ANALYSIS OF ASIMILATING PIGMENTS BY MONO AND BIDIMENSIONAL THIN LAYER CHROMATOGRAPHY TECHNIQUE

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Abstract
Considering the major importance of chlorophyll, it was chosen as the subject of this study, the extraction, separation and identification by chromatographic methods of chlorophyll types, assimilating pigments that accompany chlorophyll and chlorophyll degradation products from extracts of selected plants. The paper presents the chromatographic method for the separation of the chlorophyll pigments, focusing on the two-dimensional chromatographic technique, extraction and separation of the components of the vegetal extract and interpretation of results.

Introduction
Two-dimensional chromatography is a method applied for qualitative analysis, using a square plate on which a single spot is applied in the lower right corner, eluted with the first solvent or mixture of solvents, obtaining a first separation, and after drying, the plate rotates at an angle of 90 ° and a new elution is started with another solvent, after which the plate is compared to a standard plate on which we have a known mixture [1-4]. By two-dimensional chromatographic technique, the separation of chlorophyll (a and b) as well as other assimilating pigments extract from green leafs can be highly improved. Extracts of plants rich in chlorophyll (spinach, dill and nettle) were obtained and analyzed by thin layer chromatography using mono and two dimensional techniques. Also, chlorophyll c that is found in inferior plants was separated by mondimensional thin chromatography. Spinach (*Spinacia oleracea*) is grown for the leaves which besides the high content of chlorophyll are a source of folic acid, vitamins (B1, B2, B6, C, E, K, and PP) iron, carotene, carbohydrates and lutein. Dill (*Anethum graveolens*) has a rich content of volatile oils, mineral salts (Ca, Fe, Mg, P, K, Na, Zn, Cu, Mn). Common nettle (*Urtica dioica*) is beneficial due to detoxification and regeneration of the body [5-6]. The therapeutic properties are due to an abundance of bioactive substances with a high content of mineral salts (iron, magnesium, calcium, potassium and silicon). Spirulina (*Arthrospira platensis*) is a green-bluish microscopic alga that grows in tropical lakes [7]. Its color is due to the blue pigment that together with the chlorophyll form this color. Spirulina is an extremely rich antioxidant food containing 26 times more calcium than milk, protein, amino acids. It is a polyvitamin, contains liposoluble and water-soluble vitamins such as beta-carotene, vitamins B1, B2, B12, C, E, folic acid, nicotinic acid, vitamin H-biotin [6-10].

Experimental
Vegetal material of spinach, dry dill and dry nettle were used for extraction. 5 large leaves of plant material are triturated with 1 g of quartz sand and 0.5 g of calcium carbonate to neutralize its acidity (Fig. 1). The ground vegetable material is placed in a Berzelius beaker

and is supposed to extraction with 10 mL solvent prepared by mixing benzene: methanol in a ratio of 6: 4. Then, the mixture of vegetable matter and solvent is heated on the water bath for 5 minutes and filtered. In the case of other green plants, the volume of extraction solvent can be doubled, maintaining the proportion of components. If the filtrate is not sufficiently separate, it is centrifuged, then heated on the water bath for 5 minutes and filtered (Fig. 1).

Figure 1 Extraction stages for obtaining extracts of spinach, dill, nettle and spirulina

For the chromatographic separation of the chlorophylls and assimilating pigments, a spot is applied on the chromatographic paper using a Pasteur pipette. In two dimensional-thin layer chromatography technique, the spot must be applied at the starting line in a corner of the chromatographic plate. This is achieved by joining two lines at an angle of 90 ° in the lower left corner at a distance of 2.5 cm from the edge of the chromatographic plate. Plates will be introduced into a separation chamber that is a glass parallelepiped-shaped container, covered with a lid, ensuring a saturated atmosphere in the vapor of liquid phase (eluent solvent). The mobile phase has to be introduced in the separation chamber 30 minutes prior to the introduction of the chromatographic plate. The development system 1 consists of a mixture of gasoline: petroleum ether: acetone in a volume ratio of 50: 12.5: 2. In the first direction, the separation will take place of chlorophyll a (blue bluish stain), chlorophyll b (greenish yellow), xanthophyll (yellow spots), degradation products of chlorophyll (gray spots) and beta-carotene at the highest point (Fig 2 left). Development is considered to be completed when the solvent front has reached 1 cm from the top edge of the chromatographic paper. After development, the plate is air dried, marked the center of the pigment point, measured the distance traveled by the solvent front (X_D) and the distance traveled by each pigment (Xs) (Fig. 2). The Retardation factors (Rfs) are then calculated. In the next step, the plate is rotated at a 90 ^{0} angle and develop the solution 2, which is a mixture of 4 components: gasoline: petroleum ether: acetone: methanol in a volume ratio of : 50: 12.5: 5: 2.5 gasoline: separating chlorophyll a (bluish green) of chlorophyll b (greenish yellow) and xanthophyll (yellow).

