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THIN-LAYER CHROMATOGRAPHY APPLIED TO FOODS OF ANIMAL ORIGIN: A TUTORIAL REVIEW

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The chromatography techniques are partially characterized by presence of two phases (stationary and mobile), which allows identification, semi-quantification or quantification of important compounds in different matrices. Among chromatography methods, the thin-layer chromatography (**TLC**) must be considered for using in routine laboratories due to a number of advantages such as practicality, fast results and effectiveness, low cost, and simultaneous determination of analytes. This review describes the application of this technique to foods of animal origin, as well as compares TLC with other chromatographic methods. TLC has a strong potential as a surrogate chromatographic model for qualitative and quantitative analysis. Therefore, several modifications have been carried out to the conventional TLC system. Nevertheless, further studies should be performed in order to contribute with the scientific community and propagate the TLC method, which has performance and economic advantages compared to other chromatographic techniques.

Keywords: chromatography techniques, TLC, nutritious compounds, drug residues, deterioration substances, foods of animal origin.

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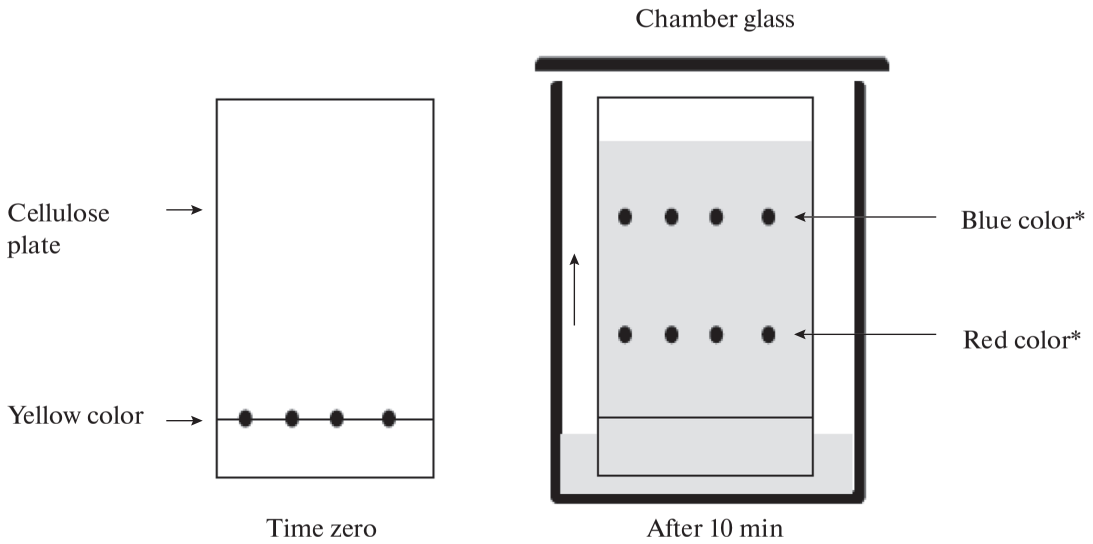
Thin-layer chromatography is a technique applied to detect several compounds in many fields, including environmental, clinical, flavors, cosmetics, and particularly for pharmaceutical and food analysis. In the food industry, TLC has been used to identify a large number of nutritious compounds such as lipids, vitamins, amino acids as well as drug residues and substances produced during the deterioration process like mycotoxins and biogenic amines [1–9]. In this context, TLC is a method widely employed in the laboratories due to its practicality, quickness, effectiveness, low cost and ability to determine many samples simultaneously when compared to other chromatographic techniques such as paper chromatography (**PC**), HPLC and gas chromatography (**GC**) [9, 10].

TLC is based on the separation of components of a mixture by differential migration on a thin layer of adsorbent retained on a flat surface. This technique allows the semi-quantification of substances by visual comparison of the spots with the same retention factor formed by samples and standard solutions and by the color intensity of the spots. Moreover, when this technique is associated to other tools such as digital image processing and UV detection, the substances can be quantified, and it is possible to obtain results similar to those from quantitative techniques [4, 11–13].

Therefore, the TLC method allows identifying, estimating (semi-quantitative analysis) and quantifying several compounds in different matrices, including those from animal origin, as well as HPLC and GC. In this context, this review contains descriptions of the methods and applications of TLC to foods of animal origin and comparison with other chromatographic methods widely used.

Chromatographic techniques. In 1903, a Russian botanist Mikhail Tswett described the separation of six pigments from vegetal leaves by using solid adsorbents. In that case, the stationary phase was calcium carbonate and the mobile phase was petroleum ether. By capillarity, the mobile phase containing the plant extract interacted with the particles of calcium carbonate. Therefore, the compounds were separated by different forces of chemical attraction of each substance in relation to calcium carbonate. This process was named chromatography (*chroma* = color and *graphein* = write) [14].

Chromatography is partially characterized by the medium on which the separation occurs (Fig. 1). This medium is commonly identified as the stationary phase. The stationary phases that are typically used include paper, thin plates coated with silica gel or alumina, or columns packed with the same substances. When the stationary phase is present as a plane surface, the methods are called planar chromatography



Scheme made from theory of colors separation using PC proposed by Mikhail Tswett [14].

The cellulose plate (stationary phase) absorbs the solvent — ethanol (mobile phase) with time and the samples are being carried by capillary. The colors are separated because have different degrees of affinity with stationary phase and mobile phase.

*The red color have higher affinity with the stationary phase whereas the blue color have higher affinity with the mobile phase.

