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CONTENTS

THE SIGNIFICANCE OF THE ISOLATION OF PLANT PIGMENTS

The main aim of this work (chapter) is to represent certain plant pigments that are significant for humans and nature. Plant pigments are natural organic compounds that carry dyes and are found in plant cells and tissues. Nature is rich with various natural dyes found in fruits and vegetables. The attractive colour of various fruits and vegetables originates from pigments.

Beautiful colours like green originate from chlorophyll, which is responsible for photosynthesis. Photosynthesis is a process of chemical reactions by which photosynthetic organisms (plants, algae, and bacteria) absorb the Sun energy, turn it into chemical energy, and use that energy for CO2 reduction in carbohydrates. Natural flavonoid compounds contain red, blue and violet colours. Various hues of yellow and orange colours originate from carotenoids such as β-carotene, lycopene and others. Anthocyanins are natural organic components with beautiful purple hues of colours. These plant pigments are significant for human health. As the role of the natural pigments as antioxidants has great significance, the main aim of this chapter is to represent the selected natural organic compounds and their structures from the organic chemistry point of view.

The difference between dyes and pigments is that these beautiful pigments to be represented in this text are given to us by nature. This text deals with their main features, structures, analysis methods and isolation procedures of the selected pigments.

Key terms/definitions

DYE is a coloured substance chemically absorbed by the substrate to which it is applied. This feature separates dyes from pigments as they are not chemically absorbed by the material they colour.

NATURAL DYES are extracted from plants, insects, or minerals. Most natural dyes are obtained from plant sources such as roots, berries, bark, leaves, and wood, as well as other biological sources such as fungi.

Both **DYES AND PIGMENTS** are coloured because they absorb only certain wavelengths of visible light. Dyes are usually soluble in water, whereas pigments are insoluble. Some dyes can be made insoluble by adding salt to get a light pigment. Most natural dyes are obtained from non-animal sources: roots, berries, bark, leaves, wood, fungi and lichens.

INTRODUCTION

Dyes and pigments are coloured substances able to absorb visible light (400-700) nm. Dyes are highly significant in human life, and they have been applied since ancient times as compounds used for colouring various objects. Dyes were obtained from various plant raw materials.

Dyed substances are classified into organic, non-organic, natural and synthetic. The most important division of dyed substances is the one that divides them into *dyes* and *pigments*. Plant pigments include many molecules with multiple functions. They act as antioxidants in the fight against free radicals and they protect plants from diseases. They have antibacterial, antiviral and antioxidant effects.

Organic dyed substances are classified by their chemical composition, their method of use and the way of releasing the absorbed energy. The great colourfulness of plants is mostly caused by three types of plant pigments. These pigments include: porphyrins, carotenoids and flavonoids.

Porphyrins are a group of organic compounds containing four porphyrin rings with a methyl group in the tetrapyrrole ring, where iron metal is bound as chelate to the central part. Green leaves contain chlorophyll, which gives them the green pigment colour. Green leaves also contain yellow and red-coloured pigments, carotenes and xanthophylls, but they are not visible due to the great presence of chlorophyll.

When the fruits and vegetables get ripe, they may be followed by chlorophyll breakdown and synthesis of red and blue anthocyanin. Table 1 contains three types of organic compounds that give natural colour to plants.

TABLE 1: Classification of plant pigments

Pigment type	Compound type	Colour (leaf, flower and fruit)
PORPHYRINS	CHLOROPHYLL	green
CAROTENOIDS	CAROTEN (α, β and γ) and lycopene XANTOPHYL	yellow, orange, red yellow
FLAVONOIDS	FLAVON FLAVONOL ANTHOCYANINE	yellow yellow red, blue and purpure

2. PORPHYRINS

Porphyrins are porphin derivatives which play an important role in biological processes. These organic molecules participate in the conversion of light into chemical energy through transferring processes of oxygen and electrons. The word *porphyrin* comes from the Greek word *porphyra,* which means purple. The compound consists of a group of macrocycles consisting of four pyrrole units conjugated through methine bridges with a highly conjugated structure. Figure 1 shows the structure of the porphyrin ring:

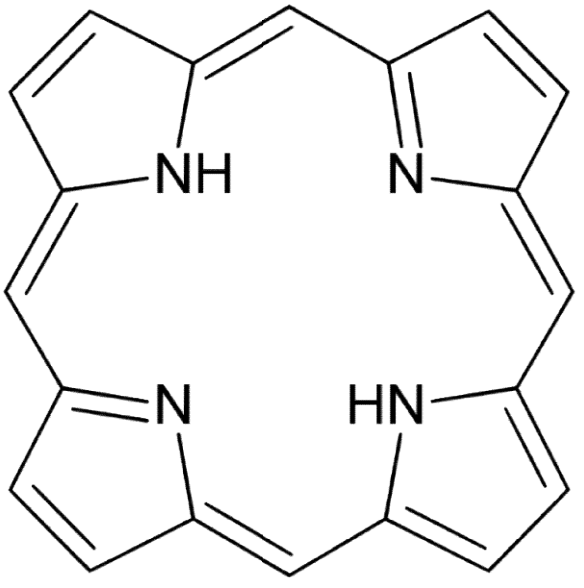

FIGURE 1: Porphyrin structure

This structure provides intense absorption in both UV and visible areas of the electromagnetic spectrum. This provides a purple colour to these structures. The porphyrin ring differs by groups bound to the pyrrole rings of porphin and by the complex-bound metal atoms in the centre. The porphyrin molecule is significant in medical chemistry and science about materials.

Chlorophyll is one of the organic molecules in a series of green pigments that is found in the organelles of living organisms. It plays an important role in plant photosynthesis. So, the green pigment present in the leaf is chlorophyll. Scientists *Pelletier* and *Caventou* were the first ones who described it. Its name comes from the Greek words *khloros* (*light green*) and *phyllon* (*leaf*). Chlorophyll structure consists of the porphyrin ring with magnesium ion in its centre.

Chlorophylls are classified into four types, and chlorophyll *a* is the first and the most important. It absorbs most energy from wavelengths including purple, blue and orange-red light. It is followed by chlorophyll *b* and *c*, and chlorophyll *d* is the fourth type found in red algae.

The complete structure of chlorophyll *a* and *b* was elucidated by *Fischer and Wenderoth*, whereas the first chemical synthesis was explained by *Woodward et al*. The most recent research indicates improvements in the process, structure, chemistry, and analytic methods.

The formation of carbohydrates from carbon dioxide and water in the presence of sunlight:

$$6CO_2 + 6H_2O \xrightarrow[\text{chlorophyll}]{\text{sunlight}} C_6H_{12}O_6 + 6O_2$$

This green pigment, chlorophyll and its derivatives are exactly used as additives to various food products. Figure 2 shows the structures of chlorophyll *a* and *b*.

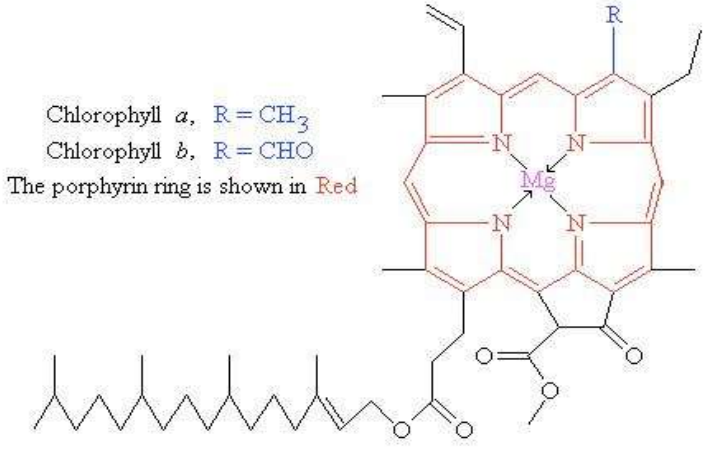

FIGURE 2: The structure of chlorophyll *a* and *b*

Porphyrin with iron metal (heme) in its centre is a complex. Red pigment, haemoglobin is a metal protein. The heme is a transporter of oxygen from the lungs into tissues.