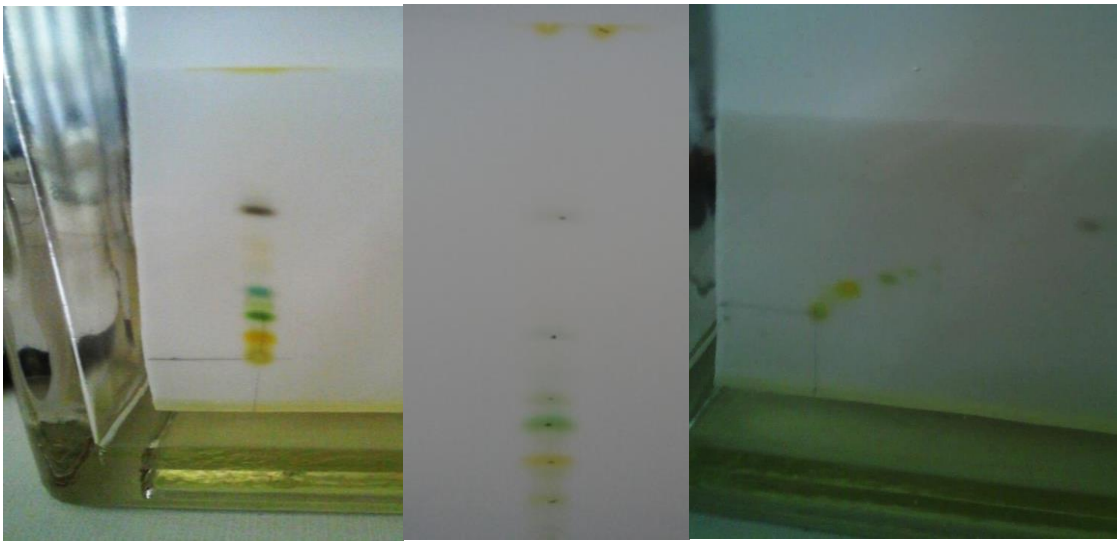

Figure 2. Developing, marking the spots, measuring Xd and Xs, developing in solution 2

In spirulina extract, besides chlorophylls a blue-green spot that was separated. It can be attributed to phycocyanin belonging to the group of phycobilin assimilating pigments. Thus, a comparative separation was performed, an extract of a higher plant and spirulina extract (obtained of spirulina powder), which is part of the group of algae. One gram of spirulina is mixed with 80% acetone, left in the dark overnight at 4 ° C for complete extraction. The extract obtained by centrifugation for 5 minutes is subjected to separation by one-dimensional chromatography on a silica gel plate. The chromatographic plate was developed with a solvent composed of petrol: petroleum ether: acetone in the same ratio as solvent 1. After development, the corresponding pigments are identified by the color and position of the obtained points. It was marked the center of the point of each pigment and calculated the value of the Rfs.

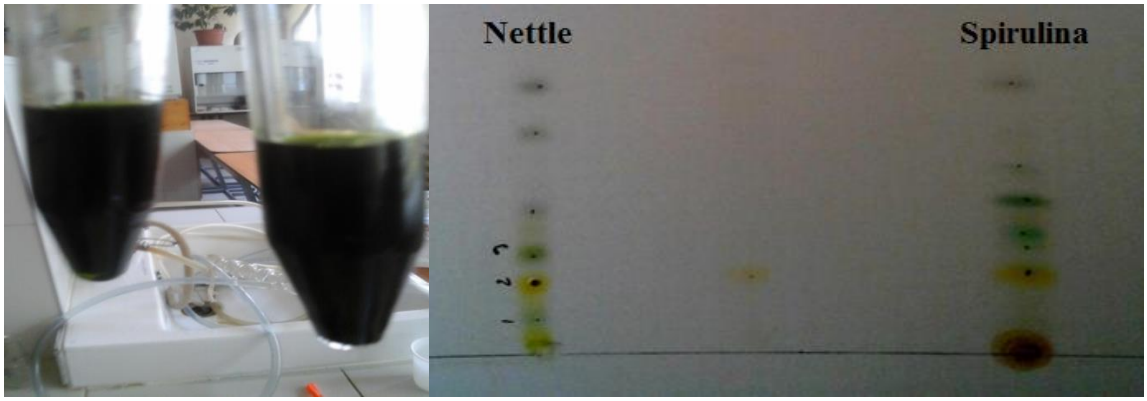

Figure 3. Spirulina extract and the chromatogram of spirulina compared to nettle extracts

Results and discussions

Chromatogram must be read immediately after the chromatographic paper has dried of, as it discolors immediately if it is exposed to light. Rfs are calculated. The maximum Rf value was obtained for carotene, with the highest yellow spot. For the other spots, the center of the pigment and the solvent front will be marked. Rfs are calculated using formula 1. Vegetable extracts have been used with different degrees of freshness: spinach, nettle and dill. Following development with solvent 1, composed of: petrol: petroleum

ether: acetone for 30 min, the following values of retardation factors (Rf) were obtained, shown in Table 1. By applying a spot of the extracted material from the vegetal mass on the chromatographic paper, it was identified by two-dimensional chromatography, chlorophyll a, chlorophyll b, carotene, xanthophyll and phytol resulting by the degradation of chlorophyll.

