**MPM containing flavonoids.**

Flavonoids are natural nitrogen-free heterocyclic compounds – the derivatives of benzo-γ-pyrene or chromones. O2 – heteroatom. The molecular framework of flavonoids consists of a C6-C3-C6 units, i.e. phenylchromone skeleton.

Flavonoids are vegetable aromatic compounds, the basic structure consists of the diphenylpropane skeleton C6-C3-C6. The flavonoid molecule consists of two phenyl residues, linked by an aliphatic three carbon chain.

A

B

Diphenylpropane

Flavonoids can be presented as chromone and chromane derivatives, containing the aryl radical in 2,3 or 4 positions.

  

γ-pyrone chromone phenylchromone or flavon

Then the structure was confirmed by the synthesis. The chromone skeleton, which is the basis of flavonoids, was obtained by S. Kostanetsky, subsequently Ruiman under the effect of H2SO4 on phenoxy-coumaric acid and distillation of the reaction products.



Phenoxycoumarin acid chromone -2-carboxylic chromon

The flavonoids are formed when the phenyl radical is attached to ring B in position C-2, and the isoflavonoids – in position C-3.

The flavonoids are classified according to the following features: the degree of oxidation of propane skeleton, the position of side phenyl radical and value of heterocycle.

According to the structural features associated with the chemical properties and biogenetic origin flavonoids are divided into the several groups:

Euflavonoids (flavonoids with phenyl radical at C-2); isoflavonoids with phenyl radical at C-3; neoflavonoids – the derivatives of 4-arylchromone (or diarylpropenine); biflavonoids.

Flavonoids can be condensed each other and other phenolic compounds (phenolcarboxylic and hydroxy-cinnamic acids, lignans, and with isoprenoids and alkaloids).

In plants flavonoids are present mainly in the form of glycosides, rarely- as aglycones. A diversity of flavonoid glycosides relates to the significant set of sugars, the position of attachment to its aglycone, and also the sugars have different values, configuration cycle and glycosidic bonds (furanose and pyranose forms of monosaccharides, D- and L-isomers, α- or β-bond and etc.).

Flavonoids are divided into О-, S-, N- and С-glycosides according to the type of bonds. О-glycosides are easily hydrolyzed by acids and enzymes. C-glycosides are difficultly hydrolyzed by acids and enzymes. That’s why its hydrolysis is carried out with Kiliani mixture (the concentrated hydrochloric acid and acetic anhydride). In plants flavonoids are present primarily as O- and C-glycosides.

Flavonoids are various organic compounds, associated with each other genetically, but have different pharmacological action. Flavonoids are widely distributed in higher plants, present in microorganisms and insects. About 40% of flavonoids is related to the group of flavanol derivatives. In plants the derivatives of flavon, flavonon, chalcone and auron are also widely distributed in plants. Flavonoids are widely distributed in plant world. They are often present in plants of ranunculaceae, legumes, rosaceae, polygonaceae, rutaceae and etc. They are not found in lower plants and animals. The amount of flavonoids reaches 0,01-1,5% in plants, and in some plants- 20-25%. They are often present in flowers, fruits and leaves, less – in shoots and underground organs (exception is the licorice root and rest-harrow). The maximum amount is present in leaves and herbs during bud-formation and flowering period, and the amount is decreased during fructification period; however it is increased in flowers and fruits.

The largest amount of glycosides accumulates in this phase, but at the end of vegetation – free aglycones.

The flavonoid classification is based on the following features, as the degree of oxidation of propane skeleton, position of side phenyl radical, value of heterocycle and etc.

According to the structural features associated with the chemical properties and biogenetic origin flavonoids are divided into the several groups:

* Euflavonoids – flavonoids with phenyl radical at C-2
* Isoflavonoids – with phenyl radical at C-3
* Neoflavonoids – the derivatives of 4-arylchromones (or diarylpropenin)
* Biflavonoids

Euflavonoids are divided into the following groups:

1. Flavone derivatives. They are yellow or colorless substances, having the double bond located between carbons 2 and 3. Most of flavone molecules contains hydroxyl groups in 5,7,3 and 4 positions. Apigenin – 5,7,4-trioxyflavone, luteloin- 5,7,3,4 – tetraoxyflavone are the compounds of this group.

 

apigenin luteolin

Each compound is often present in the free state or in the glycosidic form in flowers of different plants.

Flavones without OH-groups in the ring B are also found in nature. For example, baicalin or 5,6,7-trihydroxyflavones in flowers and leaves of scutellaria. Methylated flavones are found among the flavone derivatives. Usually, CH3–groups are in conjugated form at 3 and 4 positions. For example, akacetin or 5,7 dioxy- 4′- metoxyflavones or apigenin 4′- methylated ether in tansy. Diosmetin or 5,7,3′–trihyrdoxy 4′- metoxyflavone and 4′- methylated ether of luteolin found in mint and tansy can be presented as examples of this group compounds. In addition, the compounds containing the methyl groups in different positions are present.