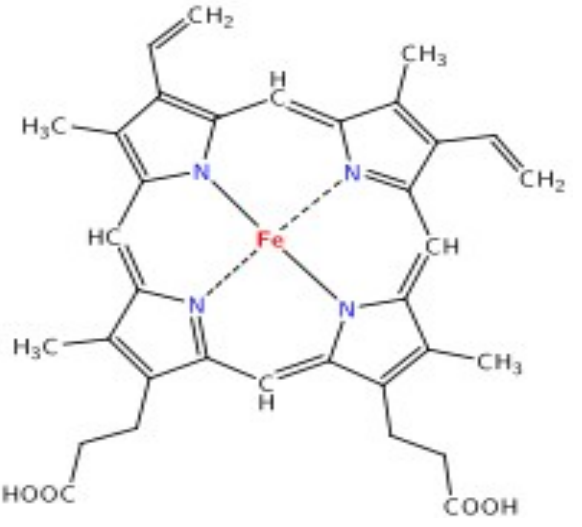

FIGURE 3: The haemoglobin structure

3. CAROTENOIDS

Carotenoids are soluble organic compounds synthesised in plants and fungi, in smaller concentrations in algae and bacteria, humans and animals. The carotenoids were named by Wachenroder who isolated the β-carotene compound from a carrot in 1831 and named it "carotene". After that, in 1837, Berzelius isolated the yellow pigments from the autumn leaves and named them "xanthophylls". Through his further research, Richard Willstatter established the empirical formula of carotenoids (C_{40}). In 1911, when innovative chromatography techniques were used, the scientist Tswett separated a fairly large number of pigments and collectively named them "carotenoids". Rich organic molecules are sources of yellow, orange and red colours of many plants. Fruits and vegetables provide 40 to 50 carotenoids, which are found in the human diet. There are over 750 different types of carotenoids. The carotenoid structure consists of covalently linked isoprene units. The main feature of these plant pigments is a linear chain of tetraterpene with 40 carbon atoms and eight bound units. They are classified into two groups:

- *carotenes* (hydrocarbons), one of their characteristics is that they dissolve in non-polar solvents, and
- *xanthophylls* with oxygen in their structure.

The most important role of carotenoids in nature is their participation in the process of photosynthesis. They are found in chloroplasts where they are bound to proteins. Due to their polyene structure, they are very unstable and oxidised, cyclised and isomerised in the presence of oxygen. They are isolated from natural material by extraction using organic solvents in an inert atmosphere and without the presence of light whereby the protein part is denatured. Such a resulting mixture is divided by Craig's method, which is based on the different solubility of carotenoids in non-polar and polar solvents. The purification of isolated fractions is usually performed by chromatographic methods. They are most often identified by characteristic maxima in the visible part of the UV spectrum.

The colorimetric methods are used for quantitative determination because carotenoids give a blue colour with sulfuric acid or antimony (III)-chloride in chloroform as a solvent. Most carotenoids have a central part of the molecule shaped as a long-conjugated chain. The ends of the molecule can be open (aliphatic) and cyclized. Compounds such as α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin and lycopene are the most common carotenoids in the diet. The corresponding organic compounds such as α-carotene, β-carotene, and β-cryptoxanthin are the provitamin A carotenoids, meaning that the body can convert them into retinol. Figure 4 shows the provitamin A structure.

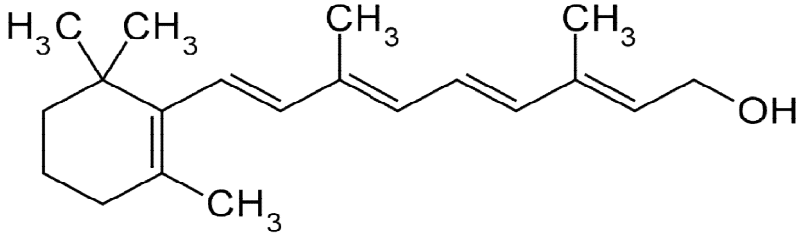

FIGURE 4: The structure of provitamin A

Other carotenoids such as lutein, zeaxanthin and lycopene are carotenoids that do not belong to provitamin A because they cannot be converted to retinol. Since provitamin A is a proven organic molecule with the antioxidant activity of these natural carotenoids (yellow, orange and red colour), these natural components are used in the food, cosmetic and animal food industries.

4. TERPENES

Natural organic compounds (terpenes) have different structures and their common unit is isoprene consisting of 5 carbon atoms. The isolation process of natural compounds takes place through distilling water vapour from plants containing fragrant oils. Organic natural components have a nice and sweet fragrance and they are used as perfumes, deodorants and medications. Natural compounds are isolated through the distillation process with water vapour from plants that contain fragrant essential oils. The precursor unit in the biosynthesis of terpenes is acetic acid. Through a series of biochemical and enzymatically controlled reactions, by binding of two molecules of acetate and a molecule of acetyl-CoA, monoterpenes are formed. The condensation of two molecules of monoterpenes produces diterpenes. Monoterpenes represent the most abundant group of natural terpenes, formed by dimerization of the isoprene units, they are components of volatile fractions of essential oils. Some of the monoterpenes include myrcene isolated from the bay leaf oil, and cis-β-ocimene, found in the hop oil. Sesquiterpenes are found in oils of various plants and resins. The greatest number of sesquiterpene compounds are constituents of essential oils.

Bisabolene is a monocyclic sesquiterpene found in the oil of camomile, lemon and spruce. An example from the diterpene group is the most important primary alcohol phytol, which is obtained by chlorophyll hydrolysis and represents the main component of vitamins E and K. Triterpenes are natural terpenes composed of C_5 isoprene units, in a "head-to-tail" manner. Instead of that, two sesquiterpenes or diterpenes are bound in a "tail-to-tail" manner. Figure 5 shows the isoprene unit:

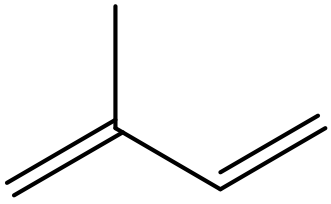

FIGURE 5: Isoprene structure

They are classified according to the general formula $(C_5H_8)n$, or according to the number of carbon atoms into:

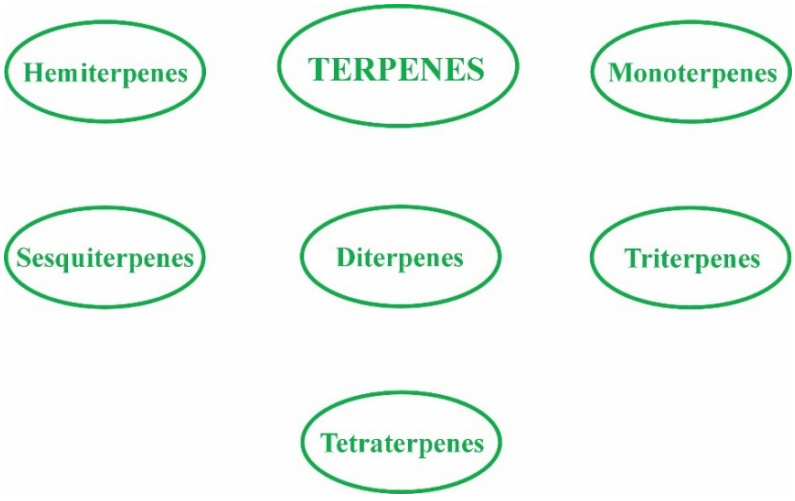

FIGURE 6: The classification of terpenes

4.1. Tetraterpenes

Tetraterpenes with 40 carbon atoms, carotenoids are yellow, orange or red pigments of plants and animals. They give colours to many sorts of fruits, vegetables, flowers and leaves. They are found in eggs, algae, fungi and feathers of many birds. By chemical structure, carotenoids are tetraterpenes and they belong to lipids. The most

important acyclic tetraterpene with eight isoprene units is lycopene, a red pigment found in tomatoes, watermelons, red grapes, etc.

4.1.1. Acyclic tetraterpene

Lycopene ($C_{40}H_{56}$) is the simplest red pigment found in tomatoes, watermelons, etc. It binds thirteen moles of hydrogen by catalytic hydrogenation giving the saturated tetracontane, $C_{40}H_{82}$. This compound is synthesised by Wurtz's reaction from two moles of dihydrophytyl bromide, and thus the carbon skeleton is determined. A further organic reaction procedure, ozonolysis produces two moles of acetone together with laevulin aldehyde, which marks the positions of double bonds. It undergoes cyclisation to form β and α-carotene. Lycopene is best known for its antioxidant properties of all other carotenoids.

FIGURE 7: Tomato red colour comes from lycopene

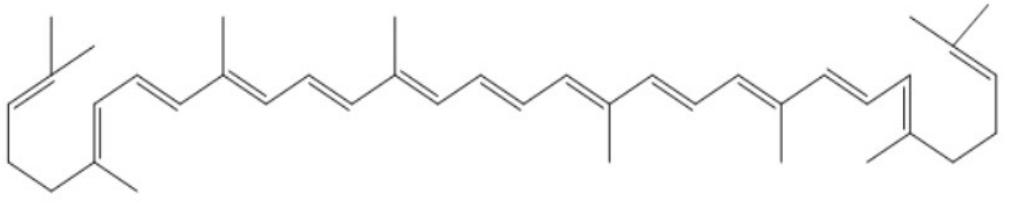

FIGURE 8: Chemical structure of lycopene

4.1.2. Monocyclic and bicyclic tetraterpenes

Carotene is a mixture of isomers as proven by chromatographic adsorption. Identification was made and by now it is divided into six carotenes: α-carotene, β-carotene, γ-carotene, δ-carotene, ε-carotene, ζ-carotene. A full identification was made only on the first three isomers, the other three were not fully proven. The cyclisation process of neurosporene, which is an organic compound of 40 carbon atoms, produces α- and β-zeacarotene, which forms γ-carotene by oxidation. Figure 8 shows the structure of neurosporene.

