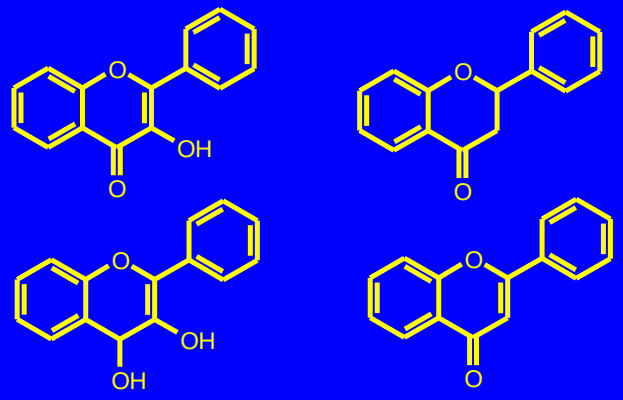
Phytochemistry in the System of Science, Methodology

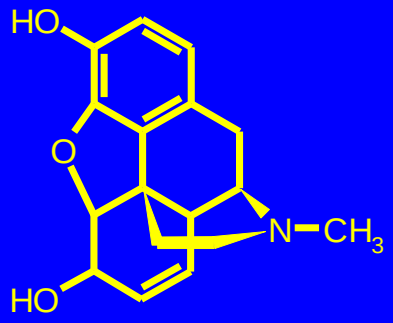
Introduction into Phytochemistry,
Historical Excurse

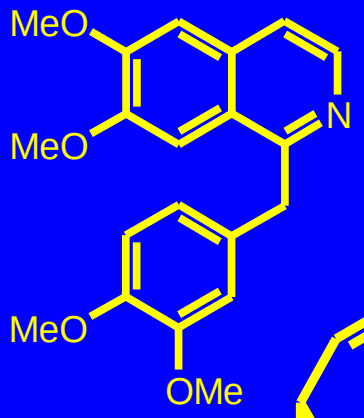
- Greek origin of word „***phytochemistry***“
- All chemical aspects touching the plant kingdom and life of plants
  - Plant nutrition and primary metabolism of plants (photosynthesis etc.) excluded to the plant physiology and plant biochemistry
  - Chemical studies of organic carbon-containing compounds created by plants (especially in so called secondary metabolism)
  - For example: comparative phytochemistry – comparing differences of natural compounds synthesis in plant species and botanical families

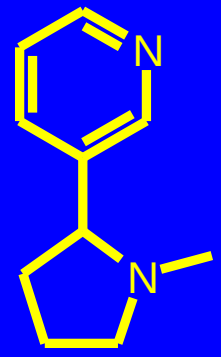
Founders

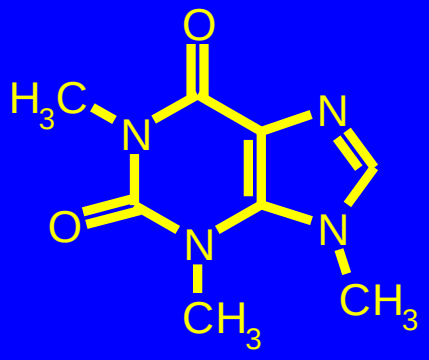
- 1664 Boyle (England) - flavonoids
- 1806 Sertürner (Deutschland) - morphine
- 1817-1820 Pelletier a Caventou (France) - quinine
- 1848 Merck (Deutschland) - papaverine
- 1829 Posselt a Reimann - nicotine and 1819 Runge - caffeine (Deutschland)

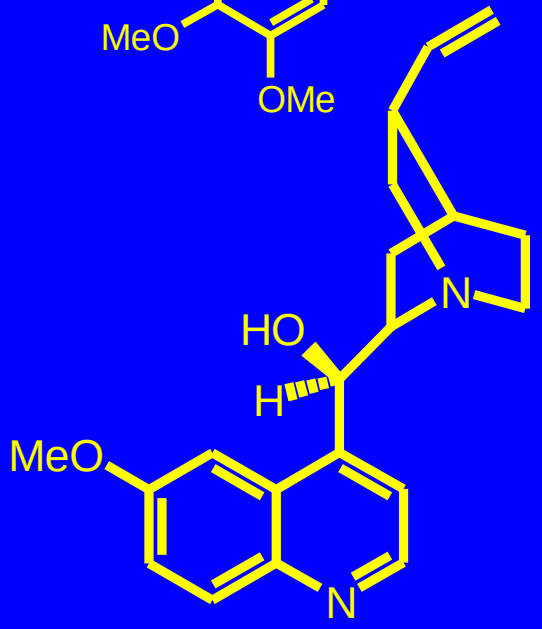












- Bigger development at century turning point
- Till the fifties slow progress
  - Compounds isolated and identified only in crystal form, identification based on chemical degradation and biogenetic principals
- 1957 5000 of natural compounds including alkaloids
- After WW2 great progress in chromatographic and spectral applications (separation and identification)
- Present time – more than 600 000 natural compounds

