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Review

Thin-layer chromatography with biological detection in phytochemistry

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ABSTRACT

Bioautography on thin-layer chromatographic (TLC) plates is a means of detecting the biological activity of a sample which has migrated on the plate with a suitable solvent. It only requires small amounts of sample and is ideal for the investigation of plant constituents, which often occur as complex mixtures. It can be used for the target-directed isolation of these constituents. In contrast to HPLC, many samples can be run at the same time on TLC. Organic solvents, which cause inactivation of enzymes or death of living organisms, can be completely removed before biological detection. Many bioassays are compatible with TLC. Antimicrobial, radical scavenging, antioxidant activities and enzyme inhibition feature among the tests that are employed.

Contents

1. Introduction

Thin-layer chromatography, combined with both biological and chemical detection methods, is an effective and inexpensive technique for the study of plant extracts. It can thus be performed both in sophisticated laboratories and in small laboratories which only have access to a minimum of equipment [1]. When TLC is combined with a biological detection method, it is known as TLC bioautography. Historically, the technique of TLC bioautography has been known since 1946 [2,3]. TLC bioautography falls under the category of so-called benchtop bioassays. These are compact and simple tests which can be performed with a minimum of sample in a short time. Bioassays are defined as tests which are used to detect the biological activity of an extract or pure substance isolated from an extract, obtained from a living organism [4], while "benchtop" refers to the smallness of space occupied by the method.

True bioautography involves growing an organism (a microorganism, for example) on the TLC plate, while autography is

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generally the application of a chemical method to detect a biological effect or process (radical scavenging activity, for example).

TLC-bioautography can be thought of as a simple on-line or in situ method which permits the separation of a complex mixture and, at the same time localizes the active constituents on the TLC plate. This contrasts with work using agar dishes, which does not distinguish between active and inactive components found together in the zones of inhibition. In this case, only the bioactive sum of a sample is indicated, and not the activities of single compounds. Unlike HPLC, a number of samples can be treated at the same time. Chromatography of the samples is under strictly identical conditions. TLC has another advantage over HPLC in that the organic mobile phase, which might cause inactivation of enzymes or living organisms, is evaporated and cannot impede the detection. HPLC on-line bioassays have to be compatible with the eluent. After the separation of sample components on TLC, these products are immobilized on the plate. They are easily accessed because they are open and, furthermore, they are available for slow manipulations, such as incubation of bacterial cell cultures. In on-line HPLC assays, there is a continuous flow of eluent and any interactions with chemicals or organisms have to be fast.

The major applications of TLC bioautography are to be found in the fast screening of a large number of samples for bioactivity and in the target-directed isolation of active compounds (bioactivity-guided fractionation) [5].

Since TLC only involves an amount of product on the plate and cannot deal with concentrations, it is only a semi-quantitative method. Supplementary tests in solution are required to provide full quantitation and give IC_{50} values of pure compounds, for example.

2. Antifungal and antibacterial assays

Much work has been done over the last 40 years on the screening of plant extracts for antifungal and antibacterial activity by TLC bioautography.

Three bioautographic techniques have been described for the investigation of antimicrobial activity [6–8]: agar diffusion, direct bioautographic detection on the TLC plate, agar-overlay. The influence of various parameters in these tests has been evaluated [9].

2.1. Agar diffusion

Agar diffusion (or contact bioautography) involves the transfer by diffusion of the antimicrobial agent from the chromatogram to an agar plate inoculated with a microorganism, and is the least-employed of the techniques.

This method was used, for example, in the testing of 22 Thai medicinal plants for the activities of their extracts against gonorrhea, caused by the Gram-negative bacterium *Neisseria gonorrhoeae*. In the bioautographic assay on a methanol extract of the stem of *Coscinium fenestratum* (Menispermaceae), after migration, the TLC plate was placed on agar inoculated with the microorganism. The plate was incubated at 37 °C for 24 h in a 5% CO_2 atmosphere and then the zones of inhibition were observed, with Ceftriaxone as positive control. Comparison with an authentic sample on the TLC plate led to the identification of berberine as the active principle [10].

Amphotericin B, a polyene antifungal agent, is used clinically for the treatment of fungal infections. As it is not a homogeneous substance and contains minor heptaenes and tetraenes, TLC autobiography is an ideal method for the quantitative determination of amphotericin B and indeed the European (Ph. Eur. 6) and United States Pharmacopoeia (USP 29) refer to agar diffusion for the analysis of the compound. Work has been performed on the optimization of the bioassay: test organisms were *Candida albicans* and *Saccharomyces cerevisae*. Of the five assay media investigated, Mueller–Hinton Agar supplemented with 2% glucose and 0.5 mg/ml methylene blue inoculated with *C. albicans* was found to give clearest areas of inhibition [11]. A detection limit of 0.8 ng per spot was obtained. Densitometric evaluation at 385 nm required ten times more substrate and 50 ng was needed to detect amphotericin B at 366 nm [12].