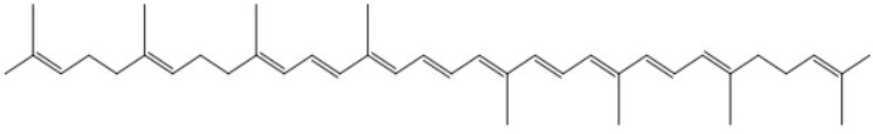

FIGURE 9: Chemical structure of neurosporene

The most important bicyclic carotenes are α- and β-carotene formed from γ-carotene. α-carotene has only one β-ionone ring, while the other half is of the α-ionone type. In γ-carotene, half of the molecule is similar to β-carotene, while the other half of the molecule is not cyclised as in the lycopene molecule.

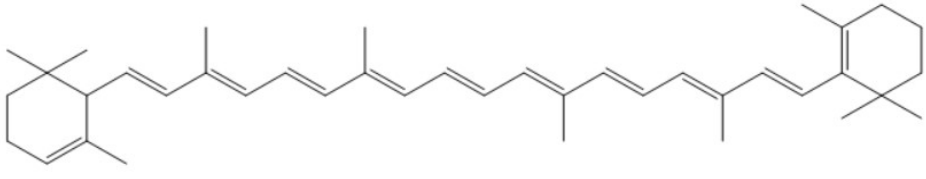

FIGURE 10: Chemical structure of α-carotene

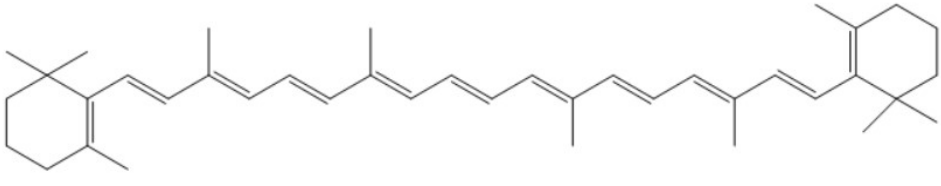

FIGURE 11: Chemical structure of β-carotene

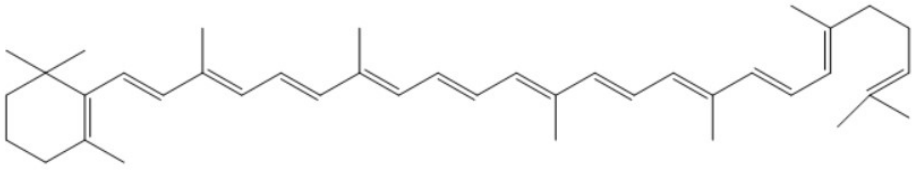

FIGURE 12: Chemical structure of γ-carotene

Along with carotenes, leaves contain yellow pigments known as xanthophylls with oxygen in their structure. Oxygen may be present in the form of a single or several hydroxyl groups, one or more carboxyl groups or hydroxyl and carboxyl groups. Hydroxyl groups can be esterified or free. Oxygen may appear in the form of 1,2- or 1,4-oxide rings.

Lutein ($C_{40}H_{56}O_2$) is a plant pigment present in green and yellow leaves and flowers. With a distinctive purple colour, it is found in chloroplasts. In combination with zeaxanthin, it makes the yolk pigment. An organic compound, lutein is a hydroxy derivative of β-carotene. As it contains oxygen in its structure together with isomer zeaxanthin it belongs to the xanthophyll group of carotenoids. In their structure, xanthophylls contain two hydroxyl groups, and they have great polarity in comparison to other carotenoids. Lutein does not have provitamin activity (vitamin A) in the human body. Taking this into consideration, it has been assumed for a long time that its potential role in the human body is unimportant.

FIGURE 13: Lutein is found in green leaves

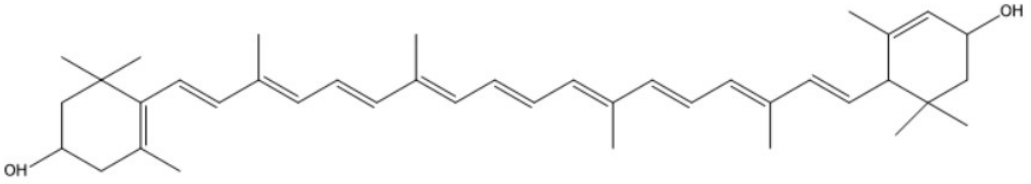

FIGURE 14: Chemical structure of lutein

Zeaxanthin ($C_{40}H_{56}O_2$) is a molecule of dihydroxy derivative β-carotene, the main pigment of the yellow corn, but also present in the yolk.

FIGURE 15: The yellow colour of corn comes from zeaxanthin

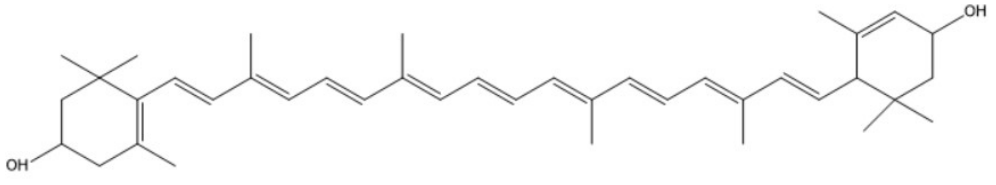

FIGURE 16: Chemical structure of zeaxanthin

Carotenoids, lutein and zeaxanthin are most abundant in dark green leafy vegetables such as kale, spinach, and lettuce, but also in egg yolks, peppers, corn and tangerines.

Cryptoxanthin ($C_{40}H_{56}O$) is a natural organic molecule and it is the main pigment in a tangerine. It is also found in green beans, peppers and corn. This compound is a mono-hydroxy derivative of β-carotene. Figure 18 shows the structure of an important pigment.

FIGURE 17: The orange colour of tangerine comes from cryptoxanthin

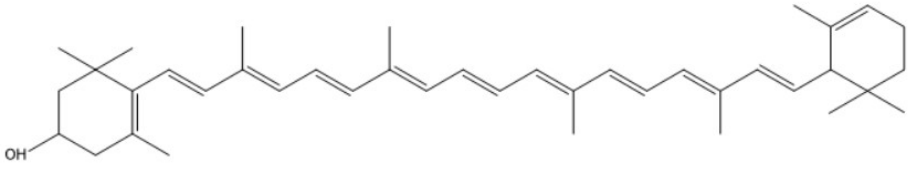

FIGURE 18: Chemical structure of cryptoxanthin

5. FLAVONOIDS

Flavonoids are natural polyphenol organic compounds, coming from the Latin word (*flavus-yellow*). Research shows that these compounds contain significant antioxidative and anticancerogenic effects. The organic compounds of this content act as enzymic inhibitors, and respirators in biosynthesis. Flavonoids are divided into: flavones and flavanones, flavanols and dihydro flavanols, isoflavones and isoflavones, chalcones and dihydro chalcones, aurones, anthocyanins, catechins, leucoanthocyanidins, biflavones and proanthocyanidins. Figure 19 shows a yellow saffron plant in yellow colour.

FIGURE 19: *Crocus flavus subsp. flavus* (Yellow saffron)

The structure of all flavonoids consists of a carbon skeleton that is comprised of two benzene rings connected with three carbon (C_6-C_3-C_6) compounds. Figure 20 shows the general formula of flavonoids.

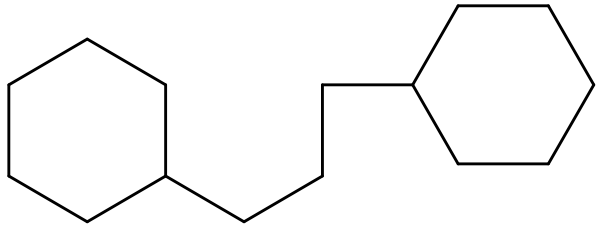

FIGURE 20: The general formula of flavonoids

Flavan is a compound formed by binding two benzene rings to three C atoms and creating a six-member ring with the bound oxygen.