Primary and Secondary Plant Metabolism

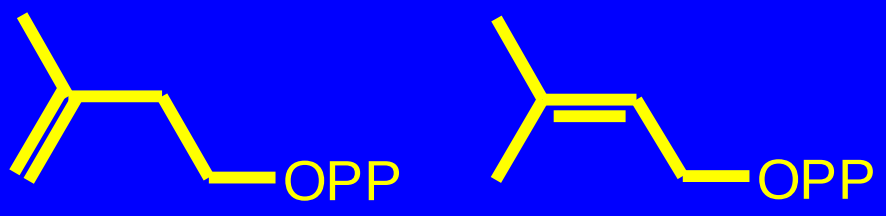
- *Primary metabolites:* ubiquitous, vital compounds
  - Simple sugars
  - Amino acids (especially proteinogenic)
  - Purines and pyrimidines of nucleic acids
  - Chlorophyll
  - Fatty acids and low-molecular carboxylic acids

- ***Secondary metabolites:*** taxonomically limited occurrence, the importance for plants not explicit, specific conditions necessary for their production.
- Production in different plant organs, transportation and chemical modifications.
- Probably not necessary for life.
- Nomenclature by different systems:
  - Trivial
  - Semi trivial
  - Chemical
  - According to the biosynthetic origin

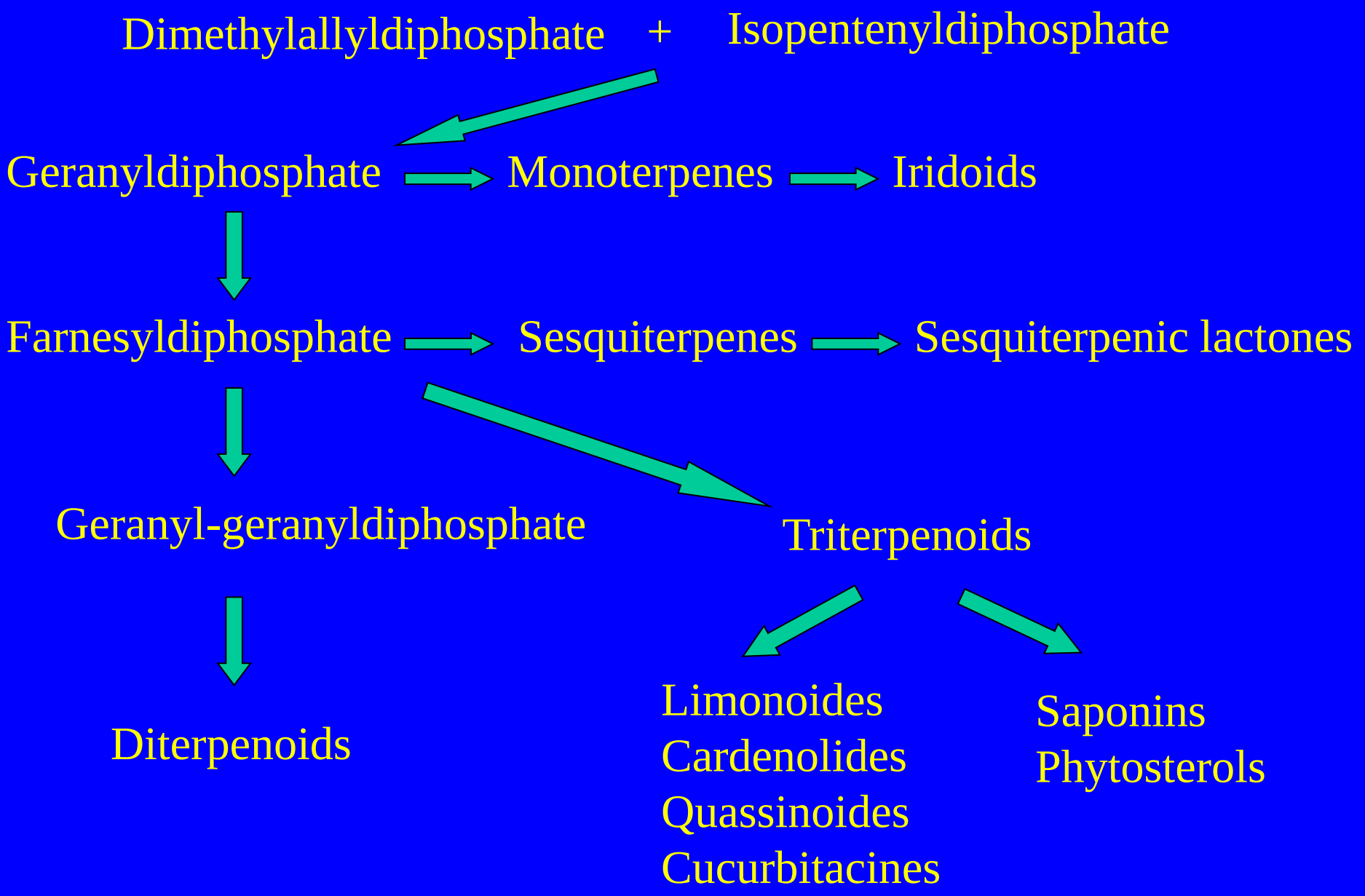
- Main classes of secondary metabolites:
  - Terpenoids
  - Alkaloids and other nitrogen-containing compounds
  - Phenolic compounds