 

baicalin acacetin



diosmetin

2. The derivatives of flavonol or 3-oxyflavon. Quercetin or 5,7,3,4-tetraoxyflavonol, kaempferol or 5,7,4-trioxyflavonol, myricetin or 5,7,3,4,5-pentoxyflavonol are referred to this group of compounds.

 

flavonol quercetin

 

myricetin kaempferol

They are yellow substances, they are often present in glycosidic form in nature. The glycosides of quercetin are monoside quercitrin, the sugar component is rhamnose, bioside rutin which has important medical significance (the sugar component is rhamnose and glucose residue) and monohyperoside (sugar component is galactose), the methyl ethers of quercetin – rhamnetin and isorhamnetin. Rhamnetin is present in fruits of common buckthorn, isorhamnetin – in violet and dlphinium.

glucose -о-rhamnose

(rutinose)

rutin

 

rhamnetin isorhamnetin

High-hydroxylated and methylated flavones are also found, digicitrin or 6,7,8,3′,4′,- penthametoxyflavanol in the leaves of digitalis can be presented as an example.

3. Flavanone derivatives. They differ from flavones by the absence of the double bond between C-2 and C-3. They are found in plant of the following families: rutaceae, rosaceae, legumes and ferns, gymnosperms in pine. Naringenin or 5,7,4-trioxyflavanon and eridictyol or 5,7,3,4-tetraoxyflavanon are referred to this group.

 

flavanone naringenin

eriodictyol

The ether of eriodictyol –hesperitin can be presented as an example of methylated ethers, which is found in the glycosidic form –hesperidin in citrus peel. Hesperidin is a bioside which consists of glucose and rhamnose. Hesperidin has rutin-like property.



hesperidin

4. Flavanonol derivatives. They differ from flavanonones by the presence of OH-groups at C-3. This group of compounds are present in plant in the free state, but rarely and in small amount. Aromadendrene or 5,7,4-trioxyflavanonol, containing in eucalyptus leaves are referred to this group.

 

flavanonol aromadendrene

5. Chalcone derivatives. Chalcone derivatives are yellow or pink substances. They differ by the absence of γ-pyrane ring in molecules. They are as the isomerization products of flavanonones. About 12 such substances are found in nature. Firstly diols are formed during heating with acids, then one molecule of water is separated and they are converted into flavanone.

Flavanones, in turn, are converted into chalcones in an alkaline medium. Hesperidin-methyl-chalcone is referred to this group.

Rhamnose-glucose-O

Hesperidin-methyl-chalcone

6. Aurone derivatives. Aurone derivatives are yellow or pink compounds. In present there are 6 types of aurones, which exist with chalcones in the family of compositae. Areosidin or 4,6,7,3,4,-pentoxyaurone, is found in helichrysum arenarium. Aureosidin or 4,6,7,3,4-pentahydroxyauron, which exist in helichrysum arenarium, is referred to this group.  

auron aureosidin

7.Anthocyanidin derivatives are red, violet and blue compounds. They give bright color to flowers and fruits and they are similar to flavones. They are as reduced compounds of flavones without keto-groups in position 4. They are referred to pyroxon bases, they easily form salts with acids. The anthocyanidin colour is changed. They can be considered as cation flavilia. Anthocyans form phenolates with alkalines due to the presence of phenolic hydroxyl groups.



Cation flavilia

Anthocyanidin colour depends on the number of hydroxyl groups, position and pH medium. As the number of phenolic groups increases the colour changes from pink-red (pelargonidin) or violet-red (cyanidin) to blue-red colour (delphinidin). Methylation of hydroxyl groups leads to reduction of colour darkness. Pink peonidin and blue-violet malvidin are the examples. Anthocyanidin colour depends on pH. Fuchsin - oxonic salts coloured into red (for example, cyanidin-chloride) is produced at pH=3 or below, violet colour (cyanidin bases) are produced at pH=8,5, phenolate cyanidin is produced at pH=11 (strongly alkaline medium) and the colour is darkened and changed to blue colour. In plants anthocyanidins are present in the form of glycosides (anthocyans) and aglycones. Usually sugar components are associated with hydroxyl groups in 3,3′ or 5 positions. Anthocyanidins are divided into the following types:

a) violet-red cyanidin. Bioside cyanin (in petals of red rose, poppy), prunicyanin (in plum fruits) and ceracyanin are often found in plants. Cyanidin is found in the form of salts, bases and phenolates in plants, and depending on this they give various colours to flowers and fruits. For example, cyanidin-phenolate gives a blue colour to cornflower, but the oxonic salts give a red colour to rose and geranium.