Fig. 1. Scheme of colors separation by paper chromatography.

(paper chromatography and thin-layer chromatography). However, when the stationary bed is within a tube, for example in HPLC, the methods are considered column chromatography. The mobile phase is the solvent that accompanies the analyte as it moves through the stationary phase. The compositions of the stationary and mobile phases define a specific chromatographic method [15].

Moreover, the chromatography separation is based on three main characteristics which are related to chemical compounds: electric charge (ion exchange), molecular size (size exclusion or gel filtration) and polarity (hydrophilic and hydrophobic interactions). Additionally, the chromatography techniques based on compound separation by polarity can present normal or reverse phases. The method with normal phase is characterized by a mobile phase with lower polarity than the stationary phase whereas the technique with reverse phase is the opposite (the mobile phase has higher polarity compared to the stationary phase). Therefore, the knowledge of the structural characteristics of the analytes is fundamental for the choice of the stationary and mobile phases used in the separation method [16].

Thin-layer chromatography first described in 1938 is similar to paper chromatography. However, instead of using paper as stationary phase, it involves adsorbent materials like silica gel, alumina or cellulose on a flat, inert thin layer substrate. Before 1960 workers prepare their own plates in the laboratory but the procedures were often messy, arduous and resulted in many rejects. After this period, the workers only prepare their own plates if they require a special type of layer that is not commercially available, because several convenient precoated plates are commercially available [2]. Currently, beyond different types of TLC plates, there are kits ready to extraction and elution (A400A19, A400B11), including Solid Phase Extraction Vacuum System (A580), standards (discs with impregnated drugs, e.g. morphine, codeine, meperidine, methadone, and propoxyphene – AN121), and chromatograms for separation of acidic, basic and neutral drugs which are sets TOX-LAB by Varian. When compared to PC, the TLC method has the advantages of faster runs and better separations, being more sensitive, more reproducible and allowing the choice among different adsorbents. Another advantage of TLC is the resolution, which is greater than in PC because the particles on the plate are smaller and more regular than paper fibers. Therefore, TLC has largely replaced paper chromatography [2, 17]. An example of the use of TLC in foods of animal origin comes from 1976, when a TLC method was developed for semi-quantification of biogenic amines in fish [7].

TLC is a popular chromatographic technique widely used for a variety of applications including analysis and purification, usually employed in the laboratory. Although the chromatographic resolution of TLC is typically lower than the resolution of HPLC, a number of advantages still make TLC an indispensable analytical tool [10].

TLC is used to separate non-volatile mixtures [17] and is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminum oxide or cellulose. This layer of adsorbent is known as the stationary phase. The samples and standards must be applied on the plate which is then placed in a chromatography recipient containing a solvent or solvent mixture (known as the mobile phase) which is drawn up the plate via capillary action. The different analytes ascend the TLC plate at different rates and the separation is achieved. In other words, like all other chromatographic methods, the separation process of complex mixtures by TLC is based on the different affinity of the analyte with the mobile and stationary phases [10].

In relation to the adsorbent material used to make the plates, silica gel is the most widely used adsorbent being employed on the separation of several compounds in several food matrices, including products of animal origin by using the adsorption mechanism. However, other adsorbents may be used such as alumina, cellulose, polyamide and others. When compared to alumina, one of the major advantages of silica gel is that it has little or no tendency to catalyze unwanted degradation [18]. Usually, aluminum oxide plates are used for the determination of basic substances, alkaloids, steroids; microcrystalline cellulose for the detection of amino acids, sugars and antibiotics; and RP-2, RP-8 and RP-18 (silica gel plates chemically modified with alkyl groups) to analyze fatty acids, carotenoids, steroids, and cholesterol and its esters [19].

Generally, the silica gel plates are commercially available, with standard particle size ranging to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate (gypsum) and water. This mixture is spread as thick slurry on an unreactive carrier sheet, usually thick aluminum foil, glass or plastic. The resultant plate is dried and activated by heating in an oven for 30 min at 110°C. The thickness of the adsorbent layer is typically around 0.10–0.25 mm for analytical purposes and around 0.5–2.0 mm for preparative TLC. Moreover, there are several sizes of plates and chromatography recipients which should be acquired according to the analytical requirements [20].

Applications of TLC in food of animal origin. TLC can be used to monitor the progress of a reaction, to identify, semi-quantify or quantify compounds present in a given mixture as well as determine the purity of a substance [6]. TLC is based on the differential migration of the components of a mixture which occurs due to different interactions between the mobile phase and the stationary phase. The wide variety of combinations among mobile and stationary phases turns this technique extremely versatile and widely applicable

[13] in environmental, clinical, forensic, pharmaceutical, food, flavors, and cosmetics fields. Within the foods of animal origin, TLC may be applied to determine a variety of compounds such as lipids, vitamins, carbohydrates, amino acids, organic acids, proteins, enzymes, nucleic acids, phosphorus compounds, growth regulators, antibiotics, synthetic and natural food dyes, mycotoxins and pesticides or insecticides in food and water [2–4, 6, 9].

For the detection (presence or absence) of the compounds, a developing solution is sprayed on the plate and the spots are visualized. When the substances are not capable of producing color, different techniques can be used such as chemical reactions, adding fluorescent indicators to the adsorbent layer during the process of preparation of the plates or spraying the plates with fluorescent solutions and observing under ultraviolet lamp.

