: Ergotism is the effect of chronic ergot poisoning, which earlier frequently occurred due to the ingestion of the alkaloids through the consumption of rye and other cereals infected with ergot; in the present, such symptoms may develop through the action of a number of ergoline-based drugs. In the Middle Ages, this gangrenous poisoning was known as *Ignis sacer* ("holy fire") or Saint Anthony's fire, named after monks of the Order of St. Anthony, who were particularly successful at treating this disease. The symptoms vary: convulsive symptoms can involve painful seizures and spasms, diarrhoea, paraesthesias, itching, headaches, nausea and vomiting, while dry gangrene can arise as a result of vasoconstriction induced by the ergotamine-ergocristine alkaloids. This affects the less vascularized distal structures, such as the fingers and toes. The symptoms include desquamation, a weak peripheral pulse, loss of peripheral sensation, oedema and ultimately necrosis of the affected tissues and death.

Separation and TLC determination of water-soluble and peptide alkaloids

The alkaloids are extracted from the plant in their base form. The addition of tartaric acid solution makes the medium acidic and converts the alkaloid bases to their salt form. In this form, all the alkaloids are present in the acidic aqueous phase. The isoelectric point of peptide alkaloids is low, and even at pH 5 they can be extracted with organic solvents. **Chloroform phase I** contains the peptide alkaloids, such as ergotamine. When the remaining aqueous phase is made alkaline, the water-soluble alkaloids are converted into their free base forms and become extractable with chloroform. **Chloroform phase II** contains only water-soluble alkaloids, such as ergometrine. The visualization of the TLC plate is carried out according to the van Urk reaction (see below).

van Urk reaction

See method in Practical 10. Two molecules of ergoline alkaloid condense with one molecule of dimethylaminobenzaldehyde to form a delocalized electron system with a violet-blue colour.



Test for sclereritrine

See method in Practical 10. As a result, the aqueous phase first turns bluish-red, but after the addition of H_2SO_4, the aqueous phase becomes colourless, while the ethyl acetate phase is yellowish-red.

Test questions 10

Essay questions:

- Describe the detection of loganin (which drug we used, how we extracted it and what we observed).
- Explain the separation of the water-soluble and peptide alkaloids of ergot. How did we check the separation with TLC? How did we visualize the TLC plate after development?
- How did we detect sclereritrine (drug, extraction and observation)?

Mechanisms and structures:

- Give examples of alkaloids from nux vomica seed (strychnine and brucine).
- Draw the strychnane skeleton, loganin and chlorogenic acid.
- Describe and give the mechanism of detection of strychnine and brucine. Start with the extraction of the drug and explain the results.
- Give examples of ergot alkaloids (ergometrine, ergotamine, ergocristine, α- and β-ergocriptine and ergocornine).
- Draw lysergic acid, isolysergic acid and the ergoline skeleton.
- Describe, draw and explain the reaction mechanism of the van Urk test (drug, extraction, reagent and observation).

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Nux vomica seed and ergot

Practical 11

Drugs with alkaloid content IV

Drugs with alkaloids derived from xanthine:

1. TLC examination of the caffeine, theobromine and theophylline content of Coffeae semen, Colae semen, Cacao semen and Theae folium
2. Micromurexide reaction
3. Quantitative determination of caffeine content by HPLC

Coffeae semen	**Colae semen**	**Cacao semen**
Coffee beans	Cola nut	Cocoa beans
Coffea arabica L.	*Cola acuminata* L.	*Theobroma cacao* L.
Rubiaceae	Sterculiaceae	Sterculiaceae

Theae folium
Tea leaf
Camellia sinensis L.
Theaceae

1. TLC examination of the caffeine, theobromine and theophylline content of Coffeae semen, Colae semen, Cacao semen and Theae folium

Extraction: Add a few drops of cc. NH_3 to the drug, and then extract it with 10 ml of methanol on a water bath for 5 min. Filter the extract through filter paper into a porcelain dish and evaporate the liquid to dryness. Dissolve the dry residue in 1 ml of methanol.

TLC determination
Sorbent: Silica gel

Spots:

Coffeae semen methanolic extract	20 µl
Colae semen methanolic extract	20 µl
Cacao semen methanolic extract	20 µl
Theae folium methanolic extract	20 µl
0.1% methanolic solution of caffeine	20 µl
0.1% methanolic solution of theophylline	20 µl
0.1% methanolic solution of theobromine test	60 µl

Solvent system:
Chloroform – methanol – cc. NH_3 (47.5:4.5:0.5)

Detection:
Spray the TLC plate with KI + I_2 solution, and after 5 min spray it again with an alcoholic solution of R-HCl.

2. Micromurexide reaction
Place 0.05 g of Theae folium on the microsublimation plate, cover it with a glass slide and heat them together over a Bunsen burner. Wash the sublimate from the glass slides with methanol into a porcelain dish and evaporate the liquid to dryness. Add 5 drops of 25% HCl and 1 drop of cc. H_2O_2 to the dry residue, and then add 2 drops of R-NH_4OH.

Write down your observations.

3. Quantitative determination of caffeine content by HPLC
HPLC analysis is performed on a reversed-phase column (stationary phase). 0.1% phosphoric acid in water – acetonitrile (9:1) is used as mobile phase in isocratic mode. The chromatographic separation is monitored by a UV detector at 274 nm. The following practical work is to be performed under the instructor's close supervision.

Preparatory work – mobile phase
Check the solvent level within the container; at least 500 ml is needed for the experiment. Place the eluent in an ultrasound water bath for degassing. Filter the mobile phase on a membrane filter (<0.5 μm pore size).

Preparatory work – HPLC instrument
Turn on the instrument (pump, detector and computer). Setting up the machine: the flow rate is 0.4 ml/min in the first 9 min, which is then increased to 2 ml/min within 2.5 min. The run time is 12 min. The temperature is 60 °C. The detection wavelength is 274 nm. After flushing the machine with the solvent, wash the stationary phase with the eluent for equilibration.

Preparation of the samples
Measure 250 mg of instant coffee, 500 mg of ground coffee or 500 mg of tea, and extract it with 100 ml of HPLC grade hot water in an ultrasound water bath for 5 min. Filter the extract through filter paper into an Erlenmeyer flask, and then filter 4 ml of the extract through a teflon filter (0.45 μm pore size).

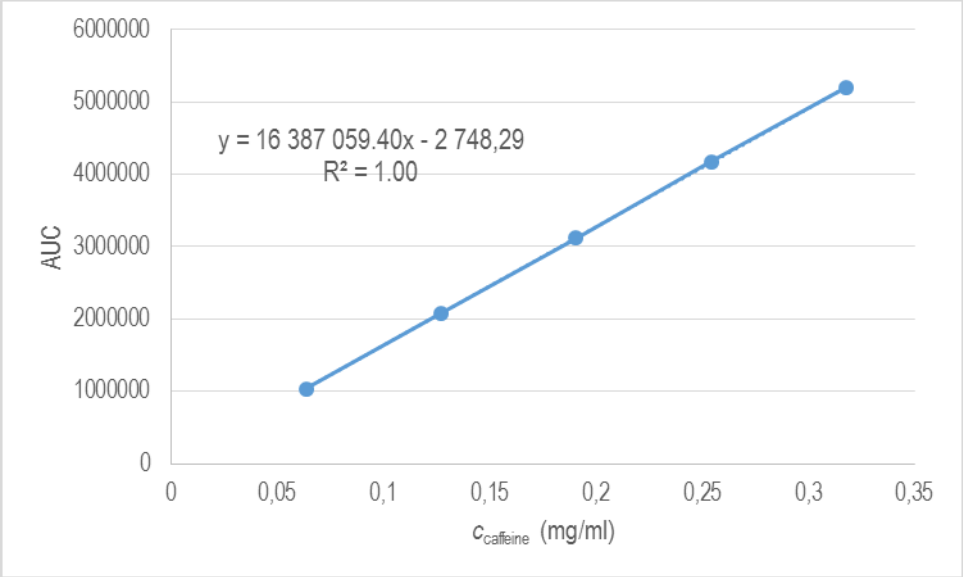
Working with the HPLC
Putting the sample solution into the Hamilton syringe (250 μl): the syringe needs to be cleaned with water between the injections of different samples. Wash the syringe with the sample three times and drain these solutions into the waste container. Then carefully suck up 100 μl of sample into the syringe. Do not allow any air bubbles to enter the syringe; if air bubbles are injected into the system, it can not work properly, and a pulse flow increases, a flow rate drops, and/or a baseline disturbance takes place. Introduce all

of the sample from the syringe into the loop when it is in the LOAD position. The calibrated volume of the loop is 20 µl, so 100 µl of sample will wash it completely, and 20 µl of sample (exactly the same as the volume of the loop) will finally be injected into the stationary phase. In the LOAD position, the loop is not in the path of the mobile phase. Then, turn the injector to the INJECT position; in this way, the sample within the loop is suddenly turned into the mobile phase stream towards the column, and the separation starts. Observe the retention time (~7.7 min) and the AUC (area under the curve) of the peak of caffeine in the chromatogram.

Calculation of the caffeine content of the sample

The AUC value of a certain peak is proportional to the amount of the compound injected into the system. For quantitative determination, a calibration curve is necessary, which has already been obtained from a set of standard samples of known concentrations. A stock solution (3.18 mg/10 ml) of caffeine was prepared, diluted and applied as standard samples. The equation of the calibration plot was obtained by performing linear regression on the AUC values of the standard samples by MS Excel. Calculate the caffeine content in 1 g of sample by using the equtation of the calibration plot given below, where *y* is the AUC of your sample, and *x* is the caffeine content expressed in mg/ml.

	AUC 1	AUC 2	AUC 3	AUC mean	$C_{caffeine}$ [mg/ml]
calibration solution 1	1031573	1039113	1042335	1037673	0.0636
calibration solution 2	2077355	2081974	2088432	2082587	0.1272
calibration solution 3	3124386	3119639	3125827	3123284	0.1908
calibration solution 4	4171081	4175574	4168844	4171833	0.2544
Stock solution	5199523	5203313	5209025	5204135	0.318

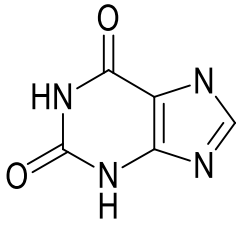


Provide the practical instructor with the following data: your name, the weight (mg) and AUC of the sample and the caffeine content (mg) calculated for 1 g of sample.

Theory 11

Drugs with alkaloids derived from xanthine

The purine nucleotides, together with the pyrimidine nucleotides are vital constitutive structural units of nucleic acids, and they also function as coenzymes. Adenine and guanine are the purines commonly involved in these roles, while xanthine and hypoxanthine are important in their biosynthesis.



xanthine

Theae folium
Tea leaf
Camellia sinensis L.
Theaceae

Tea consists of the prepared leaves and leaf buds of *Camellia sinensis* L., a shrub or tree with alternate, evergreen leaves. The tea tree is indigenous to Eastern Asia and now extensively cultivated in China, Japan, India, Sri Lanka and Indonesia. Green tea is prepared in China and Japan by rapidly drying the freshly picked leaves in copper pans over a mild artificial heat. Black tea is prepared in Sri Lanka and India by heaping the fresh leaves until the fermentation has begun, and then rapidly drying them with artificial heat. It contains 1-4% caffeine and small amounts of adenine, theobromine, theophylline, xanthine, 15% tannins of the catechin type: epigallocatechin gallate, epicatechin gallate, and epicatechin, and about 0.75% yellow volatile oil. One cup of tea contains around 30-60 mg of caffeine.

Uses: It is used mainly for preparing beverages. It has stimulating, diuretic and astringent effects.

Murexide test
See method in Practical 11. This test is used to detect xanthine derivatives. After the addition of 25% HCl and 1 drop of cc. H_2O_2, an orange precipitate forms, which dissolves in a few drops of NH_4OH to give a crimson colour.

Coffeae semen
Coffee beans
Coffea arabica L.
Rubiaceae

“Coffee bean” is the dried, ripe seed of *Coffea arabica* L. deprived of most of the seed coat. Coffee beans are generally roasted until they acquire a dark colour and a characteristic aroma. The plant is a small evergreen tree or shrub, indigenous to Ethiopia and other parts of Eastern Africa, but it is widely cultivated in tropical countries

(Indonesia, Sri Lanka, Central and South America and Brazil). It contains 1-2% caffeine and small amounts of theobromine and theophylline (all of these alkaloids as salts of chlorogenic acid), 3-5% tannins, 10-13% fatty oil and 5-8% phenolic acids such as caffeic acid and chlorogenic acid. The aroma of the coffee beverage is largely due to an oily substance known as caffeol, consisting of about 50% furfurol with traces of valerianic acid, phenol and pyrimidine. It is produced during the roasting process. One cup of coffee usually contains ca. 100 mg of caffeine.

Uses: It is mainly used for preparing beverages. It has stimulating, diuretic and astringent effects. Caffeine is a competitive antagonist of the adenosine receptors located on the presynaptic neurones and, as such, it exerts a prolonged excretion of certain excitatory neurotransmitters (e.g. noradrenaline). Caffeine also inhibits PDE (phosphodiesterase), which results in higher levels of cAMP within the cells. This potentiates the effects of excitatory neurotransmitters. The above two mechanisms result in the well-known CNS-stimulating effect of caffeine.

Cacao semen
Cocoa beans
Theobroma cacao L.
Sterculiaceae

Cocoa seeds are obtained from *Theobroma cacao* L., a tree usually 4-6 m in height. Cocoa is produced in South and Central America, the West Indies, West Africa, Ceylon and Java. The seeds can be prepared in different ways, but the following process is more or less typical. The seeds with the pulp of the fruit are allowed to ferment for 3-9 days. When the liquid drains from them, they change colour from white or red to purple. After the fermentation, the seeds are roasted to reach the desired odour and taste. The testa and the husk are then removed and the nibs or kernels are used to prepare chocolate products. Cocoa seeds contain 0.9-3.0% theobromine, 0.05-0.36% caffeine, cocoa fat (or butter), tannins of the catechin type, and about 70 different volatile oil components which are responsible for the taste and flavour.

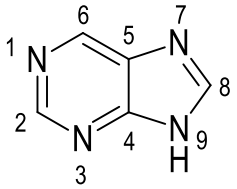
Uses: Cocoa has nutritive, stimulant and diuretic properties. Theobromine is used as a diuretic. It has less action on the CNS, but a greater effect on the urinary tract.

Colae semen
Cola nut, seed
Cola acuminata L.
Sterculiaceae

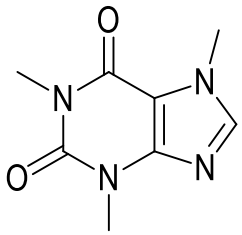
The drug is the dried cotyledon of *Cola acuminata* L. The cola tree is indigenous to West Africa and is cultivated in East Africa, Sri Lanka, Indonesia, Brazil and the West Indies. In

the tropical countries, the fresh cola nuts are chewed as a stimulant. It contains up to 3.5% caffeine, 0.5-1% theobromine, and catechin-type tannins: catechin and epicatechin.

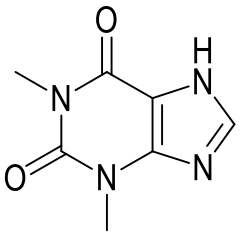
Uses: Cola possesses the CNS-stimulating action of caffeine. It is an ingredient of several beverages.



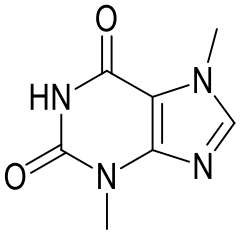
purine



caffeine



theophylline



theobromine

TLC analysis of drugs with xanthine alkaloids

The extraction of the alkaloids is started with an alkali in order to convert the components into their free base forms, to allow their extraction with an organic solvent. After development, a visualization reagent mixture, iodine + HCl is used to make the alkaloids visible. Spraying the plate with an alcoholic solution of potassium iodide and iodine and subsequently with 25% HCl gives brownish-red spots. With this TLC test, the xanthine alkaloid contents (type and relative amounts) of these drugs can be compared.

Test questions 11

Essay questions:

- TLC analysis of xanthine alkaloids. Describe how we extracted the different drugs, how we visualized the TLC plate and what we concluded?
- How did we perform the murexide test?

Mechanisms and structures:

- Draw the structures of xanthine and purine.
- Give examples of xanthine alkaloids (caffeine, theophylline and theobromine).

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Tea leaf, coffee beans, cocoa beans and cola nut

2nd Semester

Practical 1

Laboratory apparatus
Safety and laboratory procedures
Analysis of drugs with volatile oil content, I

1. General examination of volatile oils (colour, odour, taste and purity)
2. Lavandulae flos, Coriandri fructus, Menthae piperitae folium, Menthae crispae folium and Carvi fructus: TLC analysis of monoterpenes

Carvi fructus
Caraway seed (fruit)
Carum carvi L.
Apiaceae

Coriandri fructus
Coriander seed (fruit)
Coriandrum sativum L.
Apiaceae

Lavandulae flos
Lavender flower
Lavandula angustifolia L.
Lamiaceae

Menthae piperitae folium
Peppermint leaf
Mentha x *piperita* L.
Lamiaceae

Menthae crispae folium
Spearmint leaf
Mentha spicata var. *crispa* L.
Lamiaceae

1. General tests for volatile oils (colour, odour, taste and purity)

Quality test (odour and taste): Add 3 drops of volatile oil to 10 g of sugar. After mixing, the taste and odour should be identical with those of the plant part(s) from which the volatile oil is derived.

Purity test:
Detection of ethanol: Pour 1 ml of volatile oil into a reaction tube and close it with a cotton wool plug in which you have previously enclosed a few pieces of fuchsine crystals. Warm the test tube for a short time in the water bath.
Detection of fixed oil: Drop the volatile oil onto filter paper and leave it to stand. The volatile oil should evaporate completely within a short period of time, without leaving greasy or transparent marks on the paper.

Write down your observation.

2. Lavandulae flos, Coriandri fructus, Menthae piperitae folium, Menthae crispae folium, and Carvi fructus: TLC analysis of monoterpenes

Testing for carvone (spearmint and caraway seed)

Extraction: Extract 2.5 g of Menthae crispae folium and 1 g of Carvi fructus with 15 and 5 ml of chloroform, respectively, by gently shaking for 5 min. Filter the extracts and use them for chromatography.

TLC analysis
Sorbent: silica gel

Spots:

Carvi fructus chloroform extract	40 µl
0.1% methanolic carvone solution	20 µl
Menthae crispae folium chloroform extract	60 µl

Solvent system:
n-hexane – acetone (8:2)

Detection:
Spray the TLC plate with a 1% methanolic solution of 2,4-dinitrophenylhydrazine (DNPH).

Testing for linalool and menthol (lavender, coriander and peppermint)

Extraction: Extract 1 g of Menthae piperitae folium or 1 g of Coriandri fructus with 5 ml of chloroform, or 1 g of Lavandulae folium with 10 ml of chloroform by gentle shaking.

TLC analysis
Sorbent: Silica gel

Spots:

Lavandulae flos chloroform extract	20 µl
0.1% methanolic linalool solution	10 µl
Coriandri fructus chloroform extract	40 µl
0.1% methanolic menthol solution	10 µl
Menthae piperitae folium chloroform extract	60 µl

Solvent system:
n-hexane – acetone (8:2)

Detection:
Spray the TLC plate with R-vanillin − sulfuric acid reagent and place it into the heating chamber at 105 ºC for 5 min.

Theory 1

Volatile oils

Volatile oils are complex mixtures of odorous principles found in various plant species. They are called volatile because they evaporate at room temperature, but they are also referred to as **ethereal oils** or **essential oils**. They are mixtures of from ten to hundreds of components.

Common properties of volatile oils: they have a characteristic, usually pleasant odour, and they are usually colourless or pale-yellow, except for chamomile, absinth and yarrow oil (blue or greenish-blue). Their density is usually less than 1, except for clove, cinnamon and mustard oil. During their long-term storage, volatile oils may oxidize and resinify, which may result in a change in their colour or odour. To prevent this, they should be kept in a dry environment, in tightly-stoppered, full (not partly empty), dark glass containers. Volatile oils are almost immiscible with water, but they have a slight solubility in it, which is enough to form aromatic waters. On the other hand, they are completely soluble in ether, alcohol and most organic solvents.

Special occurrence of volatile oils in plant tissues: Depending on the plant family, volatile oils may occur in specialized secretory structures (since the volatile oil is not soluble in the aqueous environment of the cells, it is excreted in the form of oil drops in or close to the place of its origin), such as glandular hairs (Lamiaceae), parenchyma cells (Piperaceae), oil-tubes or vittae (Apiaceae), or in lysigenous or schizogenous passages (Pinaceae and Rutaceae).

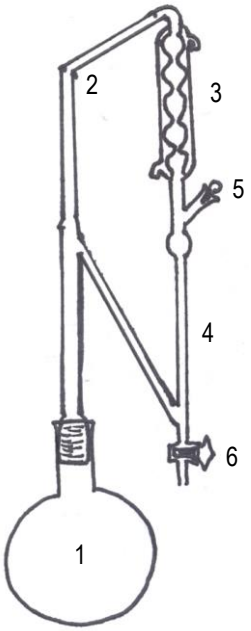
Chemical constituents of volatile oils can be divided on the basis of their biosynthetic origin into three classes:

- **Terpene derivatives** (formed by the acetate – mevalonic acid pathway)
- **Aromatic compounds** (formed by the shikimic acid – phenylpropanoid pathway)
- **Other compounds** [containing S or N atoms]

Determination of volatile oil content:

Place the amount of the air-dry drug specified by the monographs for determination in whole species or comminuted, as specified, into the distilling flask with 400 ml of water, add a few grains of pumice and mount the adapter. Through the aperture (e), let the water into the volumetric tube (d) until it flows back into the distilling flask. Then heat the contents of the flask by steadily moving the flame, and distil for 3 to 4 h after boiling has

started. The essential oil accumulates in the upper widening part of the volumetric tube and is separated from the aqueous phase, which flows back into the distilling flask. After the prescribed time is over, stop the heating. Read the volume of essential oil in the volumetric tube after 5 min. Then continue the distillation for 1 h and observe the volume of oil again as above. If the volume of the essential oil has not increased by more than 5%, the process can be regarded as finished; otherwise, the distillation must be continued. When the volumetric tube has cooled down to room temperature, the total volume of the essential oil can be read off. Calculate the volume of the oil related to the air-dry drug applied and express it as percentage.



1. glass distilling flask
2. ascending glass tube
3. vertical bulb condenser
4. volumetric tube with 0.01 ml gradation per 1 ml
5. glass-stoppered aperture
6. drain tap

Methods of obtaining volatile oils:

- **Steam distillation:** This method is typically applied if the constituents of the oils are not heat-sensitive. The drug is introduced into the distilling chamber along with water and subjected to heat until all volatile matter, both water and oil, has been condensed in the condensing chamber. Volatile oils used for medical purposes must be prepared by steam distillation.
- **Solvent extraction:** In the perfume industry, most of the volatile oil production is accomplished by extraction with solvents such as ether or benzene. The advantage is that the temperature stays constantly low during the process. As a result, the extracted oils have more natural odour as compared with distilled oils, which may undergo some chemical changes because of the high temperature used. The disadvantage of the solvent method is that it is more expensive than the distillation method.
- **Extraction with fat (enfleurage):** This is an old-fashioned method for the extraction of volatile oils from flowers (rose and jasmine). Enfleurage on a large scale is carried out in the perfume industry, because this method can preserve the typical fragrance of volatile oils. The freshly picked flowers are

strewn over the surface of a specially prepared fat base and left there for a defined time, and are then replaced by fresh flowers. At the end of the harvest, the fat, which is not renewed during the process, is saturated with flower oil. This oil is extracted from the fat with alcohol.

- **Expression:** If the volatile oil content in the drug is high, this method can also be applied (orange). It's disadvantage is that other constituents will also be present in the liquid obtained, so other purification techniques must be used in order to have the oil in the appropriate quality.
- **Enzymatic hydrolysis:** Glycosidic volatile oils are obtained by this method (bitter almond oil and mustard oil).
- **Supercritical extraction (SCE):** SCE is performed with supercritical fluids (SCFs). SCF can be any compound at a temperature and pressure above the critical values, above the critical point. The compounds in this state have both gaseous and liquid properties, behaving as a gas (they are able to ocupy the volume they are in), but they are also able to dissolve components as a liquid. The most common SCF is CO_2 under high pressure, which is an excellent solvent for apolar compounds. When the SCF is in contact with the solid drug, the volatile and apolar substances will partition into the supercritical phase. After this, the extracted components can be separated completely from the SCF by means of a temperature and/or pressure change. Advantages: low temperature, environment-friendly, and the SCF can be recycled.

Tests for quality and impurities:

Examination of a volatile oil can be performed by testing its taste, odour, volatility, density, refractive index and optical density. TLC or gas chromatography can be used for chemical characterization.

Taste and odour: *See the method in the practical part.* The taste and odour is characteristic to the oil.
Fixed oil impurity: *See the method in the practical part.* If the oil leaves a fatty spot, it contains a fixed oil impurity.
Alcohol impurity: *See the method in the practical part.* The cotton wool turns red if the volatile oil contains alcohol impurity because the evaporating alcohol dissolves the fuchsine to give a red colour.

Adulteration of volatile oils:
According to some assessments, 80% of expensive volatile oils are adulterated. Benzene, halogenated hydrocarbons, volatile oils from other plant species, added synthetic main component(s), fixed oils and alcohol may be present as adulterants.

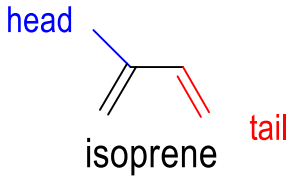
Common uses of volatile oils:

- Rubefacients in antirheumatic ointments for topical application

- Expectorants via inhalation
- Appetizers, secretolytics in the digestive system and carminatives; accordingly, several herbs containing volatile oils are utilized as spices
- Disinfectants and antiseptics (several volatile oils have bactericidal and bacteriostatic properties)
- Aromatics, flavouring of medicinal formulae

Terpenes

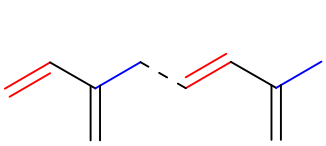
Terpenoids are products of the secondary metabolism in plants, formed from isoprene units. Even though they are built up from the same C_5 isoprene units, their chemical structures and properties show great diversity.
Terpenoids can be classified according to the number of isoprene units they were biosynthesized from.

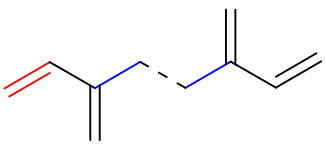


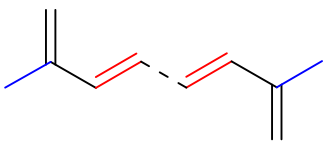
isoprene

Number of isoprene units	Common name	Compounds
1	Hemiterpenes (C_5)	Rare occurrence, mostly as substituents or precursors
2	Monoterpenes (C_{10})	Volatile oil components (less oxidized forms) Iridoids (highly oxidized forms)
3	Sesquiterpenes (C_{15})	Volatile oil components (less oxidized forms) Sesquiterpene bitters (highly oxidized forms)
4	Diterpenes (C_{20})	Resins, diterpene bitters, gibberellins, vitamin A
6	Triterpenes (C_{30})	Triterpene saponins, bitters, steroids
8	Tetraterpenes (C_{40})	Carotinoids
n	Polyterpenes (C_x)	Natural rubber

The isoprene units can be linked to each other in three different ways:

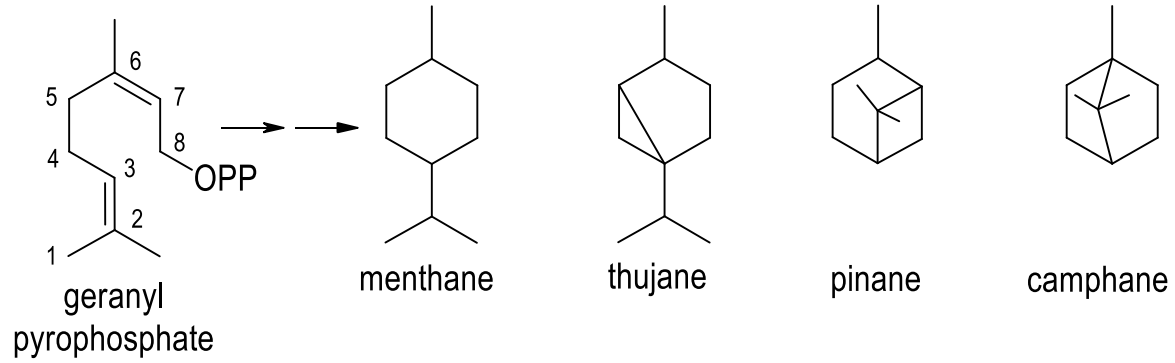

head-tail


head-head


tail-tail

Although the classification of terpenes is based on the number of their isoprene units, the properties of each component are influenced by the chemical structure. In every group, we can find saturated, unsaturated, linear or cyclic compounds, oxidized moieties (alcohols, aldehydes, ketones and acids) or their derivatives (esters and lactones), and carbohydrates can also be linked to these compounds as glycosides.

Monoterpenes. During their biosynthesis, isopentenyl pyrophosphate and dimethylallyl pyrophosphate are combined to form geranyl pyrophosphate, which may play an important role in the synthesis of most of the monoterpenes. In this group, the linkage of the two isoprene units is head—tail in most of the cases. This linkage results in the formation of geranyl pyrophosphate, which is the basis of most common monoterpenes. Through the formation of new C-C bonds or other functional groups, the following common monoterpene skeletons can be formed (the assignment is not complete):



Carvi fructus
Caraway seed (fruit)
Carum carvi L.
Apiaceae

The drug consists of the dried ripe fruits of *Carum carvi*. The plant is native to Europe and Southern Asia. Carvi fructus is slightly curved, brown and glabrous, 4-7 mm long and about 1 mm wide. The drug contains the volatile oil in the mesocarp in six large vittae. Besides the 3-7% volatile oil, the drug also contains 10-20% fixed oil, proteins and carbohydrates. The main constituent of the volatile oil is carvone, but limonene, dihydrocarvone, carveol and dihydrocarveol can also be found.

Uses: Caraway and caraway oil are used as carminatives and spices. The mechanism of action can be attributed to the smooth muscle-relaxing effect of the volatile oil components.

Menthae crispae folium
Spearmint leaf
Mentha spicata var. crispa L.
Lamiaceae

The spearmint drug is the dried leaf and flowering top of *Mentha spicata var. crispa*. It is widely cultivated in England, Southern Europe and the USA. The leaves are almost sessile and bright-green. They are collected before or at the beginning of flowering. The herb has a spicy hot taste and a characteristic odour similar to that of caraway. The drug contains 1-2.5% volatile oil, flavonoids and bitters. The main constituents of the oil are carvone, dihydrocarvone, carveol and dihydrocarveol acetate.