Terpenoids

- Biosynthetic origin in isopentyl-diphosphate and dimethylallyl-diphosphate



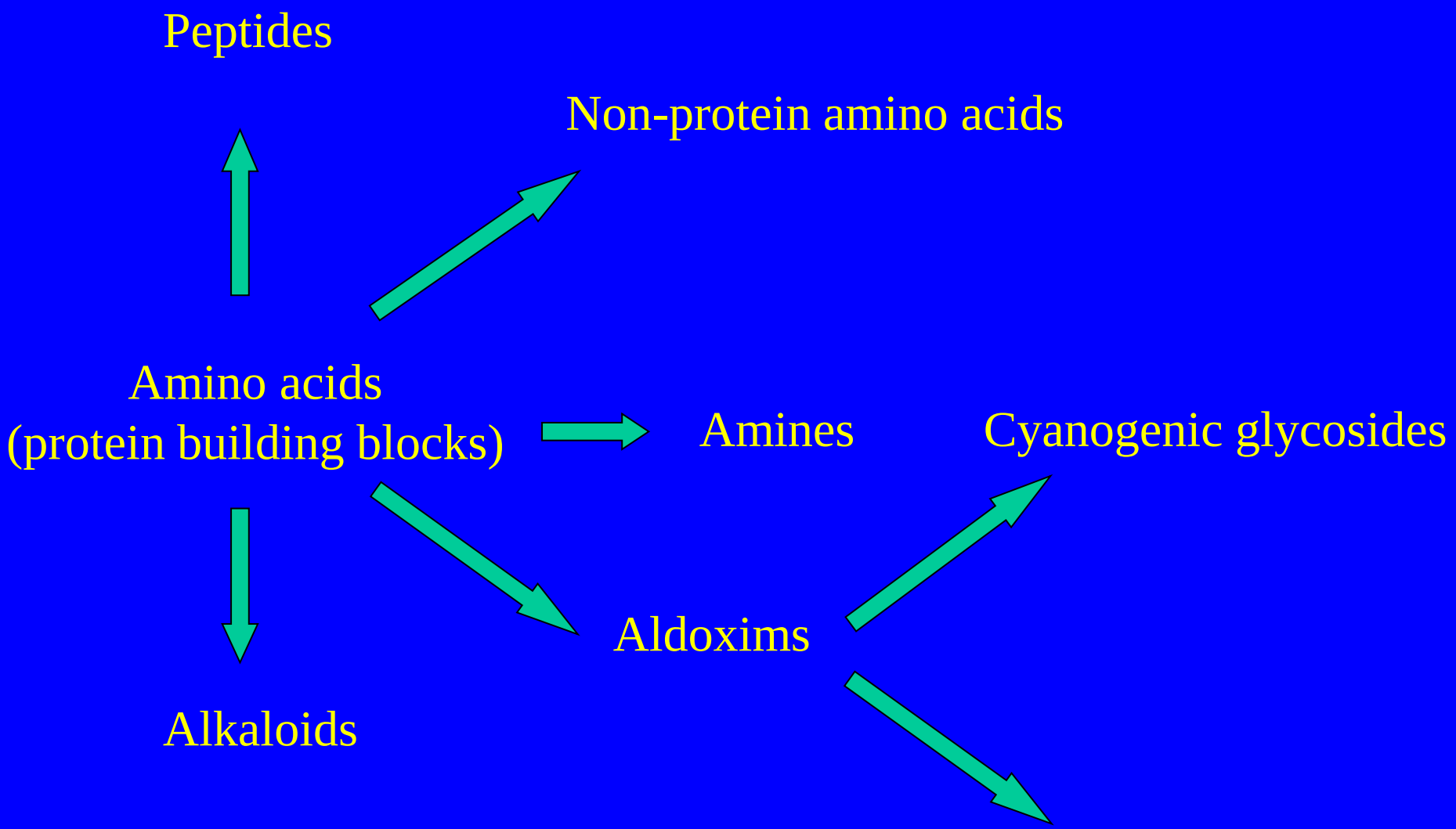
  - Lipophillic properties
  - Unsaturated carbohydrates, different oxidation pattern.
  - Individual subdivisions according to the number of C5 units
  - Approx. 20 000 compounds


Dimethylallyldiphosphate
+
Isopentenyldiphosphate
Geranyldiphosphate
Monoterpenes
Iridoids
Farnesyldiphosphate
Sesquiterpenes
Sesquiterpenic lactones
Geranyl-geranyldiphosphate
Triterpenoids
Diterpenoids
Limonoides
Cardenolides
Quassinoides
Cucurbitacines
Saponins
Phytosterols

Group of Terpenoids	*Characteristics*
Monoterpenes	Volatile compounds, essential oils
Iridoids	Usually lactones, bitter taste, often glycosides
Sesquiterpenes	Essential oils, higher boiling point
Sesquiterpenic lactones	Asteraceae family, bitter taste
Diterpenoids	Resins and plant growth hormones
Triterpenoid and steroidal saponins	Foam making compounds, hemolytic activity
Cardenolides and bufadienolides	Cardioactive glycosides, toxins
Phytosterols	Building blocks of membranes
Cucurbitacines	Bitter taste, typical for Cucurbitaceae
Carotenoids	Plant pigments

Alkaloids and other Nitrogen-Containing Metabolites

- The most known nitrogen-containing compounds
- Nitrogen part of structure (usually in cyclic system)
- Basic
- Limited distribution in species or families – chemotaxonomic limitations
- More than 10 000 compounds