For the *identification* of the substances, the *retention factor* (R_f) is used. R_f is calculated from the following formula:

$$R_f = h_x/h_0,$$

where h_x is the distance between the starting, or spotting, line (place where spots of samples are applied on the plate in a straight line) and the center of the spot of the substance; and h_0 is the development length that means the distance between the spotting line and the solvent front line (mark of the eluent front, when the eluent approaches the upper edge of the chromatographic plate).

On the other hand, for semi-quantitative *determination*, the intensity of the spot is used. A larger amount of substance in the mixture is represented by a spot with more intense color. Additionally, the size of the spot can also give semi-quantitative information (a bigger spot indicates a higher content of a particular compound in the mixture). The intensity of the spots is evaluated by comparing with the intensities of spots with known amounts visually or using a densitometer [11].

In addition, TLC can be one- or two-dimensional similar to other chromatographic techniques such as HPLC, GC and liquid chromatography. In relation to two-dimensional separations (technique performed in two steps), various different coupling strategies have been developed for the first and second steps. The procedure of two-dimensional TLC (2D TLC) begins with the development of a sample-loaded plate with the first solvent. After removing it, the plate is rotated 90° and developed with a second solvent. Developing the plate with two different solvents increases the spot capacity (analogous to peak capacity in HPLC) when compared to single TLC or one-dimensional TLC (1D TLC) [21]. 2D TLC is a powerful tool to separate very complex samples such as lipid mixtures. In general, a solvent mixture with high elution power is used first (dimension number one), followed by an eluent with lower elution power (dimension number two). This confers the advantage that the analyte is concentrated in each step, and the gradient development often helps to overcome problems related to limited resolution. Nevertheless, this method has also serious disadvantages when compared to one-dimensional TLC (1D TLC). Only a single sample can be investigated by using one plate and, thus, 2D TLC is much more time-consuming than 1D TLC. Moreover, as only a single sample can be applied, the simultaneous application of standards is impossible. This makes spot assignments as well as semi-quantitative data analysis highly difficult. Despite these disadvantages, the quality of separation and resolution are highly improved by 2D TLC method which can be used as any other chromatographic technique [22].

However, when the claim is rapid separation and visual identification, the TLC is the simplest and most economical between the chromatographic techniques. Therefore, this separation technique was suggested as screening method [7], being used in several laboratories for general analysis of foods [18], and, due to TLC effectiveness and accuracy, especially in the determination of drug residues and substances from deterioration process like mycotoxins and biogenic amines (histamine, cadaverine, putrescine, spermidine, spermine and others) which are produced from bacterial decarboxylation of the amino acids that naturally compose the foods [1, 5, 8, 9].

In this context, the laboratory practice in products of animal origin, led to the performing of TLC analyses in response to industrial demand for quality control of fresh and frozen fishes or canned fish, mainly tuna and sardines. These fishes have a significant amount of histidine and, consequently, more predisposition to histamine formation. Additionally, the biogenic amines are fairly evaluated in fermented and maturated products such as several kinds of cheese and salami, taking into account experimental studies [23]. In this case, TLC may be used to suggest contamination during the steps of industrial processing or indirectly analyze the hygienic quality of the product or raw material, which is an important characteristic in quality control of products of animal origin.

Biogenic amines. The evaluation of biogenic amines by TLC method was first described in 1976 [7]. We adapt this semi-quantitative technique to analytical needs and existent infrastructure in our laboratory [1, 5, 23]: for extraction of biogenic amines, 1 g of the sample (muscular portion) is transferred to a tube where 2 mL of methanol is added. Then, the two phases are homogenized in a vortex with the assistance of a thin spatula to increase the contact surface between the muscular portion and methanol. Next, the tube is heated in water bath to boiling and placed in a centrifuge (503 g during 2 min) for obtaining a supernatant. The standards are powdered preparations that are commercially available and only need to be diluted in methanol. For example: for the preparation of the

histamine standard it is necessary to dilute 16.6 g of this amine in 100 mL of methanol in a volumetric flask. Afterwards, 10 μL of supernatant and the standards of amine are applied on a silica gel plate. The samples are applied with the assistance of an automatic pipette and standards with aid of a microsyringe. In general, the concentrations applied depend on the analytical need of each laboratory and on the official limits of biogenic amines established by each country. In our laboratory, we use 2, 5 and 10 μL which are equivalent to 2, 5 and 10 mg per 100 g, respectively). Then the plate is placed into a chamber glass containing the eluent solution (20 mL of acetone and 1 mL of ammonium hydroxide), which must be covered immediately after the placing the plate. The eluent solution moves on the silica gel plate by capillarity and carries the amines present in the sample until it reaches about 1 cm from the upper edge. During this period, the amines move depending of their relative affinity to the stationary phase (silica gel) and mobile phase (eluent). Next, the plate is removed from the camera and submitted to drying with hot air flow until total elimination of ammonia vapors [1, 5, 23].

For visual identification of the amines, ninhidrine solution 0.3% (in methanol) must be evenly sprayed on the plate with subsequent hot air flowing. The ninhidrine solution is designed as developing solution because it is capable of forming a complex with NH_2 group of the biogenic amines generating a pink coloration. Due to this fact, the silica gel plate should not contain residues of ammonia and, therefore, the drying step must be rightly performed. Otherwise, all the plate will have a pink coloration and it will be impossible to visually identify the amines.

According to literature, the solvent acetone : ammonia (95 : 5) can be used as eluent solution and 0.2% ninhydrin solution with 2% of acetic acid in methanol as detecting agent of spots [24, 25].

