UNIVERSITY OF VETERINARY AND PHARMACEUTICAL SCIENCES BRNO

Faculty of Pharmacy

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Phytochemistry

Instructions for laboratory practice

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1. Isolation of content compounds – extraction, purification

Content compounds in plants:

- Primary metabolites (carbohydrates, aminoacids, purins and pyrimidins of nucleic acids, fatty acids, lipids, plant pigments)
- Secondary metabolites (terpenoids, nitric compounds, phenolics, acetic acid derivatives)

Content compounds division:

- Major
- Minor
- Ballast (chlorophyll, celulose, lignin)

Content compounds ar attached to plant material:

- Chemically
- Physically

Methods for obtaing content compounds:

EXTRACTION

Method using two immiscible solvents occuring in various state (solid/liquid; liquid/liquid)

Types of media for extraction:

- Liquid
- Gas
- Solid

Liquid extractions:

- Total
- Selective

Types of liquid extraction according to process

- Periodical
    - Maceration
    - Digestion
        - Infusion
        - Concoction
- Semicontinuous
- Continuous

MICRO-SUBLIMATION

The change of solids into gases followed by the desublimation is the principle of microsublimation.

PURIFICATION

Proper method of extraction leads to acquisition of content compounds in certain state of purity. For the further purification we can use these methods

- Liquid-liquid extraction
- Lyofilization
- Precipitation
- Crystallisation

Excercise No. 1:

Preparation of plant material for phytochemical analysis, maceration

The easiest method for obtaining content compounds is maceration. Main advantages of this method are simplicity of execution, cheapness (instrumental and financial), efectivness and selectivity.

Maceration is performed in dark, well-closed vessels filled with plant material and suitable solvent for 24 hours under normal conditions (temperature, pressure).

The aim of this practice is to perform maceration with *Cudrania tricuspidata* wood. The crude extract will be then used for the further excercises.

Procedure:

Fill in the vessel with approx. 100 g of dried wood of *Cudrania tricuspidata* and wash them over with 500-1000 ml of ethanol (the plant material must be fully submerged) and mix with glass stick. Close and label the vessel and leave till next laboratory practice.

Scheme:

Conclusion:

Excercise No. 2:

Isolation of plant pigments from *Urtica dioica* by Soxhlet extraction

Urtica dioica (Urticaceae), stinking nettle, contains a high amount of plant pigments in leaves, especially chlorophyll, carotenoids, flavonoids and xanthophylls. The aim of this excercise is to isolate these pigments with acetone. Later on, the pigments will be identified using thin layer chromatography (TLC) and isolated using column chromatography (CC).

Procedure:

10 g of fresh plant leaves are cut and extracted in 300 ml of acetone in Soxhlet apparatus for 4 hours. After that, the extract is dried used anhydrous sodium sulphate, filtered and the solvent is removed under vacuum to a volume of approx. 10 ml.

Scheme of Soxhlet apparatus:

Conclusion:

Excercise No. 3:

Isolation of compounds from *Species urologicae*

Species urologicae is a medicinal plant mixture in the form of tea with urinary disinfection and antiseptic properties. Methanol is used for the extraction to obtain phenolic glycosides and flavonoids.

Procedure:

1g of the tea mixture is extracted by boiling for 15 minutes with 10 ml of water and 10 ml of methanol under reflux in a water bath. The mixture is then filtered immediately through a cotton wool. Once cooled, 5 ml of lead acetate solution is added and the solution is then filtered again (using a filter paper). The filtrate is collected for further use.

Scheme:

Conclusion:

Excercise No. 4:

Micro-sublimation of caffeine

Cautious heating of coffee leads to the sublimation of caffeine at the temperature of 140-150 ºC. Caffeine can be observed as small colorless needles under the microscope.