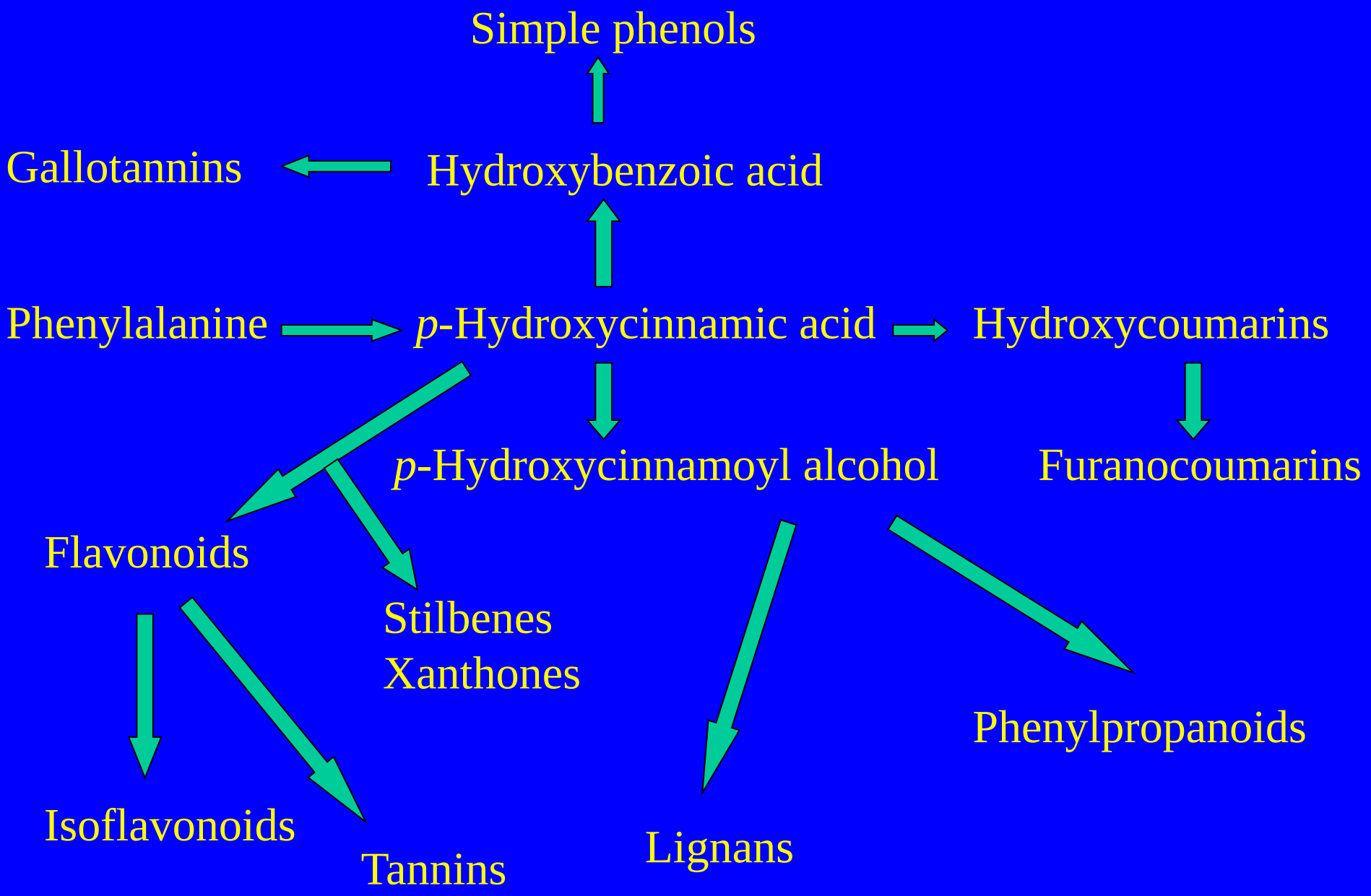

Peptides
Non-protein amino acids
Amino acids
(protein building blocks)
Amines
Cyanogenic glycosides
Aldoxims
Alkaloids
Glucosinolates

Alkaloids	
Amaryllidaceae alkaloids	Limited to Amaryllidaceae, 275 different structures
Betalains	Yellow and red pigments
Diterpenoid alkaloids	Very poisonous, mainly Ranunculaceae
Indol	Many structures, mainly Apocynaceae and Loganiaceae
Isochinoline	Widely distributed, greatest group, many subdivisions
Lycopodium	150 structures, clubmosses, mosses
Monoterpenoid	Relative to iridoids, different heterocycles
Sesquiterpenoid	Mainly orchids
Peptid	Mainly Rhamnaceae
Pyrrolidin and piperidin	Polyhydroxyalkaloids, inhibitors of enzymes, pharmacologically active
Pyrrolizidin	Senecio, Asteraceae
Chinolin	Rutaceae
Steroidal	Apocynaceae, Buxaceae, Liliaceae, Solanaceae
Tropane	Solanaceae

Non-proteinogenic amino acids	Often present in seeds, especially Leguminosae
Amines	Mainly volatile compounds, sporadic occurrence, in traces
Cyanogenic glycosid	Widely distributed in Rosaceae, low concentrations
Glucosinolates	Brassicaceae
Purines a pyrimidines	Nucleic acids, methylxanthines