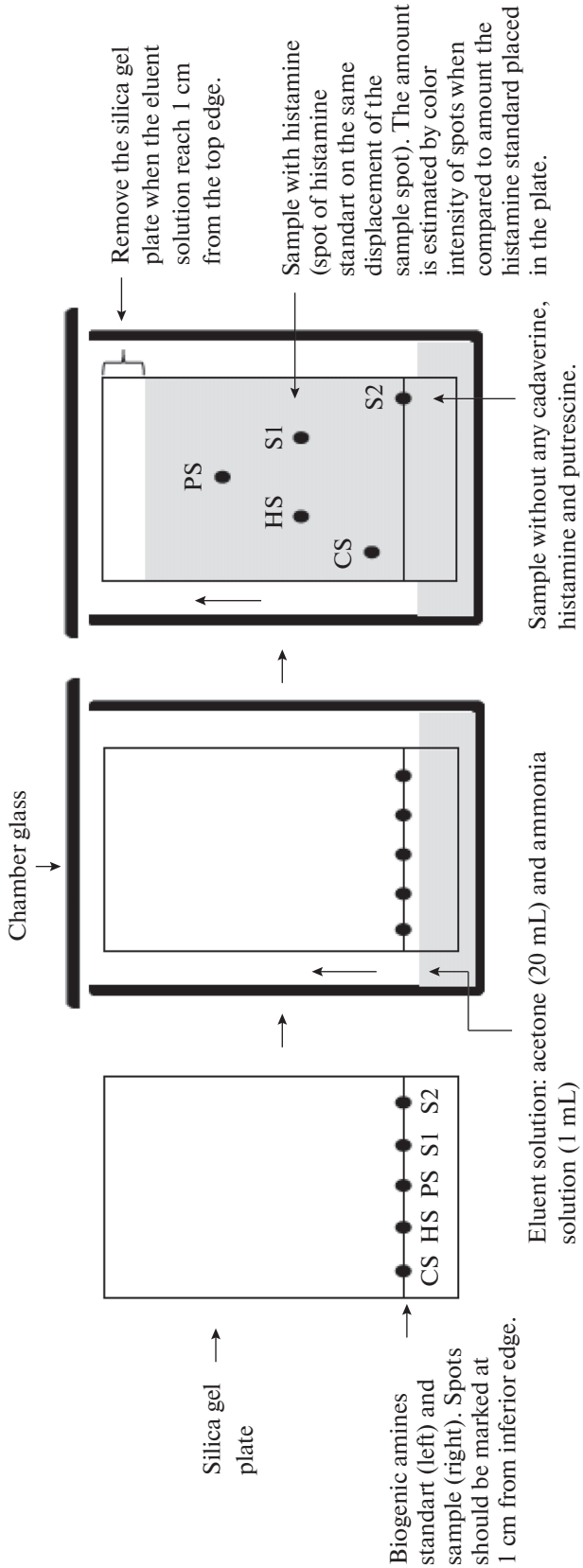
In the first moment, amine identification (Fig. 2) is performed from the visual comparison of the spots of standards and samples using differential migration as parameter. The amount of amines present in the sample is estimated by color intensity (the color intensity of the spot formed by the amine in the sample is compared to the intensities of color showed by the standards). In our laboratory, the concentration standards commonly used are 2, 5 and 10 mg/100 g. Therefore, the results are expressed as <2 mg/100 g, between 2–5 mg/100 g, between 5–10 mg/100 g, and >10 mg/100 g, where samples containing higher amounts of amines show higher color intensities.

For performing the 2D TLC method, the plate is immersed in the first eluent solution (solvent mixture) followed by drying. After the first step separation, the procedures are repeated, but the eluent solution (solvent mixture) must be chemically different to the one used in the first step [22].

Although there are still several concerns against a wider application of TLC (e.g., lower chromatographic resolution in comparison to HPLC and potential oxidation of the analyte caused by the exposition to atmospheric oxygen), there are many advantages that make TLC clearly competitive to liquid chromatography. If commercially available ready-made TLC plates are used, even a less experienced user is able to perform high quality separations because it is a convenient and simple method; the equipment needed for TLC is rather inexpensive and thus can be easily established in any laboratory; the technique is already certified in several industrial establishments; TLC can be easily used to determine different analytes quantitatively (if reliable standards are available); TLC does not provide any "memory" effects, as a completely new stationary phase is used in all the cases (in liquid chromatography remaining contributions of a previous run can be never completely excluded); TLC is less expensive regarding the required consumables and particularly more environmentally friendly due to smaller consumption of solvents than HPLC; many different samples may be simultaneously applied onto a single TLC plate; in practice, TLC is often faster than liquid chromatography although there are recent "multiplexing" liquid chromatography solutions available that enable the analysis of several samples in parallel; TLC may also be used for the analysis of "suspicious" samples that might easily damage an HPLC system. These facts are particularly important in food chemistry where often little defined mixtures have to be characterized [10].

Lipids and lipophilicity of compounds. In the field of lipids, TLC is classically used for routine separations and identification of individual lipids [26]. Regardless the subsequent analysis, the first step is normally the extraction of lipids. This seems rather trivial but it is actually a very important point because many lipids cannot be extracted easily since they are incorporated in the original biological material with other compounds [27]. For lipid extraction from animal, plant and bacterial tissues the use of chloroform and methanol (2 : 1, v/v) is recommended because the tissue water is the ternary component and its amount is very important in order to avoid loss of lipids [28]. The most popular stationary phases for lipid separation are silica gel and alumina, whereby silica gel is unequivocally the absolutely dominant phase. Additionally, silica can be modified by impregnation with other substances to provide optimum results regarding the separation of a certain lipid class [26].