Uses: As a spice, appetizer, slight spasmolytic and carminative. It is used for its taste in mouthwash formulae and toothpastes.

Menthae piperitae folium
Peppermint leaf
Mentha x piperita L.
Lamiaceae

The drug consists of the dried leaf and flowering top of *Mentha x piperita*. Peppermint is a hybrid mint, a cross between watermint (*Mentha aquatica*) and spearmint (*Mentha spicata*). The plant is indigenous to Europe, but is now widely cultivated throughout the world. It is a perennial plant; the leaves are dark-green and usually slightly hairy. The plant stores the volatile oil in these hairs/trichomes. It contains rosmarinic acid and 0.5-4% volatile oil, in which the chief components are menthol, menthyl acetate and menthone.

Uses: Peppermint is used in refreshing teas, toothpastes and mouthwashes. It is used as an amarum, appetizer, carminative and spasmolytic. These latter effects can mainly be attributed to its menthol content, which can decrease the Ca^{2+} influx into the smooth muscle cells of the gastrointestinal tract (Ca^{2+}channel-blocking effect). The drug has choleretic and cholekinetic effects due to its flavonoid content. In topical preparations, it has a cooling effect, and it can be used to relieve itching. When inhaled, menthol is a potent nose decongestant. There is evidence that the volatile oil of peppermint is effective against headache when applied to the temple.
Caution: It should be noted that children under the age of 3 years tend to be a hypersensitive to menthol, and the administration of menthol in the form of an ointment on the face, under the nose or on the chest is strongly contraindicated. In severe cases, laryngeal oedema, spasm of the respiratory muscles or respiratory paralysis may occur.

Lavandulae flos
Lavender flower
Lavandula angustifolia L.
Lamiaceae

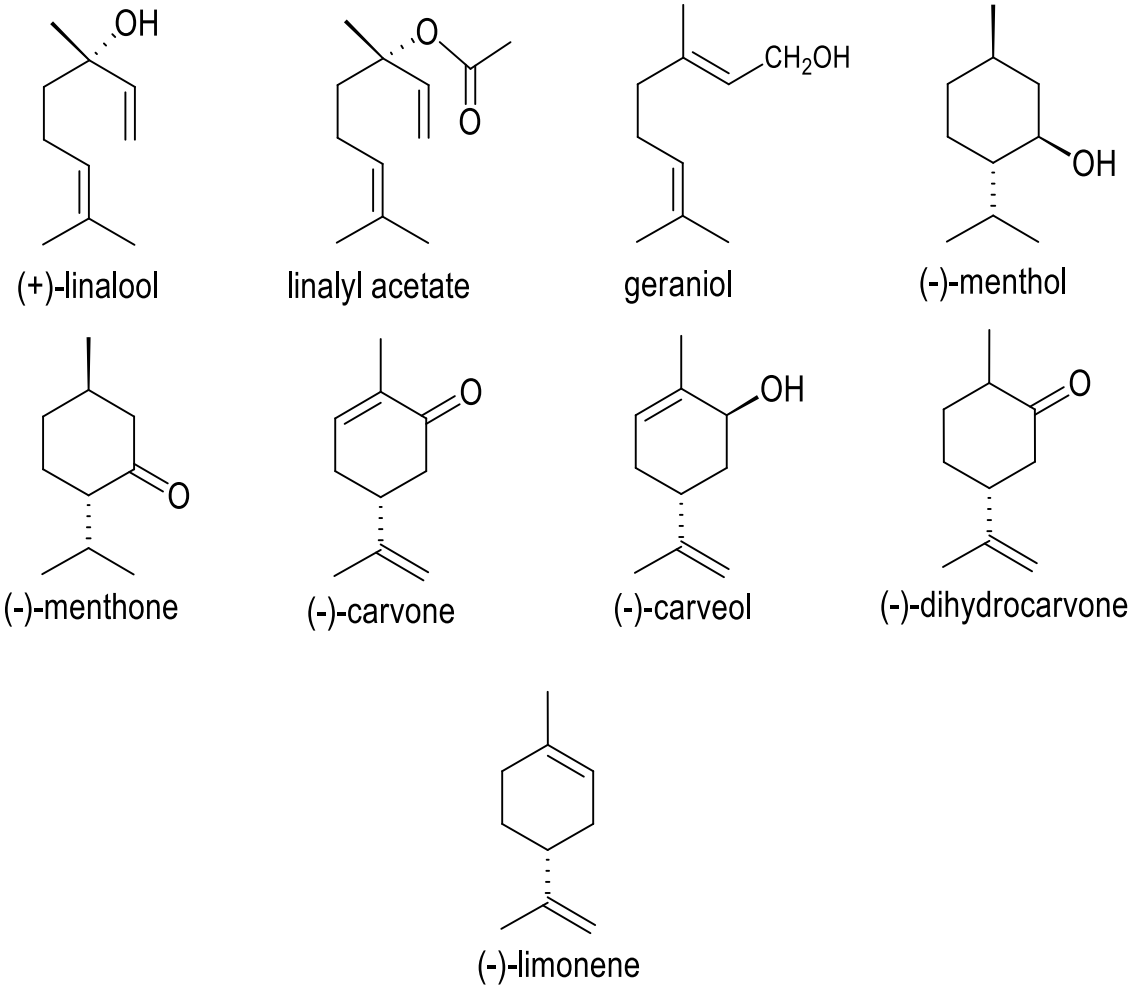
The drug consists of the flowers of an evergreen shrub, *Lavandula angustifolia*. The volatile oil is distilled from the freshly collected flowers, which have a characteristic violet colour. Lavender is native to the western Mediterranean region, but it is also widely cultivated in Europe. The drug contains 1-3% volatile oil, tannins, coumarins, flavonoids and bitters. The main constituents of the oil are linalool and linalool esters, primarily linalyl acetate and butyrate.

Uses: The drug is a constituent of teas or is used in aromatherapy to treat anxiety and sleeplessness. The main constituents of its volatile oil (linalool and linalyl acetate) are absorbed into the bloodstream and enter the CNS after oral administration or via inhalation, and potentiate the effect of GABA, an inhibitory neurotransmitter. Its volatile oil also has a spasmolytic effect, which results in a mild carminative property. It is used in ointments and lotions for rheumatic disorders. The drug has insect repellent properties too.

Coriandri fructus
Coriander seed (fruit)
Coriandrum sativum L.
Apiaceae

The drug consists of the dried nearly ripe fruits of *Coriandrum sativum*. The plant is native to south-eastern Europe, but it is also cultivated worldwide. The drug is about 2-4 mm in diameter, yellowish or brownish in colour and contains the volatile oils in one or two vittae. Coriander contains 1% volatile oil, the main constituents being linalool, geraniol, borneol, α-pinene and limonene.

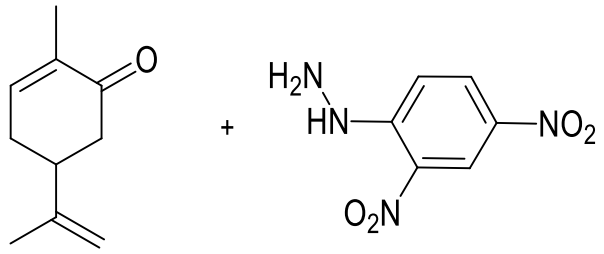
Uses: Coriander and coriander oil are used as spices and carminative drugs. They can be used in dyspepsia because of the spasmolytic and gastric juice secretion-increasing effect of the volatile oil components.



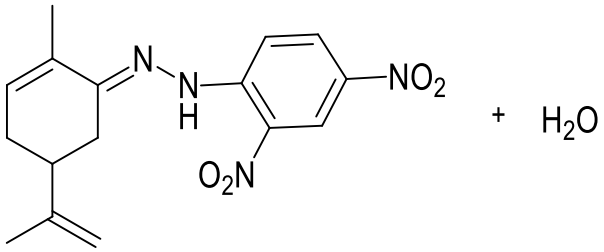
TLC analysis of monoterpenes: tests for carvone, linalool and menthol

These monoterpenes are apolar compounds and can easily be extracted with chloroform. One of the detecting reagents is 2,4-dinitrophenylhydrazine (DNPH), which is suitable for the detection of aldehydes and ketones such as carvone, which bears a keto function. The result is seen as yellowish-red spots. Since linalool and menthol possess –OH groups, the former reagent is not applicable for their detection. For the visualization of these two monoterpenes, vanillin — sulfuric acid (a 1% solution of vanillin in cc. sulfuric acid) is used. The mechanism of visualization is based on the strong dehydrating effect of cc. H_2SO_4, which results in various decomposition products, including carbocations that can bind to the vanillin (an aromatic aldehyde) content of the reagent. The conjugated double bond systems of the products give coloured spots, the colour frequently being characteristic of the compound visualized.

DNPH reaction – detection of aldehydes and ketones



carvone DNPH



carvone-dinitrophenylhydrazone (orange)

Test questions 1

Essay questions:

- Define volatile oils.
- Classify volatile oils according to the chemical structures of their constituents.
- Give the process for determination of the volatile oil content of drugs.
- Describe the methods of obtaining volatile oils from drugs.
- What are the common properties of volatile oils, and the general tests for quality and frequent impurities/adulterants?
- What are the common uses of volatile oils?
- Define terpenes, and give their classification as regards the isoprene units they contain.
- What are monoterpenes and how do they build up?
- How can we analyse monoterpenes by TLC? How did we extract monoterpenes from the drugs and visualize them on the developed TLC plates?

Mechanisms and structures:

- Draw the basic skeletons of monoterpenes (menthane, thujane, pinane, camphane).
- Give examples of monoterpenes (linalool, linalyl acetate, geraniol, menthol, menthone, carvone, carveol, dihydrocarvone and limonene).

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Caraway seed, spearmint leaf, lavender flower, coriander seed and peppermint leaf

Practical 2

Analysis of drugs with volatile oil content II

1. TLC examination of Matricariae flos
2. Testing for proazulenes by EP test (Absinthii herba, Millefolii herba and Matricariae flos)
3. Equipment for the steam distillation of volatile oils

Matricariae flos	**Absinthii herba**	**Millefolii herba**
Chamomile flower	Absinthe wormwood herb	Common yarrow herb
Matricaria recutita L.	*Artemisia absinthium* L.	*Achillea millefolium* L.
Asteraceae	Asteraceae	Asteraceae

Matricariae extractum fluidum

1. TLC examination of Matricariae flos

Extraction: Extract 1 g of Matricariae flos with 10 ml of chloroform by gentle shaking for 5 min. Filter the extract through filter paper into a porcelain dish and evaporate off half of the solvent at room temperature.

TLC determination
Sorbent: silica gel

Spots:

Matricariae flos chloroform extract	80 µl
Matricariae aetheroleum	20 µl

Solvent system:
benzene – ethyl acetate (8:2)

Detection:
Spray the developed plate with EM reagent and keep it in the heating chamber for some minutes at 105 °C.
(EM reagent = 5% *p*-dimethylaminobenzaldehyde and 10% phosphoric acid dissolved in glacial acetic acid).

2. Testing for proazulenes by EP test (Absinthii herba, Millefolii herba and Matricariae flos)

A. Extract 1 g of drug with 10 ml of chloroform by gentle shaking for 5 min. Filter the extract into a porcelain dish and evaporate the liquid to dryness on the water bath.

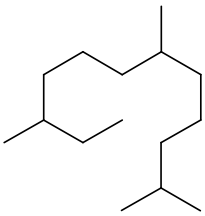
Dissolve the dry residue in 2.5 ml of EP reagent (*p*-dimethylaminobenzaldehyde dissolved in glacial acetic acid) and 1 ml of water, pour the solution into a test tube and keep it in the hot water bath for 2 min. After it has cooled, shake it with 2 ml of petrolether.

B. Put 0.5 g of drug into a reaction tube, and add 1-2 ml of EP reagent and then place the tube on the water bath for 10 min.

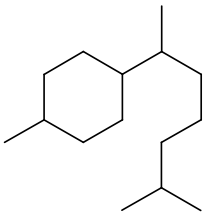
Write down your observations.

Theory 2

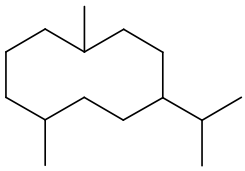
Sesquiterpenes are secondary plant metabolites built up from 3 isoprene units. The units are linked to each other through head—tail binding in most cases. As in the case of the monoterpenes, a great diversity of compounds can be formed from the basic molecule by various structural changes such as cyclization, oxidation or rearrangement. The sesquiterpenes can be linear, mono-, bi- or even tricyclic. The most important basic skeletons are:



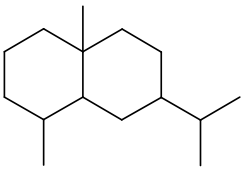
farnesane



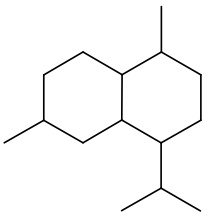
bisabolane



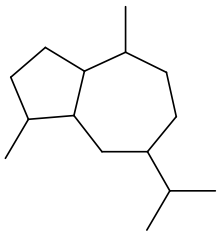
germacrane



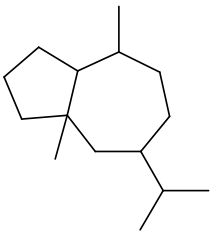
eudesmane



cadinane



guaiane



pseudoguaiane

Two classes of sesquiterpenes (sesquiterpene hydrocarbons and sesquiterpene alcohols) are components of the volatile oils of plants. They are liquid at room temperature and they can be distilled with steam.
A group of sesquiterpenes known as sesquiterpene lactones can be the components of essential oils or they can be bitter principles. Proazulenes belong in this group. These compounds are highly oxidized, they contain various functional groups (hydroxy, epoxy, aldehyde, lactone, ester groups, etc.). They are mostly crystalline solids. Their name comes from their decomposition products, the “azulenes”, a typical example of which is chamazulene (see below).

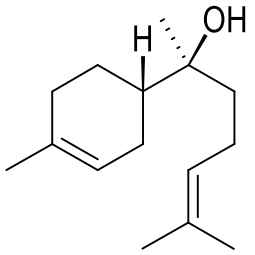
The Asteraceae family is characterized by a high accumulation of sesquiterpenes.

Common pharmacological effects of sesquiterpenes are their antibacterial, anti-inflammatory, cytotoxic and anxiolytic action. It is important to mention that sesquiterpenes may also cause allergic reactions.

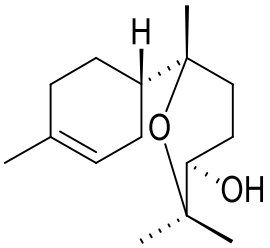
Matricariae flos
Chamomile flower
Matricaria recutita L.
Asteraceae

Chamomile flower is the dried flower heads of *Matricaria recutita.* It usually grows near populated areas throughout Europe and temperate zones of Asia. It has been widely introduced into temperate North America and Australia too. The flowers consist of white lingulate florets and yellow tubular florets. The plant blooms in early to mid-summer, with a strong, aromatic smell and a bitter taste. The drug contains 0.25-1.35% volatile oil (chamazulene, α-bisabolol, bisabolol oxide A and bisabolol oxide B), coumarins, flavones (apigenin), flavone glycosides, polyacetylenes and mucilage. The freshly distilled volatile oil has a characteristic blue colour.

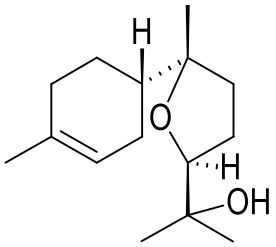
Uses: Chamomile is used medically as an anti-inflammatory agent in cases of stomach and bowel disorders (chamazulene and bisabolol are cyclooxygenase inhibitors). It is also used as a mild spasmolytic in the forms of tea or aqueous or alcoholic extracts. Topically, it is used in cases of eye or skin inflammation in the form of a compress, or as an aromatic bath in cases of haemorrhoids and gynaecological inflammations. It is also used as a mouthwash against oral mucositis.



(-)-α-bisabolol

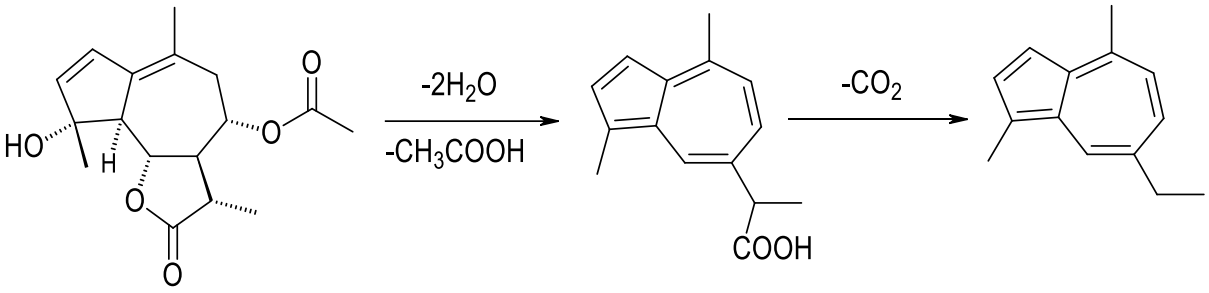


(-)-α-bisabolol oxide A



(-)-α-bisabolol oxide B

Proazulenes are sesquiterpene lactones (such as matricin or artabsin) from which azulenes (such as chamazulene) can be formed.
Chamomile contains the proazulene matricin, while chamazulene is originally not present within the drug. Matricin (guainanolide skeleton) is a non-volatile, colourless, crystalline compound which, when heated in the presence of water, decomposes to the unstable chamazulene carboxylic acid (the lactone ring breaks down with the loss of acetic acid and water). Chamazulene carboxylic acid immediately transforms to the blue chamazulene.



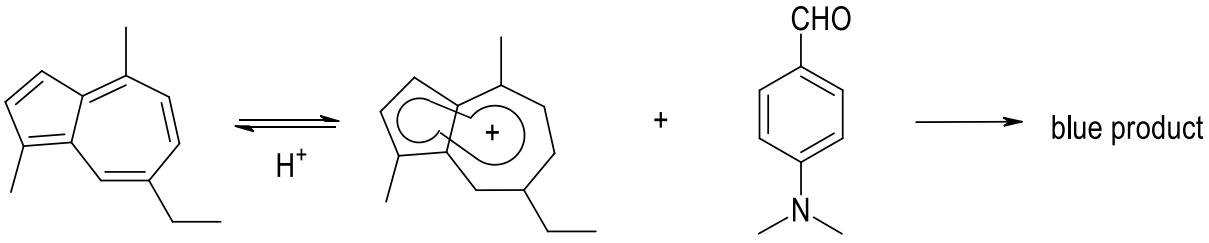
matricin chamazulene carboxylic acid chamazulene

TLC analysis of Matricariae flos

The aim of the TLC is to compare the chloroform extract of the chamomile flower and its volatile oil obtained by steam distillation. As pointed out earlier, one of the chief components of the volatile oil is the blue chamazulene, which is not present originally in the drug, but is formed during the steam distillation process. Thus, chamazulene is not detectable in the chloroform extract of chamomile flower. The visualization is based on the EP reaction described below. In order to visualize the proazulenes in the chloroform extract, the chromatogram should be sprayed with the EM reagent and placed into the heating chamber. During the heating process, the proazulenes are transformed to azulenes in the chromatogram. The azulenes formed *in situ* can then react with *p*-dimethylaminobenzaldehyde, and appear as blue, bluish-green spots on the TLC.

Detection of proazulenes (EP/EM test)

The EP reagent is the acidified solution of *p*-dimethylaminobenzaldehyde. *See the method in Practical 2.* After the addition of the reagent, the aqueous phase turns bluish-green. Petrolether helps to clarify the aqueous phase in order to give a nicer, more easily visible colour.



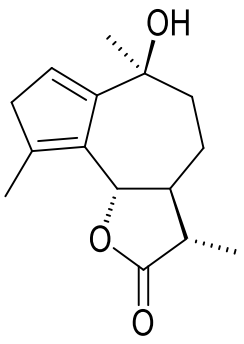
chamazulene azulenium cation *p*-dimethylamino-benzaldehyde

Absinthi herba

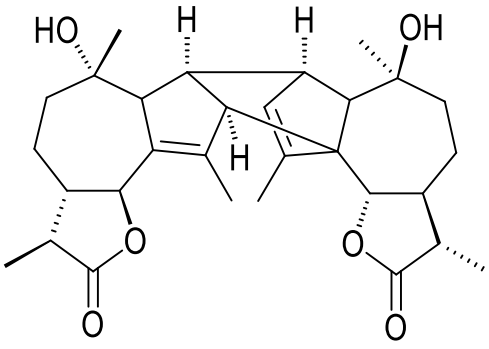
Absinthe wormwood herb
Artemisia absinthium L.
Asteraceae

The drug consists of the dried herb of *Artemisia absinthium* collected during flowering. It is a perennial plant native to Europe, Eastern Asia and Northern Africa. It grows on uncultivated ground, on rocky slopes, and at the edge of footpaths and fields. Its stems are straight, growing to 0.8-1.2 m tall, with a silvery-green colour. The leaves are covered with silky silvery-white, T-shaped hairs (keep this in mind in connection with its determination as an unknown drug powder), and it has oil-producing glands. The drug contains 0.2-0.5% volatile oil [α- and β-thujone, thujol (monoterpenes), artabsine, matricin (sesquiterpene proazulenes) and the dimer absinthine]. The volatile oil is blue or greenish-blue because of the azulenes formed from the proazulenes during distillation.

Uses: The aqueous extract is used as an amarum and appetizer because of its choleretic and gastric juice secretion-increasing effects. It is used in the folk medicine as an anthelminthic or abortive agent.
The alcoholic extract of the drug is rich in thujone, which in the event of overdosing, is a neurotoxic and convulsive agent (it blocks the $GABA_A$ inhibitory receptor in the CNS). The symptoms of "absinthism" were attributed to the high thujone content of the popular absinthe (an alcoholic short drink). In the early 1910s the absinthe trade was banned, though in 1990 the drink reappeared on the market, but only with strict restrictions concerning its maximum thujone content.



artabsin



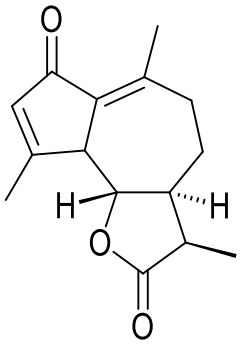
absinthin

Millefolii herba
Common yarrow herb
Achillea millefolium L.
Asteraceae

The drug is the herb of *Achillea millefolium*. The plant is native to Europe, northern Asia, North Africa and North America. Common yarrow is a perennial plant frequently found in the mildly disturbed soil of grasslands and open forests. It can reach a height of 0.2 to 1 m. Its white flowers appear in flat-topped clusters, composed of 4-6 ray flowers encircling numerous small disk flowers. The drug contains 0.1-1% blue volatile oil [pinene, sabinene (monoterpenes), sesquiterpenes, achillin and millefin (sesquiterpene lactones)], flavonoids and polyacetylenes.

Since achillin is not a typical proazulene, due to its keto function, the azulene formed and hence the volatile oil will have a greenish colour.

Uses: Used as an amarum, and an anti-inflammatory in cases of intestinal and gynaecological problems. A wound healing-enhancing effect has also been attributed to the plant.



achillin

Test questions 2

Essay questions:

- What are sesquiterpenes and how do they build up?
- Define proazulenes. What are they, where can they be found and how do they form?
- In the TLC analysis of chamomile extract and volatile oil, what did we use for extraction, how did we visualize the TLC plate and what could we conclude?

Mechanisms and structures:

- Draw the basic skeletons of sesquiterpenes (farnesane, bisabolane, germacrane, eudesmane, guaiane and pseudoguaiane).
- Give examples of sesquiterpenes (α-bisabolol, α-bisabolol oxide A and α-bisabolol oxide B).
- Give examples of sesquiterpene lactones (matricin, artabsin, absinthin and achillin).
- Give the mechanism of azulene formation (from matricin to chamazulene).
- Give and explain the mechanism of proazulene detection (EP test).

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Chamomile flower, absinthe wormwood herb and common yarrow herb

Practical 3

Analysis of drugs with volatile oil content, III

1. Caryophylli floris aetheroleum: determination of the eugenol content in a Cassia flask
2. Cinnamomi cassiae aetheroleum: determination of the cinnamaldehyde content in a Cassia flask
3. Anisi fructus and Foeniculi dulcis fructus: TLC analysis of phenylpropane derivatives

Drugs with miscellaneous terpene content

4. Determination of valepotriates
5. Definition of bitterness value

Determination of unknown drug powders

Caryophylli flos
Clove flower
Syzygium aromaticum L.
Myrtaceae

Caryophylli floris aetheroleum

Cinnamomi cortex
Ceylon cinnamon bark
Cinnamomum zeylanicum J.S.Presl.
Lauraceae

Cinnamomi corticis tincture
C. zeylanici folii aetheroleum
C. zeylanici cortices aetheroleum

Cinnamomi cassiae cortex
Cassia cinnamon bark
Cinnamomum cassia Blume.
Lauraceae

Cinnamomi cassiae cortex

Anisi fructus
Anise seed (fruit)
Pimpinella anisum L.
Apiaceae

Anisi aetheroleum

Foeniculi dulcis fructus
Sweet fennel seed (fruit)
Foeniculum vulgare subsp. vulgare var. dulce Miller.
Apiaceae

1. Caryophylli floris aetheroleum: determination of the eugenol content in a Cassia flask

Pipette 5.00 ml of accurately measured volatile oil into a Cassia flask, and then add 70 ml of 3% NaOH solution. Shake the mixture well several times and warm it by placing into

the water bath until the volatile oil which did not take part in the reaction appears on top of the liquid and separates from the aqueous NaOH phase. Cool the flask to room temperature and add more 3% NaOH until the amount of the separated volatile oil can be determined (on the gradated neck of the flask) and read the volume. Subtract this value from the starting (5.00 ml) volume, which will give the eugenol content of 5.00 ml of volatile oil. Express the result in v/v% (calculate the eugenol content for 100.00 ml of volatile oil).

2. Cinnamomi cassiae aetheroleum: determination of the cinnamic aldehyde content in a Cassia flask

Pipette 5.00 ml of volatile oil into a Cassia flask, and then add 10 ml of 30% $NaHSO_3$ solution. Shake the mixture well several times and warm it by placing into the water bath until the precipitate dissolves. If necessary, add 10 ml of 30% $NaHSO_3$ solution again and repeat the process several times until no precipitate formation can be observed. Then fill the flask with 30% $NaHSO_3$ up to the first mark on the neck and warm it in the water bath until the content separates into two phases. Cool the flask and add more 30% $NaHSO_3$ until the amount of the separated volatile oil can be easily determined (on the gradated neck of the flask) and read off the volume. Subtract this value from the starting (5.00 ml) volume, which will give the cinnamic aldehyde content of the 5.00 ml of volatile oil. Express the result in v/v% (calculate the cinnamic aldehyde content for 100.00 ml of volatile oil).

3. Anisi fructus and Foeniculi dulcis fructus: TLC analysis of phenylpropane-derivatives

Extraction: Extract 1 g of drug (Anisi fructus and Foeniculi dulcis fructus) with 5 ml of chloroform by gently shaking for 5 min.

TLC determination
Sorbent: silica gel

Spots:

Anisi fructus chloroform extract	40 µl
Methanolic anisaldehyde solution	20 µl
Foeniculi dulcis fructus chloroform extract	40 µl

Solvent system:
n-hexane – ethyl acetate (8:2)

Detection:
Spray the TLC plate with DNPH (2,4-dinitrophenylhydrazine) reagent.

Valerianae radix
Valerian root
Valeriana officinalis L.
Valerianaceae

Valerianae tinctura
Valerianae extractum hydroalcoholicum siccum

4. Test for valepotriates

Extract 0.5 g of drug with 5 ml of chloroform, filter the extract into a porcelain dish and evaporate the liquid to dryness on the water bath. Add 1 drop of glacial acetic acid and 1 drop of cc. HCl to the dry residue.

Write down your observation.

Herbs with significant bitterness values:

Gentianae radix	**Menyanthidis trifoliatae folium**
Gentian root	Bogbean leaf
Gentiana lutea L.	*Menyanthes trifoliate* L.
Gentianaceae	Menyanthaceae

5. Definition of bitterness value

Theory 3

Phenylpropane derivatives are **aromatic compounds** built up from C_6 + C_3 units. The C_6 unit is always an aromatic ring, while the C_3 unit is a linear chain. The side-chain of phenyl-propane derivatives can be shortened to C_1 because of oxidation. General classification of the phenylpropanoids:

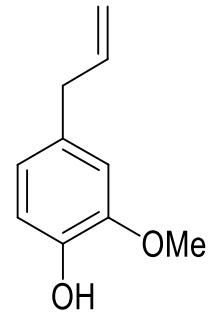
- Cinnamyl acid derivatives, e.g. *p*-coumaric acid
- Cinnamyl derivatives, e.g. cinnamyl alcohol
- Allylbenzene derivatives, e.g. safrol
- Propenylbenzene derivatives, e.g. isoanethole
- Derivatives with a shortened side-chain, e.g. vanillin

Common properties: Phenylpropane derivatives are typically not highly oxidized molecules. Because of this, they are quite apolar, with a characteristic odour, occurring as volatile oil constituents.

Caryophylli flos
Clove flower
Syzygium aromaticum L.
Myrtaceae

Clove flower is the aromatic, dried flower bud of an evergreen tree *Syzygium aromaticum*. It is native to Indonesia and is used as a culinary spice all over the world. It is harvested primarily in Indonesia, Madagascar, Zanzibar, Pakistan, Sri Lanka and India. The clove tree grows around 10-20 m high and has flowers in numerous groups of terminal clusters. During drying, the flower buds turn brown. The flower has a characteristic odour and a spicy, slightly bitter taste. The drug contains 16-25% volatile oil [of which 75-90% is eugenol (a phenylpropane derivative); the rest is eugenyl acetate, vanillin (a phenylpropane derivative) and β-caryophyllene (a sesquiterpene derivative)], 10-13% tannin, and triterpene acids such as oleanolic acid. The density of clove oil is more than 1, which means that it is heavier than water.