Procedure:

Approx. 0,1 g of the powdered test drug (coffee) is placed on the middle of a watch glass with a diameter of 7 cm. This glass is covered with a glass plate. The margins of the glass plate should exceed those of the watch glass. The upper glass is cooled (using a small beaker filled with cold water) to improve the condensation of caffeine. The system is heated for approx. 15 minutes. The upper glass is then removed, left to cool and then examined using the microscope.

Scheme of apparatus:

Scheme of caffeine crystals:

Conclusion:

Excercise No. 5:

Purification of *Cudrania tricuspidata* extract by liquid-liquid extraction

Liquid liquid extraction or shaking out is a method where the limited miscibility of two solvents is used. Solvents are chosen from the opposite sides of eluotropic line. The sample dissolved in one phase is placed into separatory funnel and the second immiscible solvent is added (the ratio of solvents is usually 1:1). The main principle of the shaking out is the diffusion of dissolved compounds between two immiscible solvents and the set of equilibrium concentration according to the solubility of the substances in solvents. After separation process, the individual phases are evaporated on a rotary evaporator.

Procedure:

Crude extract is dissolved in 200 ml of 80 % methanol. 100 ml of hexane is added and the mixture is placed into separatory funnel where two separate layers can be observed. The separatory funnel is removed from the rack and is shaked carefully while the vapor is continuously released through the tap. Place back in the rack, let settle down and then separate both layers into flasks and evaporate using rotavapor. Methanolic portion is then dissolved in 200 ml of chloroform and 200 ml of water and the shaking out process is repeated. Place back in the rack, let settle down, separate the chloroform layer and evaporate on rotary evaporator. The residual water is mixed with 200 ml of ethylacetate, similarly shaked out and both layers are separated into flasks. Ethylacetate portion is evaporated using rotary evaporator, water is removed by lyophilization. Residues will be used for chromatographic separation in the following practices.

Scheme:

Conclusion:

2. Chromatographic analysis and separation of plant extracts

Analytical method using two immiscible phases (mobile and stationary) for the separation of the molecules of analyzed sample. Each molecule has different affinity to individual phases.

- Mobile phase
- Stationary phase

Chromatographic methods can be divided into groups according to these criteria:

1) According to the character of stationary phase (SP)
    - Stationary phase is flat
    - Stationary phase is placed in chromatographic column

2) According to the character of mobile phase (MP)
    - Liquid
    - Gas

3) According to the forces causing the separation between SP a MP
    - Adsorptive
    - Distributive
    - Ion exchange
    - Exclusive
    - Affinity

4) According to supposed utilization
    - Analytical
    - Preparative

TLC: thin layer chromatography. Widespread in secondary metabolites analysis for the separation of non-volatile mixtures. Performed in planar arrangement on a sheet of glas, plastic or aluminium foil, which is coated with a thin layer of adsorbent material (stationary phase), usually silica gel, aluminium oxide (alumina) or cellulose.

To quantify the results we use the retardation factor (Rf)

Retardation factor:

Dimensionless, characteristic for each compound, changes depending on the exact conditions of the mobile and stationary phase

Calculation:

The distance traveled by the substance is divided by the total distance traveled by the mobile phase.

Methods of detection TLC:

- Non-destructive
- Destructive
  - Non-specific
  - Specific

CC: column chromatography. Type of chromatography using a column (usualy glass) filled with stationary phase. For the wet method, a slurry is prepared of the eluent (mobile phase) with the stationary phase powder and then carefully poured into the column. The molecules of the sample are divided according to the affinity to each phase.

HPLC: high performance liquid chromatography is a technique in analytical chemistry used to separate, identify and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

Excercise No. 6:

TLC of *Species urologicae* extract– identification of arbutin

Tested solution: The extract of *Species urologicae* prepared in Excercise No.2

Standart solution: Methanolic solution of arbutin (2 mg/10 ml)

Mobile phase: Ethyl acetate: methanol: H_2O (100:17:13)

Detection I: Diazotized sulfanilic acid (freshly prepared – to 5 ml of sulfanilic acid add sodium nitrite until pale yellow solution appears)

Detection II: Ethanolic solution of KOH

Procedure:

Apply cca 10 µl of the standart and 10 µl of the sample solution separately on the TLC plate. Develop the TLC plate to a distance of approx. 10 cm. Aftery drying with a hair dryer, apply detection I and II consequently by spraying. Observe red spots located at the top of the TLC plate (=arbutin)

Calculate the Rf values of standard and sample spots.