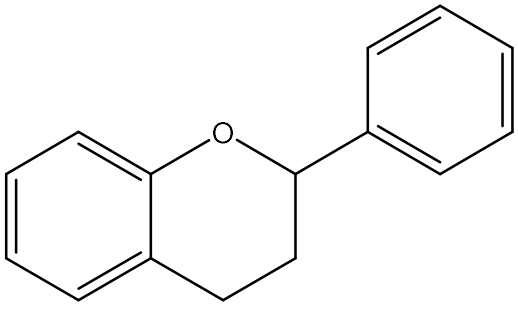

FIGURE 21: The structure of flavan

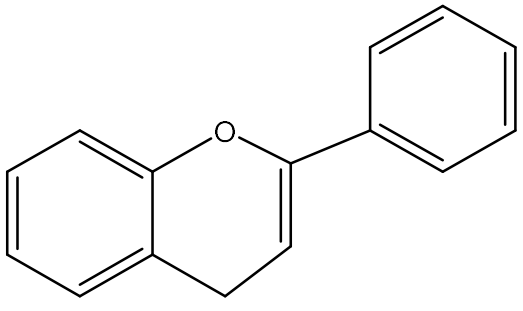

FIGURE 22: The structure of the flaven

By further process of structure forming, the heterocyclic ring has an additional oxygen atom and it is called *flavon*. The flavon molecule belongs to the group of flavonoids with a specific yellow colour. The structure of this group of flavonoids is shown below.

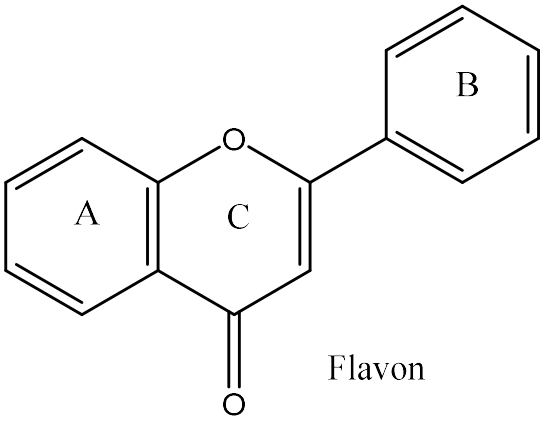

FIGURE 23: The structure of the flavon

In its structure, the *flavonol* structure has all the same elements as the flavon molecule, including the additional OH-group in the C-3 position. These compounds achieve their discolouration by adding the hydroxyl group. As they belong to the group of flavonoids, based on their structure, the important compounds include: quercetin, kaempferol and myricetin.

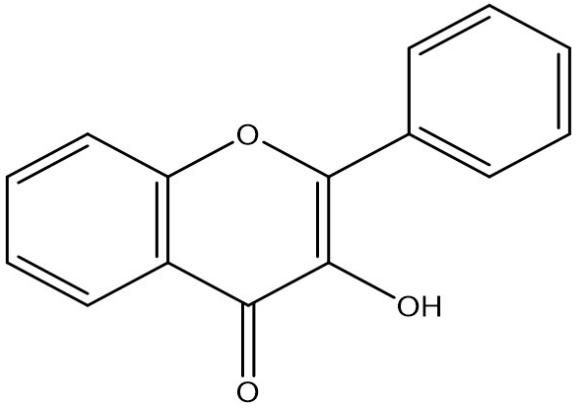

FIGURE 24: The structure of the flavonol

Quercetin, a plant organic compound is extremely significant and present in many food products such as onions, apples, various seeds, black tea etc. It belongs to the group of flavonoids that are important in the plant world. It is yellow and its basic structure consists of flavon and flavonol that can have OH, OCH_3 group or O-sugar in their structure. It

is found in the form of glycosides such as quercetin, found in yellow flowers, and the bark of oak.

FIGURE 25: Quercetin in onion

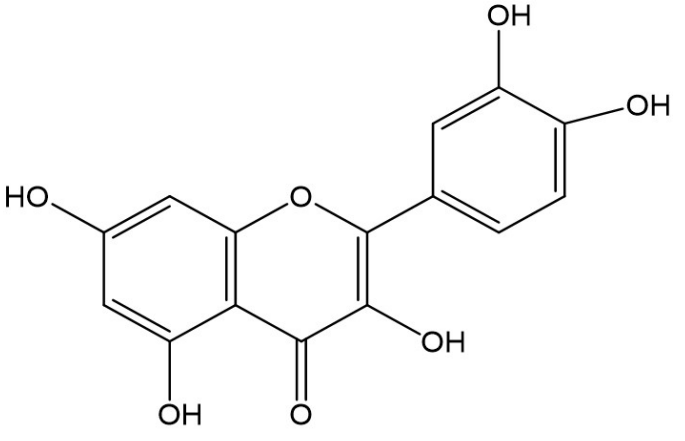

FIGURE 26: The structure of the quercetin

5.1. Anthocyanins

Anthocyanins are important glycosidic organic compounds in the plant world that have a flavyl ring in the basic structure, that is, a 2-phenylbenzopyryl ring. Coloured pigments, which are glycosylated, polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium,

consist of A, B and C rings (two benzoyl) and a heterocyclic C ring between them.

The compounds got their name from the Greek words *anthos*-flower and *kyanos*-dark blue. These are purple, blue and red pigments found in various fruits and vegetables. They are broken down into aglycones, anthocyanidins and sugars by hydrolysis.

Structural transformations of anthocyanins occur largely due to the different number of hydroxyl groups, nature, degree of methylation and number of sugars attached to the aglycone part of the molecule, substituents, nature and number of aliphatic and aromatic acids attached to the sugar. The colour of anthocyanins depends largely on their pH value. If we have the cationic form AH+ which is stable in a strongly acidic environment and is coloured red, a decrease in acidity leads to a deprotonation reaction of the AH+ form, and then the anhydrous form A is formed in an approximately neutral environment, which is coloured blue. The pseudobase form is formed in a weakly acidic environment, and through a hydration reaction it changes to form B. This form is in equilibrium with the chalcone form C, which is colourless. Many compounds have been isolated, about 540 different anthocyanins. About six basic anthocyanidin compounds, or aglycones, can be listed. They are present in the form of glycosides in the plant world, such as *cyanidin, pelargonidin, peonidin, delphinidin, petunidin and malvidin.*

Pelargonidin ($C_{15}H_{11}O_5$ *+)* is a natural, orange pigment found in berry-like fruits such as strawberries, cranberries, blackberries and others. It is also found in vegetables such as red radish, coloured in red by this plant compound. IUPAC name for plant pigment is 3,5,7-trihydroxy-283-hydroxyphenyl)-*1λ4-benzopyran-4ylium. That is an organic compound that contains a flavylium ring with hydroxy groups at positions 3,5, 7 and 4'.*

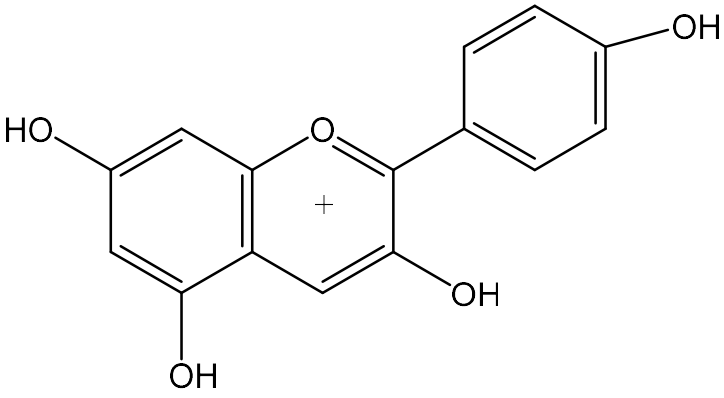

FIGURE 27: The structure of the pelargonidin

FIGURE 28: Radish, strawberry, geranium

Cyanidin ($C_{15}H_{11}O_6$ +) is a red-violet plant component found in berry-like fruits such as sweet cherries, strawberries, blackberries, aronia, cranberries, blackberries, and others. IUPAC name for this organic compound is *3,3',4'-Pentahydroxyflavylium or 2-(3,4-dihydroxy phenyl)-3,5,7-trihydroxy- 1λ⁴-benzopyran-1-ylium.*

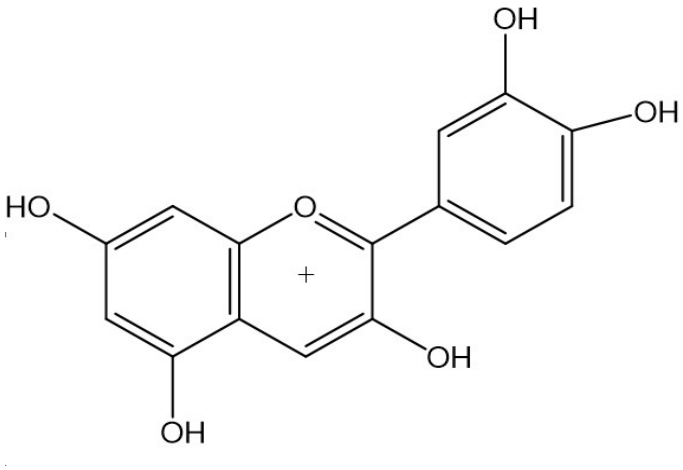

FIGURE 29: The structure of the cyanidin

FIGURE 30: Strawberry

Peonidin ($C_{16}H_{13}O_6$ +) is a natural organic compound extracted from the cyanidin structure. Peonidin is a plant pigment which leaves a violet-orange colour, such as with peony and rose, after which it was named. It is found in cranberries, blueberries, plums, grapes, and sweet cherries. The IUPAC name of this plant pigment is *2-(4-hydroxy-3-methoxyphenyl) chromenylium-3,5,7-triol.*