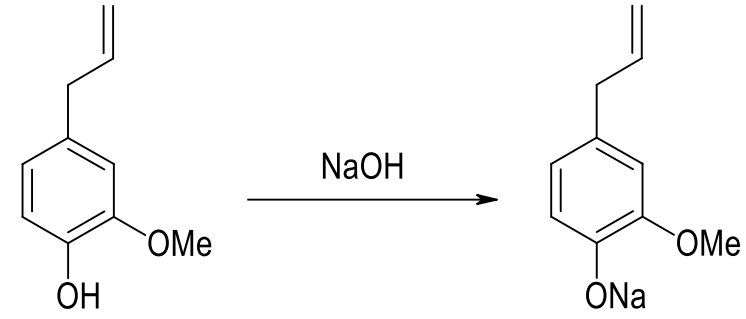
Uses: The drug and the oil are used as flavouring agents or spices. Medically, they are useful in dyspepsia, and exhibit local antiseptic and antibacterial effects (mostly used as a mouthwash or mouth gel to treat infections of the oral mucosa).



eugenol

Caryophylli floris aetheroleum: determination of the eugenol content in a Cassia flask

See the method in Practical 2. The mechanism of the reaction is the following.



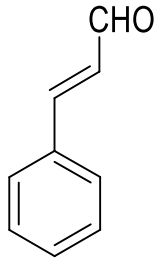
eugenol — Na eugenolate (water-soluble)

Cinnamomi cortex
Ceylon cinnamon bark
Cinnamomum zeylanicum J. S. Presl.
Lauraceae

Cinnamomi cassiae cortex
Cassia cinnamon bark
Cinnamomum cassia Blume.
Lauraceae

Ceylon cinnamon is an evergreen tree native to Ceylon but is cultivated in several tropical countries. Cassia cinnamon is native to South China. The drug consists of the dried bark of the tree, freed from the outer cork and the underlying parenchyma. It contains 1-4% volatile oil, mostly consisting of cinnamic aldehyde (64-90%). Other constituents of the oil are benzaldehyde, eugenol and *trans*-cinnamic acid. The density of the oil is 1.05-1.06. The drug also contains a considerable amount of tannins.

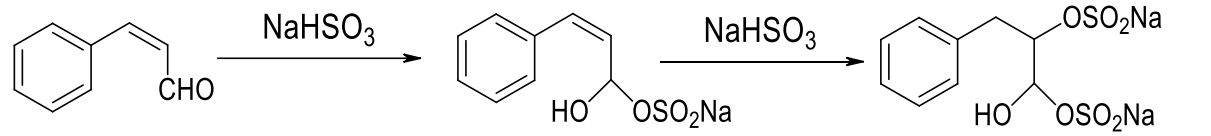
Uses: Cinnamon is used as a flavouring agent and mild astringent. The oil has carminative and germicidal properties.



cinnamic aldehyde

Cinnamomi cassiae aetheroleum: determination of the cinnamic aldehyde content in a Cassia flask

See the method in Practical 2. The mechanism of the reaction is the following.



cinnamic aldehyde

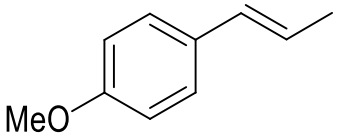
cinnamicalcohol-sulfone-Na-bisulfite
(water-soluble)

Anisi fructus

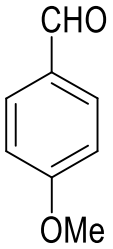
Anise seed (fruit)
Pimpinella anisum L.
Apiaceae

The drug consists of the dried ripe fruits of *Pimpinella anisum*, an annual plant indigenous to the Levant (Middle East), but it is also widely cultivated in Europe, Egypt and America. The drug consists of the greyish-brown pear-shaped cremocarps. They are 3-6 mm long and 2-3 mm wide. They have an aromatic odour and a sweet, aromatic taste. Anise fruit contains ca. 2-6% volatile oil. The chief component of the oil is *trans*-anethole (90%), but anisketone, anisaldehyde and methylcavicol can also be found. The drug itself and its volatile oil are sensitive to oxidation, where primarily anisaldehyde formation takes place as a main reaction.

Uses: Aniseed is a flavouring agent, commonly used as a spice. It has carminative, spasmolytic and expectorant activity (in animal experiments, *trans*-anethole exerted a secretolytic effect on the respiratory mucosa and bronchodilation). As a tea, it increases the amount of mother's milk during breast feeding.



trans-anethole

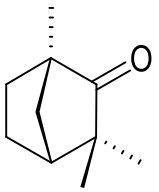


anisaldehyde

Foeniculi dulcis fructus
Sweet fennel seed (fruit)
Foeniculum vulgare ssp. *vulgare* var. *dulce* Miller.
Apiaceae

Fennel consists of the dried ripe fruits of *Foeniculum vulgare.* It is cultivated in many parts of Europe, and much is imported from India, China and Egypt. The drug consists of the whole cremocarps and partly of the isolated mericarps. It contains 2-7% volatile oil, the chief constituents being *trans*-anethole and a bicyclic monoterpene fenchone.

Uses: Because of the high *trans*-anethole content, the uses are the same as in the case of anise seed.



(+)-fenchone

TLC analysis of phenylpropane derivatives
With this TLC analysis, the anisaldehyde contents of anise seed and sweet fennel seed can be compared. The apolar components can be extracted with chloroform. The mechanism of detection is the same as in the cases of spearmint leaf and caraway seed. DNPH (2,4-dinitrophenylhydrazine) reagent is used for the detection of aldehydes and ketones, which appear as orange-red spots on the TLC plate.

Drugs with miscellaneous terpene content

As the terpenes become increasingly oxidized, they lose their characteristic odour, volatile property but become more and more bitter. If these highly oxidized terpenoids do not have significant bioactivities other than those coming from their taste, they are classified as bitters. A bitter taste exerts a reflectory increase in digestion, which makes bitter compounds effective appetizers.

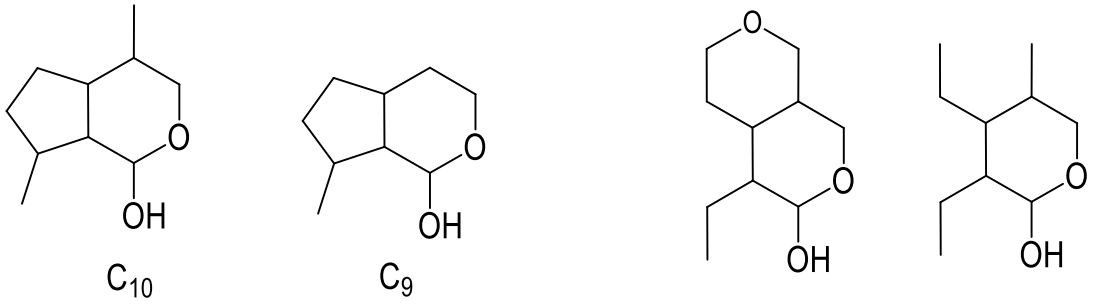
Bitters (bitter principles) are a heterogeneous group of naturally occurring compounds, marked by their strong bitter taste and therapeutic importance rather than a chemical classification.
Bitters have several groups from a chemical aspect:

- monoterpenes (iridoid and secoiridoid glycosides)
- sesqui- and diterpenes (highly oxidized components bearing a lactone ring in most cases)
- triterpenes
- flavonone glycosides, etc.

Chemical classification of bitters of monoterpenoid origin:

- **Iridoids:** monoterpenes built up from 2 dimethylallyl pyrophosphate molecules forming a cyclopentane and an oxygen heteroatom-containing six-membered (pyran) ring. The number of carbon atoms can be 10 or 9.
- **Seco-iridoids** are iridoids built up from 2 pyran rings, or in which one of the pyran rings is open, and in a few cases the oxygen heteroatom can be replaced by a nitrogen.

General structures of iridoids:



C_{10} C_9

iridoids seco-iridoids

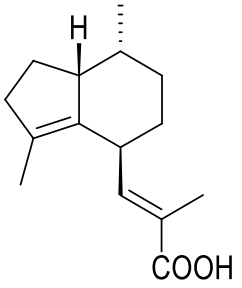
Irioid and seco-iridoid derivatives mostly occur as glycosides in plants; in 90% of the cases, one glucose molecule is bound to them. Iridoids are not stable molecules; they can easily be broken down by enzymatic cleavage. Because of their bitter taste, iridoids are good appetizers. Some iridoids possess other pharmacological effects, such as anti-inflammatory, antibacterial and antifungal properties.

Bitterness value: This is the reciprocal of the highest dilution of a chemical compound, solution or herbal extract (prepared from 1 g of dried plant material) which still has a bitter taste. In order to overcome subjective differences in personal sensitivity to a bitter taste, this value is determined by comparing the threshold bitter concentration with that of a dilute solution of quinine hydrochloride.

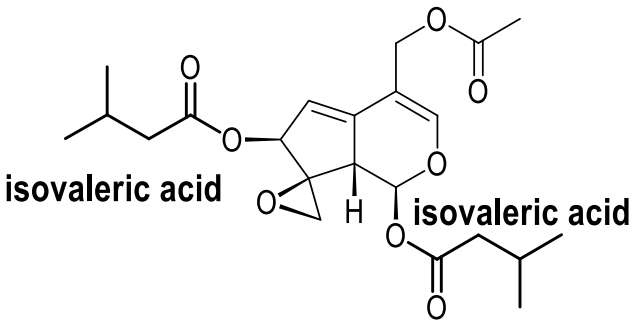
Valerianae radix
Valerian root
Valeriana officinalis L.
Valerianaceae

Valerian consists of the rhizome and roots of *Valeriana officinalis* collected in the autumn and dried at room temperature. The plant is perennial, about 1-2 m in height and is widely cultivated in Europe, Japan and the USA. The drug contains 0.5-2% volatile oil (mono- and sesquiterpenes, e.g. valerenic acid) and valepotriates (epoxy-iridoid esters), e.g. valtrate, izovaltrate, didrovaltrate and acevaltrate. These compounds are unstable: during drying and heating, the esters break down, forming baldrinal, homobaldrinal and isovaleric acid, which is known to be responsible for the unpleasant odour of the drug. Valerian also contains alkaloids and flavonoids.

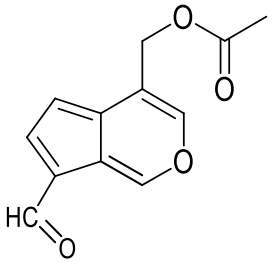
Uses: Valerian is used as a mild sedative and anxiolytic in cases of irritability, restlessness and sleeplessness. Although the mechanism of action and the group of the active agents have still not been fully clarified, it seems that baldrinal and homobaldrinal (by-products of valepotriates) can be regarded as active ingredients of the drug. It is advantage that no addiction to the valerian root has been reported among users during the centuries, and the drug may therefore be regarded as a safe sedative and anxiolytic.



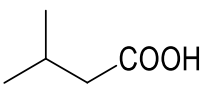
valerenic acid



valtrate



baldrinal



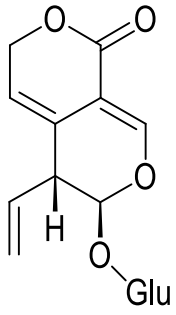
isovaleric acid

Test for valepotriates
See method in Practical 3. As a result, a greyish-blue colour can be observed.

Gentianae radix
Yellow gentian root
Gentiana lutea L.
Gentianaceae

Gentian consists of the dried fermented rhizomes and root of *Gentiana lutea,* a perennial herb about 1 m tall, found mostly in the mountains of central and southern Europe. The drug contains 2-4% seco-iridoid glycosides, such as gentiopicrin and amarogentin.

Uses: Used as an amarum and appetizer.

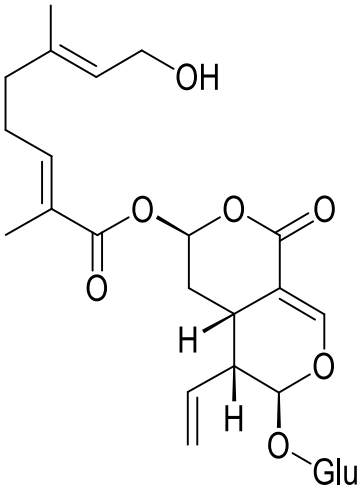


Glu = glucose

gentiopicrin

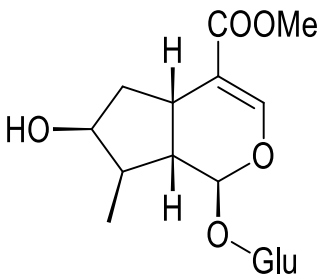
Menyanthidis trifoliatae folium
Bogbean leaf
Menyanthes trifoliate L.
Menyanthaceae

The drug consists of the dried leaves of *Menyanthes trifoliata.* This perennial plant is native to Europe, Asia and North America, growing mostly in swamps, wetlands and along slow rivers. It contains monoterpene bitters, such as the seco-iridoid foliamentin and the iridoid loganin.



foliamentin

Glu = glucose



loganin

Uses: Used as an amarum and appetizer.

Test questions 3

Essay questions:

- What are phenylpropane derivatives, how do they build up, where can we find them, and what are their common properties? Classify the phenylpropanes.
- TLC analysis of phenylpropane derivatives: what did we use for extraction, how did we visualize the TLC plate and what could we conclude?
- Describe how we performed the detection of valepotriates. What is a positive result of the test?
- Define bitters and give their characteristics and chemical classification.
- Define bitterness value.

Mechanisms and structures:

- Give examples of phenylpropane derivatives (eugenol, cinnamic aldehyde, *trans*-anethole and anisaldehyde).
- Explain the determination of the eugenol content of clove flower volatile oil. Give the mechanism.
- Explain the determination of the cinnamic aldehyde content of cinnamon bark volatile oil. Give the mechanism.
- Give the most important constituents of valerian root (valerenic acid, valtrate, baldrinal and isovaleric acid).
- Give examples of bitters (iridoids: loganin; and seco-iridoids: gentiopicrin and foliamentin).

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Clove flower, Ceylon cinnamon bark, cassia cinnamon bark, anise seed, sweet fennel seed, valerian root, gentian root and bogbean leaf

Practical 4

Drugs with triterpene saponin content

1. Liebermann-Burchard test (Primulae radix and Saponariae albae radix)
2. TLC analysis of Liquiritiae radix
3. TLC analysis of Hederae folium
4. Determination of unknown drug powders

Primulae radix
Cowslip root
Primula veris L.
Primulaceae

Liquiritiae radix
Liquorice root
Glycyrrhiza glabra L.
Fabaceae

Saponariae albae radix
Baby's-breath root
Gypsophila paniculata L.
Caryophyllaceae

Hederae folium
English ivy leaf
Hedera helix L.
Araliaceae

1. Liebermann-Burchard test (Primulae radix and Saponariae albae radix)

Extract 0.5 g of drug powder with 5 ml of chloroform and filter the extract into a porcelain dish. Evaporate the liquid to dryness on a water bath and dissolve the dry residue in 2 ml of glacial acetic acid. Pour 5 ml of cc. H_2SO_4 into a test tube and layer the extract gently on top of it.

Write down your observation.

2. TLC analysis of Liquiritiae radix

Extraction: Extract 2 g of powdered liquorice root with 20 ml of methanol by shaking for 10 min. Filter the extract and divide it into two parts. For the TLC, pour one part (2 ml) into a porcelain dish and evaporate it to dryness. Dissolve the dry residue in 1 ml of methanol for TLC. Put the remaining part of the extract into an Erlenmeyer flask, add 10 ml of R-HCl and hydrolyse it on the water bath for 1 h (to avoid evaporation of the solvent, place a glass funnel into the flask). Cool it down, transfer it into a separating funnel and extract it with 2 x 10 ml of ethyl acetate **very gently**! (Emulsion may easily occur, which disturbs the separation.) Combine the organic phases and extract them with 2 x 10 ml of water in order to eliminate the remaining acid. Pour the organic phase into an Erlenmeyer flask and dry it with anhydrous Na_2SO_4. Pour the remaining liquid into a porcelain dish and evaporate it to dryness. Dissolve the dry residue in 2 ml of methanol for TLC.

TLC analysis
Sorbent: Silica gel

Spots:

Liquiritiae radix methanolic extract (before hydrolysis)	40 µl
0.1% methanolic solution of glycyrrhetic acid	40 µl
Liquiritiae radix methanolic extract (after hydrolysis)	20 µl

Solvent system:
chloroform – methanol (95:5)

Detection:
254 nm light.

3. TLC analysis of Hederae folium

Extraction: Extract 0.5 g of drug dried at 50 ºC with 5 ml of 70% methanol while heating it for 10 min on a water bath.
Extract 1.5 g of drug (fresh) with 15 ml of 70% methanol while heating it for 10 min on a water bath.
Filter both extracts through paper and use them for TLC.

TLC analysis
Sorbent: silica gel

Spots:

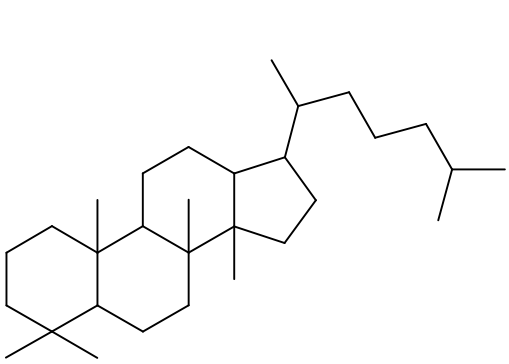
0.1% methanolic solution of α-hederin	20 µl
Hederae folium methanolic extract (fresh drug)	10 µl
Hederae folium methanolic extract (dried at 50 ºC)	10 µl
0.1% methanolic solution of hederacoside C	20 µl

Solvent system:
chloroform – methanol – water (64:30:5)

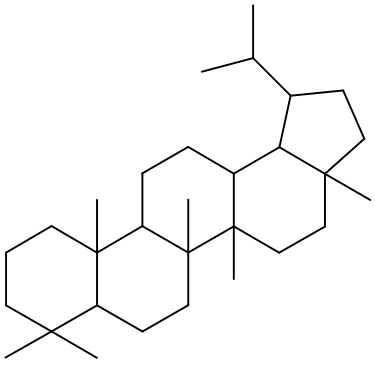
Detection:
Spray the TLC plate with R-vanillin — sulfuric acid reagent and keep it in the heating chamber for 5 min at 105 ºC.

Theory 4

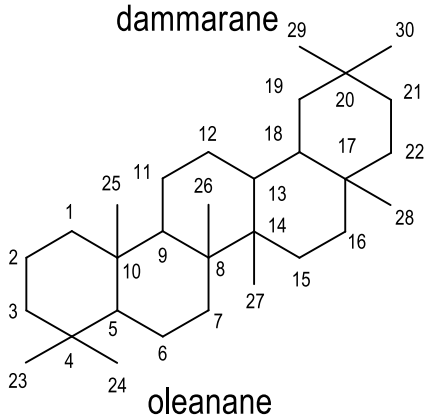
Triterpenes (C_{30}) are secondary plant metabolites built up from 6 isoprene units, by the cyclization of squalene. Triterpenes are widespread in nature. They can be found particularly in resins and may occur as either esters or glycosides. Triterpenes may be tetra- or pentacyclic, and they are highly variable, ranging from lipophilic compounds to highly oxidized compounds and hydrophilic triterpene glycosides.
Steroids with less than 30 carbon atoms (cholesterol-C_{27}, phytosterols-$C_{28\text{-}29}$, steroid saponins-C_{27} and cardiac glycosides-$C_{23\text{-}24}$) are of the same biosynthetic origin, and they are also sometimes classified as triterpenes.



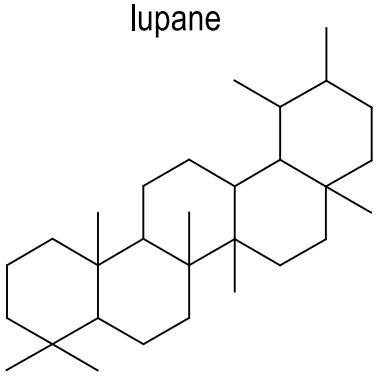
dammarane



lupane



oleanane



ursane

Saponins are compounds derived from terpenes They occur (mostly) in glycosidic form. They are water-soluble, having <u>detergent</u> (due to their ability to decrease surface tension, a **permanent foam** is produced when they are shaken with water) and <u>haemolytic</u> properties (as they can haemolyse red blood cells, they are highly toxic to humans if they directly enter the bloodstream, or to fish if they pass into the water).
<u>Saponin glycosides</u> are built up from a triterpene molecule (aglycone) and sugar components. Three different types can be differentiated, depending on the skeleton:

- triterpene saponins
- steroid saponins
- steroid-alkaloid saponins

The aglycone of triterpene saponins can be tetracyclic (with the dammarane skeleton) or pentacyclic (with the lupane, oleanane and ursane skeletons). They can be acidic, or basic. The connection of A/B, B/C and C/D rings is always *trans*, while the connection of the D/E ring can be either *cis* (ursane and oleanane skeletons) or *trans* (lupane skeleton). 1-12 sugar molecules can bind to the aglycone, mostly by linking to the (3-) hydroxy group and/or to the (28-)carboxyl group. If the formation of a glycosidic linkage occurs at only one functional group of the aglycone, the result is a monodesmoside. The molecule can also include an additional sugar chain, this structure being referred to as a bisdesmoside. The most abundant sugar components are glucose, arabinose, rhamnose, xylose and galacturonic and glucuronic acids. If the glycosidic moiety consists of more than two sugars, they always form a ramifying chain.

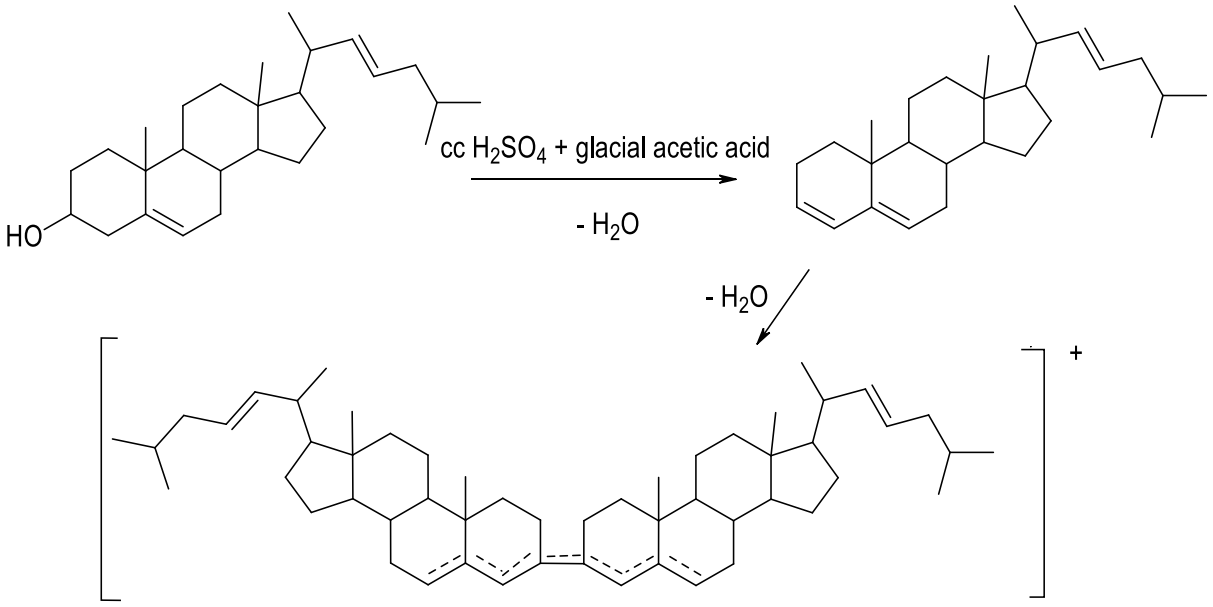
Properties:

- white powders
- high solubility in water and low solubility in apolar solvents
- the aglycones alone have high solubility in apolar solvents
- in aqueous solution, they form a precipitate with cholesterol

General uses: They are used as expectorants, but some of them also have diuretic properties. They can be effective against various bacteria, fungi and virus infections.

Detection of saponins: Liebermann-Burchard test

Unsaturated triterpenes with hydroxy groups and steroids give positive reactions in the Liebermann-Burchard test. Following the loss of two molecules of water, due to the cc. H_2SO_4, a dimer of two triterpene molecules is formed with a conjugated polyene system, which gives a brownish colour reaction.

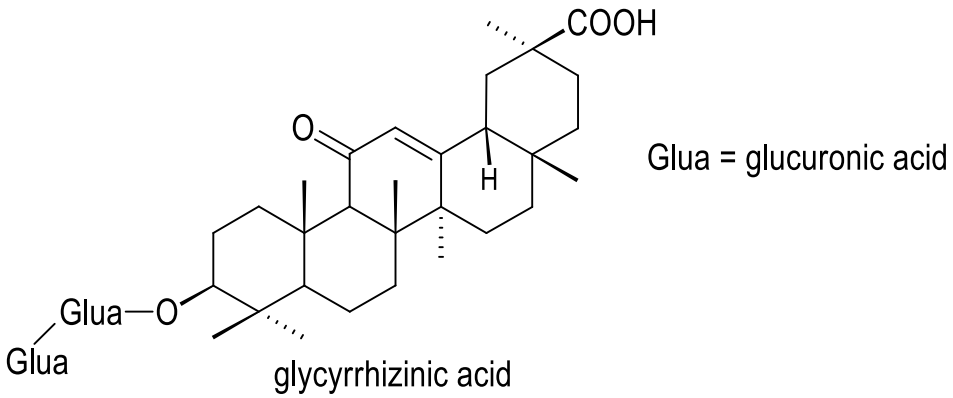


tetradiene cation

Liquiritiae radix
Liquorice root
Glycyrrhiza glabra L.
Fabaceae

The drug consists of the dried root of *Glycyrrhiza glabra.* It is around 1-2 m long and 1-2.5 cm wide. The outer surface is dark-brown and the inner surface is yellow. It contains triterpene saponins such as glycyrrhizin (3-9%), which is the Ca^{2+} and K^{+} salt of glycyrrhizinic acid, the component responsible for the sweet taste of liquorice (50x sweeter than saccharose). Its aglycone is glycyrrhetic acid. It also contains flavonoids, which confer the yellow colour to the powdered drug, and also coumarins.

Uses: It has long been used in pharmacy as a flavouring agent of various formulae, a demulcent and a mild expectorant. The drug and also the synthetic derivatives of glycyrrhizinic acid are widely used in the treatment of peptic ulcer. Glycyrrhizin and its aglycone have an anti-inflammatory effect due to the inhibition of an enzyme responsible for the breakdown of the anti-inflammatory, gastric mucosa-protecting prostaglandins. These prostaglandins (PGE_2 and PGF_2) can increase stomach mucosal cell proliferation and the secretion of protective mucus, resulting in quicker healing of the damaged stomach wall.



glycyrrhizinic acid

TLC analysis of Liquiritiae radix

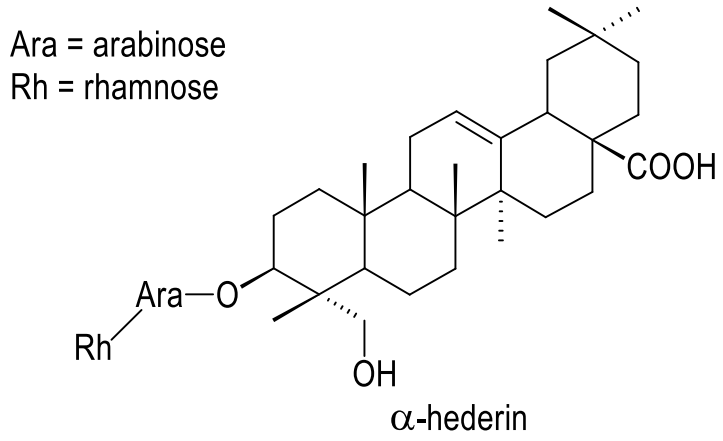
The saponine glycoside and aglycone contents of liquorice root before and after hydrolysis can be compared through this TLC determination. The visualization is carried out with 254 nm light; α,β-enones absorb UV light at this wavelength and appear as dark spots on the TLC. In the methanolic extract of liquorice root only, the saponin glycosides (mostly glycyrrhizinic acid) can be observed, but after hydrolysis its aglycone, glycyrrhetic acid, appears, which can be identified by means of the reference 0.1% glycyrrhetic acid solution.

Hederae folium

English ivy leaf
Hedera helix L.
Araliaceae

The drug consists of the dried leaf of *Hedera helix*, an evergreen climbing plant native to Europe and Asia. This plant has long been a part of the European traditional medicine and also features in Egyptian and Roman mythology. The leaves are alternate, 50-100 mm long, with a 15-20 mm petiole; they are of two types, with palmately five-lobed juvenile leaves on creeping and climbing stems, and unlobed cordate adult leaves on fertile flowering stems exposed to full sunshine, usually high in the crowns of trees or at the top of rock faces. It contains 5% saponins, mostly the bisdesmoside hederacoside C and B, which break down during processing to give their corresponding monodesmosides α- and β-hederin.

Uses: The drug has a secretolytic effect as in the case of other saponin components, but with a considerably less profound stomach-irritating side-effect. The unique characteristic of the drug is its bronchodilating and bronchus spasm-alleviating action which is mediated via its β_2-agonistic and spasmolytic properties. These effects are attributed to either hederacoside C or its monodesmoside α-hederin, but hederacoside C possesses less inconvenient side-effects.



α-hederin

TLC analysis of Hederae folium

In this TLC, the methanolic extract of English ivy leaf dried at 50 °C is compared with that of freshly collected material. The visualizing is carried out by spraying with vanillin — sulfuric acid, as discussed earlier (mint leaf and lavender flower). In the fresh drug, hederacoside C can be detected in high amounts, while α-hederin is present in only low amounts. If the leaves are dried at 50 ºC, hederacoside C disappears and only α-hederin can be detected. Ph. Hg. VIII specifies a 3% hederacoside C content for this drug. In order to meet this criterion, therefore the drug can not be dried at higher temperature.

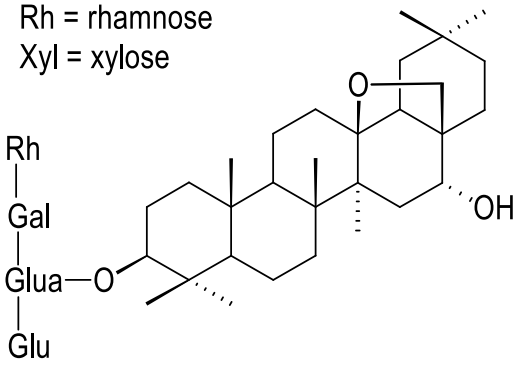
Primulae radix

Cowslip root
Primula veris L.
Primulaceae

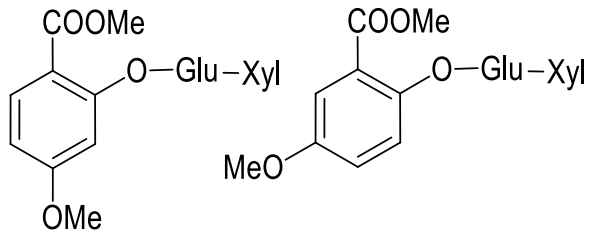
The drug consists of the rhizome and roots of the plant *Primula veris*. The rhizome is about 2-5 cm long and 5 mm wide and has a dark-brown colour. The roots are greyish-yellow, long and relatively thin. The powder of the drug is rather grey. It contains 5-10% triterpene saponins. The main component is primulic acid A, the glycosidic form of protoprimulagenin A (an aglycone). It contains phenolic components such as primverin and primulaverin, which give the characteristic smell to the drug.