 

Cyanidin Delphinidin

b) delphinidin is blue-red coloured substance, in plants it is found in the form of delphinidin (in flower of dog rose) and violanin (in the flowers of blooming violet).

c) pelargonidin is pink-red coloured substance. In plants it is found in the glycosidic form of pellargonin in geranium flower.

 

pelargonidin malvidin

Malvidin and petuanidin are the methylated delphinidin derivatives. Malvidin is found as a malvin glycoside in the flowers of malva sylvestris. Malvidin is 3′,5′-dimethyl ether of delphinidin, petunidin - 3′- methyl ether of delphinidin. Thus the peonidin and its glycoside peonin are found. Peonidin is the 3′-methyl ether of cyanidin.

The colour of most flowers mainly depends on these anthocyanidins and their derivatives.

8. Derivatives of leucoanthocyanidin or flavandiols. Hydroxyl groups are located in 3 and 4 positions in their molecules. They lose one molecule of water by the action of the acids and are converted into anthocyanidins. They are similar to catechins, and present in trees and ferns. Leucocyanidin and leucodelphinidin are often found. Leucopelargonidin is rarely found.  

leucoanthocyanidin leucocyanidin



leucodelphinidin

9. Flavan deirvatives. The double bond between oxy-group and C-2, C-3 in their molecules is not presented. The most important compounds of this group are catechins, which are included into colorless tannins and phlobaphenes. Epicatechin or 3,5,7,3′,4′ - pentahydroxyflavane can be presented as an example of this group.

 

flavan epicatechin

Catechins and leucoanthocyanidins are the precursors of tannins.

Flavonoids exist as O-glycoside and C-glycoside forms in plants. The sugar components are linked to an aglycone through carbon atom in 6 and 8 positions in C-glycosides. They are divided into C-monoglycosides, C-diglycosides, C-, O-biosides and oth. C-glycosides are very stable compounds, they are not splitted by the action of acids under normal conditions. Vitexin containing in hawthorn fruits can be presented as an example. The sugar component (glucose) of vitexin is linked to carbon atom in position 8. This apigenin is C-8-glycoside.

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vitexin

C-glycosides are formed from flavones. They exist as O-glycosides in plant world. For example, 4-rhamnoside of vitexin is found in most plants.

Isoflavonoids include 3-phenyl-α or γ—benzopyran, pyrone derivatives, rotenoids, homoisoflavonois, coumestans and oth. compounds.

  

isoflavan isoflavon isoflavonon

  

isochalcone coumaranochroman 3-phenylcoumarine

 

12-α-hydroxyrotenone coumestrol

Neoflavonoids

4-arylcoumarins, dalbergions, dalberichinols found in limited quantities of plant species are referred to this group. They are considered as diarylpropen derivatives.

  

4-benzocoumarin dalbergione 4-benzochromane

Biflavonoids

Biflavonoids (dimeric forms) are present in nature. These compounds consist of a nucleus of flavones, flavanols and isoflavanols and their unity may be in different positions.



biflavone

A diversity of flavonoid glycosides is due to the significant set of sugars and possibility of attachment to the aglycone positions, and sugars can have various value of oxide cycles, configuration of glycosidic linkages and the order of combinations between them.

In plants flavonoids are present both in the free state as as glycosides. D-glucose, D-galactose, D-xylose, D-mannose, L-arabinose, L-rhamnose; D-glucuronic acid from uronic acid are found as a sugar in flavonoid glycosides.

In present time all flavonoid glycosides are divided into 3 groups:

This (basic) group is presented as O-glycosides, in which the sugars are associated with aglycone by semi- acetal bond through oxygen atom. O-glycosides are divided into monosides, biosides, diglycosides according to the number of sugars, position and order of attachment. Monosides are simplier compounds; biosides with the same sugars can be different by the sequence and order of attachment, the value of oxide cycles and configuration of glycosidic bonds. For example, the rhamnoglycosides group in which rhamnose is associated with glucose by 2,4 or 6- carbon atom. Biosides are compounds with more complex structure. They can be converted to triosides and olygosides. The sugar residues form straight or branched chains in this compounds. In addition, the sugars can be at 2 carbon atoms and form diglycosides.

2) Second group includes C-glycosides or glycoflavonoids, which can be divided into C-monoglycosides, C-diglycosides, C-O-diglycosides, C-O-biosides. The sugar residues are attached to the aglycone through carbon atom in 6 or 8-positions.

3) Third group of flavonoid glycosides includes complex compounds. They are acylated glycosides and they are divided on the glycosides of depsinoid type and glycosides with ester bond in sugar residues according to the position of acyl substituent. In depsinoids the aglycones are usually associated with aromatic acids, but the esters with aliphatic acids are also known. Benzoic, n-oxybenzoic, protocatechiuc, n-oxycinnamonic, caffeic, ferulic, sinapic, acetic, propionic and other acids isolated from complex glycosides are identified.