$$Rf = \frac{Distance\ from\ Baseline\ travelled\ by\ Solute}{Distance\ from\ Baseline\ travelled\ by\ Solvent\ (Solvent\ Front)} = \frac{X_S}{X_D} \qquad (1)$$

Table 1. Rf values on TLC separation on silica gel plates of spinach, nettle, dill extracts - bidimensional method, (development with solvent 1; time: 30 minutes)

Identified component as spot/ Rf value	Rf values for different vegetal materials		
	Spinach, fresh X_D=12cm	Nettle ,dehidrated X_D=15cm	Dill, dried X_D=13,5 cm
Beta-carotene	0.93	0.83	0.85
Chlorophyll degraded	0.61	0.43	0.49
Chlorophyll a	0.28	0.20	0.25
Chlorophyll b	0.26	0.13	0.16
Xantophile	0.17	0.07	0.07

By rotating on a 90^0 angle the line after which the decomposition of chlorophyll into the component elements is achieved, it will become a starting line for of a new development. Another solvent, named solvent 2 with the following composition: benzene, petroleum ether, acetone and methanol was used to as mobile phase during 30 minutes. The Rf values for spinach, nettle and dill are shown in Table 2.

Table 2. Rf values at separating on silica gel plates of spinach, nettle, dill extracts by two-dimensional method with solvent 2 (development time: 30 minute)

Identified component as spot/ Rf value	Rf values for different vegetal materials		
	Spinach, fresh (X_D=11,8 cm)	Nettle ,dehidrated (X_D=13 cm)	Dill, dried (X_D=13,5 cm)
Beta-carotene	0.95	0.85	0.80
Chlorophyll degraded	0.86	0.60	0.53
Chlorophyll a	0.81	0.46	0.63
Chlorophyll b	0.67	0.35	0.42
Xantophile	0.51	0.24	0.19

By comparing the results of the two developments for the two solvents and the three plant species, the same types of pigments were identified for the three plant species. Beta carotene has the orange color with the highest Rf value for the three studied species. Chlorophyll a, bluish green has values close to elution 1, compared to solvent 2 with different but close values, the lowest values of Rfs being obtained at dehydrated nettle and the highest in fresh spinach. Chlorophyll b, greenish yellow in elution 1 has close Rf values. Elution 2 conducted to different values, the highest Rf in spinach, the lowest in the dehydrated nettle. Xanthophyll, a yellow spot has values close to solvent 1 and very different for solvent 2, the highest for fresh spinach and lowest for dehydrated nettle. Thus, using two-dimensional TLC chromatography, a better separation of the assimilating pigments was realized. In the second separation with solvent 2 the spots were purified removing the impurities. However, the

greatest drawback of two-dimensional TLC chromatography, when compared to other planar techniques, is its limitation to one sample per plate [11]. Unlike superior plants, spirulina contains beside chlorophyll a, b also chlorophyll c as well as phycocyanin pigments. By monodimensional chromatography, based on the color and position of the separated pigments, yellow-green chlorophyll, blue-green chlorophyll and carotenoid pigments were identified in the nettle (Fig. 3). In the same time, an extract of spirulina was analyzed where carotenoid pigments were identified as the highest orange color, gray degraded chlorophyll, blue-green chlorophyll, intense yellow xanthophyll. The Rfs values of the spots are shown in Table 3. It was assumed that the dark blue and blue spots that are not present in the nettle extract, are given by phycocyanin and chlorophyll c present in the composition of the spirulina extract [12-14].

Table 3 Rf values for separation on silica gel plates, the monodimensional thin layer chromatography method of nettle and spirulina powder extracts

Identified component as spot/ Rf value	**Rf values of vegetal extracts**	
	Fresh nettle (X_D 10.2 cm)	Spirulina (X_D =10.2 cm)
Carotenoids	0.72	1.00
Chlorophyll degraded	0.67	0.69
Phycocyanin		0.37
Chlorophyll a	0.14	0.26
Chlorophyll b	0.09	
Chlorophyll c		0.31
Xantophile	0.04	0.22

Conclusions

In the paper, it was aimed the identification and extraction by chromatographic methods of assimilating pigments existing in the superior green plants – spinach, dill, nettle and algae - spirulina. By separating the extract, it has been observed that in green plants besides the two types of chlorophyll a and b there are significant amounts of carotene, xanthophyll, phycobilin, which play an important role in the functioning of living organisms. Another observation is that by thermal processing and under the action of extraction solvents, a significant portion of the chlorophyll is converted into degradation products. This was observed following the separation process of the components from plant extracts on the chromatographic plate. Chlorophyll type c and phycocyanin present in algae have also been identified separately. The method used for all plant species was that of mono and two-dimensional thin layer chromatography. The presence of a significant amount of beta-carotene in all green plant species studied has been highlighted. These compounds, in the body are converted into vitamin A or retinol. For this reason, it is recommended to consume green plants, which will help maintain the health of the eyes, skin, mucous membranes, maintain the level of collagen within normal limits and contribute to the normal functioning of the immune system.

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