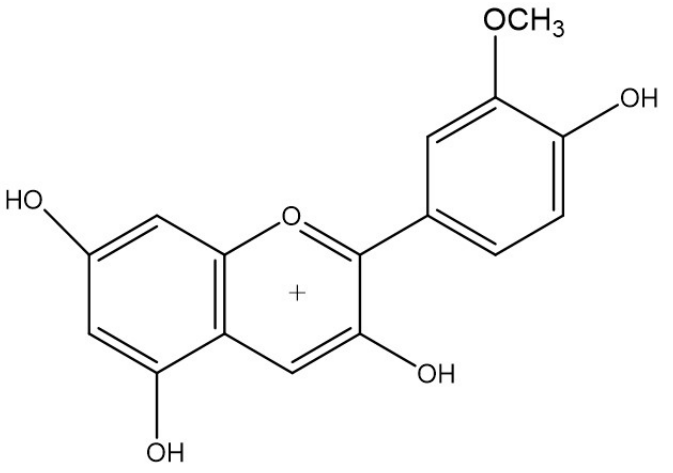

FIGURE 31: The structure of the peonidin

FIGURE 32: Cranberry, peony

Delphinidin ($C_{15}H_{11}ClO_7$) is a primary plant violet pigment that belongs to the family of anthocyanidins. The organic compound is found in fruits and vegetables such as blueberry, aubergine, vine and others. The IUPAC name of polyphenol pigment is *3,3',4',5,5',7-Hexahydroxyflavylium or 3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-1λ⁴-1-benzopyran-1-yilium.*

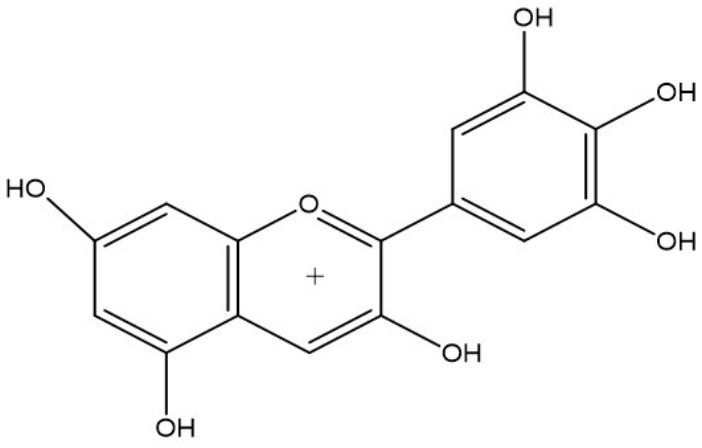

FIGURE 33: The structure of delphinidin

FIGURE 34: Blackberries

Petunidin ($C_{16}H_{13}ClO_7$) is a natural, dark-red and violet-coloured organic component. Its name comes from the word *petunia* from the flower family.

FIGURE 35: Petunia

An organic natural compound found in red berries (aronia, berries and grapes). The IUPAC of this significant compound is *3,3',4',5,7-Pentahydroxy-5'-methoxyflavylium or 2-(3,4-Dihydroxy-5-methoxyphenyl)-3,5,7-trihydroxy-1λ⁴-benzopyran-1-ylium.*

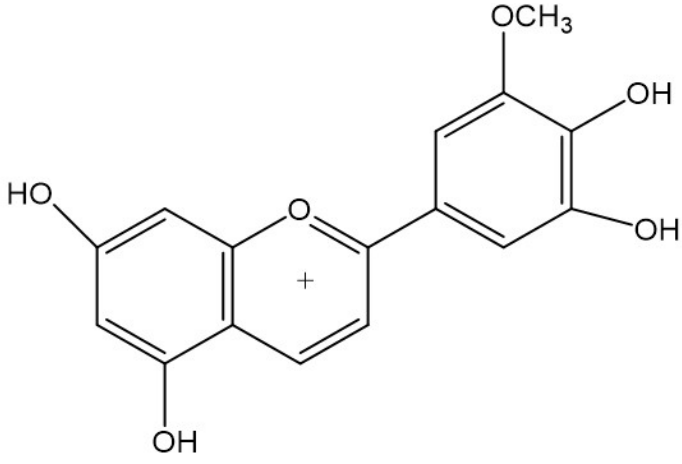

FIGURE 36: Structure of petunidin

FIGURE 37: Blackberries, petunia

A well-known plant pigment, the blue grape pigment, is malvidin-3,5-diglucoside (malvin). It is an anthocyanin cation that has two beta-D-glucoside residues present in its structure at the two positions of the C_3 and C_5 atoms. Malvin is a natural compound from the anthocyanin family found in blue grapes.

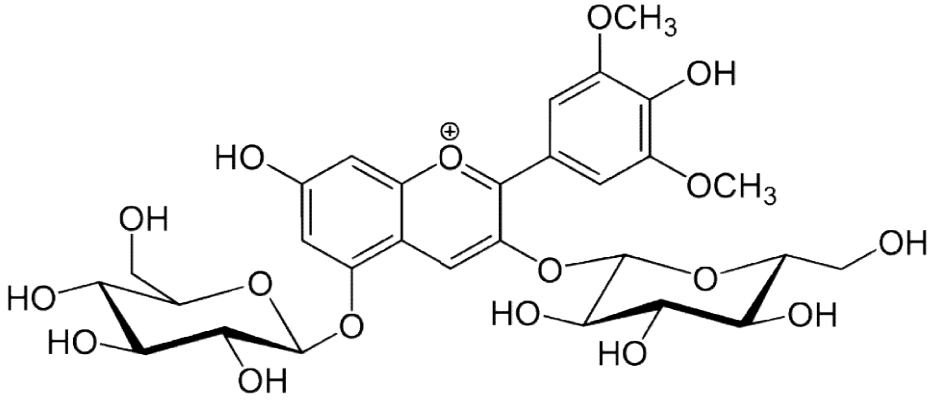

FIGURE 38: Malvin (malvidin-3,5-diglucoside)

FIGURE 39: Blue grapes

Anthocyanins are bioactive compounds that are important due to their impact on food products, overall human health and increasingly due to their potential application in the prevention of cardiovascular diseases and tumours. Natural pigments have an increasing application due to their wide range of colours and are beneficial for human health. The main problem for anthocyanins is the process from the plant material, due to their instability and low extraction yield. Various research leads to the solution of this problem and the finding of new isolation methods for better yields and stability. The most important pigments of many plants are responsible for the different colours of fruits and flowers. They are organic compounds that have strong antioxidant activity. As anthocyanins are polar organic compounds and the most suitable solvents during the extraction. Methods used in anthocyanin extraction are to be described.

6. APPLIED METHODS IN THE PROCESS OF ISOLATION AND DETECTION OF PIGMENTS

Extraction of plant pigments mostly combines solvents such as water and organic solvents. The water is used for the extraction of polar plant compounds and compounds soluble in water. If it is not possible to solve in water, plant pigments are extracted by using organic solvents. Methods used in the extraction of plant pigments include:

6.1. Extraction

Extraction is a separation method used for the isolation of chemical compounds. It is based on the distribution law, and it is defined as the separation of a substance from a solid mixture, suspension or solution by using a suitable solvent. When the desired substance is isolated, water is usually one of the components, and the other solvent is not mixed with water. The required substance must be more soluble in the solvent than in the polar phase. Extraction is classified as liquid-liquid extraction and solid-liquid extraction. When the appropriate substance is isolated, the procedure takes place in a separating funnel. Two phases that do not mix are separated there. When the solvents are separated into two layers, the ratio of solution concentration is constant regardless of the volume of the solvent and the amount of the solute. This phenomenon was expressed by Nernst, and it is known as Nernst's Distribution Law:

$$K = \frac{C_1}{C_2}$$

K represents the distribution coefficient, and c_1 and c_2 are the volume and mass concentrations. When shaking the concentrations, one needs to take care that each extraction is repeated three times so that the substance is fully transferred from one solvent into the other. The transfer is made on the contact surface between solvents. To make it as successful as possible, the contact surface needs to be enlarged by the process of shaking the funnel. Some compounds are harder to isolate during the extraction process and they require additional exposure to heat, acids, and extraction time. Such compounds include carotenoids. Methods used for carotenoid extraction are classified as follows:

- Soxhlet extraction, maceration, supported by microwaves or ultrasound,
- accelerated solvent extraction, also known as high-pressure extraction,
- pulsed-electric-field (PFF) extraction,
- supercritical-fluid extraction (SFE),
- enzyme-assisted extraction (EAE).

The carotenoid isolation is specific, and with the extraction process, it may cause the decomposition of compounds under a certain temperature. Non-polar solvents such as hexane, petroleum ether, tetrahydrofuran, etc. are used.