Physico-chemical properties

Flavonoids (from lat. Flavus –yellow) are optically active substances, according to the structure, pH and temperature they are colorless, yellow coloured and etc crystalline and have different solvency. For example, flavonoids are yellow coloured (flavones, flavanol, flavanonol), colorless (isoflavone, catechin, flavanone), and also red or blue colour (anthocyanidin).

Flavonoids give with acids red colour, with alkalines – blue colour. Aglycons of flaovonoids and their high-methylated derivatives are not soluble in water, but they are highly soluble in ether, acetone, alcohol and ethylacetate. Glycosides containing 3 sugar residues in molecule, are highly soluble in water and alcohol, but insoluble in ether. These compounds are sublimated under the influence of heat to 200 C, and they are destroyed by higher temperature.

Flavonoids have no smell. Some of them have a bitter taste. For example, flavonon-7-β-neohesperidoside is a bitter substance. Naringin and poncirin are the bitterest substances. They are 5 times more bitter than quinone hydrochloride. Its bitter taste is due to the presence of neohesperidoside.

Aglycones of flavonoids are highly soluble in diethyl ether, acetone and alcohols. They are practically insoluble in benzene and chloroform. Flavonoid glycosides are soluble in alcohol and hydroalcohol mixtures. Monosides are soluble in 95% alcohol, diglycosides – in 50% alcohol, glycosides with three and more sugars – in the weak alcohol and even in water.

In plants flavonoids in addition to catechins and leucoanthocyanidins are rarely found in the free state. Most of them are various glycosides. A diversity of flavonoid glycosides is due to the significant set of sugars and possiblity of the attachment to the aglycones at some positions. In addition to this the sugars can have different value of oxide cycles (furanose and pyranose forms of monosaccharides, D- and L-sugars, the configuration of glycosidic bonds, the order of combination between them (α or β bonds). There are O- and C-glycosides of flavonoids accoring to the type of bond. O-glycosides are highly hydrolyzed under the action of acids and enzymes, but C-glycosides are hydrolyzed under strict conditions by Kiliani mixture (the mixture of concentrated hydrochloric and acetic acids). In flavonoid glycosides the sugars are often found at C-7, then at С-41 and С-31 and rarely at С-5. С-6, С-8. Substitutions at C-3 and C-7 are common, and there are no substitutions at C-5 in free hydroxy group at C-3 among the flavonol glycosides. Acylated glycosides which acylated agents are benzoic, acetic, ferulic, caffeic, protocatechiuc and oth. acids are known in nature. Flavonoid glycosides are optically active compounds. Flavonoid glycosides are characterized by the ability for the acidic and enzymatic hydrolysis. The speed of hydrolysis is various. Flavonol-3-glycosides are highly hydrolyzed under the action of weak mineral acids (0,1-1%), flavon-7-glycosides are hydrolyzed under the 5-10% mineral acids during several hours. Flavonoid C-glycosides are not hydrolyzed by the enzymes and diluted acids. They are only hydrolyzed under the action of Kiliani mixture (the concentrated HCl and acetic acids).

Rutin and quercetin are practically insoluble in cold water, soluble in hot water, alcohol and alkaline solutions. Catechins are compounds which are highly soluble in water. Some bioflavonoids (especially in the leaves of green tea and olives) can dissolve in fats. The colour of anthocyanidin depends on the number and position of hydroxyl and metoxyl groups, and also on the ability to form complexes with metal ions. Leucoanthocyanidins are labile compounds. They oxidize till the relevant anthocyanidins under heat. In contrast to other flavonoids catechins and leucoanthocyanidins do not form glycosidic forms.

The glycosides dominates in young parts of plants, but in old parts –aglycones. It is established that in plants of southern latitudes more flavonoids are accumulated. The amount of them depends on the UV-rays. The more these rays the more flavonoids are produced. For example, the largest amount of flavonoids accumulates in plants of Caucasus and Crimea. The amount of flavonoids is increased when the height above the sea level is increased. Biological role of flavonoids in plant life has not been studied enough. Some scientists suppose that flavonoids are involved in the oxidation-reduction processes of plants, play a role as a hydrogen carrier. Some flavonoids are stimulants of growth in plants. Flavonoids are involved in pollination (attract insects), protect chlorophyll and cell plasm from destroying aciton of UV-rays.

Flavonoids prevent the oxidation of ascorbic acid and other compounds and the inactivation of enzymes. The more flavonoids are in plants, the more ascorbic acid are contained.

The collection of plant material should be carried out when it contains the maximum amount of flavonoids. Drying should be carried out quickly to prevent the destroying aciton of enazymes. The raw material is dried in shade at the temperatures of 50-60 C. The raw material should be protected from sun light which destroys the flavonoids.