Uses: It is used as an expectorant in cases of coughing, bronchitis and asthma, as a tincture or tea.

Glu = glucose
Gal = galactose
Glua = glucuronic acid
Rh = rhamnose
Xyl = xylose



primulic acid A



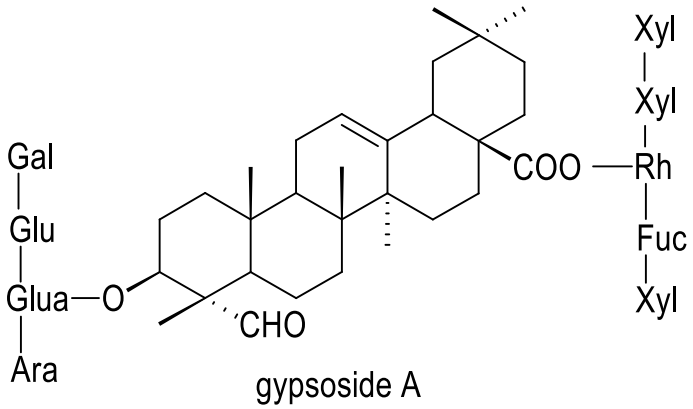
primverin primulaverin

Saponariae albae radix
Baby's-breath root
Gypsophila paniculata L.
Caryophyllaceae

The drug consists of the dried root of *Gypsophila paniculata.* It is 2-6 cm wide, around 1.5-2 m long, and light-brown in colour. It contains 6-10% triterpene saponins. The main constituent is gypsoside A, whose aglycone is gypsogenin.

Uses: It can be used as an expectorant in the form of tea or tincture.

Glu = glucose
Gal = galactose
Glua = glucuronic acid
Rh = rhamnose
Xyl = xylose
Ara = arabinose
Fuc = fucose



gypsoside A

Test questions 4

Essay questions:

- What are triterpenes, which group of compounds do they belong to, and how do they build up?
- What are the common properties and uses of triterpenes?
- How can we classify triterpenes? Detail the basic chemical characteristics of their structure.
- How can we detect triterpenes? Explain the Liebermann-Burchard reaction from the extraction of the drug to the observed result.
- In the TLC analysis of liquorice root, what did we use for extraction, how did we visualize the TLC plate and what could we conclude?
- In the TLC analysis of English ivy leaf, what did we use for extraction, how did we visualize the TLC plate and what could we conclude?

Mechanisms and structures:

- Draw the basic triterpene skeletons (dammarane, lupane, oleanane and ursane).
- Give examples of triterpene saponins (glycyrrhizinic acid, α-hederin, primulic acid A and gypsoside A).
- Draw the structures of the non-triterpenoid compounds that occurred in the practical (primverin and primulaverin).

Classification (Latin name of the drug, species and family) and uses of the following drugs:

- Cowslip root, liquorice root, baby's-breath root and English ivy leaf

Practical 5

Drugs with digitalis glycoside content

1. Keller-Kiliani test, Baljet test, Kedde test (Digitalis purpureae folium, Digitalis lanatae folium, Oleandri folium, Strophanthi semen)
2. TLC analysis of *Digitalis purpureae*
3. Determination of unknown drug powders

Digitalis purpureae folium
Purple foxglove leaf
Digitalis purpurea L.
Scrophulariaceae

Digitalis lanatae folium
Woolly foxglove leaf
Digitalis lanata L.
Scrophulariaceae

Oleandri folium
Oleander leaf
Nerum oleander L.
Apocynaceae

Strophanthi semen
Strophantus seed
Strophanthus kombe Oliver.
Apocynaceae

1. Keller-Kiliani test, Baljet test, and Kedde test (Digitalis purpureae folium, Digitalis lanatae folium, Oleandri folium, and Strophanthi semen)

Keller-Kiliani test:
Digitalis sp. and Oleandri folium: Add 10 ml of water to 1 g of drug, and then add 10 ml of boiling water to it. Leave it to stand for 20 min. Filter it into a separating funnel and extract it with 2 x 10 ml of chloroform. Combine the chloroform phases in a porcelain dish and evaporate the liquid to dryness on a water bath. Dissolve the dry residue in 3 ml of glacial acetic acid and add 1 drop of $FeCl_3$. Layer the solution gently on top of 3 ml of cc. H_2SO_4 in a test tube.
Strophantus kombe: Put 10 seeds into a mortar and crush them with a pestle as much as possible. Put the crushed material in an Erlenmeyer flask and add 5 ml of petroleum ether. Pour the petroleum ether away (this step is necessary to defat the seeds). Repeat the defatting with another 5 ml of petroleum ether, and pour the solvent away again. Extract the dried drug by shaking it with 5 ml of methanol for 2 min, then filter the extract into a porcelain dish and evaporate it to dryness on a water bath. Dissolve the dry residue in 2 ml of glacial acetic acid and add 1 drop of $FeCl_3$. Layer the solution gently on top of 3 ml of cc. H_2SO_4 in a test tube.

Baljet test:
Add 10 ml of ethyl acetate saturated with water to 0.5 g of drug and shake the mixture for 15 min. Filter the extract through cotton-wool into a porcelain dish and evaporate it to

dryness on a water bath. Dissolve the dry residue in 2 ml of water (Solution A). After this, use three test tubes for the reaction, as follows.

Test tube 1: 3 ml of Baljet reagent + 0.5 ml of 10% NaOH solution
Test tube 2: 3 ml of Baljet reagent + 0.5 ml of 10% NaOH solution + 0.5 ml of Solution A
Test tube 3: 3 ml of Baljet reagent + 0.5 ml of 10% NaOH solution + residual Solution A

Kedde test:
Add 10 ml of ethyl acetate saturated with water to 0.5 g of drug and shake the mixture for 15 min. Filter the extract through cotton-wool into a porcelain dish and evaporate it to dryness on a water bath. Dissolve the dry residue in 2 ml of water, then add 2 ml of 3,5-dinitrobenzoic acid solution (dissolved in 1% ethanol) and 2 ml of KOH solution.

Write down your observations.

2. TLC analysis of Digitalis purpureae folium

Extraction: Extract 1 g of drug with 20 ml of a mixture of 50% aqueous methanol and 10 ml of 10% basic Pb acetate solution on a water bath for 10 min. Cool the mixture down and extract it in a separating funnel with two 10 ml portions of chloroform. Combine the chloroform phases in a porcelain dish and evaporate the liquid to dryness. Dissolve the dry residue in 1 ml of a chloroform – methanol (1:1) mixture.

TLC determination
Sorbent: Silica gel

Spots:

0.1% methanolic digitoxin solution	20µl
0.1% methanolic digoxin solution	20µl
Digitalis purpureae folium extract	40µl
0.1% methanolic lanatoside B solution	20µl

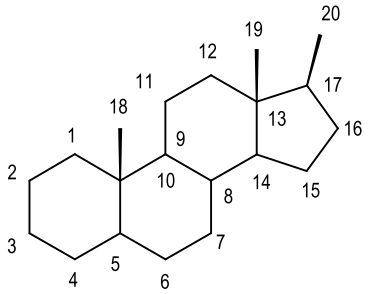
Solvent system:
ethyl acetate – methanol – cc. NH_3 (85:10:5)

Detection
Spray the TLC plate with 1% 3,5-dinitrobenzoic acid solution and, after drying, with 5% KOH solution.

Theory 5

Digitalis glycosides

Digitalis glycosides are steroid glycosides, where the aglycone is a 23 C atom-containing (cardenolide), or a 24 C atom-containing (bufadienolide) steroid molecule.



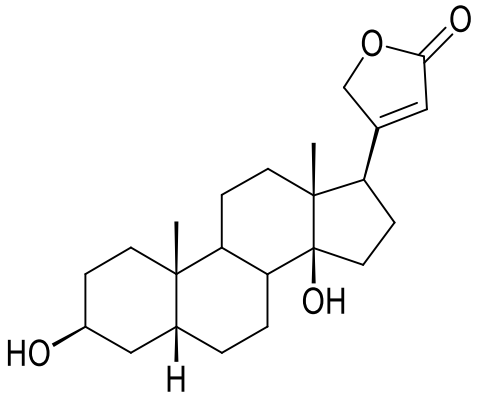
sterane skeleton

The most important functional groups of the steroid skeleton:
C-17: a lactone group (β position)
C-13: a methyl group (β position)
C-10: a methyl, methoxy or formyl group (β position)
C-3 and **C-14**: hydroxy groups (β position)

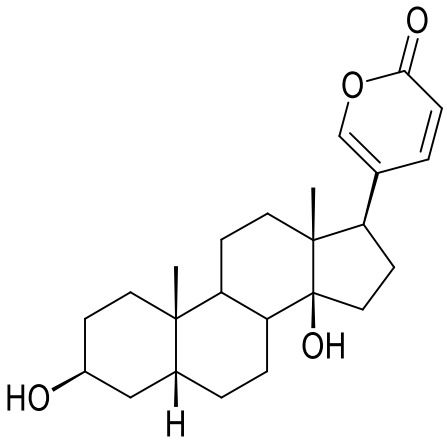
Depending on the lactone ring at C-17, digitalis glycosides can be classified as:

- Cardenolides: a 5-membered lactone ring with one double bond
- Bufadienolides: a 6-membered lactone ring with two double bonds

The connection of the B/C rings is always *trans*, while the connection of the A/B ring is mostly *cis*.



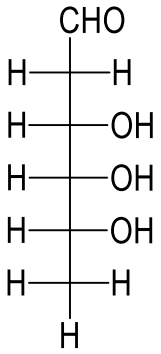
cardenolide



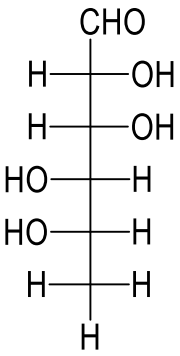
bufadienolide

Characteristics of the sugar components of digitalis glycosides:

- they are <u>always</u> connected to C-3
- the number of sugar components is between 1 and 5
- the sugar chain is <u>never ramified</u>
- the components of the sugar chain are glucose and deoxy sugars (rhamnose, fucose, digitalose, digitoxose, or cymarose)
- if both glucose and deoxy sugars are present in the chain, then the glucose is always at the end of the chain



digitoxose



rhamnose

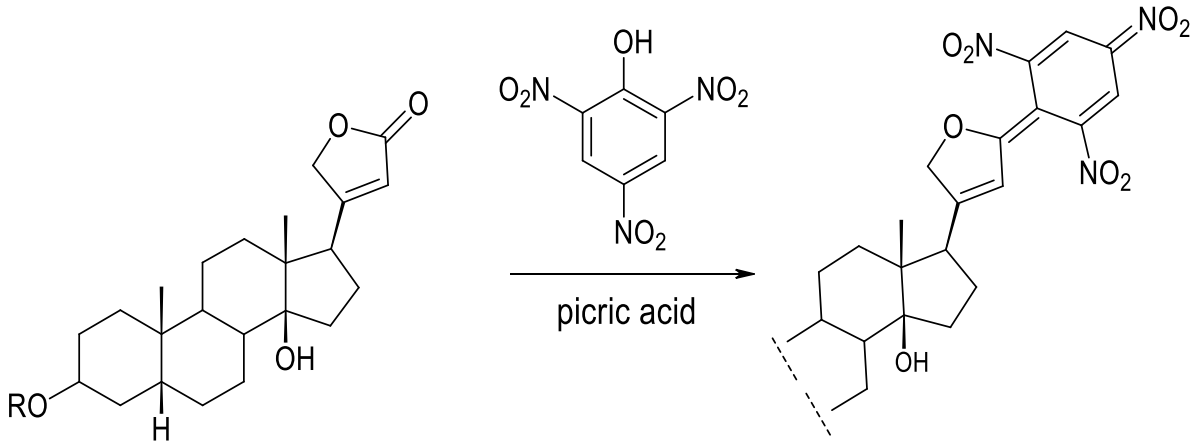
Properties and detection

Digitalis glycosides are white, crystalline powders, with a bitter taste. The aglycones can be dissolved in ethyl acetate or in a mixture of chloroform – ethanol, while the glycosides are soluble in methanol, ethanol or their aqueous mixtures.

<u>Detection of cardenolides</u>

<u>Keller-Kiliani test</u>: *See method in Practical 5.* 2-Deoxy sugar components in the glycosides can react with different colour reactions in the presence of Fe(III) chloride, glacial acetic acid and cc. sulfuric acid.

Baljet test: See method in Practical 5. The Baljet reagent is picric acid, and as a result a yellow-orange-red colour appears. The lactone ring of the aglycone forms a complex with organic nitro compounds in a basic environment, where the intensity and the shade of the resulting colour depend on the concentration of the cardenolides (Solution B should be darker than Solution A).



cardenolide

Kedde test: See method in Practical 5. With the Kedde reagent (3,5-dinitrobenzoic acid), pink-purple colour appears.

Detection of bufadienolides: Bufadienolides cannot be detected with such a specific test as in the case of cardenolides, because their lactone ring (6-membered) does not react with nitro compounds and their sugar chain does not contain deoxy sugars. They can be detected with the Liebermann-Burchard reaction (like the triterpene saponins). The problem is that other sterols and triterpenes might also react (if they are present in the solution to be tested) giving a false-positive result.

Pharmacological effect

Digitalis glycosides exert their therapeutic effect on the heart muscle. If the performance of the heart is getting weaker, the sensitivity of the heart muscle to digitalis glycosides is increasing, resulting in the following effects.

- **Positive inotropic effect:** Application of these compounds in the case of a heart insufficiency will result in a positive inotropic effect, meaning that the strength of the heart contraction is increased, the volume of the blood being pumped into the circulation therefore being higher, while the volume of the blood remaining in the left ventricle is decreased.

  Mechanism of effect: Digitalis glycosides are able to block Na^+/K^+ ATPase, which results in a high Na^+ accumulation in the heart muscle cells after the systole. This will inhibit the

concentration-dependent Ca^{2+}/Na^{+} antiporter which would transport Ca^{2+} (*accumulated in the cells during the systole*) out of the cells in exchange for Na^{+}. Due to the increased intracellular Na^{+} concentration (*because of the Na^{+}/K^{+} ATPase blockade*), the Ca^{2+}/Na^{+} pump cannot work properly, and the increased Ca^{2+} concentration therefore lasts longer, resulting in a longer and stronger heart muscle contraction.

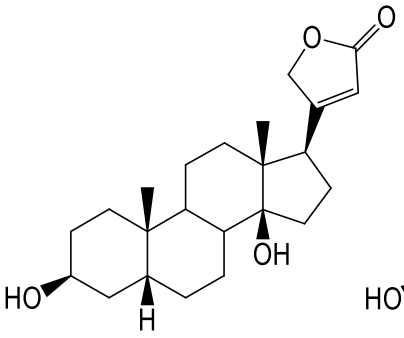
- **Negative chronotropic effect:** The digitaloids have a vagal effect (*they increase the parasympathetic nervous system activity, while affecting the vagal nerve*), which results in a decreased heart rhythm.

Thanks to their effect, digitalis glycosides can be used in cases of heart insufficiency caused by arteriosclerosis, hypertension, cardial asthma or valve problems. The glycosides are more effective than the aglycones, and bufadienolides have stronger effects than cardenolides. The strofantidine digitaloids have a rapid, but short effect, they do not accumulate and they have a weak enteral absorption, they can be applied only intravenously. As such, they are not used chronically, but they can serve as life-saving drugs in life-threatening situations. The digitalis glycosides from Digitalis sp. have a slowly developing effect. They accumulate in the body and thus, after a saturating dose, they can be used chronically in a smaller maintenance dose. Due to the narrow therapeutic range and many potential side-effects of these compounds, digitalis glycoside-containing plants are generally not safe as phytotherapeutics; the purified compounds are used instead, strictly as prescription medicines.

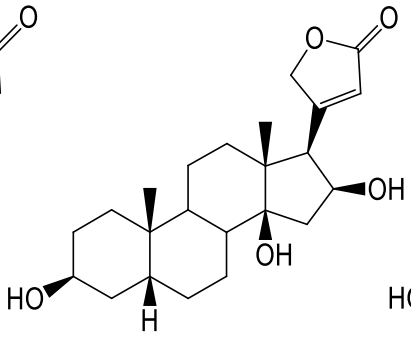
Digitalis purpureae folium
Purple foxglove leaf
Digitalis purpurea L.
Scrophulariaceae

The drug consists of the dried leaf of *Digitalis purpurea*. The leaves are 10 to 30 cm long and 4 to 10 cm wide and dark-green in colour. Both of the surfaces are hairy. These hairs can be pubescent or glandular. The pubescent hairs typically consist of 3 to 5 cells, among which one or more are frequently collapsed. The drug is collected during the flowering season and is dried at 40 °C at most because digitaloids are heat-sensitive. It contains 0.2-0.4% cardenolide glycosides, steroid saponins, pregnane derivatives and flavonoids.
The glycosides of foxglove leaf can be classified according to their aglycones. The sugar moieties are always connected to the –OH group at C-3. The primary glycosides are those with 3 digitoxose + glucose sugar components, but in the medicine the secondary glycosides, such as digitoxin, gitoxin, gilatoxin, are used which contain only 3 digitoxose units in the sugar chain.

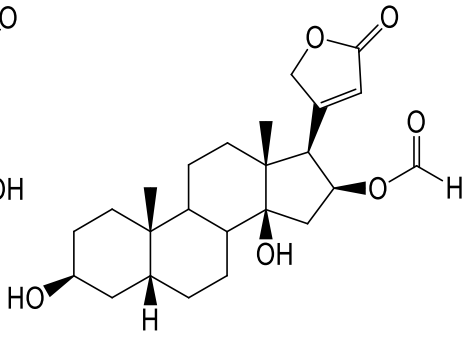
Uses: Purified digitoxin is used in tablet form in cases of heart insufficiency.



digitoxigenin



gitoxigenin



gitaloxigenin

Glycoside	Aglycone	Sugar moiety
Purpureaglycoside A	Digitoxigenin	3 x digitoxose + glucose
Digitoxin		3 x digitoxose
Purpureaglycoside B	Gitoxigenin	3 x digitoxose + glucose
Gitoxin		3 x digitoxose
Purpureaglycoside C	Gitaloxigenin	3 x digitoxose + glucose
Gitaloxin		3 x digitoxose

TLC analysis of *Digitalis purpureae*

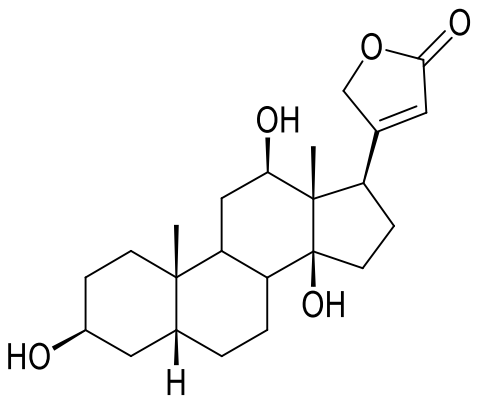
With this TLC, the digitalis glycoside content of the purple foxglove leaf can be tested, in comparison with the applied reference compounds. Among these, digitoxin and digoxin contain chains of 3 sugars, whereas lanatoside B contains 4 sugars, resulting in a higher polarity and a lower retention factor. The visualization is based on the Kedde reaction; compounds of interest appear as pink spots.

Digitalis lanatae folium

Woolly foxglove leaf
Digitalis lanata L.
Scrophulariaceae

The drug consists of the dried leaves of *Digitalis lanata*, which are up to 30 cm long and 4 cm wide. It contains 0.4-1% cardenolide glycosides, saponins, pregnane glycosides and flavonoids.
The digitalis glycosides of the woolly foxglove leaf can be classified according to the aglycone content; as a result of great variability, mainly in the sugar side-chain, more than 70 different cardenolides have been isolated from the plant so far.

Uses: The drug is utilized for the industrial scale production of the important cardenolides. Lanatoside C, digoxin and acetyldigoxin are used in medical practice.



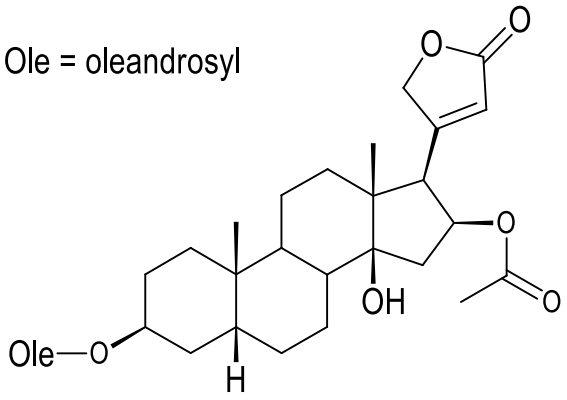
digoxigenin

Glycoside	Aglycone	Sugar moiety
Lanatoside A	Digitoxigenin	2 x digitoxose + acetyl-digitoxose + glucose
Acetildigitoxin		2 x digitoxose + acetyl-digitoxose
Digitoxin		3 x digitoxose
Lanatoside B	Gitoxigenin	2 x digitoxose + acetyl-digitoxose + glucose
Acetyldigitoxin		2 x digitoxose + acetyl-digitoxose
Gitoxin		3 x digitoxose
Lanatoside C	Digoxigenin	2 x digitoxose + acetyl-digitoxose + glucose
Acetildigoxin		2 x digitoxose + acetyl-digitoxose
Digoxin		3 x digitoxose

Oleandri folium
Oleander leaf
Nerum oleander L.
Apocynaceae

The drug consists of the dried leaf of *Nerum oleander*. The species is a shrub or a small tree with indeciduous and lanceolate leaves and pink flowers. It grows wild in the Mediterranean area and is widely cultivated in the north. The leaves contain 1.5% cardenolides, mostly oleandrin and also bufadienolides in trace amounts.

Uses: The drug has been used for the extraction of digitalis glycosides.

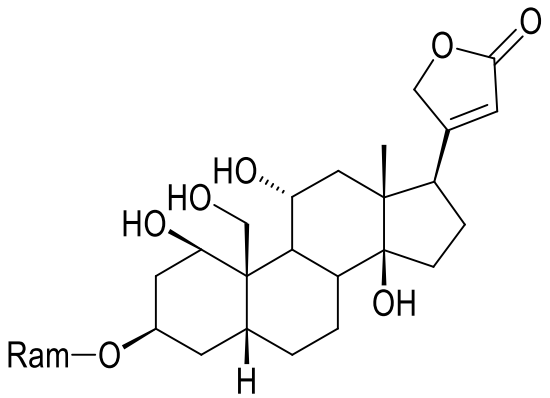


oleandrin

Strophanthi semen
Strophanthus seed
Strophanthus kombe Oliver
Apocynaceae

The drug consists of the dried ripe seeds of *Strophanthus kombe,* freed from the awns. The plant is native to Southern Africa. The drug is 10-18 mm long, 3-5 mm wide and 2-3 mm thick, with a brownish-greenish grey colour. It contains 8-10% cardenolide glycosides which are the mixture of strophantidine and strophantidol. The sugar components are cymarose and glucose. Other constituents are saponins and fixed oil.
Another species, *S. gratus*, contains 4-8% of G-strophanthin or, as it is mostly known, ouabain. An extract of these plant seeds is/was used as an arrow poison.

Uses: They are used in cases of acute heart insufficiency because they exert their effect more quickly than the glycosides of the Digitalis species. They are mostly used in life-saving interventions.



G-strophanthin

Ram = rhamnose

Test questions 5

Essay questions:

- What are digitalis glycosides, how do they build up and what are the principal chemical characteristics of their structures?
- What are the chemical characteristics of their sugar components?
- What are their common properties?
- Describe the ways to detect digitalis glycosides (cardenolides - Keller-Kiliani, Baljet, and Kedde tests; bufadienolides - Liebermann-Burchard test).
- Explain the pharmacological effects of digitalis glycosides.
- In the TLC analysis of purple foxglove leaf, what did we use for extraction, how did we visualize the TLC plate and what could we conclude?
- Pair the aglycones with their respective glycoside counterparts (e.g. lanatoside C – digoxigenin).

Mechanisms and structures:

- Draw the basic skeletons of digitalis glycosides (cardenolides and bufadienolides).
- Explain and give the mechanism of the Baljet test, from the extraction of the drug to the observed result.
- Give examples on cardenolide aglycones (digitoxigenin, gitoxigenin, gitaloxigenin, digoxigenin).
- Give examples of digitalis glycosides (oleandrin and G-strophanthin).

Classification (Latin name of the drug, species, and family) and uses of the following drugs:

- Purple foxglove leaf, woolly foxglove leaf, oleander leaf, and strophanthus seed

Practical 6

Drugs with triterpene saponin content II
1. TLC analysis of Calendulae flos
2. TLC analysis of Hippocasteni semen
3. TLC analysis of Urticae herba et radix
4. Determination of unknown drug powders

Calendulae flos	**Hippocastani semen**	**Urticae radix et herba**
Pot marigold flower	Horse chestnut seed	Nettle root and herb
Calendula officinalis L.	*Aesculus hippocastanum* L.	*Urtica dioica* L.
Asteraceae	Hippocastanaceae	Urticaceae

1. TLC analysis of Calendulae flos

Extraction: Extract 0.5 g of Calendulae flos with 10 ml of dichloromethane for 15 min at room temperature. Filter the extract into a porcelain dish and evaporate it to dryness on the water bath. Dissolve the residue in 1 ml of methanol.

TLC determination
Sorbent: Silica gel

Spots:

Calendulae flos methanolic extract	40 µl
Methanolic solution of faradiol	30 µl

Solvent system:
n-hexane – acetone (8:2)

Detection:
Spray the TLC plate with vanillin – sulphuric acid and heat it in the heating chamber for 5 min at 105 ºC.

2. TLC analysis of Hippocasteni semen

Extraction: Extract 1 g of Hippocastani semen with 7.5 ml of 70% aqueous methanol for 10 min on the water bath. Filter it on filter paper to a porcelain dish and evaporate it to dryness. Dissolve the dry residue in 5 ml of methanol.

TLC determination
Sorbent: Silica gel

Spots:

Hippocasteni semen aqueous methanolic extract	20µl
Methanolic aescin solution	10µl

Solvent system:
Buthanol – acetic acid – water (5:4:1)

Detection:
Spray the TLC plate with vanillin-sulphuric acid and heat it in the heating chamber for 5 minutes on 105ºC.

3. Quantitative determination of the β-sitosterine content of Urticae radix by TLC densitometry

Sample preparation: Measure 1.00 g of drug on an analytical balance into a beaker and extract it with 10 ml of methanol in an ultrasound bath for 10 min. Filter the extract through filter paper into a porcelain dish, wash the beaker and the filter paper with 1 ml of methanol, and evaporate the combined filtrates to dryness on the water bath. When the porcelain dish has cooled down, add 2.00 ml of methanol with a pipette to the dry residue to dissolve it, and apply 10 µl of the solution to the TLC plate with a Hagedorn pipette. Write down the amount of the measured drug (g).

Calibration: β-Sitosterine standard solution (STD; exact concentration written on the flask) is available for use. By using a micropipette, prepare a 5-fold (STD5x) and a 10-fold (STD10x) dilution from it (200 µl STD + 800 µl methanol, and 100 µl STD + 900 µl methanol, respectively) into the capped vials provided by the instructor. Apply the following volumes of these solutions and your sample of unknown β-sitosterine content (see above) with a Hagedorn pipette to the TLC plate.

- STD10x: 5 µl
- STD10x: 10 µl (2 x 5 µl)
- STD5x: 10 µl (2 x 5 µl)
- STD: 5 µl
- Nettle root extract: 10 µl (2 x 5 µl)

Solvent system:
Toluene – ethyl acetate (7:3)

Visualization of spots:
Dip the dried plate into a mixture of MeOH + 10 v/v% H_2SO_4 and dry it for 1 min at 110 °C.

Evaluation: Digital photographs of the prepared TLC plates must be evaluated by utilizing CpAtlas software under the instructor's guidance.

Data to register:

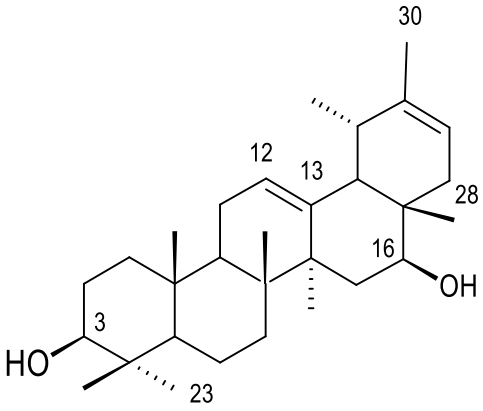
Sample	c (µg/µl)	$V_{applied}$ (µl)	$m_{\beta\text{-sitosterine}}$ (µg)	Peak area
STD10x				
STD10x				
STD5x				
STD				
Extract	-		?	

Theory 6

Calendulae flos
Pot marigold flower
Calendula officinalis L.
Asteraceae

The drug consists of the dried flower of *Calendula officinalis*. It is native to the Mediterranean region. Because of the characteristic yellow colour of the flowers, it has been used as a food-colouring agent, especially by poor people, as a substitute for the more expensive saffron. The drug contains triterpenoids such as faradiol, ursadiol, α- and β- amyrin, saponins, flavonoids, carotenoids, essential oil and coumarins.

Uses: The drug has anti-inflammatory, immunomodulatory and antibacterial properties. Because of the non-trivial acute toxicity of the extracts, marigold preparations must be reserved for topical use. Traditional and authorized indications are the following: inflammation of the skin and mucosal membranes, bruises, burns and scratches.