6.2. Solid-solid extraction (refluxing)

Solid-solid extraction is a method used for the extraction of substances with reflux or heating. Through the process of heating and continuous heating, the desired substance from the solid sample turns into the solvent due to the penetration of the solvent inside the sample particles. The sample and the solvent are located in a round-bottom flask on which a reflux condenser is placed. For even boiling under reflux, boiling stones are inserted into the round flask before the heating begins. The mixture is heated to the boiling temperature of the solvent and refluxed for a certain time, that is, the solvent vapours escape from the flask to the cooler, and the vapour condensate slowly drips back into the solvent. In the end, the soluble substance is extracted from the solid without much solvent consumption.

After that, filtration or decanting is mandatory.

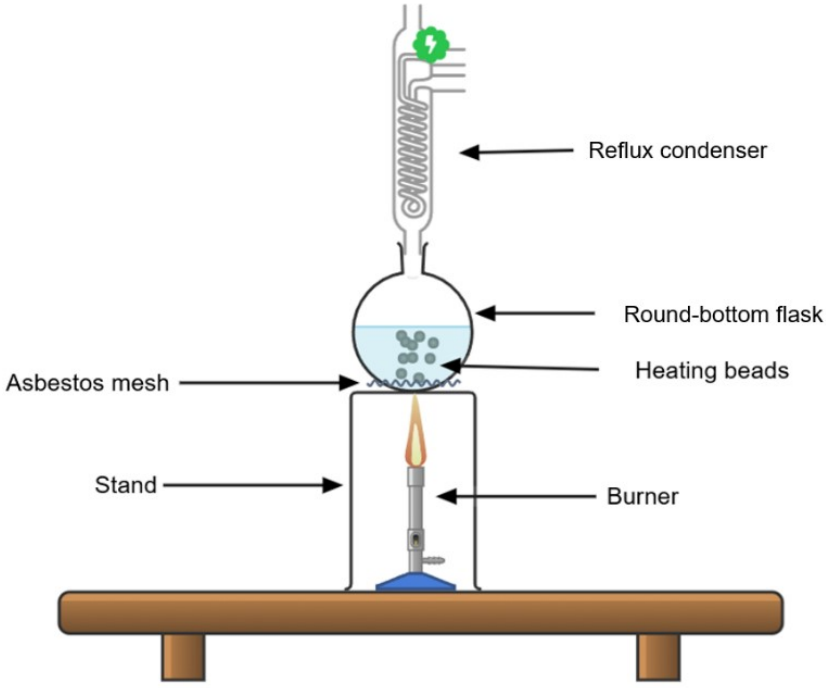

FIGURE 40. Refluxing apparatus

6.3. Soxhlet extraction

Soxhlet extraction is an innovative method that implies the extraction of a substance by a suitable solvent. The apparatus consists of a Soxhlet's extractor between the cooler and the round-bottom flask that holds solvent. The extractor contains a cylinder where the solid sample for extraction is placed. During the process of extraction, the solvent is heated until the boiling temperature and evaporates. Vapours go through the extractor tube, reach the cooler, and the condensate slowly returns to the solid sample. By further processing, the solvent fills the cylinder with the sample, while simultaneously the soluble substances are extracted in the solvent. When the cylinder is almost filled with solvent volume, the solvent with extract passes back to the flask through the siphon tube. The process is repeated through cycles and the extract is accumulated in the flask.

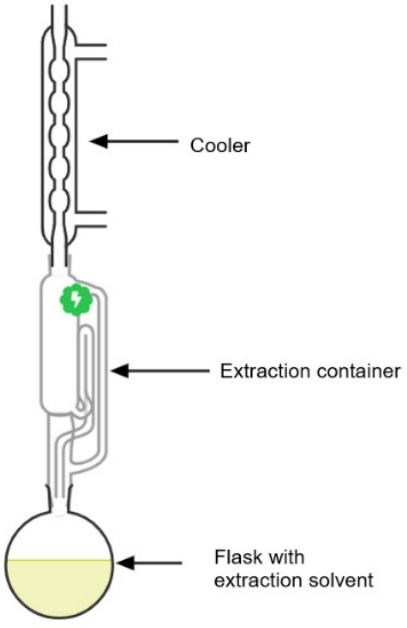

FIGURE 41: Apparatus for Soxhlet extraction

6.4. Column chromatography

Column chromatography is performed in a column filled with a solid adsorbent, and the solvent flows through the column under the influence of gravity. Silica gel or alumina is most often used as the solid, stationary phase, while an organic solvent or mixture of solvents is used as the mobile phase. The substance applied to the column is adsorbed onto the stationary phase, and the solvent dissolves the substance and washes it out of the column. As the substance travels through the column, a continuous series of adsorptions and dissolutions occur. The first part dissolves in the solvent, and the second part is adsorbed on the column, and thus an equilibrium between the eluent and the adsorbent is established.

Different substances have different partition coefficients, so they travel at different speeds through the column, and this is the basis for the extraction of the components. This creates zones of pure compound on the column, which are then eluted one after the other. The eluate is collected in fractions, which are then combined. The speed at which a substance will travel through the column depends on the strength of the

substance's binding to the stationary phase and its solubility in the eluent. The solid adsorbent is usually polar, so polar substances are adsorbed more strongly than less polar ones. Therefore, when a non-polar solvent is used, non-polar substances travel faster, while polar substances remain bound to the stationary phase. In addition to polarity, the order of elution is also affected by molecular mass. Substances with a lower molecular mass will travel faster than those with a higher molecular mass. Before extracting a mixture on a column, it is necessary to find suitable solvents that optimally extract the components. Thin-layer chromatography (TLC) is used for this.

A suitable solvent is one in which the difference in Rf values of the individual components is 0.1, and the range of Rf values of the least polar and most polar substances is from 0.2 to 0.8. If no such solvent is available, a mixture of solvents is used, or the solvent is changed during the extraction. Non-polar substances are usually removed from the column first, which means that elution usually begins with a non-polar solvent, and then polar substances are eluted by gradually increasing polarity. Filling the glass tube with adsorbent, i.e. preparing the chromatographic column, is extremely important for good extraction. The adsorbent must be uniformly distributed in the pipe, otherwise, the solvent flow rate is not uniform, and as a result, stretched and irregular zones are formed. Whether the column is filled with a suspension of the adsorbent in the solvent or the adsorbent is added unsuspended to the column with the solvent, it is important that the solvent continuously flows through the column during filling, as this reduces the possibility of air bubbles in the adsorbent column. The sharpness of the zone in the column depends on the concentration of the sample solution and the method of adding that solution.

The surface of the adsorbent must not be shaken because an irregular zone may be formed, which during elution gives stretched and poorly extracted zones of individual components. Therefore, a piece of filter paper or a thin layer of clean sand is usually placed on the upper surface of the adsorbent.

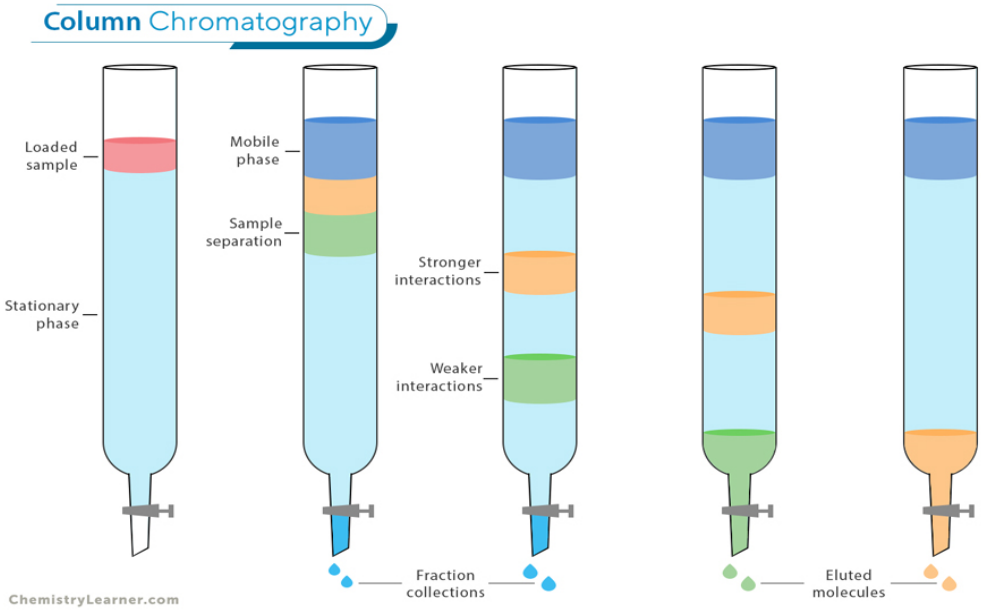

FIGURE 42: Apparatus for column chromatography

6.5. Thin-layer chromatography (TLC)

Thin-layer chromatography, like column chromatography, is based on the extraction of substances between a solid adsorbent and a liquid mobile phase. It is performed on a glass, metal or plastic plate coated with a thin layer of solid adsorbent. A very small amount of the dissolved sample is applied to the adsorbent by capillary action. The plate is immersed in the eluent so that the applied sample remains above the solvent level, as the solvent evaporates. The eluent "climbs" the adsorbent due to capillary forces and carries substances from the applied mixture at different speeds. The place where the sample is applied is called the start, and the front is the zone of the greatest distance of the mobile phase from the start. When the solvent front approaches the upper edge of the plate, it is removed from the solvent and zones are detected that determine the location of each component. The speed of the substance passing through the plate is proportional to the distance travelled. The speed is indicated by the Rf factor, which is defined as the ratio of the distance travelled by the substance from the start (x) to the distance of the front from the start (y):

$$R_f = \frac{x}{y}$$

If the components are not coloured, they must be made visible so that the Rf value can be measured. The most common methods for detecting components are ultraviolet (UV) light, reversible iodine addition, and sulfuric acid spraying. A plate with defined sites of adsorbed substances is called a chromatogram. Thin-layer chromatography has a variety of uses. It is used to determine the identity of substances based on the Rf value. It can also determine the number of components in a mixture. It is used to monitor reactions, for preparative purposes, and to find a suitable solvent that will extract the components well.