Problems can arise from the differential diffusion of compounds from the chromatogram to the agar plate. This is especially true for water-insoluble samples.

Almost all bioautographic work is performed on TLC plates. However, in a paper on the use of HPTLC-bioautography for the action of antibiotics on *Bacillus subtilis*, it is stated that developing times are shorter and that consumption of solvent is reduced [13].

2.2. Direct bioautography

In direct bioautography, the developed TLC plate is sprayed with, or dipped into, a fungal or bacterial suspension.

The direct bioautographic method is applicable to microorganisms that can grow directly on the TLC plate and suitable precautions are required to prevent unwanted dispersion or spread of the organism, especially when these are pathogenic bacteria and fungi. Direct bioautographic procedures have been described for spore-producing fungi such as *Aspergillus*, *Penicillium* and *Cladosporium* [14] and also for bacteria [15].

The effect of varying different parameters in the antibacterial assays has been investigated [16]. Of the three bacterial test strains employed in this work (*B. subtilis*, *Staphylococcus aureus* and *Escherichia coli*), *B. subtilis* gave the clearest inhibition zones and best reproducibility. More precise results were obtained when the bacteria were suspended in the nutrient medium which was poured over the TLC plates than when they were distributed over the solidified nutrient medium which was already on the plates. Variation of the culture medium and stains also gave differing results, with *p*-iodonitrotetrazolium violet (INT) proving to be the most suitable detection reagent [16].

The isolation of antimicrobial compounds from the root bark of *Cordia gilletii* (Boraginaceae) has been employed to optimize the culture medium. The authors make the point that the application of the microorganism requires a medium fluid enough to disperse the microorganism but viscous enough to adhere to the TLC plate and maintain sufficient humidity for bacterial growth. For this reason, they tested combinations of Mueller–Hinton (MH) broth and agar, to discover a medium sufficiently fluid to prepare bacterial suspensions at 37 °C, yet which solidified at ambient temperature. The mixture of MH broth and MH agar in the proportions 90:10 fulfilled this condition [17].

In the assay for antifungal activity with *Cladosporium cucumerinum*, TLC plates are first run in suitable solvents with the samples under test. *Cladosporium cucumerinum* spores are then mixed with liquid nutritive media (Sabouraud maltose broth, for example) containing antibiotics (chloramphenicol, streptomycin or similar) to avoid bacterial contamination. The suspension is then sprayed on the TLC plates so that they are just humid. After spraying, the plates are incubated at room temperature in the dark for 3 days in polythene boxes lined with moist chromatography paper. Growth of the fungus is seen as a grey coloration on the plate, while inhibition zones are white (Fig. 1). For conservation of the plates and record purposes, they are sprayed with ethanol to kill the fungus, dried and then covered with a plastic sheet [14]. A more efficient documentation method is photography.

A large number of applications of the direct bioautographic method have been reported (e.g. [18]).

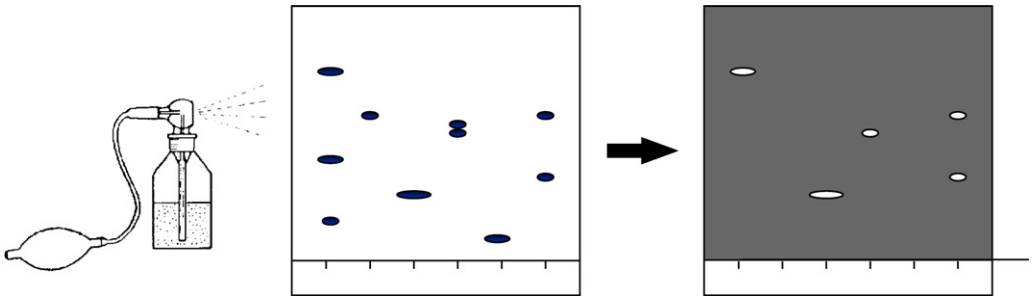

Fig. 1. Direct TLC bioautography for the detection of antifungal activity against the plant-pathogenic fungus *Cladosporium cucumerinum*. After migration of the samples, nutritive medium is inoculated with the microorganism and then sprayed on the TLC plate. White inhibition spots are visible after incubation.

Using TLC bioautography with the plant-pathogenic fungus *Cladosporium cucumerinum*, a bioactivity-guided approach was employed for the isolation of naphthoquinones from *Diospyros usambarensis* (Ebenaceae). Twigs of this plant are used as chewing sticks for cleaning teeth in Africa. Five naphthoquinones (7-methyljuglone, isodiospyrin, mamegakinone, 2-methoxy-7-methyljuglone, 3-methoxy-7-methyljuglone) were isolated, with 7-methyljuglone showing the highest activity (just 0.025 μg is required on the TLC plate for inhibition of the fungus) [19].