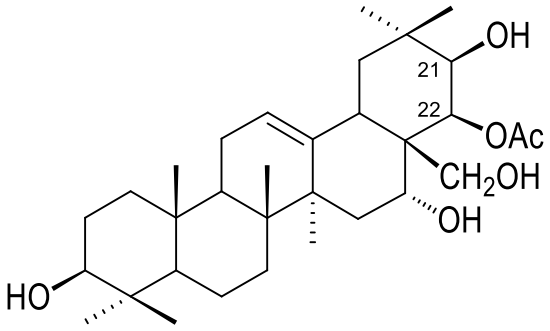


Faradiol

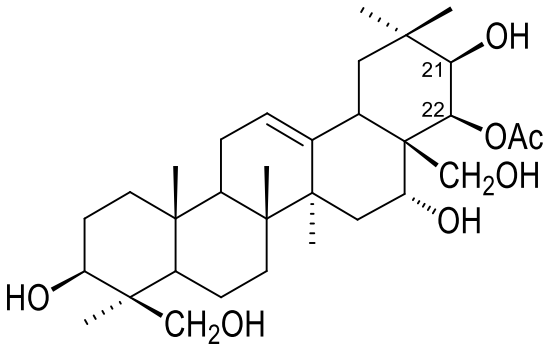
Hippocastani semen
Horse chestnut seed
Aesculus hippocastanum L.
Hippocastanaceae

The drug is the dried seed of the tree *Aesculus hippocastanum.* The plant is native to the Balkan regions. It contains 3-5% aescin (a mixture of saponin esters) where the aglycones can be protoaescigenin or barringtogenol C. Their common structural property is that the -OH groups at the C-21 and C-22 position are esterified by different organic acids. The drug also contains a considerable amount of flavonoid glycosides.

Uses: The triterpene saponins of the drug have beneficial effects in chronic venous insufficiency by increasing the venal tone and by decreasing the permeability and increasing the resistance of the capillaries. Consequential improvement of both the micro- and macro-circulation makes the drug and its preparations suitable for the treatment of varicose veins, haemorrhoids and crural oedema.



potoaescigenin



barringtogenol C

TLC analysis of Hippocastani semen

With this TLC examination, the aescin content of horse chestnut seed can be observed. Saponin esters are more polar components than their aglycone counterparts, and can therefore be extracted with methanol. The visualization technique with vanillin – sulfuric acid was described earlier.

Urticae radix et herba

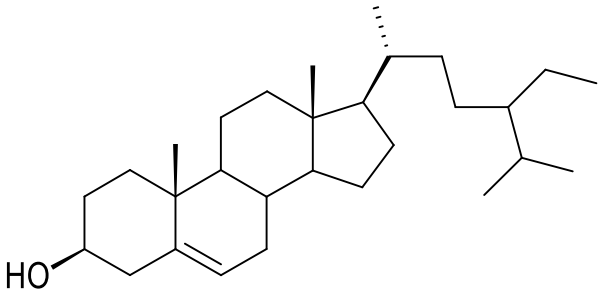
Nettle root and herb
Urtica dioica L.
Urticaceae

The drug consists of the dried roots and herb of *Urtica dioica*. The plant is native to the northern hemisphere and, due to its widespread occurrence, can be collected from wild populations. It has broad and diverse utilization possibilities, such as industrial fibre, chlorophyll extraction, and animal feeding. Its herb contains flavonoids and their glycosides, cinnamic acid derivatives, organic acids (e.g. ascorbic acid), volatile oil, fibre, biogenic amines (acetylcholine, histamine, and serotonin), leukotrienes and a considerable amount of potassium. The root contains sterols such as β-sitosterol, 7α- and 7β-hydroxy-β-sitosterol and their 3-*O*-glycosides, lignans and polysaccharides.

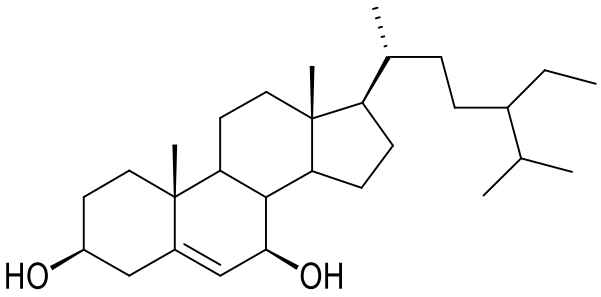
Uses: From a medicinal point of view, the aqueous or aqueous alcoholic extract of the herb has a diuretic effect probably due to its high potassium content. It is able to decrease the levels of inflammatory cytokines (TNF-α, and interleukins) in the organism

resulting in an anti-inflammatory effect. Folk medicine applies the fresh plant directly to the skin to reduce the pain in rheumatic problems. Touching the nettle herb results in a reddening of the skin, accompanied by itching due to the biogenic amine content of the plant. The long-lasting hyperaemia, which is attributed to the leukotrienes, helps to relieve rheumatic symptoms.

The root has a beneficial effect in benign prostatic hyperplasia (BPH). The mechanism of the effect is most probably that it blocks the activity of **aromatase** (*an enzyme which converts the androgens to oestrogens in the human body; in elderly men, the balance between these hormones shifts towards an increased level of oestrogens and a decreased level of testosterone; the oestrogens have anti-apoptotic activity in the case of the prostate cells which might result in their uncontrolled proliferation*) and **5α-reductase** (*an enzyme that converts testosterone into the more potent dihydrotestosterone; if the dihydrotestosterone level in the body increases it may lead to BPH in men*).



β-sitosterol



7β-hydroxy-β-sitosterol

TLC densitometric determination of the β-sitosterol content of Urticae radix

Densitometry utilizes the fact that the intensity of the spots on a TLC plate not only depends on the nature of the compound, but is also proportional to the amount present on the plate. With an appropriate calibration of known amounts of the same compound, quantitative data can be obtained from a TLC. By utilizing a high-resolution digital image of the plate and appropriate software, spot intensities allow calculation and quantification of the β-sitosterol content of the original sample from which the extract was prepared.

Test questions 6

Essay questions:

- In the TLC analysis of pot marigold flower, what did we use for extraction, how did we visualize the TLC plate and what could we conclude?
- In the TLC analysis of horse chestnut seed, what did we use for extraction, how did we visualize the TLC plate and what could we conclude?
- What is TLC densitometry? How did we quantify the β-sitosterol content of nettle root?

Mechanisms and structures:

- Give example of triterpene saponins (faradiol, protoaescigenin, and barringtogenol C).
- Give examples of plant steroids (β-sitosterol, and 7β-hydroxy-β-sitosterol).

Classification (Latin name of the drug, species, and family) and uses of the following drugs:

- Pot marigold flower, horse chestnut seed, and nettle root and herb

Practical 7

Drugs with anthraquinone content

1. Gel chromatographic separation and TLC analysis of the anthraquinone derivatives of Frangulae cortex
2. Bornträger test (Frangulae cortex, Rhei rhizome, Sennae folium, and Aloe)
3. Separation of the free and glycosidic anthranoids of Aloe and Sennae folium
4. Rosenthaler and Schouteten tests (Aloe)

Frangulae cortex
Glossy buckthorn bark
Rhamnus frangula L.

Rhamnaceae

Frangulae corticis extractum siccum normatum

Rhei radix
Rhubarb root
Rheum palmatum L.
Rheum officinale L.
Polygonaceae

Sennae folium
Senna leaf
Cassia angustifolia L.
Cassia senna L.
Caesalpiniaceae

Sennae folium extractum siccum normatum
Sennae fructus acutifoliae

Aloe
Aloe
Aloe barbadensis Mill.
Aloe ferox Mill.
Liliaceae

Aloes extractum siccum normatum

1. Gel chromatographic separation and TLC analysis of the anthraquinone derivatives of Frangulae cortex

Extract 50 g of drug powder with 500 ml of 70% aqueous methanol on the water bath for 15 min. Evaporate the extract to dryness under vacuum. Swell 10 g of Sephadex LH-20 gel in methanol and then pour it into a glass column previously plugged at the bottom with a small piece of cotton-wool. Dissolve 0.1 g of the dried extract in 2.0 ml of methanol and pipette it gently onto the top of the column. Elute the column with methanol. Collect 20 fractions. The volume of the first is 20 ml, while the volumes of the others are 2.5 ml. Check the fractionation procedure with TLC.

TLC determination
Sorbent: Silica gel

Spots:

Methanolic solution of Glucofrangulin B	10 μl
Methanolic solution of Frangulin B	10 μl
Collected fractions (1-20)	10 μl

Methanolic solution of frangulaemodin 10μl

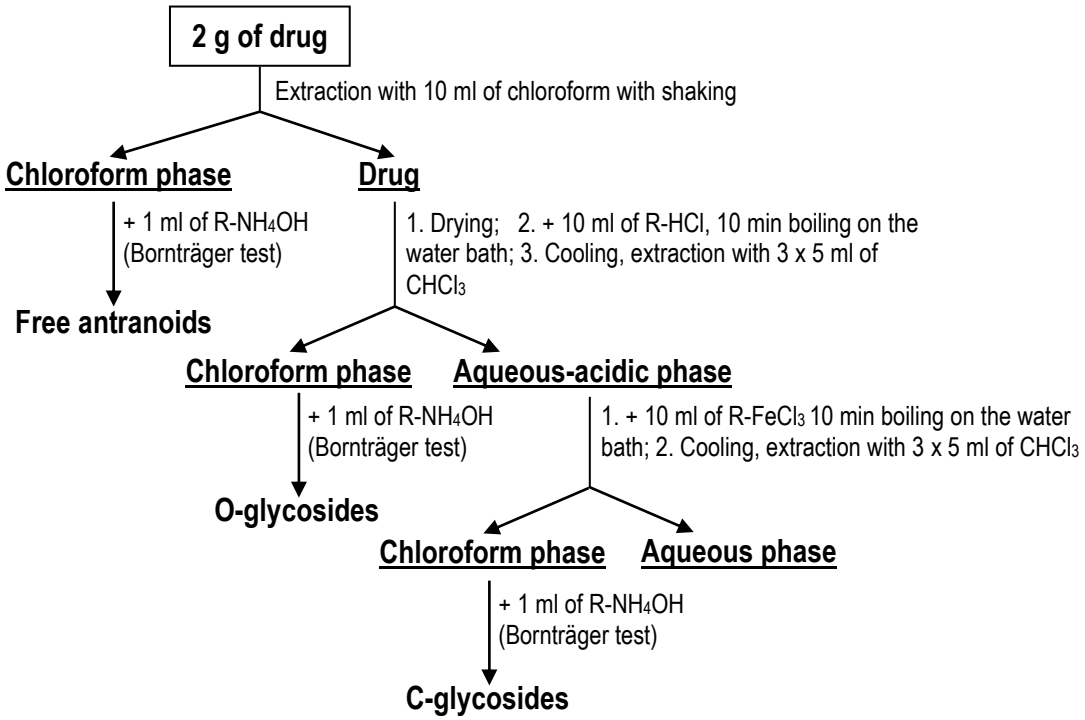
Solvent system:
Chloroform – methanol – water (90:20:2)

Detection:
366 nm light

2. Bornträger test (Frangulae cortex, Rhei rhizome, Sennae folium, and Aloe)
Extract 0.5 g of the drug with 10 ml of chloroform by shaking. Filter the extract into a test tube and add 1 ml of R-NH_4OH.

Write down your observation.

3. Separation of the free and glycosidic anthranoids of Aloe and Sennae folium



4. Rosenthaler and Schouteten tests (Aloe)
Rosanthaler test: Add 5 ml of Br_2-containing water to 5 ml of Aloe extract (made with hot water). Filter the liquid within 15 min.
Schouteten test (detection of barbaloin): Add 5 ml of borax solution to 5 ml of Aloe extract (made with hot water). Check the liquid after 2 min in 366 nm light.

Write down your observations.

Theory 7

Anthraquinone derivatives

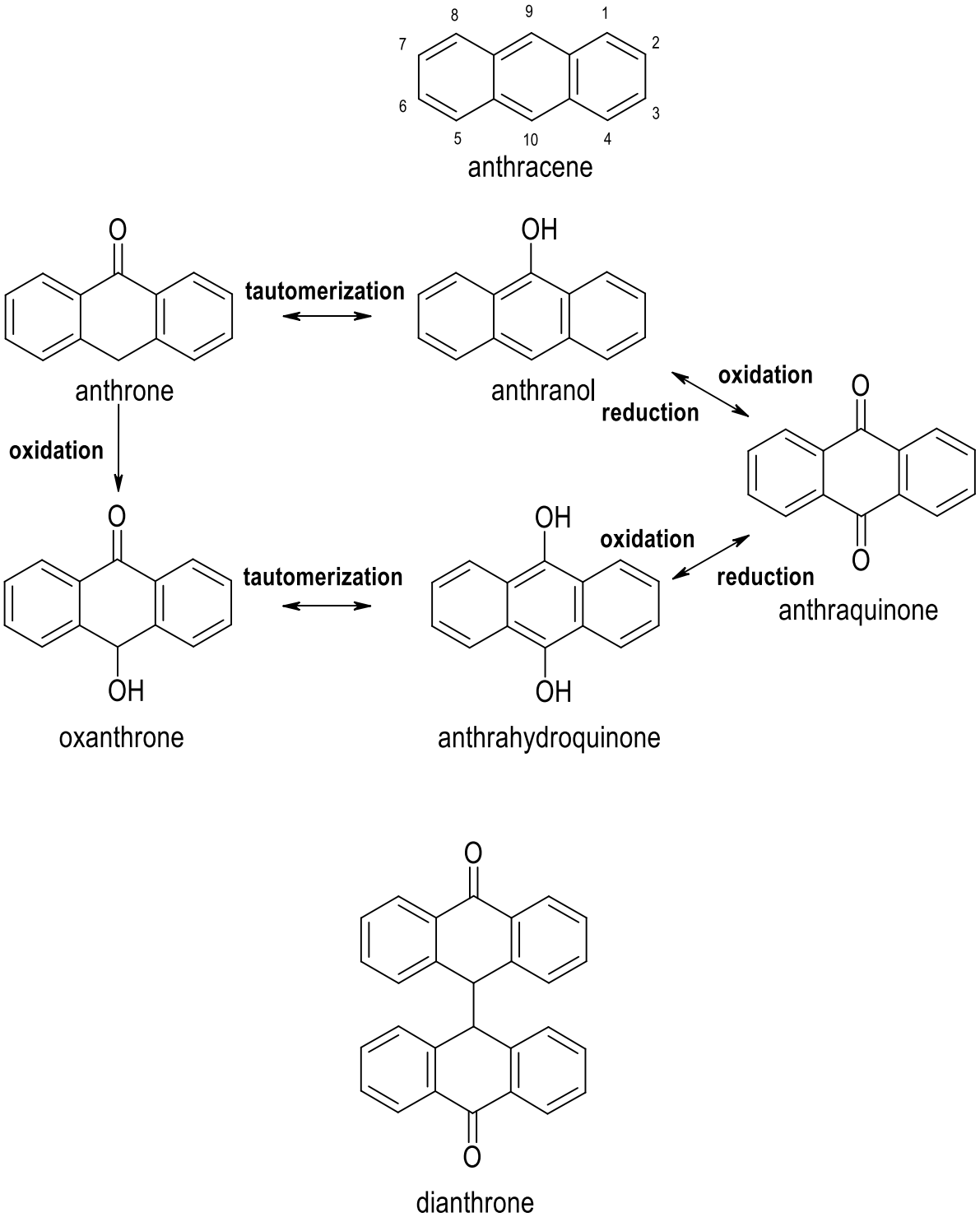
Phenols and phenolic glycosides are widespread in nature and can be found in almost every class of natural compounds which have aromatic unit(s). The following components belong in this group (here we list only those groups which will be dealt with in the pharmacognosy practicals):

- anthraquinones
- the A and B rings of the flavonoids
- tannins
- coumarins

Anthraquinone derivatives are oxidized forms of anthracene. Anthraquinones are mostly 1,8-dihydroxy derivatives and are present in plants in glycosidic forms (O- or C-glycosides). The reduced forms occur naturally, while the more oxidized forms develop during storage and drying (exposure to heat).

Classification of anthraquinone derivatives according to their oxygen content:

- Anthraquinone (aglycone, mono- and diglycosides) → most oxidized form
- Oxanthrone
- Anthrahydroquinone (not stable form)
- Anthron
    - aglycone (also called emodin)
    - anthrone glycosides (O- and C-glycosides)
    - dianthrones and dianthrone glycosides
        - isodianthrone (two identical monomers)
        - heterodianthrone (two different monomers)
- Anthranol → most reduced form

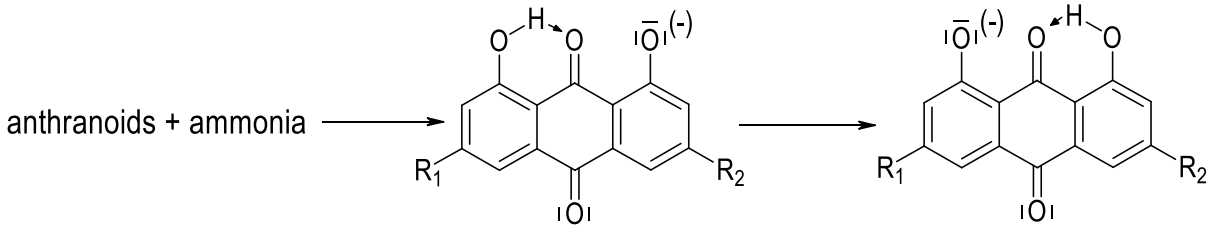


Physical and chemical properties of anthraquinone derivatives:

Solubility: The aglycones (depending on the number of -OH groups in the molecule) dissolve in moderately polar solvents, while the glycosides dissolve in polar solvents such as alcohol, water or their mixtures.

Detection: One of their characteristic properties is their colour reaction with bases. This colour can vary from deep-red to yellow (see the Bornträger test, below).

Bornträger test
See method in Practical 7. The mechanism of the reaction is that the phenolic -OH group is ionized in the alkaline environment and delocalization of the negative charge takes place due to the mesomeric effect. The colour that appears depends on the substituent at C-3: if this substituent is $-CH_3$, the developing colour is deep red, while if it is $-CH_2OH$, the colour will be orange.



Separation of the free and glycosidic anthranoids of Aloe and Sennae folium
The Bornträger reaction is suitable for the detection of aglycones. The glycosidic forms can be tested after their hydrolysis. Hydrolysis of the O-glycosides is relatively easy; it can be performed by adding HCl and applying water bath heating. For the hydrolysis of the C-glycosides, however, more aggressive conditions are necessary (HCl + $FeCl_3$ + warming).

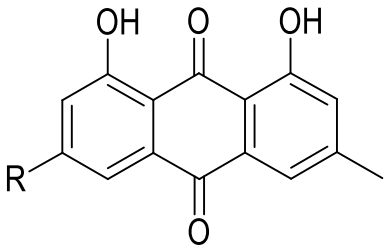
Uses: The anthraquinone derivatives have a laxative effect, causing the retention of Na^+ and water in the large intestines and increasing the motility, which results in intense diarrhoea within 6-8 h. The glycosides are more potent than the aglycones and the glycosides of the anthrones, and dianthrones are the most effective. Since anthranol derivatives can exert a severe smooth muscle spasm as a side-effect, the collected drugs are exposed to heat during drying and are stored for at least one year before use, so that these constituents can be transformed to the safer, more oxidized derivatives.
Caution: The anthraquinone derivative-containing drugs increase the blood circulation in the hypogastrium, which may result in abortion, and their use during pregnancy is therefore contraindicated!

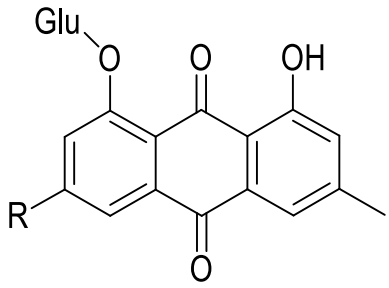
Frangulae cortex
Glossy buckthorn bark
Rhamnus frangula L.
Rhamnaceae

The drug consists of the dried (at 70-80 ºC) bark of *Rhamnus frangula*, after one year of storage. The shrub is native to Europe and Asia. The outer side of the bark is greyish-brown, while the inner side is reddish in colour. The drug contains 6-9% anthraquinone

derivatives such as frangulaemodin (an aglycone), frangulin A and B (monoglycoside derivatives), glucofrangulin A and B (diglycosides) and frangula A- and glucofrangulin A-dianthrones (dianthrones).

Uses: A laxative.





	R		**R**
frangulaemodin	-OH	glucofrangulin A	-rhamnose
frangulin A	-O-rhamnose	glucofrangulin B	-apiose
frangulin B	-O-apiose		

Gel chromatographic separation and TLC determination of the anthraquinone derivatives of Frangulae cortex

Reminder
Gel chromatography: The liquid or gaseous phase passes through a porous gel, which separates the molecules according to their sizes. The small pores exclude the larger molecules, but allow smaller ones to enter deeper into the gel's structure. Hence, smaller molecules have a larger distribution volume, which leads to their slower migration through the column. This results in an elution sequence according to molecular size: the largest first, and the smallest last.

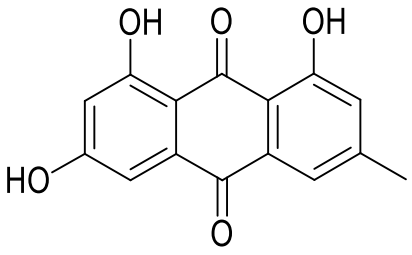
Sephadex LH-20 dextran gel is used to separate the different anthraquinones present in the extract of Frangulae cortex. The elution sequence follows the sizes of the molecules, which means that the larger dianthrone glycosides and dianthrone aglycones will elute first, followed by the smaller anthraquinone glycosides, and finally the aglycones. In zhe case of Glossy buckthorn bark, glucofrangulin B contains two sugars (apiose and glucose) and therefore elutes first, followed by frangulin B (only one sugar, apiose) and finally frangulaemodin (an aglycone).
Note: Due to the high polarity of the sugar components, the sequence will be the opposite on a silica TLC plate; a higher number of sugars confers higher polarity, and the highest retention (i.e. the lowest R_f value) will therefore be observed for glucofrangulin B.

Components are visualized on the basis of their fluorescency 366 nm light.

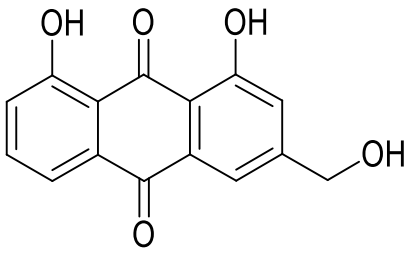
Rhei radix
Rhubarb root
Rheum palmatum L.
Rheum officinale L.
Polygonaceae

The drug comprises the dried, peeled rhizome of *Rheum palmatum* or *Rheum officinale*. The plant is native to the northern and western parts of China and several regions of Asia and Europe. The rhizome of the 6-8-year-old plant is collected in the autumn and dried either in the sun or by heating. It contains 3-10% anthraquinone glycosides, such as rheumemodin, aloeemodin, and rhein glycosides, 4-8% heterodianthrone glycosides and around 5-10% tannins of the catechin type (see Practical 10).

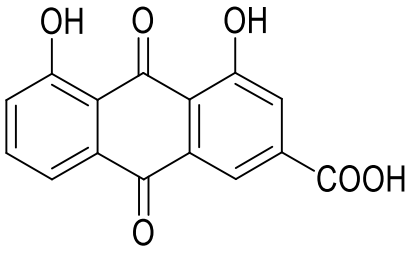
Uses: Because of the high tannin content, the drug has an obstipant effect in small doses (0.1-0.3 g), and because of its anthraquinone content it has a laxative effect in higher doses (1-4 g).



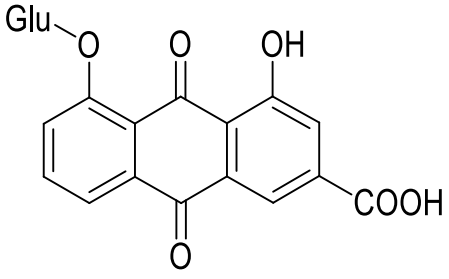
rheumemodin (=frangulaemodin)



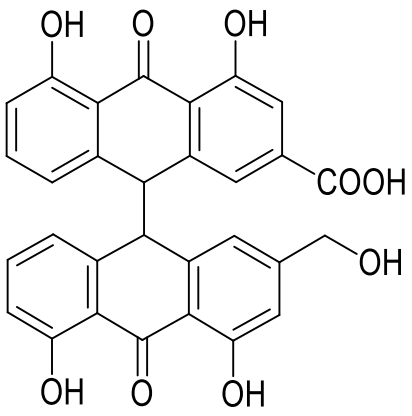
aloeemodin



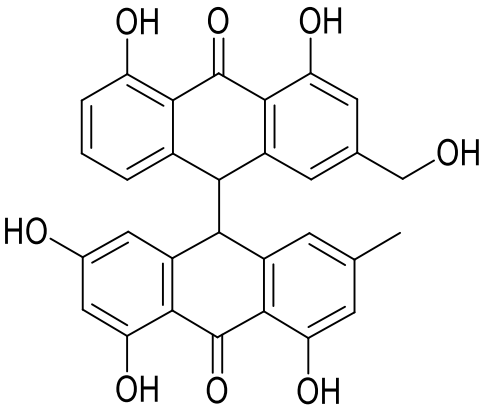
rein



reinanthrone-8-glucoside



rein-aloeemodin dianthrone

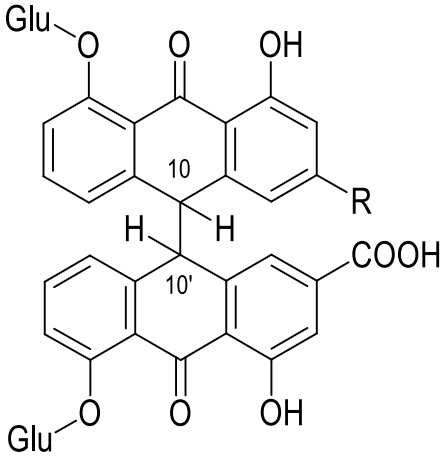


aloeemodin-reumemodin dianthrone

Sennae folium et fructus
Senna leaf and pods
Cassia angustifolia L.
Cassia senna L.
Caesalpiniaceae

Senna pods are the dried ripe fruits of *Cassia angustifolia* and *Cassia senna*; both the leaves and pods of these species are utilized as phytomedicines. The pods are collected with the leaves and separated after drying. The plant is cultivated in India and Africa. The pod is thin like a parchment, 4-5 cm long and 2 cm wide, reniform and yellowish-brown in colour. It contains 1-5% iso- and heterodianthrone glycosides such as sennoside A, B, C, D, aloeemodin and rhein glycosides. Other constituents are flavonoids, tannins and resin.

Uses: A strong laxative.



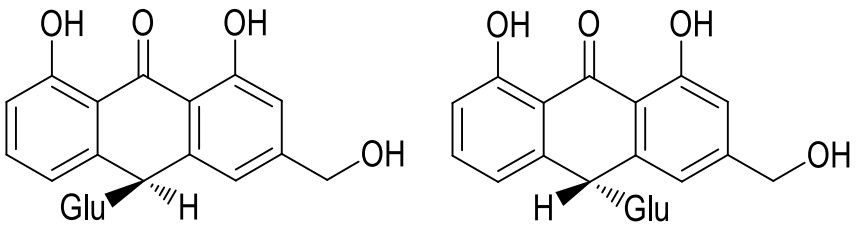
	Configuration		
R	**C10**	**C10'**	**Name**
-COOH	R	R	Sennoside A
-COOH	R	S	Sennoside B
$-CH_2OH$	R	R	Sennoside C
$-CH_2OH$	R	S	Sennoside D

Aloe
Aloe
Aloe barbadensis Mill.
Aloe ferox Mill.
Liliaceae

Aloe is the solid residue of the juice obtained by evaporation of the liquid which drains from the transversely cut leaves of various species of Aloe. These Aloe species are native to South Africa. The drug has a black or dark-greenish colour, and if sun-dried, it is pale (*Aloe hepatica*), while, if dried over a fire, its surface is shiny (*Aloe lucida*). The powder of this drug is brownish-yellow and has a bitter taste. It contains 20-40%

anthraquinone derivatives, mainly anthrone C-glycosides such as aloeemodindianthrone, and aloin A and B glycosides. Another important constituent is 30-80% resin.

Uses: A strong laxative.



aloin A aloin B

aloin A + aloin B = barbaloin

Rosenthaler and Schouteten tests: Make an aqueous extract of the drug with hot water (1:200), cool it and filter it.

Rosenthaler test

See method in Practical 7. The colour of the precipitate formed is characteristic of the *Aloe* sp. In our case, the precipitate is vivid-yellow.

Schouteten test (detection of barbaloin)

See method in practical 7. As a result, a green fluorescence appears.

Test questions 7

Essay questions:

- What are anthraquinone derivatives, which compound group do they belong to, and in what forms do they occur in plants?
- Classify anthraquinone derivatives according to their oxygen content.
- Give the physical and chemical properties, uses and detection (Bornträger test) of anthraquinone derivatives.
- How did we separate the free and glycosidic anthranoids of the drugs? Describe the process from the extraction to the detection of the different anthraquinone derivatives.
- Explain the gel chromatographic separation of the anthraquinone derivatives of glossy buckthorn bark. What is the elution sequence of the different anthranoids? Why? How could we check the separation with TLC? how did we visualize the TLC plate and what could we conclude?
- How did we perform the Rosenthaler test (from the extraction to the result)? What is it used for?
- How did we perform the Schouteten test (from the extraction to the result)? What is it used for?

Mechanisms and structures:

- Give the classification of anthraquinone derivatives according to their oxygen content (anthrone, anthranol, oxanthrone, anthrahydroquinone, anthraquinone, dianthrone).
- Describe and explain the Bornträger test from the extraction of the drug to the observed result.
- Give examples of the anthraquinone derivative content of glossy buckthorn bark (frangulaemodin, frangulin A and B, and glucofrangulin A and B).
- Give examples of the anthraquinone derivative content of rhubarb root (rheumemodin, aloeemodin, rhein, rheinanthrone-8-glucoside, rhein-aloeemodin dianthrone, and aloeemodin-rheumemodin dianthrone).
- Give examples of the anthraquinone derivative content of senna leaf (sennoside A, B, C, and D).
- Give examples on the anthraquinone derivative content of aloe (aloin A and B,and barbaloin).

Classification (Latin name of the drug, species, and family) and uses of the following drugs:

- Glossy buckthorn bark, rhubarb root, senna leaf, and aloe

Practical 8

Drugs with flavonoid content, I

1. TLC analysis of the flavonoid aglycones from Tiliae flos
2. TLC analysis of the flavonoids from Sambuci flos and Hyperici herba
3. Determination of unknown drug powders

Tiliae flos
Small-leaved linden flower
Tilia cordata Mill.
Tiliaceae

1. TLC analysis of the flavonoid aglycones from Tiliae flos

Extraction: Extract 1 g of drug with 10 ml of methanol by heating on the water bath for 10 min. Filter it and put 1 ml of the extract into a test tube for later TLC (extract I). Add 10 ml of 2n H_2SO_4 to the remaining 9 ml of the extract and hydrolyse it on the water bath for 30 min. Cool it down, pour it into a separating funnel and extract it with 2 x 10 ml of ethyl acetate. Combine the organic phases and extract them with 2 x 5 ml of water in order to remove the remaining acid. Dry the organic phase with anhydrous Na_2SO_4, filter the solution and evaporate it to dryness. Dissolve the dry residue in 1 ml of methanol (extract II) for TLC.