*Isolation of flaovnoids from plant material.*

There are some qualitative reactions for the determination of flavonoids in the extracts from medicinal plant material. To this end, cyanidin reaction, and also the reactions with borhydrate sodium, iron (III) chloride, zirconium chloride, alkaline solution and etc. are used.

Cyanidin reaction (or probe Chinode) is based on the reduction of flavonoids by hydrogen atom to anthocyanidins in acidic medium with bright-pink formation.

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Cyanidine chloride

Chalcons, aurons, catechins don’t give a cyanidine reaction, but they can form coloured oxonium salts in acidic medium. An equal volume of n-octanol is added to coloured solution of the product of cyanidin reaction and it is shaked, the glycosides are remained in water, but the aglycons pass into the layer of organic solvent (Briant reaction). In addition to this the reactions with borhydrale sodium, iron (III) chloride, zirconium chloride, alkline solution and etc. are used.

The extraction with ethanol, methanol, acetone, ethylacetate and oth. solvents is carried out for isolation of flavonoids from plant material.

The solvent is removed during the distillation of obtained extraction. A hot water is added to the residue and after cooling down the nonpolar ballast compounds are removed (chlorophyll, resins, fixed oils and etc.) from water phase by chloroform or tetrachloride carbon. Flavonoids are extracted by ethyl ether (aglycones), ethylacetate (mainly monosides) and n-butanol (biosides, triosides and etc.) from aquesous phase. Thus, fractions consisting of the sum of flavonoids are obtained. The physic-chemical properties arre also used for identification of flavonoids: 1) determination of melting point; 2) determination of specific rotation ([a]D glycosides); 3) comparison of UV-, IR- mass-, NMR-spectra with the the known standards.

UV-spectroscopy is used for the establishment of free hydroxyl groups in flavonoid molecule by adding different reagents (acetate sodium, methylate sodium, boric acid with acetate sodium, aluminium chloride and etc.). During the addition of these reagents the absorption maximum is shifted which is characterized for hydroxyl groups in different positions.

*Qualitative reactions on flavonoids*

The common reaction which is specific for all groups of flavonoids does not exist. However the following reacitons are often used:

1. Cyanidin reaciton or Chinode method. The reaction is based on the reduction of flavonoids to anthocyanidins by hydrogen atom in acidic medium forming bright-pink colour.

In the presence of hydrochloric acid flavonols, flavanones and flavones give red or orange colour due to the anthocyanidin formation.





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Cyanidin chloride

Chalcones and aurones do not give the cyanidin reaction, but they give red colour due to the formation of oxonic salts by the addition of the concentrated hydrochloric acid (without magnesium).

The colours which are formed between flavonoids and magnesium, HCl, are presented in the table.

Table. The colours which are formed between some flavonoids and magnesium, HCl.

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| --- | --- | --- |
| Number | Compound | Colour |
| 1.  2.  3.  4.  5.  6.  7.  8.  9.  10.  11. | 7,8-dimethoxyflavone  5,7,8-trimethoxyflavone  5,7-dioxy-6,8,4'-trimethoxyflavone  7,8,4'-trioxyflavone (apigenin)  7,8,3',4'-tetraoxyflavone (luteolin)  7,8,3',4',5'- pentaoxyflavone  5,7,3',4',5'- pentaoxyflavone  5,7,8,4'- tetraoxyflavone  5,7,8,3',4'- pentaoxyflavonone  5,7,4'- Trioxyflavonone (naringenin)  5,7,3',4'- Tetraoxyflavonone (eriodyctiol)  7,8,3'-Trioxy-4'-methoxyflavanone (hesperidin) | Pink  Pink-red  Red  Red-pink  Pink  Red-pink  Bright red  red  «-------------»  Bright violet-red  Violet-red  «-----------------» |

In cyanidin rection flavones have from red to orange colour, flavonols – red to crimson, flavanones – crimson to bright-red colour.

2. Cyanidin reaction by Bryant. This reaction enables to determine the aglycone or glycosidic nature of the researched substances. An equal volume of n-octanol is added to the coloured product of cyanidin reaction and shaked. Glycosides remain in water, but aglycones pass to the layer of the organic solvent.

3. The reaction with iron (III) chloride. The colours from green (flavonols) to brown (flavanones, chalcones, aurons) and red- brown (flavones).

4. Boric-limonic reaction (Wilson reaction). Борно-лимонная реакция (реакция Вильсона).

Flavonoids in which the hydroxyl and carbonyl groups are separated by carbon atom form the complexes with boric acid which are not destroyed by limonic and oxalic acids. 5-oxiflavones and 5-oxyflavonoles react with boric and limonic acids, forming bright-yellow colour with yellowish-green fluorescence in UV-light (formation of bathochromic complex).