Phenolic compounds

- Aromatic compounds with one or more hydroxyl groups
- Possible and common in glycosidic form
- Biosynthesis mainly from phenylalanine via shikimic acid


Simple phenols
Gallotannins
Hydroxybenzoic acid
Phenylalanine
p-Hydroxycinnamic acid
Hydroxycoumarins
p-Hydroxycinnamoyl alcohol
Furanocoumarins
Flavonoids
Stilbenes
Xanthones
Phenylpropanoids
Isoflavonoids
Tannins
Lignans

Subclass of phenolic compounds	*Characteristics*
Anthocyans	Red and blue pigments
Anthochlors	Yellow pigments - chalcons and aurones
Benzofurans	Distributed through some higher plants and lichens
Chromones	Small group
Coumarins	700 compounds, widely distributed
Flavonoids	May compounds, widely distributed
Isoflavonoids	Mainly Leguminosae, rare as glycosids, estrogen-like effect
Lignans	Wood and bark
Phenols a phenolic acids	Universal for plant
Phenylpropanoids	Many structures, form glycosides, as aglycones part of essential oils
Quinoid compounds	Benzoquinons, naftoquinons
Tannins	Tannins hydrolyzable, condensed
Xanthones	Pigments, mainly Gentianaceae

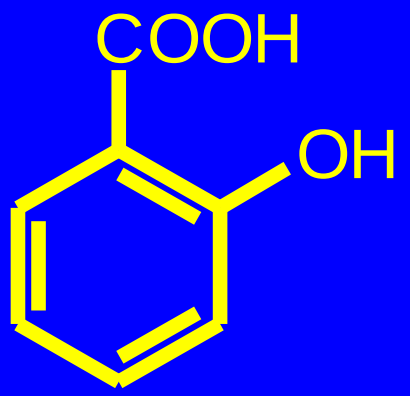
Functions of Secondary Metabolites

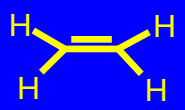
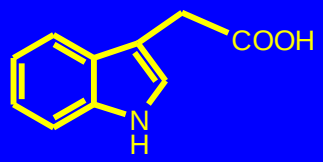
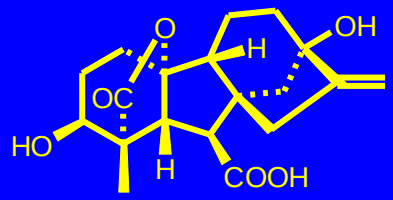
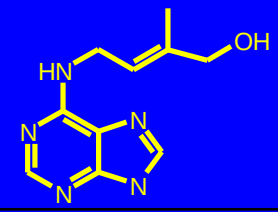
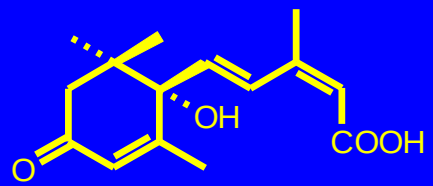
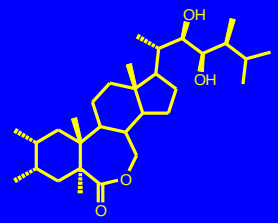
- Difficult to establish the true function
- Multifunctional
- Occurrence both as secondary and primary metabolites

- Common the occurrence of group of very similar compounds, difficult to establish function of selected metabolite
- Advantage of presence of group of similar compounds before presence of single metabolite

Functions of Secondary Metabolites

- Multifunction
  - **Salicylic acid:** signal stress molecule, metabolic signal for pollen production, protection against herbivores, allelopathic effect



Ethylen		Simple structure, hormone of ripening, seed germination, inflorescence development, stress response
Indol-3-acetic acid		30 different conjugates, „auxine“, tropism, elongation, induction of cambial cells division
Gibberellines		102 of structures, elongation and growth of plant organs, stratification and vernalization
Cytokinines		14 structures, cell division, cell differentiation, inhibition of abscission
Abscisic acid		Seed ripening, inhibition of seed germination, durability to lack of water
Brassinosteroids		More than 40 structures, stem elongation, increase of ethylene production, wood growth, stamina growth
Polyamines	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	4 structures, interaction with RNA, effect on DNA and RNA replication, growth and development of cells, ripening, flowering

Plant Pigments and Odors

•Mainly as attractants for pollinators (bats, birds, bees, butterflies and others)

•Different pollinators – different attractants

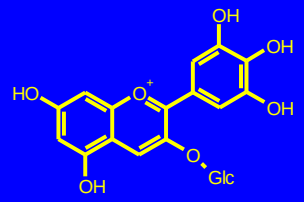
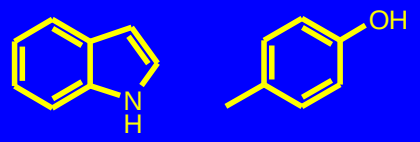
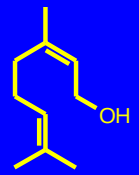
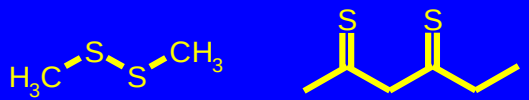
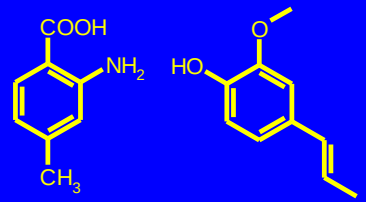
bees – blue and yellow (flavonoids and anthocyanins), soft odors

bats – night pollinators – fruit odors, sulphuric odors (for example methylsulphides)