TLC analysis
Sorbent: Silica gel

Spots:

Methanolic extract of Tiliae flos (extract I)	40 μl
0.1% methanolic solution of quercetin	20 μl
Hydrolisate (extract II)	20 μl

Solvent system:
Toluene – ethyl acetate – formic acid (5:4:1)

Detection:
254 nm and 366 nm light

Sambuci flos
Common elderberry flower
Sambucus nigra L.
Caprifoliaceae

Hyperici herba
St. John's wort herb
Hypericum perforatum L.
Hypericaceae

2. TLC analysis of the flavonoids from Sambuci flos and Hyperici herba

Sambuci flos: Extract 1 g of drug with 20 ml of methanol by heating the mixture on the water bath for 5 min. Filter the extract into a porcelain dish and evaporate it to dryness. Dissolve the dry residue in 1 ml of methanol for TLC.
Hyperici herba: Extract 1 g of drug with 20 ml of methanol by heating on the water bath for 10 min. Filter the extract into a porcelain dish and evaporate it to dryness. Dissolve the dry residue in 1 ml of methanol for TLC.

TLC determination
Sorbent: Silica gel

Spots:

Sambuci flos, methanolic extract	20 μl
0.1% methanolic solution of rutin	20 μl
Hyperici herba, methanolic extract	20 μl
Methanolic solution of hyperoside	20 μl

Solvent system:
Ethyl acetate – formic acid – water (85:10:5)

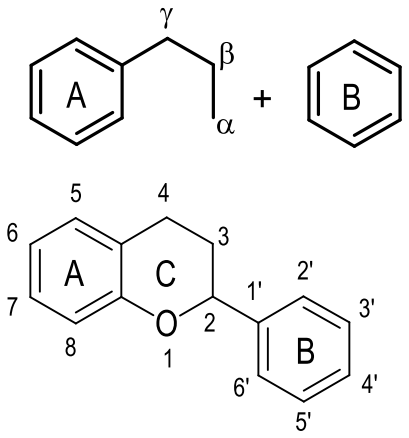
Detection:
Spray the TLC plate with a 1:3 mixture of oxalic acid and boric acid, and heat it for 5 min at 105 ºC.

Theory 8

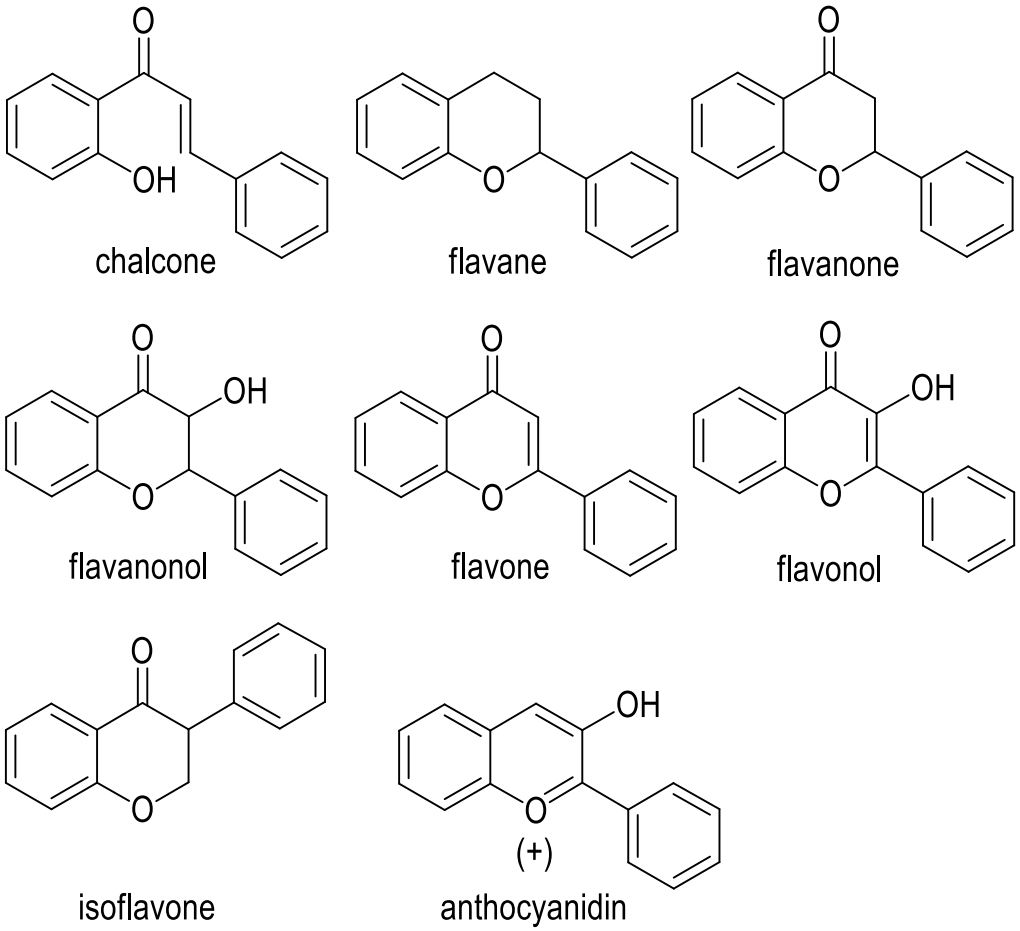
Flavonoids

The name flavonoid originates from the characteristic yellow colour (flava = yellow) of these compounds, which are among the most widespread secondary plant metabolites. In plants, they occur both in the free state and as glycosides, representing the largest group of naturally occurring phenols. Their skeleton contains 15 C atoms, formally derived from diphenylpropane ($C_6C_3C_6$). The two aromatic rings give the A and B rings of flavonoids, but in most cases the propane (C_3) moiety forms a third ring (C) fused to the A ring, and in this case the already existing B ring binds to this newly formed C ring.
The binding of the A / C and B rings may occur on the α, β or γ carbon atoms.

- binding on the α C atom → flavonoids
- binding on the β C atom → isoflavonoids
- binding on the γ C atom → neoflavonoids



The flavonoid skeletons can be divided into further subgroups, such as:

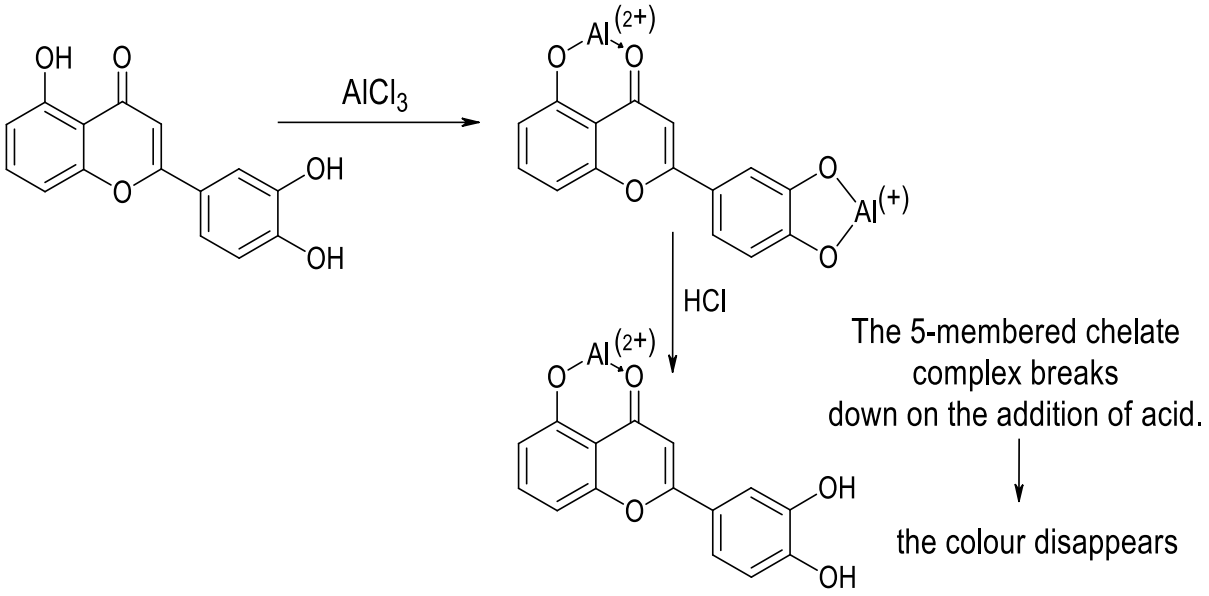


Physical and chemical characteristics, properties:

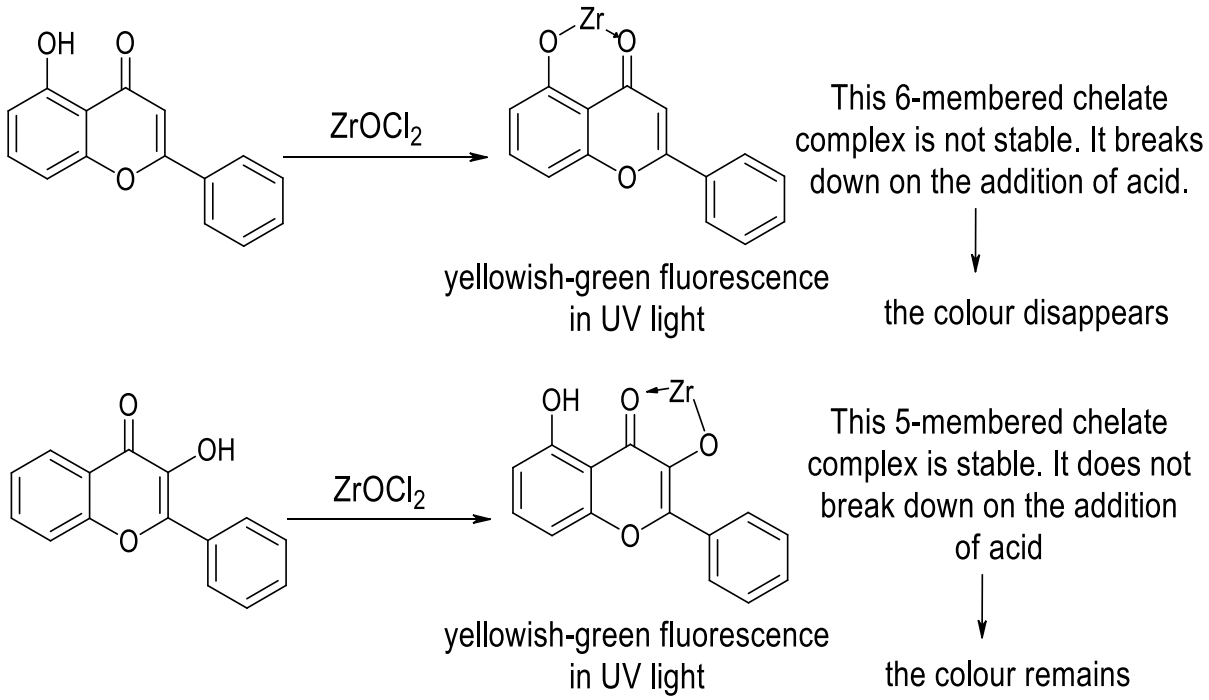
Flavonoids usually have a characteristic yellow-orange colour (the anthocyans are red at acidic pH, and bluish at alkalic pH). Flavonoids occur in a wide structural diversity in nature. They can have several –OH or $–OCH_3$ substituents, additional rings, C-methyl, prenyl, etc. groups. They occur in various forms: as aglycones (mostly in dead tissues), as glycosides (in live tissues), they can form esters or dimers or trimmers, or they can be bound to other secondary metabolites (lignans, for example). The aglycones are soluble in ethyl acetate or (the less polar ones) in chloroform. The glycosides are soluble in water or aqueous alcoholic solvents. The UV absorption of flavonoids makes their detection simple during chromatographic separations.

Reactions:

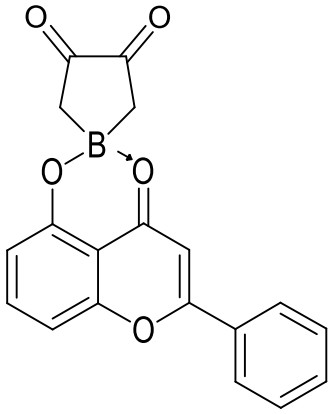
- **R-$FeCl_3$**: blue, green or greyish complex formation → if the 5–OH group is free and there is an oxo group on C-4
- **$AlCl_3$ + HCl**: a yellowish-green fluorescent reaction in UV light.



- **$ZrOCl_2$ (zirconium oxychloride) + citric acid**: a yellowish-green fluorescent reaction in UV light → if there is a free –OH group on C-3 or C-5. On the addition of citric acid, the colour disappears or remains depending on the position of the free –OH group.



- **Oxalic acid + boric acid**: yellow; under UV light, a yellow fluorescence → in the cases of an –OH group on C-5 or flavonols.



- **Basic Pb acetate**: orange or brownish-yellow colour
- **NH_3 or NaOH**: a stronger yellow colour

Note: The above reactions, particularly in combination with the changes seen in the UV-Vis spectra, provide structural information on the substitution pattern of flavonoids.

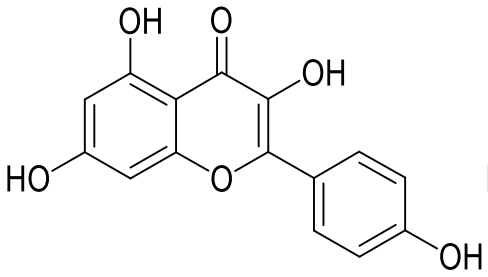
Uses: As a result of their poliphenolic structure, flavonoids are usually strong antioxidants. They can interact with intracellular redox processes on multiple levels (scavenging reactive oxygen species, complexing transition metals, inhibiting redox enzymes, etc.). Moreover, various specific effects can be attributed to selected flavonoids, as follows.

- In cardiovascular problems:
  - capillary resistance-increasing and permeability-decreasing effects ("vitamin P", rutin)
  - coronary dilating and positive inotropic effect (procyanidins from Crataegus sp., Ginkgo, and Arnica sp.)
- In varicose vein problems: an anti-inflammatory effect, improvements of the micro- and macrocirculation and decrease of oedema (rutascorbin and hydroxyethylrutoside)
- Spasmolitic effect: apigenin (Matricaria sp.), isoliquiritigenin (*Glycyrrhiza glabra*)
- Hepatoprotective and choleretic effect: Silybum sp.
- Oestrogenic effect: isoflavonoids (e.g. from *Glycine max* – soybean) and prenylflavonoids (e.g. from *Humulus lupulus* – hops)
- Diaphoretic effect: e.g. Tilia sp. and *Sambucus nigra*
- Diuretic effect: e.g. *Betula pendula* and *Solidago virga-aurea*
- Anti-allergic effect: Citrus flavonoids, quercetin, and floretin

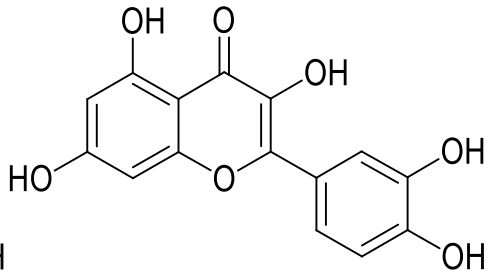
Tiliae flos
Small- or big-leaved linden flower
Tilia cordata Mill., *Tilia platyphyllos* Scop.
Tiliaceae

The drug consists of the flowers of the tree *Tilia cordata*. The tree is about 30 m high, with a large, spreading crown. The cymes are obliquely erect, with 4-15 small whitish-yellow flowers. It contains >1% flavonoids (quercetin and kaempferol glycosides), >10% mucilage, volatile oil and tannins of catechin type.

Uses: As a tea, it is used as a diaphoretic agent (causing sweating in cases of fever) in common cold, bronchitis or pneumonia. Because of its mucilage content, it also has an immunostimulatory effect.



kaempferol



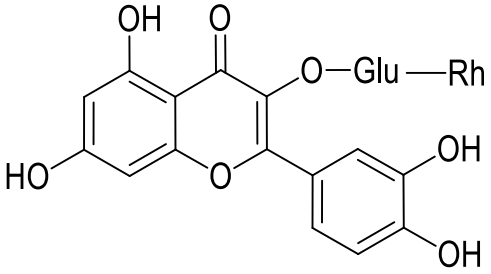
quercetin

TLC analysis of the flavonoid glycosides and aglycones from Tiliae flos
In the methanolic extract of the linden flower, flavonoid glycosides are present, while quercetin is hardly or not detectable. After acidic hydrolysis with H_2SO_4 on the water bath, the flavonoids lose their sugar moiety and become free aglycones. The aglycones are less polar than the glycosidic components, and can be extracted with ethyl acetate from an aqueous solution. The TLC analysis shows that flavonoid aglycones (such as quercetin) appear in the ethyl acetate phase, and flavonoid glycosides in the methanolic extract. All flavonoids appear as fluorescent spots under 366 nm light.

Sambuci flos
Common elderberry flower
Sambucus nigra L.
Caprifoliaceae

The drug consists of the dried flowers of the shrub or small tree *Sambucus nigra*. The plant is native to Europe. In florescence, the flowers are 10-24 cm in diameter, white, corymbose with 4-5 primary rays. It contains flavonoids (quercetin, kaempferol, and rutin), volatile oil and mucilage.

Uses: It can be used as a diaphoretic in the form of a tea in common cold; it has immunostimulatory, mild anti-inflammatory and diuretic properties.



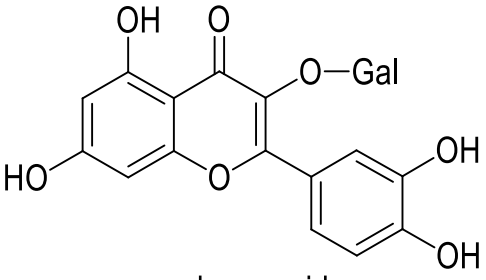
Glu = glucose
Rh = rhamnose

rutin

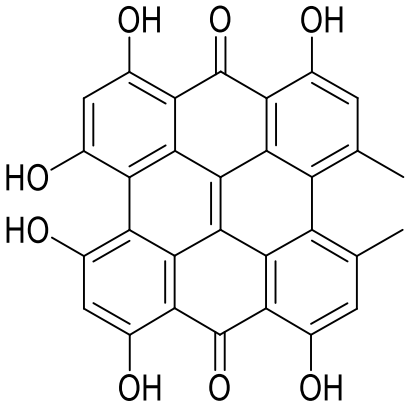
Hyperici herba
St. John's wort herb
Hypericum perforatum L.
Hypericaceae

The drug consists of the dried herb of the perennial plant *Hypericum perforatum*. The plant is native to Europe, Western Asia, and North Africa. The leaves are 8-30 mm long, with numerous translucent dots, hence the name "perforatum" (perforated). It contains flavonoids such as hyperoside, biapigenin, rutin, naphthodianthrone derivatives such as hypericin, and phloroglucinol derivatives such as hyperforin.

Uses: The drug has antidepressant and hypnotic properties which can be attributed to the mixture of the components present in the drug. Hypericin has a confirmed MAO-inhibitory effect resulting in the prolonged presence of the excitatory neurotransmitters in the body. Hyperforin selectively blocks the reuptake of dopamine, noradrenaline, and serotonin into the neurons, also resulting the prolonged presence of these neurotransmitters within the CNS. Flavonoids may also be important in the mechanism of action; they may increase the absorption of hyperforin in the gastrointestinal tract. The extract of the drug is used to treat mild depression. (*In depression, the amount of and the balance between different neurotransmitters are altered, leading to the characteristic pathological symptoms.*)



hyperoside



hypericin

TLC analysis of the flavonoids from Sambuci flos and Hyperici herba

After the methanolic extraction, rutin is detectable in the elderberry flower extract, and hyperoside in the St. John's wort herb extract. After the chromatogram is sprayed with an oxalic acid – boric acid (1:3) mixture and placed it in the heating chamber, flavonoid glycosides appear with a yellow colour.

Test questions 8

Essay questions:

- What are flavonoids, how do they build up and in which forms do they occur in plants?
- Give the physical and chemical properties and detection methods of flavonoids.
- Give the common medical uses of flavonoids.
- In the TLC analysis of the flavonoids of linden leaf, what did we use for extraction, how did we visualize the TLC plate and what could we conclude?
- In the TLC analysis of St. John's wort herb and common elderberry flower, what did we use for extraction, how did we visualize the TLC plate and what could we conclude?

Mechanisms and structures:

- Draw the basic flavonoid skeletons (chalcone, flavan, flavanone, flavanonol, flavone, flavonol, isoflavone, and anthocyanidin).
- Explain the reaction of flavonoids with $AlCl_3$ + HCl; give the mechanism.
- Explain the reaction of flavonoids with $ZrOCl_2$ (zirconium oxychloride) + citric acid; give the mechanism.
- Explain the reaction of flavonoids with oxalic acid + boric acid; give the mechanism.
- Give examples of flavonoid aglycones (kaempferol and quercetin).
- Give examples of flavonoid glycosides (rutin and hyperoside).
- Give the structure of the non-flavonoid hypericin.

Classification (Latin name of the drug, species, and family) and uses of the following drugs:

- Small-leaved linden flower, big-leaved linden flower, common elderberry flower, and St. John's wort herb

Practical 9

Drugs with flavonoid content, II

1. Isolation and TLC determination of the purity of hesperidin from Aurantii epi- and mesocarpium
2. Detection of procyanidins from Crataegi folium cum flore and Crataegi fructus (Bate-Smith reaction)
4. Determination of unknown drug powders

Aurantii amari epicarpium et mesocarpium
Bitter orange peel
Citrus aurantium L.
Rutaceae

Aurantii amari epicarpii et mesocarpii tinctura

1. Isolation and TLC analysis of the purity of hesperidin from Aurantii epi- and mesocarpium

Extraction and isolation: Add 25 ml of methanol to 5 g of powdered drug in an Erlenmeyer flask, and extract it for 25 min on the water bath. Filter the extract through fluted filter paper. Put 1 ml of the filtrate into a test tube for the TLC. Distil the remaining part of the extract under vacuum until it reaches a "syrup-like" consistency, and then add 20 ml of 6% acetic acid. Leave the mixture for 15 min and then filter the precipitate through filter paper. Wash the filtered precipitate with 10 ml of 6% acetic acid, then 10 ml of water and finally with 10 ml of isopropanol. Scrape the washed precipitate into a test tube and dissolve it in 3 ml of hot methanol.

TLC analysis
Sorbent: Silica gel

Spots:

Aurantii amari epicarpium et mesocarpium methanolic extract	20 μl
0.1% methanolic solution of hesperidin	20 μl
Hot methanolic solution of the precipitate	20 μl

Solvent system:
Ethyl acetate – formic acid – acetic acid – water (100:11:11:27)

Detection:
Spray the TLC plate with $AlCl_3$ solution and investigate it under 366 nm light

Crataegi folium cum florae
Crataegi fructus
Crataegus monogyna Jacq.
Common hawthorn leaf, flower and fruit

Crataegus laevigata (Poiret) DC.
Midland hawthorn leaf, flower and fruit
Rosaceae

Crataegi folii cum florae extractum siccum

2. Detection of procyanidins from Crataegi folium cum flore and Crataegi fructus (Bate-Smith test)

Extract 0.5 g of drug with 10 ml of 2M HCl on a water bath for 30 min (hydrolysis). After the mixture has cooled, filter it into a reaction tube, add 5 ml of *n*-butanol and shake the mixture well. The butanolic upper phase will change colour because of the presence of the procyanidins.

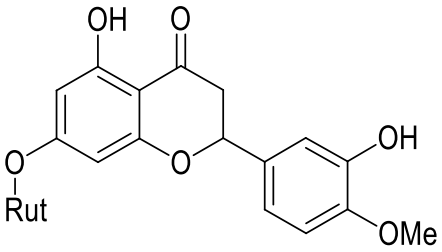
Write down your observations.

Theory 9

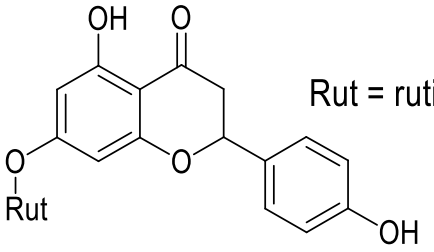
Aurantii amari epicarpium et mesocarpium
Bitter orange peel
Citrus aurantium ssp. *aurantium* L.
Rutaceae

The drug consists of the dried epicarp and mesocarp freed from most of the white parenchyma of the fruit of *Citrus aurantium* ssp. *aurantium.* The tree originates from India and is closely related with lemon and orange trees, whose fruits are eaten worldwide. It contains flavonoids such as naringin and neohesperidin (in the yellow part) and limonoids (in the white part), which give the bitter taste to the drug. It contains 1-2% volatile oil (mostly limonene and geranial) which gives the characteristic odour to the drug.

Uses: The drug is used in the treatment of dyspepsia and as an appetizer because of its bitter taste.



hesperidin



naringenin rutinoside

Rut = rutinose (= rhamnose + glucose)

Isolation and TLC determination of the purity of hesperidin from orange peel
Extract the drug with methanol and then filter it through fluted filter paper. This kind of filter paper provides a larger surface, which speeds up the filtration. Through the addition of 6% acetic acid, the pH is set to 4-5, when hesperidin precipitates in the form of an amorphous powder, while most of the other flavonoids remain in the filtrate. (*The precipitation is easier if the liquid is thicker, so most of the liquid is evaporated before this step.*) Filter the mixture and wash the precipitate with 6% acetic acid, then water and isopropanol to remove the other precipitated flavonoids; hesperidin dissolves only in hot methanol. Dissolve hesperidin in hot methanol and apply it to the TLC. In the methanolic extract of the drug, other flavonoids also appear, while in the methanolic solution of the precipitate only hesperidin should be visible. Spraying the TLC plate with $AlCl_3$ reagent results in yellowish-green fluorescence in 366 nm light.

Procyanidins are dimeric or oligomeric compounds which can be hydrolysed to catechin and anthocyanidin with acids. In most cases, they are not glycosides, but colourless compounds with tannin-like properties.

Crataegi folium cum florae
Crataegi fructus
Crataegus monogyna Jacq.
Common hawthorn leaf, flower and fruit

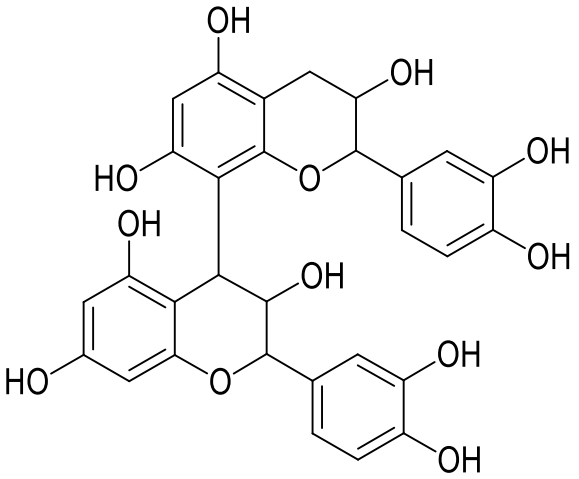
Crataegus laevigata (Poiret) DC.
Midland hawthorn leaf, flower and fruit
Rosaceae

The drug consists of the dried twigs, leaves, flowers and fruits of different *Crataegus* sp. The shrub is native to Europe. The leaves are obovate to rhombic; the fruits are dark or bright-red. The drug contains flavonoids such as vitexin, isovitexin, hyperoside (*O*-glycosides), tannins and dimeric procyanidins (procyanidin B2 and B5).

Uses: The aqueous or alcoholic extracts of the drug dilate the blood vessels, and especially the coronary vessels. They have positive inotropic, antiarrhythmic and blood pressure-decreasing effects. The mechanisms behind the effects are the following:

- Na^+/K^+ ATPase-blocking effect
- ACE-inhibiting effect (angiotensin convertase enzyme)
- K^+-channel-activating effect
- increasing the NO production of the endothelial cells, resulting in blood vessel dilation

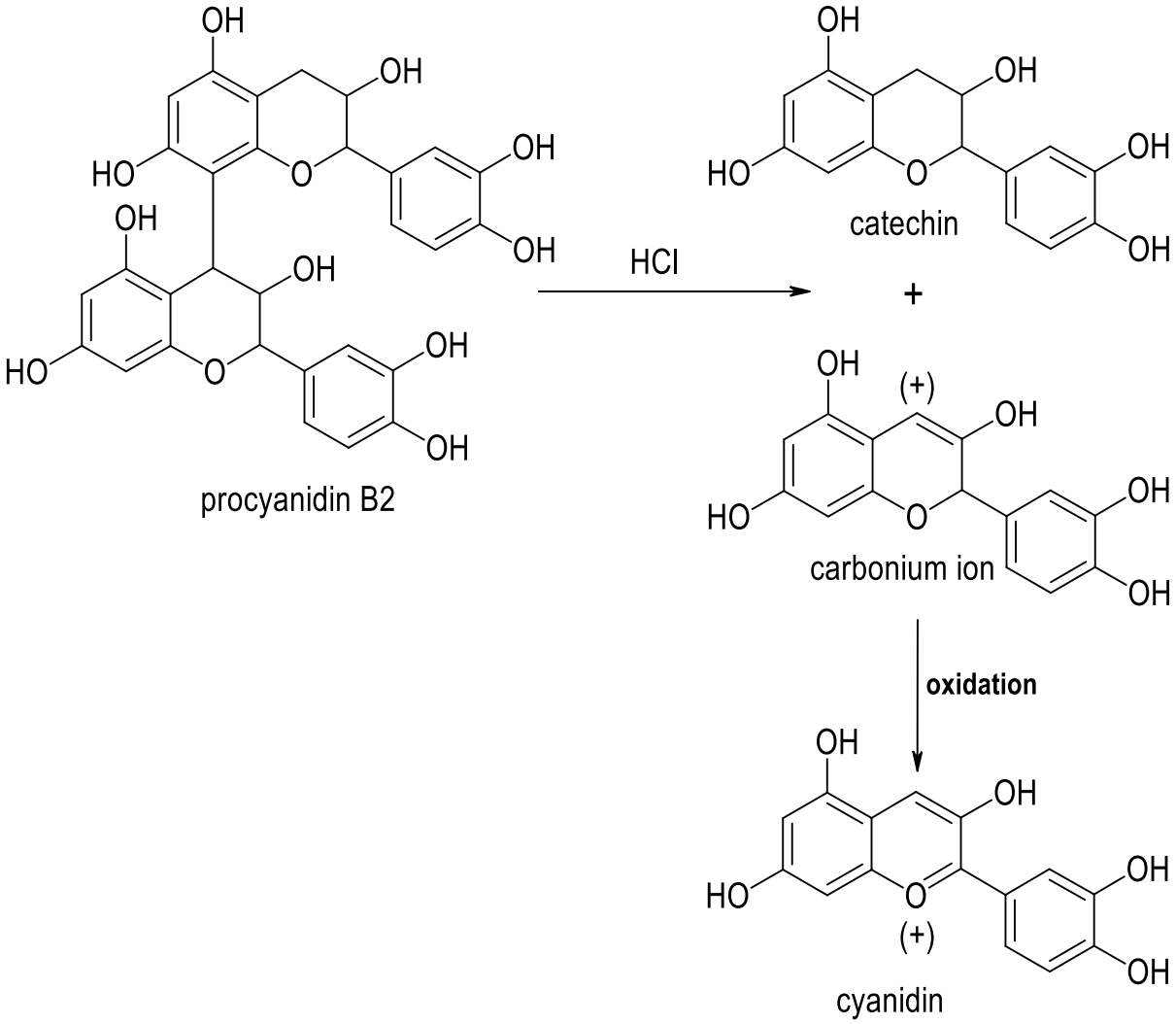
The drug decreases the levels of low-density and very low-density lipoproteins (LDL and VLDL) in the blood, and blocks the synthesis of cholesterol.
These effects lead to the drug being used in the early phase of heart insufficiency. It might also be beneficial in angina pectoris and coronary sclerosis.