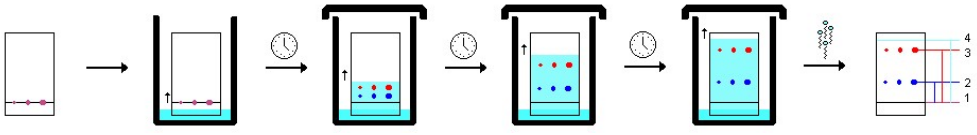

FIGURE 43: Thin-layer chromatography scheme

6.6. High-Performance Liquid Chromatography (HPLC)

One of the most significant methods for the extraction of polar organic components from the mixture is *high-performance liquid chromatography (HPLC)*. The mixture extraction principle of liquid chromatography is based on a different distribution of components between stationary and mobile phases. The stationary phase can be solid or liquid, the mobile phase can be liquid, i.e. the solvent. Depending on the stationary and mobile phase, we differentiate between chromatography with normal phase, the stationary phase is polar such as silica gel. The reverse phase uses non-polar solvents such as hexane, tetrahydrofuran, etc. For the analysis to be successful, it is necessary to establish an appropriate balance of intermolecular forces. The expression of intermolecular forces describes the expression of the relative polarities of the reactants. It refers to the relative polarities of organic functional groups and they go in increasing order (*aliphatic hydrocarbons* < *olefins* < *aromatic hydrocarbons* < *halides* < *sulphides* < *ethers* < *nitro compounds* < *esters* < *aldehydes* ≈ *ketones* < *alcohols* ≈ *amines* < *sulfones* < *sulfoxides* < *amides* < *carboxylic acids* < *water*). This can be said to

be *the rule of similarity*. To achieve the best chromatographic extractions, polarity matches are made between the stationary phase and the sample. In several situations, the stationary phase cannot meet the conditions for the successful extraction of sample components, while sample times become too short for practical application. Figure 44 shows an example of a chromatogram of the organic natural compound *delphinidin*.

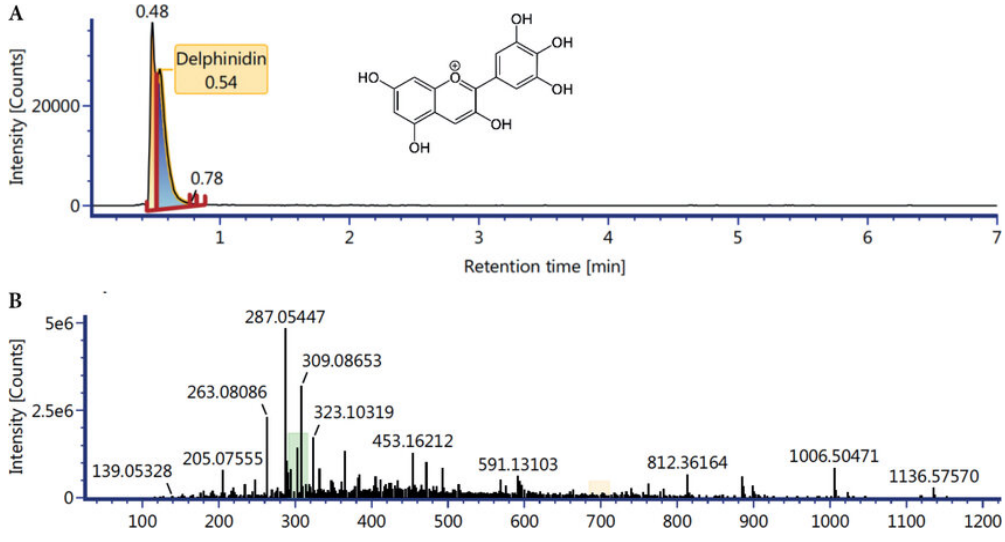

FIGURE 44: Example chromatogram of delphinidin

6.7. Centrifugal Partition Chromatography (CCC)

Centrifugal liquid-liquid chromatography, including counter-current chromatography (CCC) and centrifugal partition chromatography (CPC), is a chromatographic method used to partition solutes between two immiscible liquid phases without solid support. One of the two immiscible phases is retained in a column by a centrifugal force field and is called the stationary phase. The other phase is the mobile phase, which is filtered through the stationary phase. It is used as a method to isolate the largest amount of a particular molecule with the highest degree of purity in the shortest time and without the use of a column or silica media. The CPC method has similarities to the HPLC method, with the same goal of a thorough analysis of the sample.

FIGURE 45: Example of analysis with the CCC method

7. EXPERIMENTAL PART

7.1 Isolation of lutein from lemon, carrot or beetroot

Five grams of samples were mixed with different solvents, 4:1 included (water, hexane, ethanol, acetone 85% and isopropanol). The mixture was placed in water at 40-50°C for (1, 2, 3, and 4 hours). The samples were then filtered and absorption was measured at 447 nm. From previously prepared samples, 50 mg of extract was taken and mixed with different solvents, 2:1 (methanol: petroleum ether; petroleum ether: methanol, diethyl-ether: methanol) volume 150 ml, 30 minutes with shaking every 5 minutes. Now the NaCl solution is added in a ratio of 1:1. The solution is transferred to a separatory funnel. The formation of two layers is observed; light yellow at the top and dark green at the bottom.

The green layer is removed, because it does not contain lutein, and a 10% KOH is added to the yellow layer. The solution is left overnight, which is long enough for the saponification reaction to complete. The saponification mixture is placed back into the separatory funnel and an equal volume of petroleum ether is added. Allow to stand for 5 minutes at room temperature, followed by the addition of distilled water. The mixture is shaken vigorously and left to stand for 15-20

minutes. Two layers are formed again; the bottom layer is to be thrown away. The mixture is washed a couple of times to remove the base and the used solvents. To determine the pigment, the absorbance on the spectrophotometer is 447 nm. The pigment is left and dried until dry.

FIGURE 46: Lutein in powder form

7.2. Isolation of β–carotene from carrot

Required accessories and materials:

- Knives or graters
- Plate
- Weighing boats
- Technical scale
- Round-bottom balloon
- Condenser
- Water bath
- Bottle for vacuum filtration
- Buchner funnel
- Filter paper
- Erlenmeyer flask
- Extraction funnel

Required chemicals:

- Sample – carrot

- Dichloromethane
- Methanol
- Distilled water
- Natrium-chloride, NaCl
- Anhydrous sodium sulphate, Na_2SO_4

The extraction of carotenoids is difficult because they are very unstable substances that are subject to isomerisation and autoxidation under the influence of light, oxygen and heat, during which they decompose. Grate 10 grams of carrots, then reflux with 12.5 ml of methanol and 25 ml of dichloromethane under reflux in a round-bottomed flask over a water bath at a temperature of 50-55°C for five minutes with occasional shaking. Polar organic substances are extracted into methanol, and non-polar carotenoids into dichloromethane. The cooled mixture is filtered through a Büchner funnel in a vacuum flask. The procedure is repeated with the remaining carrot sample and 25 ml of dichloromethane.

Both filtrates were combined and gently shaken three times in a separatory funnel with 25 ml of water to remove the remaining polar substances from the dichloromethane. If an emulsion is formed during churning, add a little NaCl to separate the layers more easily. The lower, orange dichloromethane layer containing the dissolved carotenoids is dropped into an Erlenmeyer flask and dried in anhydrous Na_2SO_4. The upper aqueous layer, which contains polar substances, is discarded.