5. Reaction with SbCl3.

5-oxyflavones and 5-oxyflavonoles which react with SbCl3, form yellow or red coloured complex compounds:



6. Flavones, flavanones, flavanols give a yellow colour with ammonia solution, sodium hydroxide, potassium hydroxide and etc., that turns orange or red colour. Chalcones or aurones immediately give yellowish-orange, orange-red colours. Pure catechins do not give a colour. However the presence of small amount of impurity (the oxidation products) causes the formation of yellow colour. Anthocyanins give blue or violet colour in the presence of ammonia or sodium carbonate.

7. Catechins and the derivatives of phloroglucinol and resorcin form crimson-red colour with 1% vanilin solution and the concentrated HCl.

8. Flavones, chalcones, aurones contianing free orto-hydroxyl groups in ring B, form bright-yellow and red coloured precipitations, but anthocyanins form red or blue coloured precipitations by the spraying the alcohol solution with lead acetate medium.

9. Flavonoids give coloured complexes with 5% alcohol solution of aluminium chloride and 2% ethanolic solution of zirconium (III) chloride. Flavonoids with two oxygroups at C-3 and C-5 form chelates due to the hydrogen bonds between carbonyl and hydroxyl groups..

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10. Antocyans with sodium carbonate give various coloured reactions. Pelargonin (pelargonidin-3,5-diglycoside) with aqueous solution of sodium carbonate give purple colour, pelargonidin-3-glucoside (for example, callistephin) – red-purple, peonin (peonidin-3,5-diglycoside) – blue, cyanin (cyanidin-3,5-diglucoside) – intensive blue, malvin (malvidin-3,5,-diglucoside) – blue-green, enin (malvidin-3-diglucoside) – blue-purple.

11. Flavanones and flavanonoles are reduced by sodium borhydrate with the formation of dark-red, purple or blue coloured solutions.

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*Chromatographic analysis of flavonoids.*

The paper, thin layer and gas-liquid chromatography are widely used for the detection of flavonoids in plant material. The detection of components on the chromatogram is carried out by the visual observation in UV-light. Flavones, flavanol-3-glycosides, flavanones and chalcones are detected as dark-brown spots, flavanoles and its 7-glycosides- yellow or yellowish-green, xanthones – orange colour. After visual observation in UV-light the chromatograms are sprayed with the certain reagents (heat at 105 C for 3-5 min; 5% alcohol solution of AlCl3; SbCl3 solution in tetrachloride carbon, 5% or 10% alcohol solutions of alkaline), which enables to receive the area with brighter fluorescence in UV-light. The colour of spots of some flavonoids on chromatogram is presented in the table.

Table. The colour of spots of flavonoids on chromatogram.

|  |  |  |  |
| --- | --- | --- | --- |
| Compound | The colour of spots in UV-light | | |
| before | with AlCl3 solution | with KOH solution |
| Catechins | Without colour | Without colour | colourless turning into yellow |
| Flavanoles | yellow | Bright-yellow | yellow |
| Flavones | brown | Yellow, yellowish-green | Yellow, yellowish-green |
| Flavanones | Without colour | Pale-yellow | Yellowish-orange |
| Chalcones | Shade of yellow | Yellowish-orange | Orange-red |
| Aurones | Shade of red | Orange-red | Shade of red |

The paper and thin layer chromatography and different systems of solvents are widely used for the quantitative and qualitative determination of flavonoids. Silica gel, poliamide, cellulose and etc. are used as adsorbents in thin layer chromatography.

Chromatographic analysis is used to determine the number of flavonoid spots after detection of chromatograms with chromogenic reagents. Ammonia vapour, 10% alcohol solution of alkaline, 5% solution of aluminium (III) chloride, 5% solution of antimony chloride in tetrachloride carbon, diazotized sulfanilic acid and oth. are used as chromogen reagents. After the spraying the chromatogram with these reagents it is possible approximately to determine the type of flavonoid and the position of hydroxyl groups. Chromatograms are sprayed with Wilson reagent (0,5 g of boric acid and 0,5 g of anhydrous limonic acid in 20 ml of anhydrous methanol) and dried at 100-110 C, greenish-yellow fluorescence indicates the presence of 5-oxyflavones and 5-oxy- and 5-methoxyflavones.

The paper chromatograms are sprayed with 2% solution of zirconium chloride in methanol: yellow colouration (in non-electric light) or green fluorescence (in UV-light) indicates the presence of 5-oxyflavones and 5-oxyflavonoles; disappearance of yellow colouration or greenish-yellow fluorescence indicates the presence of flavonoles and 5-oxyflavones.