Calculation of retardation factor of the standard and sample solution:

Conclusion:

Excercise No. 7:

TLC of *Urtica dioica* extract – plant pigments identification

Tested solution: Extract of *Urtica dioica* prepared in Excercise No.2

Mobile phase: Diethyl ether: petroleum ether (1:1)

Procedure:

Apply a suitable amount of the sample solution on the TLC plate (the application must be well visible as a spot on a plate). Develop the TLC plate to a distance of approx. 10 cm. After drying at room temperature, separated spots of plant pigments are visible using UV/Vis lamp.

R_F = 1,0	Carotenoids (α-,β-carotene)	yellow
R_F = 0,48	Pheophytin	green-brown
R_F = 0,35	Chlorophyll A	green-blue
R_F = 0,20	Chlorophyll B	green-yellow
R_F = 0,11-0	Xanthophylls	bright green, yellow

Calculate the retardation factor values of spots.

Calculation of Rf values of sample spots:

Conclusion:

Excercise No. 8:

Preparative TLC of *Paulownia tomentosa* extract– isolation of flavonoids

Chromatographic methods can be used for both analytical and preparative purposes. The analytical TLC is used for quantification or identification of the constituents (comparison with standart solution), the aim of preparative TLC is to isolate the entity from the mixture.

The aim of this practice is to isolate flavonoid constituents from the mixture (fraction obtained by CC separation) using preparative TLC. Isolated compounds will be used for further HPLC analysis.

Tested solution: 10 mg of fraction obtained by CC separation, diluted in 0,5 ml of methanol

Mobile phase: Chloroform: ethyl acetate (9:1)

Procedure:

Apply the sample solution as a „strip“ on the start of the TLC plate using pasteur pipette. After drying, put the TLC plate into a beaker with methanol and develop for a while to settle the strip of the sample at the start. Dry the plate again and then develop to a distance of approx. 10 cm. After drying in room temperature, detect by using UV/Vis lamp and mark the separated strips with a crayon. Scratch the defined area of the most visible strip using the kopist and transfer the silica to the eppendorf with 0,5 ml of methanol. After centrifugation transfer the liquid with care to the vial with insert and analyze using HPLC.

Conclusion:

Excercise No. 9:

Isolation of carotenoid pigments from *Urticae folium* extract using column chromatography (CC)

Extract from Folium urticae dioicae contains chlorophyll, which is a mixture of two compounds: blue-green chlorophyll A nad bright green chlorophyll B. Furthermore, extract contains yellow pigments – xanthophylls and carotenoids. Single pigments can be separated using column chromatography on silica gel.

The task of this excercise is to isolate yellow carotenoid pigments using column chromatography. Isolated pigments will be used for further spectrometric analysis.

Tested solution: Extract *Urticae folium* previously prepared in excercise No.2

Stationary phase: silica gel

Mobile phase: Diethyl ether : petroleum ether (1:1)

Procedure:

Column preparation:

Close the tap of the column, use a piece of cotton wool to close the conus of column. Fill column with approx. 10 mL of mobile phase to replace air from the cotton wool. Put approx 30 g (60 mL) of silica gel into a beaker and add approx. 50 mL of mobile phase to make a bubble-less slurry. Then our the suspension carefully without bubbling into the column. The particles of suspension should homogenously settle. Place carefully a disk of filtration paper on the top of the column (the paper should have the same diameter as the diameter of the column).

Sample preparation:

20g of fresh leaves were extracted in acetone in Soxhlet apparatus for 4 hours. Extract was dried using anhydrous natrium sulphate, filtered and the volume was reduced into 10 mL.