For example, silver nitrate ($AgNO_3$) is primarily used to separate lipids with different fatty acyl compositions based on the degree of unsaturation because Ag^+ forms a complex with the electrons of the double bonds of unsaturated fatty acids leading to their decreased mobility [29]. In contrast, boric acid (H_3BO_3) is primarily useful for the detection of the different isomers of diflunisal acyl glucuronide as well as for the



Scheme made from theory of biogenic amines separation using TLC proposed by Schutz, Cgang e Bjedanes [7].

The silica gel plate (stationary phase) absorbs the solvent (mobile phase) which carries the amines. The separation of biogenic amines is based on the different interactions these substances with silica gel plate and with mobile phase.

CS — Cadaverine standard; HS — Histamine standard; PS — Putrescine standard; S1 — Sample one; S2 — Sample two.

Fig. 2. Separation of biogenic amines by thin-layer chromatography.

separation of isomeric phospholipids. H_3BO_3 forms complexes with compounds containing vicinal hydroxyl groups and leads to a slower migration of these compounds [30]. The lipid separation occurs according to polarity differences caused by differences of the head groups of the phospholipid of interest [31]. The separated lipid fractions can be easily visualized by staining, for instance, with dyes that bind specifically to characteristic functional groups such as amino acid or carbohydrate residues. This is a significant advantage in comparison to HPLC where the derivatization process is normally more difficult. A lot of different reagents are nowadays available (often even as ready-made spray agents) at moderate prices. These agents can be classified according to their specificity and their destructive or non-destructive capacity [32].

Fortunately, it turned out that basically all naturally occurring lipid classes can be analyzed by TLC. Thus, each potential user has to choose a method [26]. According to these authors' knowledge, TLC and HPLC will provide similar results, demonstrating the potential of the TLC method. For example, HPLC was used to determine species of aflatoxins in rainbow trout muscle and liver, where acetonitrile–water (9 : 1), purified on an immune affinity column was used for the extraction. The chromatographic conditions were: mobile phase water–methanol–acetonitrile (60 : 20 : 20, v/v/v) and column ODS [33]. On the other hand, aflatoxins were determined in pasteurized milk, yoghurt, white cheese, butter, ice cream samples by two-dimensional TLC. The extraction was performed with saturated sodium chloride and chloroform. The extracts were applied in silica plates, and diethyl ether–methanol–water (94 : 4.5 : 1.5, v/v/v) and chloroform–acetone–methanol (87 : 10 : 3, v/v/v) were used as mobile phase [34]. The recovery values were 71.4–93 and 81.4–89.5% to HPLC and TLC methods, respectively [33, 34].

Other authors [35] evaluated true vitamin B_{12} in Pacific bluefin tuna (*Thunnus orientalis*) liver by silica gel 60 TLC and by reversed-phase HPLC and found that the retention time (10.4 min) of the compound was identical in both chromatography methods.

The TLC method can also be used for the determination of lipophilicity from a single TLC run; however, it is necessary to add an optimal concentration of a well-chosen modifier to the mobile phase [3]. The mobile phase composition determines the retention time of solutes in both normal-phase and reversed-phase chromatography. The alteration of the composition and the nature of mobile phases enable tuning the retention of the separated analytes and optimization of the chromatographic process. For example, in reversed-phase chromatography an important constituent of the mixed mobile phase is the highly polar solvent (e.g. water). On the other hand, a less polar solvent (e.g. methanol and acetonitrile) is used as an organic modifier, which is added to control the process of solute elution [36]. When compared to other modifiers such as acetonitrile, acetone, dioxane, 2-propanol, and tetrahydrofurane, methanol is theoretically and practically the most adequate modifier for lipophilicity estimation, because this reagent has the lowest slope and intercept. Beyond the organic modifier addition, this technique requires applying reference compounds [3].

Vitamins. Vitamin A is a natural antioxidant that occurs in huge amounts in the human eye lens where it prevents oxidation processes induced by sunlight. The oxidation susceptibility of vitamin A and its derivatives confers many analytical problems and is most probably the reason why TLC is even nowadays frequently used because it can be performed faster than HPLC. Nevertheless, care must be taken to minimize oxidation of vitamins, and this can be reached by developing the plate at 0°C under a N_2 atmosphere [37]. The TLC plates of silica and alumina can be used to separate vitamin A from its oxidation products and other vitamins. The separation and quantitation of vitamin A on silica plates was developed with a mixture of acetone–methanol–benzene [38] and benzene–petrol ether–acetic acid. Under the applied conditions, all water-soluble vitamins remained at the origin and did not interfere with the more lipophilic vitamins. All vitamin A derivatives exhibit strong UV absorption and thus can be directly detected at 254 nm without the need of staining procedures, representing a significant advantage [39]. Additionally, there are studies on quantitative measurement of vitamin A in matrices of complex compositions by TLC technique. For example, the vitamin A esters can be separated from vitamin A alcohol by developing on alumina layers with 5% diethyl ether in petrol ether [40].

According to studies in the literature, the TLC method that provides the best results for the determination of vitamin A uses Silica Gel 60 F254 on plastic foil as stationary phase and hexane–ether (9 : 1, v/v) or benzene–chloroform (1 : 1, v/v) as mobile phase, and is photo documented in UV at 254 nm [41].