•Compounds always in combinations (anthocyanins + flavonoids in blue, carotenoids + flavonoids in yellow)

- Extra large panel of compounds as plant odor components
- Monoterpenes, sesquiterpenes, benzoates, derivatives of anthranilic acid, simple sulphuric compounds, simple esters, amines

- Different panel of compounds in leaves, flowers and fruits
  - Different purposes of presence – color and odor should repel
  - Sometimes opposite – attraction because of seed spreading

Delphinidin-3,5-diglucoside		Blue color, attractive for humble bees
Pelargonidin-3,5-diglucoside		Scarlet color
Indol and p-cresol		
Geraniol		Attractive for drones
Dimethyldisulphid, 2,4 dithiopentan		Attractive for bats
Methylanthranilate and isoeugenol		Synergy of compounds in butterflies attration

Protection Against Herbivores

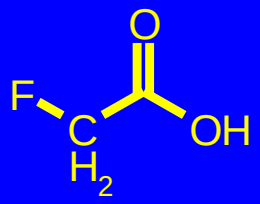
Toxic for:

- Species - salicin
- All species - oubain
- For plant alone - cyanogenic glycosides

- Concentration increases after stimulus - elicitation
- Appreciated insecticides (rotenone, pyrethroids)

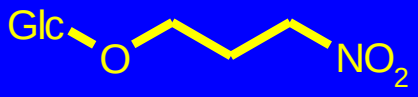
- Differences in activities of similar structures (monoterpenoids more effective when oxygenated, pyrrolizidine alkaloids when hydroxylated and in frm of esters)

Fluoracetic acid



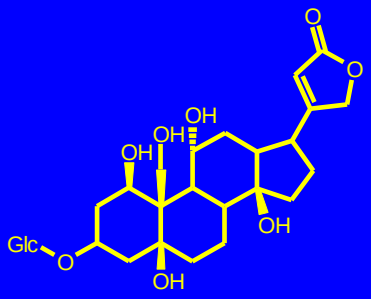
Dichapetalum cymosum, interaction with Krebs cyclus, respiratory poison for mammalians

Miserotoxin



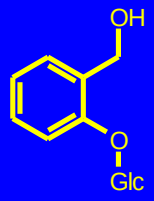
3-nitro-1-propanol-β-D-glucosid, *Astragalus atropubescens*, toxic for cattle, mitochondrial poison

Oubain



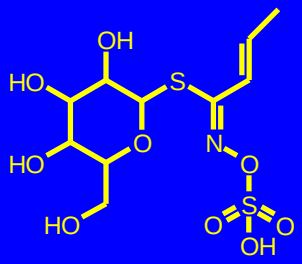
Acokanthera oubaio, cardial poison for mammalians

Salicin



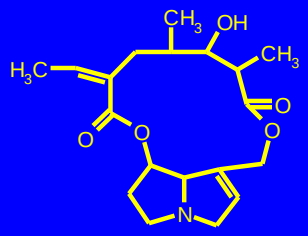
Salix spp.

Sinigrin



Brassica, toxic especially for cattle

Senecionin



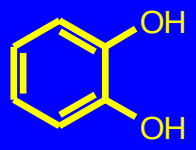
Senecio, hepatotoxic, mainly for cattle

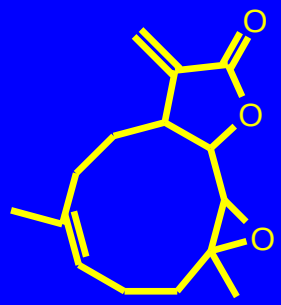
Antifungal effect

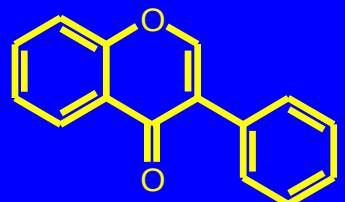
- Different types of barrier: defensive proteins, lignification, membranes and wax-compound layers, low molecular compounds
- 1. constitutive
- 2. synthesis *de novo* as response on infection – so called phytoalexins (more than 300 compounds identified)

- Attacking fungi product toxins – toxicity possible for plant and also for consumer

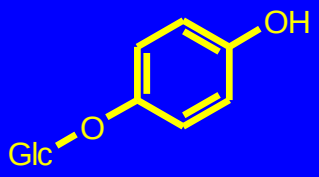
- Antifungal effect
  - Compounds located on the surface
    - » catechol, parthenolide, luteon