Procyanidine B2

Detection of procyanidins (Bate-Smith test)

See method in Practical 9. Because of the acidic environment, the *n*-butanolic (upper) phase turns red due to the anthocyanidin formed.



Test questions 9

Essay questions:

- In the isolation and TLC determination of bitter orange peel, what did we use for extraction, how did we isolate the flavonoids, how did we visualize the TLC plate and what could we conclude?
- What are procyanidins?
- What is the mechanism of the pharmacological effect of hawthorn leaf, flower and fruit?

Mechanisms and structures:

- Give the structure of procyanidine B2.
- Give the mechanism and explain the Bate-Smith test for the detection of procyanidines.

Classification (Latin name of the drug, species, and family) and uses of the following drugs:

- Bitter orange peel, common hawthorn leaf, flower and fruit, and midland hawthorn leaf, flower and fruit

Practical 10

Drugs with tannin content

1. General tannin tests
2. Quantitative determination of tannins from Quercus cortex by spectrophotometry

OR

Drugs with hydroquinone derivative content

3. TLC analysis of the methanolic extract and the sublimate of Uvae ursi folium
4. Analysis of catechin derivatives and phenolic components of Uvae ursi folium
5. Determination of unknown drug powders

Galla
Nut galls
Quercus infectoria Oliv.

Fagaceae

Quercus cortex
Oak tree bark
Quercus robur L.
Quercus petraea L.
Fagaceae

Ratanhiae radix
Rhatany root
Krameria triandra R. and P.

Krameriaceae

Agrimoniae herba
Common agrimony herb
Agrimonia eupatoria L.
Rosaceae

1. General tannin tests

Extraction: Wet each drug powder with 70% aqueous methanol and make an extract from each of them with water by heating on the water bath for 5 min. Adjust the amount of drug and water so that the final concentrations of the extracts are as follows: 0.2% Galla extract, 1% Ratanhiae radix extract, 2%, Quercus cortex extract, 3% Agrimoniae herba extract. Cool the extracts and filter them through paper. Use 5 ml from each for the following reactions:

	1-2 drops of R-$FeCl_3$	**R-alcoholic Pb acetate**	**1% quinine – HCl**	**1% gelatine solution**
Galla				
Quercus cortex				
Ratanhiae radix				
Agrimoniae herba				

The colour that appears immediately in response to the dropping, and the colour, the amount and the consistency of the precipitate formed are characteristic of the drug.
Note: The above reactions will help you when you need to distinguish these drugs from each other as unknown drug powders.

Write down your observations.

2. Quantitative determination of tannins from Quercus cortex by spectrophotometry

Boil 2.00 g of accurately measured [write down the precise measured mass (***m***)] drug powder for 2 x 15 min with 2 x 40 ml of water. Collect the extract in a 100 ml volumetric flask and, after cooling, make the volume up to the mark with water (**Solution I**). Pipette 2 ml of Solution I into a 25 ml volumetric flask and make the volume up to the mark with water (**Solution II**). Pipette 4 ml from Solution I into a 10 ml volumetric flask and add 0.4 g of powdered skin to it, then make the volume up to the mark with water. Shake it for 30 min, then filter through paper, pipette 5 ml from the filtrate into a 25 ml volumetric flask and make the volume up to the mark with water. The next step should be performed precisely 120 s before the spectrophotometric measurement. Ask for help from the instructor.

- **Measurement 1:** Pipette 1.00 ml from the prepared solution into a beaker and add 5.00 ml of reagent (50 ml of 2% Na_2CO_3 dissolved in 0.1 M NaOH solution + 1 ml of 0.5% Cu(II) sulfate dissolved in 1% Na citrate solution) and 0.5 ml of 2-fold diluted Folin reagent to it, and immediately homogenize the solution by shaking. Measure the absorbance after 120 s at 750 nm (A_I). The blank solution is a mixture of 1.00 ml of water, 5.00 ml of reagent and 0.5 ml of 2-fold diluted Folin reagent, which you also need to prepare.
- **Measurement 2:** Pipette 1.00 ml from **Solution II** into a beaker, and add 5.00 ml of reagent and 0.5 ml of 2-fold diluted Folin reagent. Measure the absorbance as described above (A_{II}).

The tannin content of the drug powder can be determined by subtracting the polyphenol content which was not bound to the powdered skin (**Measurement 1**) from the total polyphenol content (**Measurement 2**). The following equation expresses the absorbance that corresponds to the tannin content of the drug:

$$A = A_{II} - A_I$$

The concentration of the tannins (in this case expressed as equivalents of pyrogallol) can be determined from the following equotation:

$$\% = \frac{100}{m} \times \frac{50 \times 25 \times 6.5}{100} \times \frac{A}{0.425} \times 0.000625$$

Uvae ursi folium
Bearberry leaves
Arctostaphylos uva-ursi L.
Ericaceae

3. TLC analysis of the methanolic extract and the sublimate of Uvae ursi folium

Extraction: Add 25 ml of methanol to 2 g of powdered drug in an Erlenmeyer flask and extract it for 5 min at room temperature. Filter the extract through filter paper into a porcelain dish and evaporate the filtrate to dryness on the water bath. Dissolve the dry residue in 10 ml of a methanol – water mixture (8:2), transfer the solution into a separating funnel, and extract it with 2 x 5 ml of ethyl acetate. Dry the combined organic phases with anhydrous Na_2SO_4, and then evaporate the solution to dryness in a porcelain dish. Dissolve the dry residue in 1 ml of methanol.
Sublimation: Sublimate the powdered drug over a flame onto a glass slide, and then wash it off with 1 ml of methanol.

TLC analysis
Sorbent: Silica gel

Spots:

Sublimate of Uvae ursi folium	20 μl
0.1% methanolic solution of hydroquinone	10 μl
Methanolic extract of Uvae ursi folium	20 μl

Solvent system:
n-hexane – acetone (1:1)

Detection:
Spray the TLC plate with a solution containing 1% Fe(III) chloride and 1% potassium ferricyanide ($K_3[Fe(CN)_6]$).

4. Tests for catechin derivatives and phenolic components of Uvae ursi folium

Catechin derivatives: Add a few drops of hydrochloric acid-containing solution of vanillin to the powdered drug.
Phenolic components: Add 10 ml of water to 0.5 g of drug and warm the mixture on the water bath for 5 min. After it has cooled, filter the extract into a test tube and add 1 drop of $FeCl_3$.

Write down your observations.

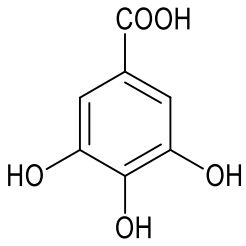
Theory 10

Tannins

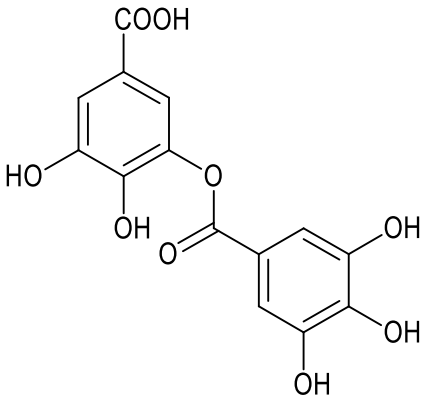
Tannins are polyphenolic secondary plant metabolites which are able to precipitate proteins from their solution, prevent their putrefaction and convert raw skin to leather. In plants, these compounds can usually be found in dead or dying cells and are believed to contribute to the protective function of barks and heartwoods: due to their ability to precipitate proteins, tannins can inhibit the functions of many enzymes, including those of plant pathogens. When applied to living tissues, this so-called astringent action forms the basis of the therapeutic use of tannins.

Physical and chemical properties:
Tannins are non-crystallisable compounds which form colloidal solutions with water; when tasted, these make a puckering, "dry-mouth" sensation similar to that caused by steeped black tea or red wine. Tannins are liable to self-condensation: they readily polymerize an in oxidative environment, including air. They precipitate solutions of gelatin, alkaloids and proteins; they form dark blue or greenish-black precipitates with iron salts and can also be precipitated by Cu^{2+}, Pb^{2+} or Sn^{2+}. Chemically, tannins can be divided into the following groups:

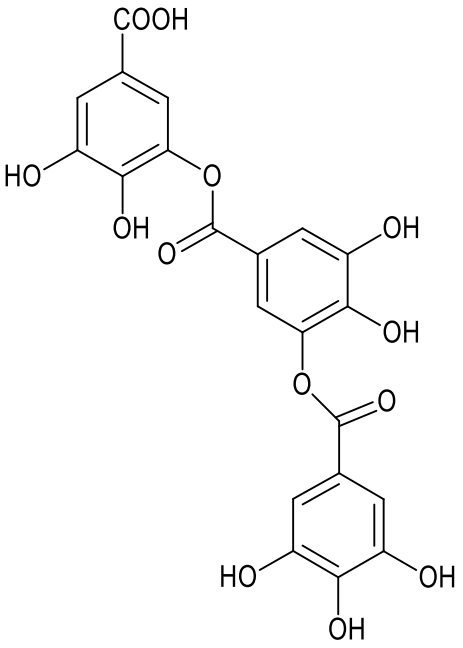
- **Hydrolysable tannins**: They can be hydrolysed by acids or enzymes.
    - ***Gallitannins***: They are formed from gallic acid and a sugar component (mostly glucose) containing ester-type bonds in the meta position, with usually 2 or 3, but at most 4 gallic acid moieties. They can be found, for example, in rhubarb and chestnut.



gallic acid

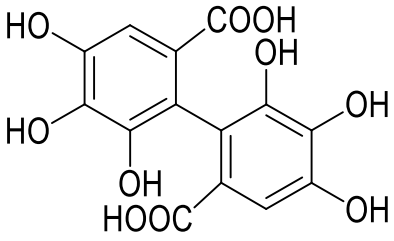


meta-digallic acid

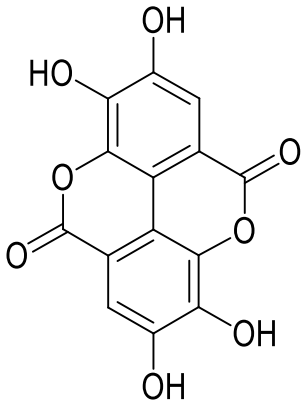


meta-trigallic acid

- ***Ellagitannins*** are formed from ellagic acid and a glucose molecule. Ellagic acid is originally not present in the drug, but is formed upon during the drying procedure. Two molecules of gallic acid are bound together with a C-C bond resulting in the formation of hexahydroxydiphenic acid, from which ellagic acid is formed by the elimination of 2 molecules of water. Such tannins can be found in eucalyptus and oak bark.

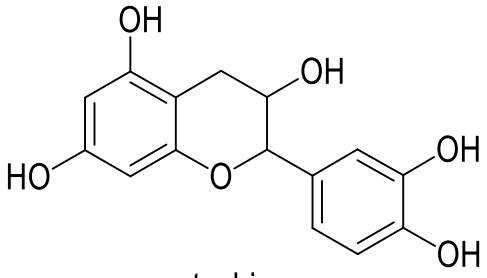


hexahydroxydiphenic acid



ellagic acid

- **Condensed, non-hydrolysable tannins (proanthocyanidins or catechin-type tannins)** are related to flavonoids expressing a “polymeric” flavan-3-ol structure. They are present in cinnamon, rhatany, cocoa, cola and tea.



catechine

- **Pseudotannins** are compounds with lower molecular weights. They are present in rhubarb, guarana, coffee and nux vomica.

Detection: Moisten the drug with 70% aqueous methanol and extract it with water by heating. Cool the mixture and filter the extract. Divide it into four equal volumes and add the following reagents to the fractions:
1% gelatin solution: precipitate formation
R-alcoholic Pb acetate: precipitate formation
1% quinine – HCl: precipitate formation (alkaloid)
1-2 drops R-$FeCl_3$: formation of a colourful complex

- bluish complex: hydrolysable tannins
- greenish complex: non-hydrolysable tannins

Uses: Because of their astringent property, tannins can be used to treat inflammations of various mucosal membranes (oral mucosa, gums, throat, stomach and the bowels). They precipitate the proteins on the surface of the inflamed mucosa, forming a protective layer on it. They have mild local anaesthetic, antibacterial and antifungal properties. Due to their effect on the intestinal mucosa, they are also used as constipating agents. Tannins can be used as antidotes in cases of heavy metal or alkaloid poisoning. Topically, they can act as styptics in mild injuries such as minor wounds and burns. Long-term oral administration of large doses of these compounds, however, is not advisable, due to the risk of liver toxicity.

Galla
Nut galls
Quercus infectoria Oliv.
Fagaceae

The galls are formed on the young twigs of dyer's oak, *Quercus infectoria*, as a result of the deposition of the eggs of an insect, *Cynips gallae tinctoriae*. The abnormal development of the tissue around the eggs is due to the activity of an enzyme, which stimulates the division of the plant cells. The galls have a high tannin content, probably due to a defensive reaction of the plant against the invader. Galla contains 40-70% of gallic acid derivatives.

Uses: As above.

Quercus cortex
Oak tree bark
Quercus robur L.
Quercus petraea L.
Fagaceae

The drug consists of the dried bark of the trees *Quercus robur* and *Q. petraea*. Quercus species are native to the northern hemisphere from the cold latitudes to tropical Asia and the Americas. The drug contains 8-20% of the catechin type of tannins and gallic acid derivatives.

Uses: As above.

Quantitative determination of tannins from Quercus cortex by spectrophotometry
The amount of tannins in the drug is quantified through a spectrophotometric determination of the polyphenol content. In orded to be able to measure tannins selectively over other polyphenols (e.g. flavonoids), two parallel measurements are necessary (**Measurement 1** and **Measurement 2**). In **Measurement 1**, the administered powdered skin binds all of the tannins in the solution, but does not interfere with other polyphenolic components. The measured absorbance (A_I) then refers to the concentration of other polyphenols. In **Measurement 2**, the absorbance (A_{II}) refers to the total polyphenol content of the solution including tannins and other polyphenolic compounds. By subtracting A_I from A_{II}, we obtain the absorbance relating to the polyphenols bound to proteins in Measurement 1, i.e. tannins: $A_{II} - A_I = A$.
The following equation can be utilized to calculate the percentage tannin content of the drug, expressed in pyrogallol equivalents.

$$\% = \frac{100}{m} \times \frac{50 \times 25 \times 6.5}{100} \times \frac{A}{A_0} \times m_0$$

A_0 = 0.425
m_0 = 0.000625 g
Dilution = 50 x 25 x 6.5

where A_0 is the absorbance of a solution containing an amount m_0 of pyrogallol.

Ratanhiae radix
Rhatany root
Krameria triandra R. and P.
Krameriaceae

The drug consists of the dried roots of a small shrub, *Krameria triandra*, native to Bolivia, Chile and Peru. The roots are almost straight, and emerge from a thick and knotty crown. The bark is dark reddish-brown and contains 8-15% of catechin-type tannins.

Uses: As above.

Agrimoniae herba
Common agrimony herb
Agrimonia eupatoria L.
Rosaceae

The drug consists of the dried herb of *Agrimonia eupatoria*, native to the whole of Europe. The plant is about 15-150 cm high; the leaves are serrate or crenulate, dark-green above and whitish-greyish beneath. The petals are golden-yellow in colour. It contains 4-10% of catechin-type tannins and flavonoids.

Uses: As above.

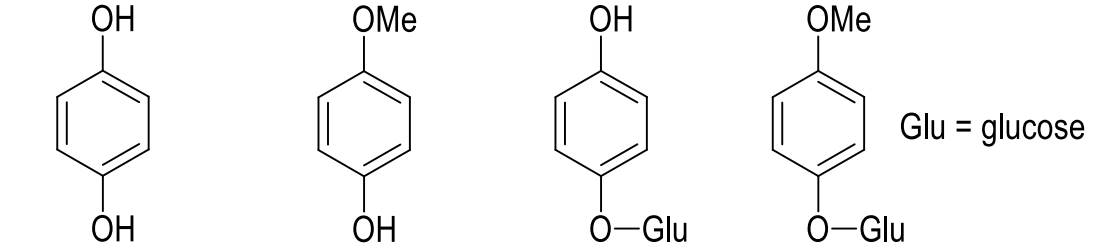
Drug with hydroquinone derivative content

Uvae ursi folium
Bearberry leaves
Arctostaphylos uva-ursi L.
Ericaceae

The drug consists of the leaves of *Arctostaphylos uva-ursi*. The shrub is native to the northern parts of Europe; its leaves are 12-30 mm long and 4-12 mm wide, obovate, dark-green and shining above, paler beneath. It contains 5-12% of hydroquinone derivatives (methylhydroquinone, arbutin, and methylarbutin) and 15-20% of catechin-type tannins.

Uses: The drug has antibacterial, disinfectant and diuretic effects within the urinary tract and it can be used in cases of bladder and renal inflammation. Arbutin is readily hydrolysed to glucose and hydroquinone after oral ingestion (on the action of the gastric acid and/or the glucosidase enzymes of the gut bacteria), and the absorbed hydroquinone is excreted into the urinary tract in the form of sulfate and glucuronide

conjugates. At alkaline pH, these conjugates release hydroquinone, which exerts its antibacterial effect. Since its activity depends on the alkaline pH of the urine, it is advisable for the patient not to eat too much meat or other acidifying foods during the treatment. As the drug is rich in tannins, its long-term use is not recommended because of the a risk of liver toxicity.



hydroquinone methylhydroquinone arbutin methylarbutin

TLC analysis of the methanolic extract and the sublimate of Uvae ursi folium

Extraction: Extraction with methanol yields several constituents, including tannins and hydroquinone derivatives, in the extract. The tannins are relatively more polar, which makes solven – solvent extraction suitable for the separation of hydroquinones from them (the ethyl acetate phase will be rich in hydroquinone derivatives and the aqueous methanolic phase in tannins).

Sublimation: Hydroquinone can sublimate, and direct heating of the drug will therefore result in the sublimation of these molecules (in the form of brownish vapour). The sublimate will condense on the cooler glass slide and the hydroquinone can be washed off with methanol.

Detection: The visualization on the TLC plate is based on the Prussian blue reaction. In this reaction, Fe^{3+} ion react with $[Fe(CN)_6]^{4+}$ ions forming a characteristic blue complex, called Prussian blue $Fe_4[Fe(CN)_6]_3$. The chromatogram is sprayed with a mixture of $FeCl_3$ and $K_3[Fe(CN)_6]$, which contains Fe^{3+} and $[Fe(CN)_6]^{3+}$ ions, which do not give a blue complex. However, hydroquinone has a reducing property and can reduce $[Fe(CN)_6]^{3+}$ to $[Fe(CN)_6]^{4+}$, which can participate in the Prussian blue reaction. As a result, the blue complex is formed at the spots on the TLC plate where hydroquinone is present.

Detection of other components

Catechin derivatives

See method in Practical 10. A reddish colour appears around the pieces of the drug.

Phenolic components

See method in Practical 10. A bluish-violet colour appears because of the formation of a Fe^{3+} complex (general tannin tests).

Test questions 10

Essay questions:

- What are tannins, which group of compounds do they belong to and in which forms do they occur in plants?
- Give the chemical classification of tannins.
- Give the physical and chemical properties and uses of tannins.
- Describe how we can detect tannins.
- How did we determine the tannin content of oak tree bark? Describe the process from the extraction to the calculation of the tannin content of the drug.
- In the TLC analysis of the extract and the sublimate of bearberry leaves, what did we use for extraction, how did we sublimate hydroquinone, how did we visualize the TLC plate and what could we conclude?
- How did we test for catechin derivatives and phenolic constituents of bearberry leaves? Explain this from the extraction to the observations.

Mechanisms, structures:

- Draw the key structures of the different types of tannins (gallic acid, meta-digallic acid, meta-trigallic acid, hexahydroxydiphenic acid, ellagic acid, and catechin).
- Draw the structures of hydroquinone derivatives (hydroquinone, methylhydroquinone, arbutin, and methylarbutin).

Classification (Latin name of the drug, species, and family) and uses of the following drugs:

- Nut galls, oak tree bark, rhatany root, common agrimony herb, and bearberry leaf

Practical 11

Drugs with triterpene saponin content, III

1. TLC analysis of ginsenosides (A-I) from Panax ginseng tea, capsule and alcoholic extract
2. Determination of unknown drug powders

Ginseng radix
Ginseng root
Panax ginseng C. A. Mey.
Araliaceae

1. TLC determination of the ginsenosides (A-I) from *Panax ginseng* tea, capsule and alcoholic extract

Extraction:
Tea bag: Cut open a tea bag and put the drug powder into an Erlenmeyer flask. Extract it with 2 x 20 ml of methanol on the water bath for 2 x 10 min. Combine the methanolic extracts in a porcelain dish and evaporate them to dryness on the water bath. Dissolve the dry residue in 5 ml of methanol, add 10 ml of acetone (a precipitate forms) and filter the mixture through paper. Use the filtrate for the TLC.
Capsule: Open the capsule and put the contents into an Erlenmeyer flask. Extract the contents with 20 ml of methanol on the water bath for 10 min. Filter the extract into a porcelain dish and evaporate it to dryness. Dissolve the dry residue in 1 ml of methanol for TLC.

TLC determination
Sorbent: Silica gel

Spots:

Methanolic extract of ginseng tea	10 μl
Methanolic extract of ginseng capsule	10 μl
Original alcoholic ginseng extract	10 μl

Solvent system:
Dichloromethane – methanol – water (70:50:10)

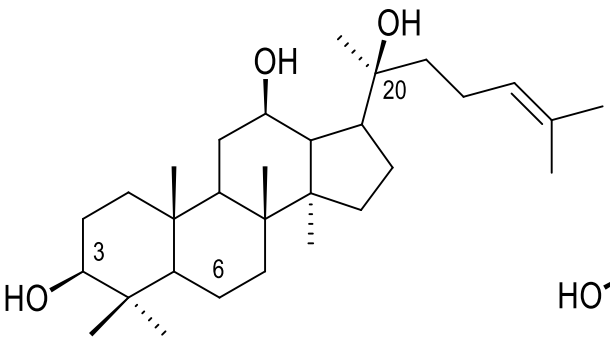
Detection:
Spray the TLC plate with vanillin – phosphoric acid and put it into a heating chamber at 120 ºC for 1-2 min.

Theory 11

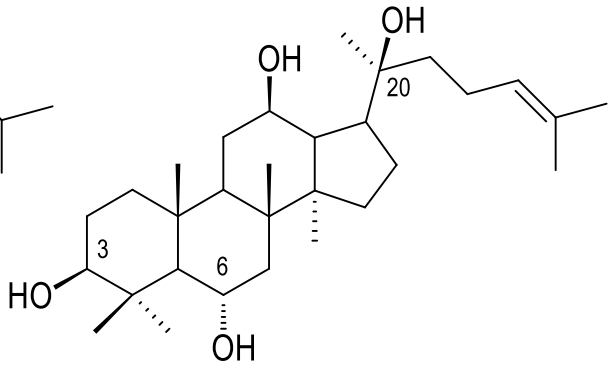
Ginseng radix
Ginseng root
Panax ginseng C. A. Mey.
Araliaceae

The drug consists of the dried root of *Panax ginseng*. Ginseng root has been part of traditional Eastern Asian Medicine for more than 2000 years. The plant is native to China and Korea, but, because of the intensive collection, the natural sources are constantly diminishing. Cultivation of the plant started some 800 years ago, which makes ginseng one of the oldest cultivated plants. It is a perennial plant about 60 cm high. Medicine uses the root of the plant, which must be at least 4 years old. The root is around 20 cm long and in most cases bears branches. Because of these branches, the root frequently has a "humanoid" shape and it was probably this similarity that led to the idea in Chinese medicine that ginseng can be used to strengthen the whole body. Ginseng root contains several triterpene glycosides, called ginsenosides after the plant. The aglycones of these ginsenosides are protopanaxadiol and protopanaxatriol. More than 20 ginsenosides are known at present, denoted by letters (e.g. ginsenoside R_0, R_a, R_b, R_c…) and differing from each other only in the sugar chains connected to C-3, C-6 and/or C-20. The drug also contains polysaccharides (panaxans and ginsenans) and polyacetylenes.

Uses: Ginseng is an adaptogen plant (*adaptogens are components which can increase or strengthen the defence mechanisms of the body against different stressors*). It has both CNS-strengthening and immunostimulant effects, and it is used to improve the physical and mental state in stressful periods or after severe illnesses. In animal experiments, ginseng increased the level of ACTH, a hypophyseal hormone responsible for controlling the steroid hormone production of the adrenal glands. It also has cholesterol and blood sugar level-decreasing and blood pressure-normalizing effects (*administration of ginseng causes decrease of a high and increase of a low blood pressure*). These effects can also be attributed to the general adaptogen properties of ginseng.



protopanaxadiol



protopanaxatriol

TLC analysis of the ginsenosides (A-I) from *Panax ginseng*-containing products

Extraction of the tea and capsule is similarly performed with methanol in both cases. Since the tea contains other constituents, the addition of acetone is necessary in order to precipitate polar contaminating components. Visualization with vanillin – phosphoric acid is widely used for the detection of ginsenosides, which appear as pink spots in each preparation. In the tea extract, components derived from the other plant constituents may disturb the observation, but the presence of ginsenosides can still be confirmed.

Test questions 11

Essay questions:

- In the TLC analysis of the ginsenosides from ginseng root-containing products, what did we use for extraction, how did we visualize the TLC plate and what could we conclude?

Mechanisms and structures:

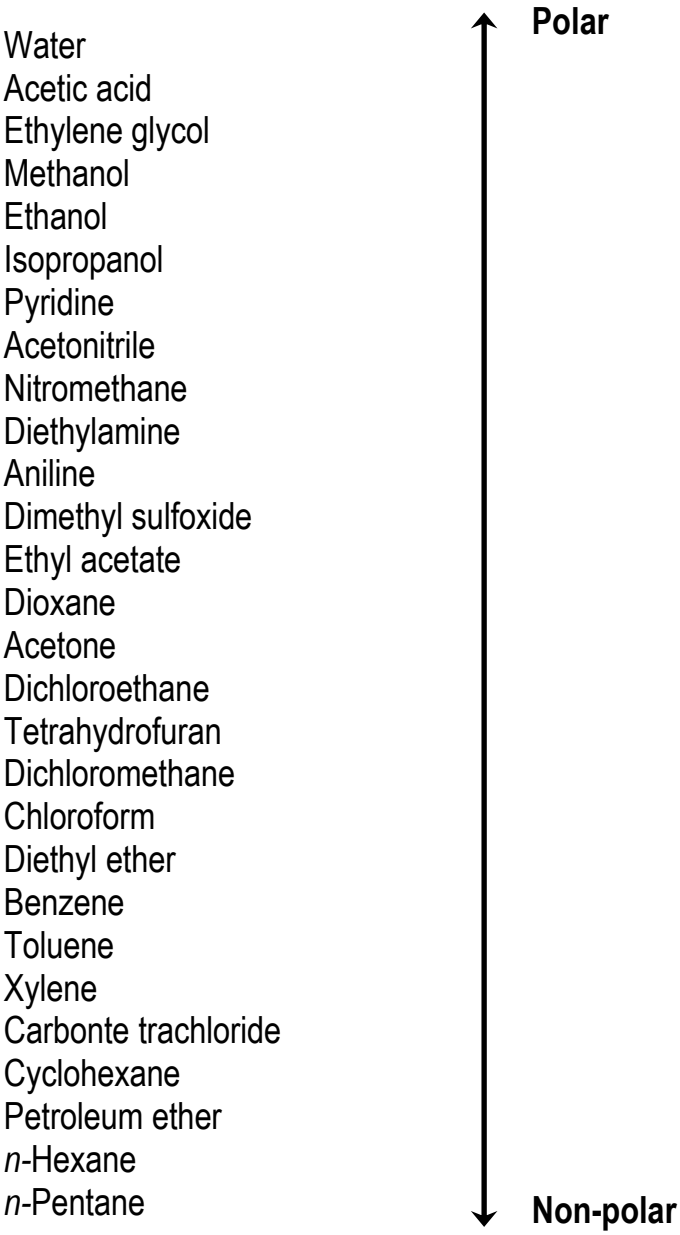
- Give examples of the aglycones of ginsenosides (protopanaxadiol and protopanaxatriol). To which position(s) of these triterpenes can sugar chains be connected?