Vitamin D is considered one of the most important vitamins, and it has been stated that insufficient nutritional supply of this vitamin would lead to increased mortality rates. TLC on silica gel plate is widely used to separate minimum amounts of vitamin D and its analogues, mainly for the pre-purification of hydrolyzed samples in order to facilitate vitamin D separation from cholesterol by means of GC [42].

Vitamin E is an antioxidant that seems to play important roles in many physiological processes and its lack or excess is also related to many diseases [43]. The single TLC method (1D TLC) is enough to promote the separation process of this vitamin, but it is very accident-sensitive because complex mobile phases containing four or five different solvents have to be used. Therefore, the use of 2D TLC method was recommended to more complex matrices such as extracts from human plasma or red blood cells. In general, the silica gel plates were developed in the first dimension

with chloroform to achieve a separation of the various homologues. For differentiation of α- and β-isomers it is often necessary to use the 2D TLC with the first eluent based on petroleum ether and diisopropyl ether as second TLC run. The commonest mode of detecting vitamin E on TLC plates is based on quenching the fluorescence of supports impregnated with a fluorescent indicator. Alternatively, these compounds can be visualized by nonspecific procedures such as charring after spraying with sulfuric acid, perchloric acid, nitric acid or 10% copper and sulfate in 8% phosphoric acid [44].

Vitamin K is an essential cofactor in mammals and has important Ca^{2+} binding properties. Usually, silica gel plate and reverse phase TLC with methylene chloride and methanol (70 : 30) is used as direct method of detection and indication of vitamin K [45].

The water-soluble vitamins (C, B complex and H) play an important role in human health, and their lack or excess produces specific diseases. Therefore, the analysis of these compounds is indispensable for monitoring their content in pharmaceuticals and foods in order to prevent some human diseases [46]. The methods related to the analysis of an individual vitamin and its degradation compounds are limited and there are only a few papers that report on the simultaneous determination of almost all hydrophilic vitamins [47]. Because hydrophilic vitamins have different chemical characteristics, the most difficult problem in the simultaneous determination of all these compounds is to find an optimum stationary phase–mobile phase system. Additionally, the structural analogues are difficult to separate in one chromatographic run, and this is the case of investigations about hydrophilic vitamins. However, the TLC and high performance thin layer chromatography (**HPTLC**) are the methods of choice mainly when many samples must be compared and these techniques give the possibility to perform two-dimensional separations either by use of the same stationary phase with different mobile phase systems [48] or by using a stationary phase gradient [49]. Different combinations of adsorbents have been reported: silica gel + alumina, cellulose + silica gel, polyamide + silica, polyamide + cellulose [50]. Due to different mechanisms of interaction, the separations on such adsorbent combinations enable obtaining a complete resolution of very complex samples (multicomponent mixtures) as products of animal origin.

Researchers tested the determination of water soluble vitamins by using stationary phase gradient. The silica gel strips were connected face-to-face (1 mm overlap) with cellulose strips along the shorter side (1.5 cm) of the strip. The silica gel was scraped from an area of 1 × 1.5 cm before connecting making sure that the edges had no irregularities resulting from partial loss of adsorbent, because such irregularities may lead to deformation of the zones during their transfer to the second layer. It is important that both plates are turned face to face and pressed together; the layers should overlap. To achieve a close contact between the layers, the strips were compressed together with clamps. A mixture of methanol–benzene–formic acid (6 : 4 : 1, v/v/v) was used as mobile phase and the spots were detected under UV illumination at 254 nm [12].

These authors concluded that a complete separation of the water-soluble vitamins investigated was possible by TLC with stationary phase gradient using silica gel and cellulose plates. Moreover, TLC with stationary phase gradient is a relatively simple and inexpensive method enabling the combination of a large variety of stationary and mobile phases for the analysis of various mixtures. Another advantage of the proposed method is the fact that spots from a plate developed on stationary phase can be transferred to the second plate, without the scraping of bands, extraction, and re-spotting.

Dyes. The synthetic food dyes can also be successfully determined by the TLC technique. Although there is no ideal method for the detection of these compounds, TLC is the best solution to qualitative determination of dyes in a mixture because it allows obtaining satisfactory results in a short time. Moreover, the TLC technique guarantees the correct interpretation of results of analysis because it involves few elements: shape and color of spots, displacement on the plate and light resistance (natural dyes exposed to the light fade in the course of time). For TLC the sample preparation must be carefully purified in order to assure satisfactory separation. Sugars, fats and other substances should be removed by using solid phase extraction (**SPE**) because partial removal of these compounds causes the blurring of spots [4], in general, by SPE-C_{18} which allows three efficient steps: extraction, concentration and purification of the analytes. Before extraction of synthetic food dyes, SPE-C_{18} must be conditioned and cleaned with 2 mL of isopropyl alcohol and then with 5 mL of acetic acid; 3 mL of the homogenized and degassed samples were passed into SPE-C_{18} to remove aqueous fraction containing sugars. For extraction, 10 mL solution of 18% (v/v) isopropyl alcohol was used. In TLC analysis, 30 μL of each sample extracted and 5 μL of food colorant standards (0.4 mg/mL) were applied to the silica gel chromatography plates. The mobile phase used was 8 mL of isopropyl alcohol and 3 mL of ammonium hydroxide [51]. These authors used the same extraction method in both TLC and HPLC, and found that retention time and UV spectra obtained with HPLC-PDA analysis of the standard and samples confirmed the results obtained with TLC analysis. Other authors [52] proposed an extraction method in spiked sausages of different matrices (beef, pork, chicken and fish) with synthetic dyes. Sample of 10 g was extracted with 15 mL volume of methanol–water (95 : 5) by microwave extraction under 100 psi and 50 W at 80°C for 5 min. Then the samples were cooled down to room

temperature and centrifuged at 15000 rpm for 3 min at 4°C. The supernatants were passed into SPE-C_{18} with 4 mL volume of methanol–acetic acid (95 : 5) and flow rate of 1 mL/min. The collected eluates were evaporated at 50°C in a rotary vacuum evaporator under nitrogen, resuspended in 0.5 mL methanol, and filtered with 0.22 μm membrane filter.