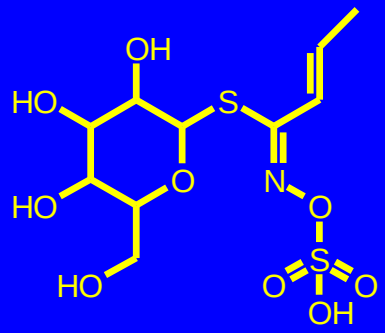


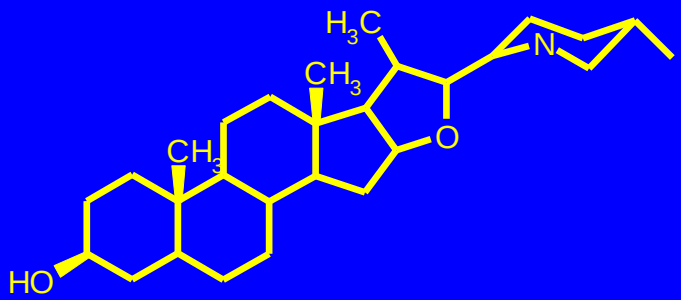




  - Vacuole located compounds
    - » arbutin, sinigrin, tomatin, avenacin, avenacosid







- Compounds with activity of animal hormones
  - steroids:
    - » ecdysons
  - terpenoids
  - estrogen-like compounds
    - » formononetin
- Plant compounds entering the symbiotic relationships
  - Lichens and root parasites
  - Chemical changes after infliction of microbes and parasites

Methods of Isolation and Analysis of Plant Secondary Metabolites

- Reasons for isolation and separation of compounds:
  - Initial extract shows promising biological activity
  - Chemotaxonomic studies
  - Isolation of commercially interesting compounds, for example for breeding
- Necessity clearly select targets
- To found an idea about chemical character of target compounds
  - Polarity, presence of acidic and basic functionalities, chemical relationship of single compounds

- To found an idea about physical character of target compounds
  - Melting points, boiling points, UV absorption and other spectral characteristics

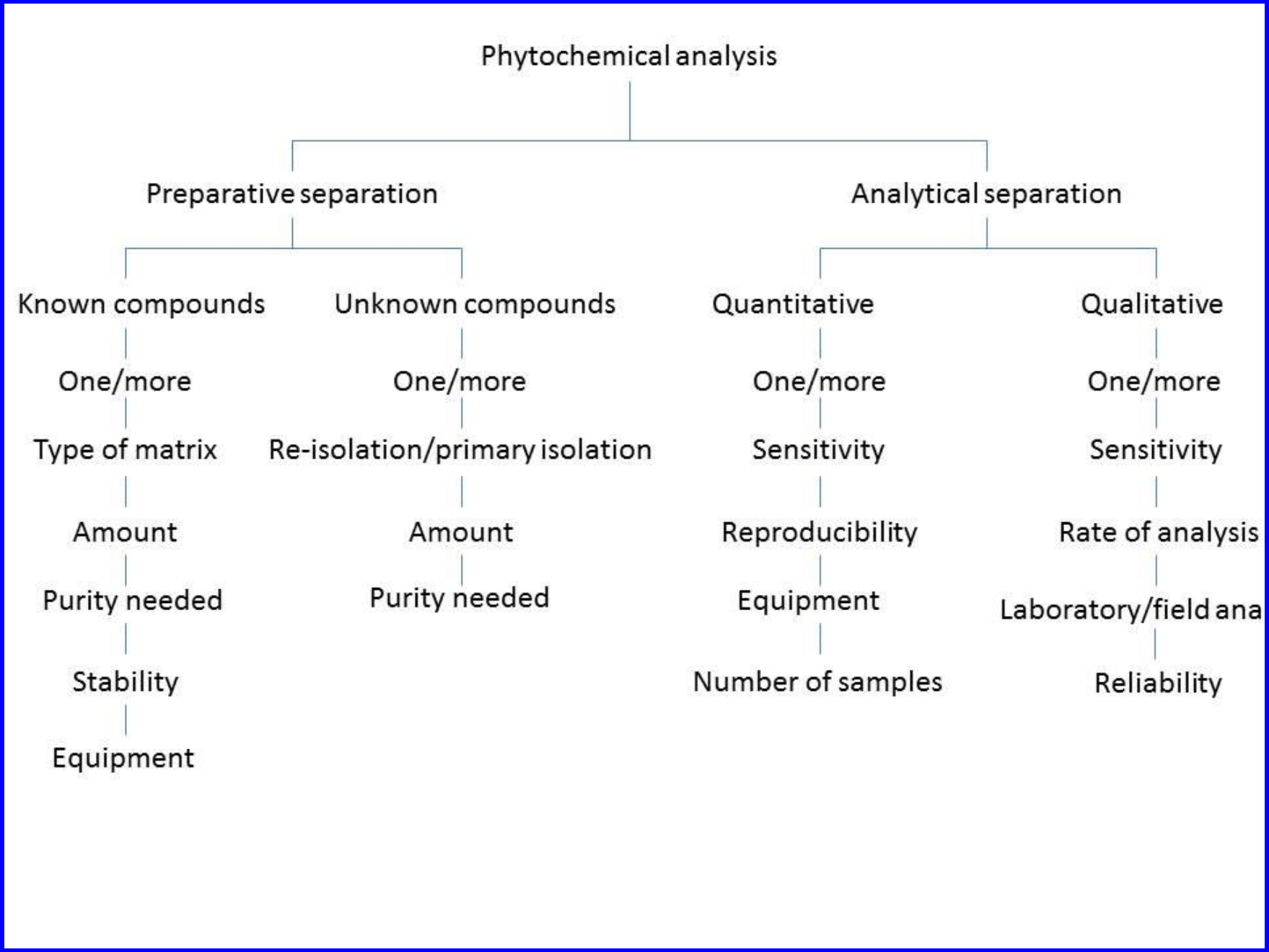
- Make a thorough literature research.