Sample application:

Open the column tap and let the level of mobile phase to reach the top of the sorbent and close the tap. Draw approx. 0,2 mL of sample solution into pipette or pasteur pipette and apply carefully on the top and let it soak into the sorbent. Use 1 mL of mobile phase to overlay the top and let it soak into the sorbent again. Repeat this twice while the tap column is open and closed again. Be aware of drying of the surface of the sorbent. Carefully fill the column with the mobile phase and open the valve to flow rate of mobile phase approx. 40 drops/min.

Isolation of carotenoid pigments:

Mobile phase is gathered into test-tubes as 10 mL fractions. Carotenoid pigments move with the top of the mobile phase, therefor we can combine several fractions at the beginning into

beaker and re-use them as a mobile phase again. At the moment when the yellow band moves to the end of the column (3 cm) we can start with the collection of the fractions containing carotenoids. Purity of isolates can be checked via TLC on silica, mobile phase diethyl ether-petroleum ether 1:1.

Spots on TLC and column

$R_f = 1,0$	Carotenoids	Yellow
$R_f = 0,48$	Pheophytine	Green-brown
$R_f = 0,35$	Chlorophyll A	Green-blue
$R_f = 0,20$	Chlorophyll B	Green-yellow
$R_f = 0,11\text{-}0$	Xanthophylls	Bright green, yellow

Scheme of CC apparatus:

Conclusion:

Excercise No. 10:

HPLC analysis of *C. tricuspidata* extracts

Instrument: HPLC system Agilent 1100 series with DAD detector

Tested solution: hexane, chloroform, and ethylacetate portions of ethanolic extract of *C. tricuspidata* obtained by liquid-liquid extraction in previous excercises

Mobile phase: methanol (MeOH), 0,2% HCOOH, acetonitrile (ACN); gradient elution

Stationary phase: HPLC column Ascentis® Express RP-amide 100 × 2,1 mm, particle size 2,7 μm

Temperature: 40 °C

Pressure: maximum 400 bar

Flow: 0,3 ml/min

Injection: 1 μl

Gradient elution:	0. minute	10 % ACN, 90 % 0,2% HCOOH
	36. minute	100 % ACN
	36.–40. minute	100 % ACN

Detection: DAD**,** observing wavelengths 254 and 280 nm.

Procedure: Put 1 mL of each sample (concentration approx. 1 mg/mL) into the vials using automatic pipette, mark the vials and place them into the HPLC sampler. According to the teacher's instructions turn on all the units of the HPLC system and fill in all necesary acquisition parameters (name of the sequence, total number of vials, name of the sample, method etc.). Once all these steps are ready, start the acquisition.

Scheme of HPLC apparatus:

Basic HPLC characteristics:

Conclusion:

3. Identification of content compounds- spectroscopic techinques (UV, IR, NMR, CD)

Excercise No. 11:

Determination of carotenoid pigments in *Urticae folium* using UV/VIS spectrophotometry

Carotenoid pigments (mixture of α-carotene and β-carotene) were isolated from the *Urticae folium* extract. Their content in the sample can be easily determined by using spectrophotometry in the area of the visible light. Sample absorption is directly related to the concentration of carotenoid pigments according to the Lambert- Beer law: ($A = \varepsilon \times b \times c$), where A is absorbance, ε is molar extinction coefficient , b is the path of light and c is concentration.

The aim of this excercise is to determine the carotenoid content in the sample

Procedure:

Sample prepared by column chromatography is transferred quantitatively into 25 ml volumetric flask and the mixture of diethy lether-petroleum ether (1:1) is added. Absorbance of the sample is then measured at λ=460 nm. Carotenoid content is deduced from the calibration curve and re-calculated to carotenoid content in the drug (%).

Calculation:

Conclusion:

Závislost absorbance na koncentraci karotenoidů při 460nm