Another example of sample purification is boiling the acetic acid solutions of the samples with white wool and then stripping dyes from it by boiling with dilute ammonia. This kind of sample treatment allows removing substances which may cause problems on the separation of dyes by the TLC method and is also useful for other chromatographic techniques [4].

Mycotoxins, pesticides and others. *Mycotoxins* are toxic chemical products formed as secondary metabolites by a few fungal species that readily colonize crops and contaminate them with toxins in the field or after harvest. The analysis of these compounds is common in animal feed (grains). In relation to mycotoxin determination, TLC, HPLC and GC represent a broad range of chromatographic techniques used for practical analysis and detection of a wide spectrum of mycotoxins. However, the most popular method used for rapid detection of some mycotoxins in animal tissues is TLC, which offers the ability to screen large numbers of samples economically for both semi-quantitative and quantitative purposes. This is due to its high throughput of samples, low operating cost and ease of identification of target compounds [9].

Aflatoxin B_1 (**AFB_1**) is found in liver tissues and its determination is useful to diagnosis of aflatoxicosis in chicken [53]. This procedure includes an extraction step using methanol–acetone (50 : 50) and 10 g liver tissue, purification by column chromatography, concentration, and spotting on TLC plates coated with silica gel. For plates developed with ether–methanol–H_2O (95 : 4 : 1) in the first direction and chloroform–acetone–isopropanol (87 : 10 : 3) in the second direction were used. Quantification is performed comparing the fluorescence of extract and AFB_1 standard spots visually with trifluoroacetic acid–hexane (1 : 4) spray. Aflatoxin M_1 (**AFM_1**) may be found in the milk of animals that are fed with AFB_1. This fact is important for the public health since the dairy products are present in human diet. The procedure for AFM_1 determination consider an extraction step from 50 mL milk samples with 125 mL chloroform, purification by silica gel column chromatography, purification, evaporation and resuspension in 100 mL of chloroform using an injection syringe. 2D thin layer chromatography included two mobile phases of ether–methanol–water and chloroform–acetone–methanol [54]. Finally, two methods (acetone as extraction solution and lead acetate or ferric gel for cleanup) were evaluated in determination of mycotoxin M_1 in eggs by TLC [55]. However, emulsion occurred in both methods, leading to the loss of toxin. This behavior it was attributed to high content of lipids and proteins.

Pesticides are widespread throughout the world and play a beneficial role in agriculture against different pest that destroy crops. Their presence is common in the environment and depends on geographical area, season of the year, number of farms, and quantity and intensity of use of plant-protection agents [56–58]. The most important food of animal origin related to pesticide contamination (organophosphorus, organochlorine, carbamates and pyrethroids) is honey [57]. Methodologies for determining pesticides in bee products are principally based on gas and liquid chromatography with mass spectrophotometer detector [58]. However, the use of TLC is widely used for cheap and easy implementation [59]. Authors [60] proposed a TLC method for detection of pesticides (simazine and atrazine) from honey samples. These herbicides are non-toxic to bees. However, the bees, feeding on nectar and pollen from contaminated blossom can introduce them into honey. These authors suggest an ultrasonic solvent extraction at 30 kHz working frequency and 400 W power at 25°C in honey samples (10 g) pretreated at 33°C. The extracts are passed through Whatman 40 filter, the filtrates are transferred into 100 mL separating funnel and allowed to separate. Next the extract is evaporated at 35°C to dryness, and the residues were dissolved in 1 mL of methanol.

In relation to amino acids, a ternary mixture butanol–glacial acetic acid–water (60 : 19 : 21, v/v/v) was described as the optimum mobile phase for the separation and identification of seven amino acids on microcrystalline cellulose [61].

TLC has also been extensively used in the analysis of carbohydrates and various stationary phases are used to separate these compounds. Silica gel, alumina, Kieselguhr and cellulose are the most popular stationary phases. The separation of sugars in biological samples has been recommended on silica gel with ethyl acetate–isopropanol–water (60 : 30 : 10, v/v/v) and isopropanol–*n*-butanol–0.5% aqueous boric acid (50 : 30 : 30, v/v/v) followed by *n*-butanol–acetone–0.5% boric acid (40 : 50 : 10, v/v/v), respectively [19].

TLC analysis can be coupled with other chromatography methods such as HPLC and GC–mass spectrometry (**MS**) [62, 63]. Next context, authors [62] isolated several phthalides derivatives from *Chuanxiong*. An ethanol extract of *Chuanxiong* was dissolved in 50 mL of hot water and extracted three times with 50 mL of ethyl ether and ethyl acetate, respectively. After, the samples were evaporated to obtain the ethyl acetate and ethyl ether extract, which was separated by TLC (normal-phase plates, ethyl ether–hexane, 1 : 5, v/v), and the spots were visualized under UV light at 254 nm. Each spot was removed from the plate (silica gel 60) and extracted with methanol and then each fraction was analyzed by GC–MS (HP-5 MS capillary column, injection volume of 1 μL, 1.0 mL/min as column flow rate of the carrier gas He, mass selective detector HP 5973) and HPLC (Zorbax SB-C18 ODS

column, flow rate 0.7 mL/min, 20 μL as injected sample volume, mobile phase water and methanol, where 55% methanol was used for 10 min and then increased to 70% methanol within 2.0 min, which was maintained to the end, and detection at 270 nm).