‘It is not necessary to develop a bicycle again.‘

- 1. Cultivation of medicinal plants, collection/harvest, drying, storage of drugs

The term „drug“ means unmodified or modified conserved or fresh plants or plant parts, animals or their parts or metabolic products of plants, animals and microorganisms.

- 2. Quality control
- 3. Isolation and separation of physiologically active compounds
- 4. Identification of physiologically active compounds


Phytochemical analysis
Preparative separation
Analytical separation
Known compounds
Unknown compounds
Quantitative
Qualitative
One/more
One/more
One/more
One/more
Type of matrix
Re-isolation/primary isolation
Sensitivity
Sensitivity
Amount
Amount
Reproducibility
Rate of analysis
Purity needed
Purity needed
Equipment
Laboratory/field ana
Stability
Number of samples
Reliability
Equipment

Preparative Separation

- Search in the available literature – make a literature research on the topic
- Optimization of methods
- Look for all known properties of target compounds.
- First make a trial with small amount of sample.

- Use sensitive methods.
- Partial material, do not work on the whole amount at once.
- Make a taxonomical search.

Analytical Separation

- Search in the available literature – make a literature research on the topic
- Choose optimal method, modify or confirm the functionality/ reliability.

- If necessary pre-purify sample.

Phytochemical Literature

- Chemical abstracts, Biological abstracts
- Beilstein abstracts
- Phytochemical Analysis - phytochemical metods
- Journal of Natural Products, Phytochemistry, Planta Medica
- Journal of Chromatography, Journal of Liquid Chromatography, Chromatographia.

Cultivation of Medicinal plants, Collection/Harvest, Drying, Storage of Drugs

- Small consumer/individual person – collection of plants in wild nature contra mass production for business

- Active principles usually present unevenly in all parts of plant.
- Proper term of harvest.
  - Concentration of content compounds changes in the life of plant, seasonal variations (concentration could change in seasons, weeks, hours).

- Menthol in spearmint only in older plants, young plant contain precursors only.
- Camphor collected from 40 years old trees.
- variability in the season:
  - » Subterranean parts collect in autumn.
  - » Seeds and fruits from beginning to full maturity.
- Daily variability of content compounds depend on external factors:
  - » Changes of light and dark
  - » Temperature
  - » Humidity
- Collection of wild plants is the oldest way of harvest. Drug collected in this way has not standard content of active compounds.

- Standard cultural cultivation of medicinal plants is better for standard content of active compounds.
- Usage of pharmaceutical industry.
- Main conditions for successful propagation:
  - Choice of location.
  - Choice of cultivar.
  - Time of harvest.
  - Usage of breed cultivars with high resistance against diseases and noxious animals.
  - Resistance to frost and drought.
  - Economical aspects.

- Phytotherapeutic usage:
  - Fresh plants:
    - » To get compounds which could decompose by drying and storage.
    - » By-pass of energy-consumpting drying
    - » Homeopathy
  - Conserved plants:
    - Rapid processing.
    - Stopping of undesired changes: treatment of alcohol vapors on harvested material, lyophilization- freeze drying.
    - In some cases – active compound synthesis arises after collection – post mortal synthesis - cardioactive glycosides.

- Drying:
  - Fastest and most economic way of conservation.
  - Bad drying can destroy material
  - Removal of water – we stop the invasion of microbes and fungi, stopping of unwanted metabolic procedures which could change compounds.
- Good drying procedure did not affect the level of active compounds.

- Well defined drying temperature:
- Usual drying temperatures
  - Leaves, herbs, flowers 30-40 °C.
  - Roots and bark 30- 65 °C.
  - Volatiles contaning plants less than 35 °C.
  - Use of short-termed higher heating to stop enzymatic procedures and decomposition of unwanted compounds.
    - Sometimes opposite – wanted procedures, by enzymatic process could be created active content compounds – fermentation.
    - Fermentation can change the „design“ of drug, taste, color etc. (black tea).

- Time of drying:
  - Herbs, leaves, flowers several days.
  - Roots several weeks.
- Way of drying:
  - Directly on sun light.
  - Natural heat.
  - Well air conditioned room.
  - Special drying cabinets with controlled temperature.

- Regular Conditions of Storage:
  - Bad conditions – excessive humidity causing the invasion of moulds (*Penicillium*, *Rhizopus* and *Eurotium*).
    - Important to maintain low air humidity – max. 65 %.
  - Direct sun light is bad.
  - Fluctuation of temperature.
    - between 5-15 °C.
  - Some drugs must be preserved against malefactors, especially insects, different types of Některé drogy je také potřeba chránit proti škůdcům, zejména hmyzu, různým typům roztočů, červotočů a pod.

Extraction Techniques

- Spontaneous isolation without extraction.
- Usually necessary at least one step of sample extraction and one step of sample purification.
- Not only separation from high molecular compounds, but also removal of low molecular compounds which can disturb the final steps of separation and purification.
- *Matrix effect.*