Classification (Latin name of the drug, species, and family) and uses of the following drugs:

- Ginseng root

Appendix

Sequence of polarity of solvents



TLC visualizing reagents

Reagent	Preparation	Method	Colour	Compounds
Thymol + sulfuric acid	0.5 g of thymol in 95 ml of ethanol + 5 ml of 97% sulfuric acid	1. Spray the TLC plate 2. Heat for 5 min at 120°C	pink	sugars
Iodine + starch solution	Solid iodine is spread in a chromatographic tank	1. Place the TLC plate into a tank and expose it to iodine vapour 2. Dry the plate and spray it with starch solution	blue	compounds with conjugated double bonds (e.g. fixed oil)
Dragendorff	Solution A: 0.85 g of basic bismuth nitrate in 10 ml of glacial acetic acid and 40 ml of water under heating. Solution B: 8 g of potassium iodide in 30 ml of water. Stock solutions A and B are mixed	Spray the TLC plate	orange	alkaloids
van Urk	Dissolve 0.2 g of 4-dimethylamino-benzaldehyde in a mixture of 35 ml of water and 65 ml of *cc.* sulfuric acid then add 0.15 ml of a 10% aqueous iron(III) chloride solution.	1. Spray the TLC plate 2. Heat for 5 min at 105 °C	violet-blue	indole alkaloids, ergot alkaloids
Iodine + HCl	Solution A: 1 g of potassium iodide and 1 g of iodine in 100 ml ethanol. Solution B: 25 ml of 25% HCl with 25 ml of ethanol.	Spray the TLC plate with solution A, followed by solution B	blue-grey	purines
Vanillin + sulfuric acid	1 g of vanillin in 100 ml of ethanol then add 2 ml of cc. H_2SO_4.	1. Spray the TLC plate 2. Heat for 5 min at 120 °C	various colours	essential oils, triterpenes
EM reagent	5 g of p-dimethylamino-benzaldehyde dissolved in a mixture of 10% phosphoric acid and glacial acetic acid	Spray the TLC plate	blue	proazulenes
2,4-Dinitrophenyl hydrazine (DNPH)	0.1 g of DNPH in 100 ml of methanol, followed by the addition of 1 ml of 36% hydrochloric acid	Spray the TLC plate	orange-red	ketones, aldehydes
Kedde	5 ml of freshly prepared 3% ethanolic 3,5-dinitrobenzoic acid with 5 ml of 2M NaOH	1. Spray the TLC plate with Kedde reagent 2. Let it dry 3. Spray the TLC plate with KOH solution	pink-purple	cardenolides
Oxalic acid + boric acid	Mix 3 ml of oxalic acid and 9 ml of boric acid solution	1. Spray the TLC plate 2. Heat for 5 min at 120 °C	yellow	flavonoids
$AlCl_3$	Dissolve 0.5 g of $AlCl_3$ in 100 ml of ethanol	1. Spray the TLC plate 2. Check under 366 nm light	yellow fluorescence	flavonoids
Prussian blue	1% Fe(III) chloride + 1% potassium ferricyanide ($K_3[Fe(CN)_6]$) in 100 ml of water	Spray the TLC plate	blue	reducing components (e.g. hydroquinone)
Vanillin + phosphoric acid	1 g of vanillin in 100 ml of 50% phosphoric acid	1. Spray the TLC plate 2. Heat for 5 min at 120 °C	various colours	essential oils, triterpenes

Guidelines for the determination of unknown drug powders

The following information will guide you through the identification of unknown drug powders. It summarizes what was learned in each practical, but does not substitute it. First identify the colour of your unknown drug powder. Remember that colours are often very subjective and the following colours are therefore only approximations for guidance.

White powder

1. Add some water and I_2 to the powder
→ turns blue: starch
→ add HCl → bubble formation: **starch contaminated with $CaCO_3$**

2. Determine the shape of the starch particles under the microscope:

- **wheat starch**
- **potato starch**
- **maize starch**
- **mixed starch (potato and maize)**

Yellowish-white powder

1. Add some water to the powder and shake the mixture well. Check the consistency:
→ a gel-like solution: polysaccharide
→ Add I_2
→ turns yellow with small blue spots in it: **tragacanth gum**
→ turns yellow without blue spots in it: **acacia gum**

→ permanent foam formation → drugs with saponine content:

- **baby's-breath root**

Yellow powder

1. Add some water to the powder and shake the mixture well. Check the consistency:
→ permanent foam formation → drugs with saponin content
→ add 80% H_2SO_4 to the drug: it turns orange + add R-NaOH to the drug: it turns brown

- **liquorice root**

Yellowish-greenish-brown

1. Carry out a Bornträger test for anthranoids
→ if it is positive: **glossy buckthorn bark**

Green powder

1. Extract the drug with diluted H_2SO_4, filter the extract into a test-tube and carry out a general alkaloid reaction with one of the three general alkaloid reagents (Mayer, Dragendorff or Wagner) or a Vitali test. Always carry out a blank test (with diluted H_2SO_4 and the reagent used, in another test tube so as to be able to compare the colours at the end of the reaction).

→ if it is positive, clarify the drug on the water bath with R-NaOH, and then check the shape of the Ca-oxalate crystals under the microscope.

→ prism: **hyosciamus leaf**

→ cluster: **stramonium leaf**

2. If the drug is not positive for alkaloids, extract it with chloroform and carry out a Kedde, Baljet or Keller-Kiliani test in a test tube

→ if it is positive: **purple foxglove leaf**

3. If the drug does not contain digitaloids, carry out a Bornträger test for anthranoids

→ if it is positive: **senna leaf**

4. If the drug does not contain anthranoids, add a few drops of a hydrochloric acid-containing solution of vanillin to the powder of the drug

→ if a reddish colour appears: **bearberry leaves**

5. If the drug is none of the above:

→ clarify it on the water bath with R-NaOH and search for trichomes

→ star-shaped: **marshmallow leaf (Althaea)**

→ T-shaped: **absinthe herb**

Light (slightly yellowish)-brown

1. Carry out a Bornträger test for anthranoids:

→ if positive: **rhubarb root**

2. If negative for anthranoids, extract the drug with water on the water bath for 5 min, and then add 1-2 drops of R-$FeCl_3$ to check for tannins.

→ if positive: **nut gall**

Yellowish-brown

1. Check the powder: if it contains small black pieces:

→ carry out a Bornträger test for anthranoids:

→ if positive: **aloe**

Reddish-brown powder

1. Extract the drug with diluted H_2SO_4, filter the extract into a test tube and make a general alkaloid test with one of the three general alkaloid reagents (Mayer, Dragendorff or Wagner).

AND/OR

2. Carry out a Grahe probe

→ if positive: **cinchona bark**

3. If it is not positive, extract the drug with water on the water-bath for 5 min, and then add 1-2 drops of R-$FeCl_3$, alcoholic Pb acetate, 1% quinine – HCl or 1% gelatin solution to check for tannins.
→ if it is positive, check the colour and consistency of the precipitates: **rhatany root** or **oak tree bark**

Greyish-brown powder

1. Check whether the drug contains larger pieces or only fine powder:
→ small root-like pieces:
→ add a few drops of R-NH_4OH to the drug and extract it with chloroform for 5 min. Then filter the extract into a porcelain dish and evaporate it to dryness. Carry out a rubremetin and/or Frohde test.
→ If it is positive: **ipecacuanha root**
→ small stone-like pieces:
→ add a few drops of R-NH_4OH to the drug and extract it with chloroform for 5 min. Then filter the extract into a porcelain dish and evaporate it to dryness. Carry out a test for strychnine and/or brucine.
→ If it is positive: **nux vomica seed**
→ a fine powder:
→ make an aqueous solution and shake it well
→ if it forms a permanent foam: **cowslip root**

Examples of MTOs

1st semester, I. MTO from Practicals 1-6.

Test **10 points**

Simple choice

1. Which of the following components is not a furocoumarin?
   A. Bergapten
   B. Rutarin
   C. Ribalinium
   D. Rutamarin

2. Chose the monosaccharide units and types of glucosidic bonds that form amylopectin:
   A. α-D-glucose, 1-6 glucosidic bonds
   B. α-D-glucose, 1-4 glucosidic bonds
   C. β-D-glucose, 1-4 glucosidic bonds
   D. α-D-glucose, 1-4 and 1-6 glucosidic bonds
   E. α-D-glucose and β-D-glucose, 1-4 glucosidic bonds

3. In TLC chromatography, the mobile phase moves because of:
   A. gravity
   B. high pressure
   C. capillary action
   D. electric interaction

Multiple choice

4. The effectiveness of an extraction can be influenced by:
   A. the size of the drug grains/powder
   B. temperature
   C. pH
   D. the applied chromatographic methods
   E. the characteristics of the extracting solvent

5. What are the main characteristics of gums and mucilages?
   A. Their water-based solutions give a permanent foam after shaking
   B. They are mixtures of different homo-polysaccharides
   C. They can form precipitate with alcohol when dissolved in water-based solution
   D. They are produced spontaneously or after injury by higher plants

E. They can be precipitated with salts of heavy metals (e.g. Pb^{2+})

6. Characteristics of fixed oils:
   A. They are solid, semi-solid or liquid components
   B. They are always solid components
   C. They are hydrophilic components
   D. They are liable to rancidity, which can be detected with a HCl + ether solution of phloroglucinol
   E. They are ethers of alcohols and long-chain fatty acids.

7. What are the advantages of using two-dimensional thin-layer chromatography?
   A. A higher amount of sample can be tested than in conventional TLC
   B. It is relatively simple to perform
   C. It has a higher resolution than conventional TLC
   D. It can be used for preparative purposes
   E. It is a suitable technique for testing more than one plant extract in parallel

8. Which reagents are suitable for the identification of vitamin C?
   A. 0.01M iodine solution
   B. Behrens reagent
   C. H_2O_2 and 5 drops of 1% alcoholic benzidine solution
   D. Fehling's reagent
   E. $NaHCO_3$ + $FeSO_4$

Find the pair:

9. Compounds and their identifying reagents

A. Cellulose	1. Basic lead acetate (Pb acetate)
B. Starch	2. Iodine – zinc chloride solution
C. The Ca^{2+} salt of arabic acid	3. Acidified alcohol + ammonium oxalate
D. Arabin	4. H_2O_2 + alcoholic benzidine
E. Peroxidase enzyme	5. Iodine solution

10. Compounds and their TLC visualizing reagent

A. Rutamarine	1. Thymol – sulfuric acid
B. Monosaccharides	2. Iodine vapour and starch
C. Unsaturated fatty acids	3. 366 nm light
D. Arabinose	4. Thymol – sulfuric acid

Questions

1. Classify the different chromatographic methods and specify the mechanism of separation involved and the nature of the stationary phase. **15 points**
2. Describe and explain the mechanism of detection of rancidity. **10 points**
3. Describe the method used to isolate rutamarin from Rutae herba by preparative TLC. **5 points**
4. Drug classification. Give the **Latin names** of the species, family and drug of the following drugs. Describe their effects or common uses. **5 points**

   Common rue herb, tragacanth gum, marshmallow root, virgin castor oil, rose hip
5. Draw the structures of the following compounds: **5 points**

   furocoumarin skeleton, D-glucose, linamarin, cellulose, ricinolic acid

1st semester, II. MTO from Practicals 7-11

Test **10 points**

Simple choice

1. In most cases, alkaloids can be found in plants in the following form:
    A. esters with mineral acids
    B. salts with different acids derived from the plant
    C. free bases
    D. esters with different acids derived from the plant
    E. alkaloid hydrochlorides

2. What is the Mayer reagent?
    A. An acetic acid solution of potassium tetraiodobismuthate(III)
    B. An aqueous solution of potassium iodide + I_2
    C. An aqueous solution of potassium tetraiodomercuriate(II)
    D. A sulfuric acidic solution of ammonium molybdate
    E. A sulfuric acid solution of potassium dichromate

3. With which test can you identify the active components of Belladona?
    A. The marquis reaction
    B. The talleiochin reaction
    C. The administration of cc. HNO_3
    D. The grahe test
    E. The vitali test

Multiple choice

4. Identify the correct statements that correctly finish the following sentence: “Cinchonae cortex…”
A. …is used against malaria
B. …contains quinine and quinidine
C. …has an expectorant or a nausea/vomiting-inducing effect, depending on the dose
D. …contains alkaloids derived from tryptophane
E. …contains alkaloids with a tropane base

5. Which of the following statements concerning the non-water-soluble alkaloids of Secale cornutum are true?
    A. They are lysergic acid amides
    B. They are lysergic acid esters
    C. The can contract the uterus
    D. They have an ergoline skeleton
    E. Their synthetic derivatives have a blood pressure-decreasing effect

6. What type of compounds can be found in Theae folium?

A. Purine alkaloids
B. Alkaloids with a xanthene skeleton
C. Tannins (of catechin type)
D. Chlorogenic acid
E. Volatile oil

7. Which of the following compounds are opium alkaloids?

A. Thebaine
B. Thein
C. Papaverine
D. Meconic acid
E. Narcotin

8. Identify the correct statements that correctly finish the following sentence: “Capsici fructus…”

A. …contains pseudoalkaloids derived from phenylalanine
B. …is used topically in cases of hair loss
C. …is used internally to help digestion
D. …contains carotenoids, which can be extracted from the plant with aqueous solvents
E. contains protoalkaloids

Find the pair

9. Drugs and effect

A. papaverine	1. a cough suppressant
B. morphine	2. a spasmolytic
C. quinidine	3. a painkiller
D. quinine	4. an expectorant
E. codeine	5. against malaria
F emetine	6. an antiarrhythmic

10. Active agents and determining reagents

A. cephaelin	1. cc. formaldehyde + cc. H_2SO_4
B. morphine	2. ammonium molibdate + cc. H_2SO_4 (Frohde reagent)
C. ergotoxin	3. *p*-dimethylaminobenzaldehyde + H_2O_2
D. hyoscyamine	4. $K_2Cr_2O_7$ + 80% H_2SO_4
E. strychnine	5. cc. HNO_3 + methanol + KOH

Questions

1. Define alkaloids, and give their classification, their general chemical properties and the methods for their extraction. **10 points**
2. Describe and explain the mechanism of the rubremetin test. What kind of drug did we use, how did we extract it and what was the observation? **10 points**
3. Describe and explain the separation of morphine from the other opium alkaloids. **5 points**
4. How did we detect loganin (drug, extraction, and observation)? **5 points**
5. Drug classification. Give the **Latin names** of the species, family and drug of the following drugs. Describe their effects or common uses: **5 points**

   belladonna leaf, paprika fruit, opium, strychni semen, cocoa seed
6. Draw the structures of the following compounds: **5 points**

   lysergic acid, strychnine, codeine, quinine, theophylline

2nd semester, I. MTO from Practicals 1-5

Test **10 point**

Simple choice

1. In which chemical group can the compound lanatoside C be categorized?

A. Monoterpene
B. Sesquiterpene lactone
C. Triterpene steroid glycoside
D. Triterpene saponin
E. Triterpene steroid

2. Which of the followings have a triterpene skeleton?

A. lupane
B. menthane
C. germacrane
D. thujane
E. guaiane

3. Which of the following reagents are suitable for the detection of menthol on a TLC?

A. Iodine chamber + starch solution
B. EM reagent
C. Vanillin – sulfuric acid
D. 2,4-Dinitrophenylhydrazine
E. 3,5-Dinitrobenzoic acid + alcoholic KOH solution

Multiple choice

4. Which of the following statements are correct?

A. Gitaloxin is an aglycone
B. Digitoxin is an aglycone
C. Lanatoside B is a glycoside
D. Gitoxigenin and digitoxigenin differ only in their sugar moieties
E. Purpureaglycoside B and lanatoside B differ only in their sugar moieties

5. What are the common medical properties of sesquiterpene lactones?

A. Expectorants

B. Anti-inflammatory
C. Appetizers
D. Diuretics
E. Carminatives

6. Which of the following statements are true with respect to saponins?

A. Their skeleton can be a tetracyclic steroid
B. 1-12 sugar molecules can bind to them
C. The saponin glycosides are soluble in apolar solvents
D. Their sugar chain is never ramified
E. They can be detected with the Keller-Kiliani test

7. Which of the following statements are true with respect to cardenolides?

A. They can be detected with 3,5-dinitrobenzoic acid + alcoholic KOH solution
B. They have a five-membered lactone ring with one double bond attached to C-17
C. They have a six-membered lactone ring with two double bonds attached to C-17
D. They have a pentacyclic skeleton
E. They can be detected with the Keller-Kiliani, Baljet and Kedde tests

8. Valerianae radix contains:

A. valtrate
B. primverin
C. foliamentin
D. baldrinal
E. isovaleric acid

Find the pair

9. The drug and its effect:

A. Absinthii herba	1. an appetizer
B. Saponariae albae radix	2. a heart strengthener
C. Carvi fructus	3. a sedative and hypnotic
D. Valerianae radix	4. a carminative
E. Strophanthi semen	5. an expectorant

10. The drug and its detecting test:

A. Digitalis purpureae folium	1. glacial acetic acid and cc. HCl
B. Valerianae radix	2. glacial acetic acid and cc. H_2SO_4

C. Liquiritiae radix	3. EP reagent + heating
D. Millefolii herba	4. Fe(III) chloride, glacial acetic acid and cc. H_2SO_4

Questions

1. Define terpenoids. Describe how they are formed, where can they be found, and how they can be classified? **5 points**
2. Define digitalis glycosides. Describe their basic skeleton and important functional groups, their common chemical properties and their pharmacological effects. Specify which types of substituents can be found on the skeleton and on which carbon atom. Indicate the connection types between the rings, and draw the two main types. Describe the characteristics of their sugar chains. **15 points**
3. Describe and draw a schematic representation of the EP test procedure. What is it used for? **10 points**
4. Drug classification. Write the **Latin names** of the species, family and drug of the following drugs. Describe their effects or common uses: **5 points**

caraway seed, absinthe wormwood herb, liquorice root, cassia cinnamon bark, oleander leaf

5. Draw the structures of the following compounds: **5 points**

thujone, farnesan, matricin, lupane skeleton, gitaloxin

2nd semester, II. MTO from Practicals 6-11

Test **10 point**

Simple choice

1. To which class of compounds does arbutin belong?

A. methylhydroquinone
B. hydroquinone-O-monoglycoside
C. methylhydroquinone-O-monorhamnoside
D. hydroquinone diglycoside
E. hydroquinone

2. How can hypericin be classified?

A. a flavone-glycoside
B. a flavonol-glycoside
C. an anthraquinone-O-glycoside
D. a naphthodianthrone
E. a heterodianthrone

3. How can procyanidines be detected?

A. The *n*-butanolic solution of their acidic extract turns red.
B. A yellow colour appears after their reaction with HNO_3 at room temperature.
C. Their alkaline solution turns green during heating.
D. Their chloroform extract turns red, orange or yellow when mixed with diluted ammonia.
E. A blue or green colour is visible after reaction with $AlCl_3$.

Multiple choice

4. Flavonoids can be detected by using:

A. saturated borax solution
B. $AlCl_3$ + HCl + UV light.
C. R-$FeCl_3$
D. basic Pb acetate
E. quinine – HCl solution

5. Which of the following are products of the acidic hydrolysis of procyanidins?

A. catechin/epicatechin
B. flavanediol
C. chalcone
D. anthocyanidin
E. flavonol

6. Which of the followings are hydrolysable tannins?

A. gallitannins
B. catechin type tannins
C. ellagitannins
D. gallic acid derivatives
E. pseudotannins

7. Which of the following are flavonoid-containing drugs?

A. linden flower
B. St. John's wort herb
C. lavender flower
D. cowslip root
E. bitter orange epicarp

8. Choose the statements that can correctly complete the following sentence: "Bitter orange peel is used…"

A. …in cases of dyspepsia
B. …as an appetizer
C. …as a diuretic
D. …as a diaphoretic
E. …in cases of a high blood lipid level

Find the pair

9. The type and the components:

A. dianthrone glycoside	1. frangulaemodin
B. anthraquinone diglycoside	2. glucofrangulin B
C. anthraquinone O-glycoside	3. frangulin A
D. aglycone	4. sennoside C

10. The drug and its effect:

A. Common hawthorn flower, fruit	1. diaphoretic

B. Senna pods	2. cardioprotective
C. Nut gall	3. laxative
D. Common elderberry flower	4. astringent
E. St. John's wort	5. urinary disinfectant
F. Bearberry leaf	6. antidepressive

Questions

1. From which drug and how can tannins be extracted? Indicate the reagents to use. How can tannins be detected? **5 points**
2. Define anthranoid derivatives; describe their characteristic features: chemical classification, common properties, common use and method of detection. **15 points**
3. In the TLC analysis of linden leaf, what did we use for extraction, how did we visualize the TLC plate and what could we conclude? **5 points**
4. Draw the complex formed during the detection of flavonoids with an oxalic acid + boric acid mixture. **5 points**
5. Drug classification. Write the **Latin names** of the species, family and drug of the following drugs. Describe their effects or common uses: **5 points**

rhubarb root, common hawthorn flower, hyperici herba, nut galls, pot marigold flower

6. Draw the structures of the following compounds: **5 points**

frangulaemodin, rutin, procyanidin B2, ellagic acid, and protopanaxatriol

Classification, constituents and uses of drugs for *in toto* drug determination

1st semester

Acaciae gummi
Acacia gum, *Acacia senegal* (Mimosaceae)
Constituents: polysaccharides
Uses: a vehicle in pharmaceutical technology

Agar
Agar, *Gelidium species* (Rhodophyceae)
Constituents: polysaccharides
Uses: in microbiology (a substrate)

Althaeae folium
Marshmallow leaf, *Althaea officinalis* (Malvaceae)
Constituents: polysaccharides, flavonoids
Uses: an immunostimulant, against colds, and coughs

Belladonnae folium
Deadly nightshade / Belladonna leaf, *Atropa belladonna* (Solanaceae)
Constituents: alkaloids (L/D-hyoscyamine = atropine)
Uses: a parasympatholytic

Cacao semen
Cacao seed, *Theobroma cacao* (Sterculiaceae)
Constituents: alkaloids (xanthine derivatives – theobromine, theophylline, small amounts of caffeine), fixed oil (cocoa butter)
Uses: an industrial source of cocoa butter (stock for suppositories)

Cinchonae cortex
Cinchona bark, *Cinchona pubescens* syn. *C. succirubra* (Rubiaceae)
Constituents: alkaloids (quinine, quinidine)
Uses: quinine: an antimalarial agent; quinidine: an antiarrhythmic effect

Coffeae semen
Coffee seed, *Coffea arabica*, (Rubiaceae)
Constituents: alkaloids (xanthine derivatives – caffeine, theophylline, and theobromine)
Uses: a CNS stimulant

Colae semen
Cola seed, *Cola acuminata,* (Sterculiaceae)
Constituents: alkaloids (xanthine derivatives – coffeine, theophylline, theobromine)
Uses: CNS stimulant

Conii fructus
Hemlock seed/fruit, *Conium maculatum* (Apiaceae)
Constituents: alkaloids (coniin), fixed oil, no volatile oil
Poisonous! Looks like anise seed, but without its smell.

Gossypii lana
Cotton wool, *Gossypium hyrsutum* (Malvaceae)
Constituents: almost 100% cellulose
Uses: for bandages

Hyoscyami folium et semen
Hyoscyamus leaf and seed, *Hyoscyamus niger* (Solanaceae)
Constituents: alkaloids (L/D-hyoscyamine = atropine)
Uses: a parasympatholytic

Ipecacuanhae radix
Ipecacuanha root, *Cephaëlis ipecacuanha, Cephaëlis acuminata* (Rubiaceae)
Constituents: alkaloids (emetine and cepheline)
Uses: in small doses an expectorant, in higher doses an emetic

Lini semen
Linseed, *Linum usitatissimum* (Linaceae)
Constituents: polysaccharides, fixed oil and cyanogenic glycosides
Uses: an immunostimulant (polysaccharides) and in dermatological applications (oil)

Ricini semen
Castor bean, *Ricinus communis* (Euphorbiaceae)
Constituents: fixed oil (rich in ricinolic acid), toxic protein (ricine), and alkaloid (ricinine)
Uses: a laxative (only the cold-expressed oil can be used; the seed is highly toxic!)

Rosae pseudo-fructus
Rose hip, *Rosa canina* (Rosaceae)
Constituents: vitamins C and A, and carotinoids
Uses: a vitamin supplement

Secale cornutum
Ergot, *Claviceps purpurea* (Clavicipitaceae)
Constituents: ergot alkaloids (e.g. ergotamine and ergometrine)

Uses: an industrial source of ergot alkaloids; ergometrine: an oxytocin-like effect, ergotamine: a sympatholytic and vasoconstrictor

Stramonii folium
Stramonium leaf, *Datura stramonium* (Solanaceae)
Constituents: alkaloids (L/D-hyoscyamine = atropine)
Uses: a parasympatholytic

Strychni semen
Strychnos seed, *Strychnos nux-vomica* (Loganiaceae)
Constituents: alkaloids (strychnine and brucine)
Uses: a tonic on skeletal muscles; in higher doses it causes strong spasms (highly toxic!)

Theae folium
Tea leaf, *Camellia sinensis* (Theaceae)
Constituents: alkaloids (theophylline, caffeine and theobromine)
Uses: a CNS-stimulant effect

Tragacantha
Tragacant gum, *Astragalus gummifer* (Fabaceae)
Constituents: polysaccharides
Uses: a vehicle in pharmaceutical technology

2nd semester

Absinthii herba
Absinthe herb, *Artemisia absinthium* (Asteraceae)
Constituents: volatile oil (mono- and sesquiterpenes, a high amount of thujon), and bitter compounds (sesquiterpenes, absinthine, and artabsine)
Uses: an appetizer, a choleretic, and against gastric disorders
Thujone is toxic, and highly abortive in pregnant women!

Aloe capensis
Aloes, *Aloe capensis* (Liliaceae)
Constituents: anthraquinones
Uses: a laxative

Anisi fructus
Anise seed/fruit, *Pimpinella anisum* (Apiaceae)
Constituents: volatile oil (monoterpenes, *trans*-anethol and anisaldehyde)
Uses: an expectorant, spasmolytic, antibacterial and spice

Aurantii amari epi et mesocarpium
Orange peel, *Citrus aurantium ssp. amara*, (Rutaceae)
Constituents: bitters, flavonoids and volatile oil
Uses: an appetizer and against gastric disorders

Calendulae flos
Marigold flower, *Calendula officinalis* (Asteraceae)
Constituents: triterpene saponins and flavonoids
Uses: an anti-inflammatory and for treating wounds

Carvi fructus
Caraway seeds/fruit, *Carum carvi* (Apiaceae)
Constituents: volatile oil (monoterpenes, mainly carvone and limonene)
Uses: a spasmolytic, carminative, antibacterial and spice

Caryophylli flos
Clove flower, *Syzygium aromaticum* (Myrtaceae)
Constituents: volatile oil (phenyl propane derivatives, mainly eugenol)
Uses: an antibacterial, mild local anaesthetic and spice

Cinnamomi cassiae cortex
Cassia cinnamon, *Cinnamomum cassia* (Lauraceae)
Constituents: volatile oil (phenyl propane derivatives, mainly cinnamic aldehyde)
Uses: an antibacterial, appetizer and spice

Coriandri fructus
Coriander seed/fruit, *Coriandrum sativum* (Apiaceae)
Constituents: volatile oil (monoterpenes, mainly linalool)
Uses: an appetizer, antibacterial and spice

Crataegi folium cum flore
Hawthorn leaf and flower *Crataegus monogyna, Crataegus laevigata* (Rosaceae)
oligomeric Constituents: procyanidins
Uses: a mild cardiotonic

Digitalis purpureae folium
Purple foxglove leaf, *Digitalis purpurea* (Scrophulariaceae)
Constituents: cardiotonic steroid glycosides
Uses: against heart insufficiency (not used in modern phytotherapy; an industrial source of digitalis glycosides)

Foeniculi dulcis fructus
Fennel seed/fruit, *Foeniculum vulgare* subsp. *vulgare* var. *dulce* (Apiaceae)

Constituents: volatile oil (monoterpenes)
Uses: a spasmolytic and expectorant, and against gastric disorders

Frangulae cortex
Glossy buckthorn bark, *Rhamnus frangula* (Rhamnaceae)
Constituents: anthraquinones
Uses: a laxative

Galla
Galla, *Quercus infectoria* (Fagaceae)
Constituents: tannins
Uses: an astringent

Hippocastani semen
Chesnut seed, *Aesculus hippocastanum* (Hippocastanaceae)
Constituents: triterpene saponins (aescin)
Uses: a veno-protective, against oedema and for treating varicose veins

Hyperici herba
St. John's wort herb, *Hypericum perforatum* (Hypericaceae)
Constituents: anthraquinone derivatives (hypericin) and flavonoids (hyperoside)
Uses: against mild and medium depression

Lavandulae flos
Lavender flower, *Lavandula angustifolia* (Lamiaceae)
Constituents: volatile oil (monoterpenes, mainly linalyl acetate and linalool)
Uses: against nervous sleeplessness and nervous gastrointestinal problems, and an antibacterial

Liquiritiae radix
Licorice root, *Glycyrrhiza glabra* (Fabaceae)
Constituents: triterpene saponins (glycyrrhyzine)
Uses: an expectorant and a sweetener, and against peptic ulcer

Matricariae flos
Chamomile flower, *Matricaria recutita* (Asteraceae)
Constituents: volatile oil (sesquiterpenes, mainly α-bizabolole, chamazulene – a metabolite of matricin), sesquiterpene lactones (proazulenes, e.g. matricin), polyacetylenes, flavonoids and polysaccharides
Uses: an antibacterial, spasmolytic and anti-inflammatory

Millefolii herba

Achillea herb, *Achillea millefolium* (Asteraceae)
Constituents: volatile oil (mono- and sesquiterpenes), sesquiterpene lactones (proazulenes) and flavonoids
Uses: an anti-inflammatory, spasmolytic and antibacterial

Primulae radix
Primrose root, *Primula veris,* (Primulaceae)
Constituents: triterpene saponins
Uses: an expectorant

Quercus cortex
Oak bark, *Quercus robur*, *Quercus petraea* (Fagaceae)
Constituents: tannins
Uses: an astringent

Rhei radix
Rhubarb root, *Rheum palmatum*, *Rheum officinale* (Polygonaceae)
Constituents: anthraquinones
Uses: a laxative

Sambuci flos
Elderberry flower, *Sambucus nigra* (Caprifoliaceae)
Constituents: flavonoids and polysaccharides
Uses: a diaphoretic and against colds, coughs and bronchitis

Saponariae albae radix
Bany's breath root, *Gypsophila paniculata* (Caryophyllaceae)
Constituents: triterpene saponins
Uses: an expectorant and diuretic

Sennae folium
Senna leaf, *Cassia senna*, *Cassia angustifolia* (Caesalpiniaceae)
Constituents: anthraquinones
Uses: a laxative

Tiliae flos
Linden flower, *Tilia cordata, Tilia platyphyllos* (Tiliaceae)
Constituents: flavonoids and polysaccharides
Uses: a diaphoretic and against colds, coughs and bronchitis

Uvae ursi folium
Bearberry leaf, *Arctostaphylos uva-ursi* (Ericaceae)
Constituents: hydroquinone derivatives

Uses: a disinfectant of the urinary tract

Valerianae radix
Valerian root, *Valeriana officinalis* (Valerianaceae)
Constituents: epoxy-iridoid derivatives (valepotriates) and volatile oil (monoterpenes)
Uses: a mild sedative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