After spraying the paper chromatogram with 5% solution of SbCl3 in carbon tetrachloride (reagent Martini-Bettolo) the yellow or yellow-orange colour indicates the presence of flavones, flavanols, flavanones and isoflavonones; red or red-purple colour – chalcones. Chromatograms are sprayed with freshly prepared of diazotized sulphanilamide, the immediately produced orange-red colouration indicates the presence of 7-oxyflavones, 7-oxyisoflavones; orange-red colouration produced in 1-2 min is explained by the presence of 7-oxyflavonones. Some phenolic compounds undergo the diazotization reaction, that’s why this reaction is carried out after the establishemnt of flavonoid type. Taking into account the properties of raw material the flavonoid compounds are isolated with the selective extraction from the medicinal plant material. It is sprayed with petroleum ether or tetrachloride carbon for removing the lipophilic substances from the plant material. Then the material is extracted with diffrent ethanol or methanol concentration, hot water and etc. The mixture of chloroform and alcohol is used for the extraction when the material contains mainly metoxylated flaovnoids. Obtained extarctions are evaporated and the lyophilization is used. Then the residue is treated with chloroform, ethyl ether, ethylacetate or used for the obtaining of individual substances. Sometimes the precipitation methods with different lead salts are used for the purification and separation of flavonoids. Compounds containing orto-hydroxyl groups form the precipitation with lead acetate, but containing single hydroxyls form the precipitation with basic lead acetate. They can be received in crystalline form after the destroying of lead pecipitation – flavonoids by the solution of hydrogen sulfide (the derivativs of luteolin, apigenin and etc.). The control of flavonoid separation is carried out by TLC or paper chromatography.

Measuring the absorption of light by flavonoids in UV-light provides valuable input for their identificaiton. The position of maximum absorption with the reaciton of chromogen reagents indicates the number of hydroxyl groups and their position in flavonoid molecule.

At present HLPC (high-performance liquid chromatography) is used for the qualitative and quantitative determination of flavonoid extract.

Several methods are used for the quantitative determination of flavonoids, however the spectral methods are becoming more common.

Preparative column chromatography is a comfortable method for the separation of flavonoids into individual components. The polyamide powder- capron, cellulose, silica gel, magnesol powder and etc. are used as adsorbents.

The elution of substances from chromatographic column is carried out by the mixture of chloroform and its ethylacetate or methanol or ether, the mixture of benzene with its ethylacetate or methanol, and polar solvents: aqueous solutions of increasing concentration ethanol, methanol or the mixture of ethylacetate-methanol-acetone-water.

Column chromatography is widely used for the separation of sum of flavonoids into individual substances. Polyamide, sometimes silica gel and cellulose are used as adsorbents in column chromatography. The separation is conducted better in polyamide column. Elution of aglycone is carried out with alcohol and chloroform, glycosides – the mixture of water and alcohol in different concentration. Re-chrommatography, preparative thin layer or paper chromtography are used for the isolation of individual flavonoids. Complex researches are carried out for the determination of chemical structure of individual flavonoids. The determination of the melting point, specific rotation of glycosides, different chemical transformations: acylation, alkylation, hydrolysis and oth., spectra methods of analysis (UV-, IR-, NMR-, mass-spectra). The acylation reaction is used for the quantitative determination of hydroxyl groups, its position and quantitative determination of free hydroxyl groups. Mineral acid or enzymatic hydrolysis are carried out for the determination of the number of sugar residues and position of the attachment to aglycone in glycosides of flavonoids. The character and position of the attachment of substituents, configuration of bonds are determined by the spectral methods of analysis.

UV-spectra of flavonoids are characterized by the presence of 2 absorbance maximum. Flavonoid glycosides (for example, rutin) have 2 absorbance maximum “shoulder” at 258 and 361 nm.

The positions of maximum and “shoulder”,  and etc. are used for the identification of substance. This value for rutin is 325,5, for monoglycosides quercitrin – 350 nm. UV-spectroscopy is used fir the establishment of free hydroxyl groups in flavonoid molecule by the addition of different precipitation and complexing reagents (for example, sodium acetate, sodium methylate, boric acid with sodium actetae, aluminium chloride and etc.). The addition of these reagents the absorbance maximum is shifted which characteristic for the hydroxyl groups in different positions.

The bands which are characteristic for the different groups are in IR-spectra of flavonoids. For example, 3200-3500 sm-1 (phenolic and alcohol OH-groups), 1660 sm-1 (carboxylic group), 1610, 1580, 1510, 1460 sm-1 (aromatic C=C-bonds).

Flavon and flavonones in UV-light give the absorbance maximum. Two intensive absorption bands at 240-260 nm (high-frequency), at 330-375 nm (low-frequency) are characteristic for them.