Authors [63] also determined triglycerides (**TG**) in goat's milk by coupling TLC and GC–MS. Silica gel was incubated with 20% aqueous solution of $AgNO_3$ overnight and then activated at 100°C for 30 min. The plate was placed twice in a saturated chamber with chloroform and, after drying, sprayed with a 0.15% ethanolic solution of 2',7'-dichlorofluorescein. The spots were visualized under UV light, and after removing of spots 20 μL of TG trinanoin C27 (3.15 mg/100 mL) was added as internal standard. For TGs extraction, 20 mL of diethyl ether protected with butylhydroxytoluene was used, followed by filtering and solvent evaporation under reduced pressure. The residue was resuspended in 140 μL hexane and 0.2 μL from that solution was used for the determination of TGs and fatty acids by GC–MS. A capillary column, supplied by Restek, Rtx-65 TG (35% dimethyl-, 65% diphenyl polysiloxane) was used. The chromatographic conditions were as follows: the initial temperature (220°C) was raised to 320°C at a rate of 15 grad/min and then to 355°C at a rate of 370 grad/min holding this temperature for 20 min. The injector and detector temperatures were 355 and 370°C, respectively. The carrier gas was helium, pressure at the top of the column was 25 psig, the split ratio was 1 : 4 and the calculated flow rate was 0.8 mL/min. Electron impact spectra were recorded at 70 eV. Full spectra (50–1000 amu) were recorded at a scan speed of 2 s/decade over the entire elution profile. Data were analyzed using an ICIS II Data System from Finnigan MAT. The authors [63] concluded that the combination of the $AgNO_3$–TLC technique and capillary GC successfully determined the degree of unsaturation of goat's milk.

High performance thin layer chromatography. The TLC technique seems to be a little forgotten and underestimated method but in the authors' opinion it is worth to discover it anew. It is believed that TLC will have an important development associating it with other methodologies [4, 64] or making a number of improvements to the original method to automate the different steps, to increase the resolution achieved by TLC. This method is referred as high performance thin layer chromatography and allows higher accuracy on the quantification of several compounds [4, 22, 64].

HPTLC is widely used for the identification and quantification of organic and inorganic compounds in pharmacology, medicine, biochemistry and food analysis, including products of animal origin because of its obvious advantages over conventional TLC such as rapidity and high efficiency of separation. In other words, HPTLC is an enhanced form of TLC. A number of improvements can be made to the basic TLC to automate different steps, increase the resolution and allow more accurate quantitative measurements [21]. HPTLC can be used to determine all the compounds that are detected by TLC. The most important differences between both methods (TLC and HPTLC) are the different particle sizes and the more uniform layer of the stationary phase of the commercially pre-coated HPTLC plates resulting in a greater precision of applying samples and processing the obtained data. These facts are responsible for the increase of efficiency and sensitivity of the HPTLC method [65].

The automation of the HPTLC is useful to overcome the uncertainty in droplet size and position in case the sample is applied to the TLC plate by hand. One recent approach to automation has been the use of piezoelectric devices and inkjet printers for applying the samples [21]. In other words, all steps from application to mixing solvents, development and drying can be automated, although usually both techniques (TLC and HPTLC) are confused and used as synonyms [26].

The effectiveness of HPTLC was described in the determination of several compounds such as lipids [26], dyes by digital processing of images that allowed quantitative determination with high precision [4], amino acids with leucine-anthracene reagent by HPTLC associated spectrophotometry [66], drug residues in meat products such as tetracyclines where the UV light was used to detect these compounds [67], and simultaneous determination of vitamins A, E, and B by HPTLC in one chromatographic run using fractional elution, using benzene as the first mobile phase and a 0.02 M aqueous micellar solution of sodium dodecyl sulfate as the second eluent [68].

Moreover, electrospun glassy carbon nanofibrous stationary phases (electrospinning process applied to stainless steel plates using an electrode from the high voltage power supply) for HPTLC were described. The optimized mobile phase used for the laser dye was 2-propanol whereas 20 mM borate buffer formed in 90 : 10 (v/v) water to acetonitrile was used for amino acid analysis. A digital documentation system with UV lamp was used for the visualization of both compounds (laser dye and amino acids). This technique showed very differing selectivity for the analysis of a set of 6 laser dyes and complete resolution of ternary mixtures of laser dyes and essential amino acids [69].

Thus, methods of TLC and its refined version HPTLC are even nowadays indispensable tools of modern analytical chemistry [70]. The multiple developments and its combination with other analytical techniques have dramatically increased the use of thin-layer chromatography for the characterization of complex mixtures such as products of animal origin [19].

* * *

The TLC has a strong potential as a surrogate chromatographic model for qualitative and quantitative analysis. To convert these opportunities into practice, several modifications have been carried out on the conventional TLC system. Therefore, further studies on

TLC and HPTLC should be performed in order to contribute with the scientific community and for enhancing the thin-layer chromatography method, facilitating the laboratory practices due to performance and economic advantages compared to other chromatographic techniques such as paper chromatography, HPLC and gas chromatography.

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