FIGURE 47: Isolated β-carotene

7.3. Isolation of lycopene from tomatoes

Required accessories and materials:

- Erlenmeyer flask with ground stopper (1000 ml)
- Buchner funnel
- Vacuum bottle (1000 ml)
- Filter paper
- Separating funnel (1000 ml)
- Round-bottomed balloon (50 ml)
- Tripod with ring
- Test tubes (2 pieces)

Required chemicals:

- Methanol
- Petroleum ether
- Anhydrous sodium sulphate
- Benzene
- Chloroform
- Antimony (III) chloride

Tomato concentrate (50 g), 90% aqueous methanol (50 ml) and petroleum ether (50 ml) are shaken vigorously for 15 min in a sealed Erlenmeyer flask with a ground stopper on a magnetic stirrer. The suspension is filtered through a Buchner funnel, the filtrate is kept and the precipitate is returned to the Erlenmeyer flask and shaken with 90% aqueous methanol (50 ml) and petroleum ether (50 ml) for 15 minutes. The suspension is filtered, and the filtrates are combined and transferred to a separatory funnel, where two layers are observed: the upper dark red (petroleum ether) layer and the lower (methanol) orange layer. The methanol layer was separated and the petroleum ether layer was washed with water (50 ml) and dried over anhydrous Na_2SO_4.

After drying, the solvent is removed on a rotary vacuum evaporator, leaving a dark red oil that is treated twice with benzene (treatment with benzene consists of dissolving the oily residue and evaporating the solution to syrup consistency). The obtained oil is dissolved in 2 ml of benzene and heated to boiling, and then warm methanol (3–4 ml) is added, during which red lycopene crystals are extracted.

Colour reactions of lycopene:

- Lycopene dissolved in cc H2SO4 gives a blue colour.
- A chloroform solution of lycopene with antimony (III) chloride gives a blue colour which is unstable and fades quickly.

7.4. Isolation of chlorophyll from spinach

1. The first step in the process of isolating spinach is to wash the spinach leaves with water and dry them with a towel. Take 2 g of spinach leaves and cut into small pieces. Put it all in a mortar with a pestle and chop the leaves, then add 3 mL of acetone. Decant the solution through a Bucher funnel with a piece of cotton wool into a screw cap tube and squeeze the residue to obtain more extract.

2. Then pour 3 ml of acetone into the jar and grind the rest again. Pour the liquid portion into a screw cap tube through the filter funnel. After that, pour 3 ml of acetone and 5 ml of hexane into the beaker and grind the rest again, and then filter the extract into a test tube with a screw cap. The leftovers should be very pale green.

3. After that, add 3 ml of salt water to the test tube and put the cap on, vigorously shake the test tube with the cap to wash out the chlorophyll-acetone-hexane solution. Occasionally open the cap of the test tube to release the pressure. Then return the test tube to the test tube rack and allow the mixture to extract. The top layer should be dark green and the bottom transparent.

4. If you notice that the emulsion is difficult to extract, add a little salt solution to make it easier to extract. Using a dropper, extract and collect the organic strain in another clean tube with a stopper or discard the lower layer. Finally, add enough anhydrous sodium sulphate (Na_2SO_4) to the solution containing the chlorophyll inside the screw-cap tube to dry the solution. Then filter the solution using filter paper into a 25 ml conical flask.

Thin Layer Chromatography (TLC)

1. Draw a line with a pencil 1 cm from one edge of the plate. Use the capillary tube to withdraw some of the chlorophyll solution from the conical flask and gently apply the solution to the starting line of the TLC plate. Allow the solvent to evaporate completely. Then apply more samples to the same spot. Repeat the above procedures until the coloured stain is visible.

2. Place a large strip of filter paper around the inside wall of the glass container and a round piece of paper on the bottom.

3. Add a mixture of acetone and hexane in a volume ratio of 2:8 to the glass container. The depth of the solution in the container should be less than 1 cm. Cover the tank cap for a few minutes to allow the solvent vapours to saturate the air in the tank.

4. Using tweezers, place the TLC plate vertically in a glass container containing acetone and hexane for the TLC to work. When the solvent almost reaches the top of the TLC plate, remove the plate and label the front side of the solvent.

5. Try other mixtures of acetone and hexane (3:7 and 4:6) to see which mixture gives the best resolution. Find the Rf values.

7.5. Isolation of pelargonidin 3,5-O-Diglucoside

Material:

- Plant material
- Extraction of orange-red pomegranate flowers

Required chemicals:

- Methanol (CH_3OH)
- Hydrochloric acid (HCl)

Isolation procedure:

- Young orange-red pomegranate flowers are picked, cleaned and cut into pieces. They are stored at 4°C. At the beginning of the experiment, 250 g of flowers are to be weighed and 250 mL of methanol with 50 mL of 0.1% methanolic HCl (green solvent) is to be added, and sonicated for 30 minutes and left for cold maceration at 4°C overnight. Tannins (punicalagin) were deposited at the bottom of the beaker and gently removed from the supernatant. The finished extract is centrifuged at 3000 rpm for 15 minutes to extract the supernatant. Using the column chromatography, the extract was purified. Using a rotary evaporator, the extracts produced were concentrated to dryness

at 40°C under reduced pressure and then stored at 2°C in hermetically sealed glass vials with screw caps.

7.6. Isolation of quercetin from apples

Material

- Apple peel

Chemicals used:

- Methanol
- Ethanol
- Chloroform
- Ethyl-acetate
- Green solvent – water

The isolation process:

- Apple peel was taken and processed into a dry powder. For extraction of flavonol molecules using different solvents, dehydrated apple peel (0.5 g) was mixed with a solvent (25 mL) in glass-stoppered Erlenmeyer flasks (125 mL capacity). In the research, two extraction factors were examined: the percentage of aqueous methanol as the extraction solvent and the duration in the ultrasonic bath. For each treatment, extraction solvent (50 mL) was used to extract phenolic molecules from dehydrated apple peel (1 g) using glass-stoppered Erlenmeyer flasks. In another study, several levels of methanol acidification were tested for quercetin and the extraction of quercetin glycosides from dehydrated apple peel powder (1 g). The extraction solvent used in this experiment was 50 mL of methanol containing 0%, 0.01%, 0.1%, 1%, and 2% (v/v) HCl. To compare different solvents, 100% water, methanol, acetone, ethyl acetate, and chloroform were used, and the mixture of apple peel powder and solvent was mixed well, placed in an ultrasonic bath and exposed for 60 minutes (four times for 15 minutes with an interval of 10 minutes). The temperature of the ultrasonic bath was between 20 and 28 °C. Three replicates of each solvent were prepared. In further research, the levels of methanol used were 60%, 70%, 80%, 90% and 100% (v/v). Three replicates of each methanol concentration were prepared using deionized water. The apple

peel powder and extraction solvent were vortexed and the flasks were placed in an ultrasonic bath at intervals from 15 min to 75 min, with a 10 min break for sampling.

7.7. Isolation of β-cryptoxanthin from fresh tangerines

Material

- Fresh tangerine

Chemicals used

- Hexane
- 1M NaCl (~10% (v/v)
- Methanol KOH
- 10% diethyl ether
- Acetone

Isolation process:

- In the first step of the organic compound isolation, 1 kg of fresh tangerine was cleaned and homogenised with 1 L of hexane using a mechanical homogenizer. The isolation and purification of the compound were done at a rapid pace to avoid degradation and isomerization.
- Homogenized samples were transferred to 200 mL centrifuge bottles and centrifuged at 7000 × g for 10 minutes at 4 °C, the supernatant containing carotenoids was then removed. The pelleted sample was repeatedly (2-3 times) extracted with hexane, until colourless. The collected supernatants were combined, and then the lower and upper hexane phases were extracted. The pelleted sample was repeated (2-3 times) and extracted with hexane until they became colourless. The collected supernatants were pooled, and then the lower and upper hexane phases were extracted. To easily extract the upper hexane from the lower aqueous phase, ~10% (v/v) 1 M NaCl was used. The hexane part was evaporated in a vacuum-rotary evaporator at 35 °C.
- The resulting extract was dissolved in 20 mL of hexane, mixed with an equal volume of 10% methanolic KOH (KOH; w/v), flushed with nitrogen gas (N2) (to minimize oxidation) and incubated at 55 °C for 45 min for saponification. The mixture was transferred to a separatory funnel, extracted three times with

hexane containing 10% diethyl ether and the above lipophilic hexane solutions were combined and then washed three times with water to remove traces of KOH. Diethyl ether was added to improve the polarity of the hexane solution and improve the solubility of unesterified β-cryptoxanthin. The lipophilic hexane solution was dried under vacuum (<35 °C) using a rotary evaporator, and the residue was redissolved in 10 mL of acetone.

7.8. Purification of β-cryptoxanthin

After the β-cryptoxanthin isolation, thin-layered chromatography (TLC) was performed

- TLC plates were chromatographed by a mixture of acetone/hexane (1:3; v/v).
- The top spot of β-cryptoxanthin (Rf = 0.6) was scraped off, eluted with acetone and centrifuged at 10,000xg for 5 min. The supernatant was collected, dried under nitrogen and stored at -20°C until the analysis.

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