Table. UV- absoprtion spectra of some flavonoids (in ethanol).

|  |  |  |
| --- | --- | --- |
| Flavonoid compound | λmax, (nm) | E, lg |
| Flavone  3',4'-dioxyflavone  5,7-dioxyflavone (chrizin)  5,7-diacetylflavone  Apigenin-7-apioglucoside (apiin)  Apigenin-7-rhamnoglucoside  5,7,4'-trioxyflavone (apigenin)  5,7,4'-trimethoxyflavone  Luteolin-7-glucoside  5,7,3',4'-tetraoxyflavone (luteolin)  5,7,3,4-tetraacetylflavone  3-oxyflavone (flavonol)  3,5,7-trioxyflavone (galangin)  3,7,3',4',-tetraoxyflavon (fisetin)  3,5,7,2'-tetraoxyflavon (datiscetin)  3,5,7,4'-tetraoxyflavone (kaempferol) | 297,5; 250  345; 245  330; 270  302,5; 255  341; 267  335; 270  340 265  325; 265  350; 259  355 258  300; 258  347,5;305; 239  360; 267,5  370; 315;  252,5  360; 262,5  370; 310;  267,5  375; 258;  380; 263;  375; 255;  252; 269;  254  310  361; 272  259  352; 260  361; 310;  258 | 4,20; 4,07  4,28; 4,17  3,90; 4,42  4,43; 4,18  4,29; 4,17  — —  4,31 4,25  4,33 4,25  — —  4,28 4,22  4,35 4,30  4,04 3,86;  4,14  4,07; 4,23  4,43; 4,42;  4,33  3,99; 4,14  4,28; —  4,12  4,32; 4,32  4,15; 4,22  3,27; 4,37  4,34; 4,29;  4,37  4,34 4,15  4,34  4,24; 4,35  4,28; 3,96;  4,35 |

The control of flavonoid separation is carried out by thin layr and paper chromatography, with appropriate reagents.

The measurement of light absorption by flavonoids in the UV region of the spectrum provides valuable material for their identification. The position of the maximum absorption in reactions with chromogenic reagents indicates the number of hydroxyl groups and their position in the flavonoid molecule.

At present, HPLC (high-performance liquid chromatography) is used for quantitative and qualitative determination of total flavonoids.

Several methods are used for the quantitative determination of flavonoids, but spectral methods of analysis have been increasingly used and applied.

*Quantitative determination of flavonoids.* The universal method of quantitative determination of flavonoids does not exist. In each case, they are treated individually, using weight, photometric, polarographic, potentiometric, spectrophotometric, complexometric and other methods. Spectral methods have become more and more widely used in the practice. These methods are classified according to the formation of coloured products: reduction in acidic medium or with sodium borhydrate; complex formation reactions with metals; compounds with diazonium; interaction with alkalines and etc.

In recent years different physic-chemical methods have become more widely used. These methods are faster and more accurate than gravimetric and titrimetric methods and allow to detect a significant amount of flavonoids. Photoelectrometry, spectrophotometry, densitometry with paper and thin layer chromatography are referred to these methods. Chromatography is used for purification and separation of flavonoids into individual components.

**Biosynthesis of flavonoids**

Biosytnehsis of flavonoids follows the mixed pathway. Ring A is synthesized via actetae pathway, but ring B – through shikimic acid (scheme).

Biosynthesis of the ring B. The shikimic acid which produced by the glycolytic decomposition of sugars passes through the series of intermediate compounds consistently with the participation of ATP and it is converted into prephenic acid. Prephenic acid is a key intermediate substance for the biosynthesis of flavonoids, coumarins, aromatic aminoacids and other compounds.

Scheme. Biosynthesis of the ring B of flavonoids.

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Shikimic acid Prephenic acid

C

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tyrosine p-coumaric acid

Formation of ring A and flavonoid (chalcone).

CH2

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Triacetic acid p-coumaric acid

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Intermediate compound Chalcone

It is able to convert into a number of products. For example, p-coumaric acid formation. Firstly the amination of prephenic acid and its decarboxylation occur. Tyrosine, in which dezamination leads to p-coumaric acid, its formula can be written in two ways, its second designation clarly presents ring B, or rather the structural fragment –С3-С6.

Biosynthesis of ring A. Triacetic acid reacts with p-coumaric acid, and the chalcone is formed as a result of chain closure and enolisation. Chalcone is a precursor of all other group of flavonoids; during the oxidairton of chalcones flavones, flavonoles and etc. are formed; during the reduction – anthocyanidins, catechins, leucoanthocyanidins (scheme).

Scheme. Intertaction in the biosynthesis of flavonoid compounds.

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O

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C

H

C

Dihidrochalcone

Aurones

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O

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O

H

O

O

H

H

Isoflavones

Flavanones

Chalcone

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Flavanoles

Flavanoids

Flavones

Flavanoids

(Leucoanthocyanidins)

Flavonoids (Catechins)

Anthocyanidins