Quinones have figured prominently in the search for natural products with potential use in agricultural fungal pathogen control. For example, one study reports on the activity by direct bioautography of 1,4-naphthoquinones, 1,2-naphthoquinones, 1,4-benzoquinones and anthraquinones against the fungal pathogens *Colletotrichum acutatum*, *Colletotrichum gloeosporioides* and *Colletotrichum fragaria* [20]. Species of *Colletotrichum* cause typical symptoms of anthracnose in plants. As in the previous reference [19], juglone was one of the best antifungal agents.

The BioLuminizer™ system (commercialized by Camag, Muttenz, Switzerland), which is a general detection method for bioactive substances, uses *Vibrio fischeri*, a non-pathogenic Gram negative marine bacterium. This has an intrinsic bioluminescence, emitting a greenish light as a product of cellular respiration at a critical cellular density. Luciferase, the bioluminescence catalyst, is expressed and catalyses an oxidation reaction that releases excess energy in the form of light [21]. After migration of the sample, the TLC plate is coated with the bioluminescent bacteria which produce dark spots on a luminescent background with toxic or bioactive compounds [22]. This method is suitable for the detection of toxins and chemical adulterants in food, beverages, cosmetics, waste water and drinking water, in picogram quantities.

The bioautographic method can be extended for use in 2D-TLC experiments. The advantage of this method is that migration in each of the two dimensions can be brought about with solvents of very different polarities. Applications to the discovery of inhibitors of the plant pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides* have been described [23].

The BioArena system of Tyihak is a combination of overpressure layer chromatography (OPLC) with bioautography. Separation in an OPLC system results in better-defined spots than in conventional TLC and HPTLC separations [24]. A number of applications have been reported, including the investigation of *Chelidonium* alkaloids [25].

2.3. Agar-overlay

The agar-overlay technique is a hybrid of the two other methods and it is applicable to a broad spectrum of microorganisms. It provides well defined zones of inhibition and is not sensitive to contamination. A suspension in a suitable broth of certain microorganisms in agar is gently smeared on the developed TLC plate and then incubated for several hours in a humid atmosphere, allowing for growth of the microorganisms. Active compounds are transferred by a diffusion process from the stationary phase to the microorganism.

For Gram-negative bacteria, an agar solution containing the red-colored bacterium *Serracia marcescens* can be employed. The red-colored gel is incubated overnight at room temperature and inhibition zones appear as white or pale yellow areas on a red background [26].

With other, colorless, microorganisms, zones of microbial growth inhibition are visualized with the aid of a dehydrogenase activity-detecting reagent (tetrazolium salt). Metabolically-active microorganisms convert the tetrazolium salt into the corresponding intensely colored formazan (Fig. 2).

If phenol red is incorporated into media containing 0.6% agar and the plates are sprayed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium chloride (MTT), clearer results are obtained, with dark red colored inhibition zones appearing against a blue background [27]. A comparison of different tetrazolium salts has been carried out and it has been established that one of the most sensitive tetrazolium salts is *p*-iodonitrotetrazolium violet (INT) [28].

The agar-overlay assay has been used for yeasts such as *C. albicans* and can also be applied to bacteria such as *B. subtilis* [29], *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [27].

In the case of the yeast, *C. albicans* is maintained on Sabouraud agar slants. A colony is removed and broth cultured in Sabouraud liquid medium (50 ml). The medium is shaken at room temperature. After 12 h, the medium becomes cloudy. New medium is inoculated with culture 7 h before the test in order to reach the exponential growth phase of the microorganism. Malt agar is used as the solid medium for the overlays. Molten medium (50 ml) is maintained in a water bath at 45 °C. A portion of the liquid culture (0.5 ml) of *C. albicans* is introduced, to afford a concentration of 10^5 cells/ml in the inoculum. When the TLC plate has been developed with the samples, approximately 10 ml of the inoculum is distributed over the plate (10 cm × 10 cm) with a sterile pipette. After solidification of the medium, the plates are incubated overnight at 30 °C in polyethylene boxes lined with moist chromatography paper. The chromatograms are sprayed with an aqueous solution (2.5 mg/ml) of thiazolyl blue (methylthiazolyl-tetrazolium bromide, MTT) and incubated for 4 h at 30 °C. Clear inhibition zones are observed against a purple background [29].

In one application with Brazilian plants, aqueous extracts of the vegetable material were prepared and their antifungal activities were established on agar plates. The active extracts were then migrated on TLC plates with the solvent n-butanol–acetic acid–water 8:1:1. Acid-containing solvents and butanol have to be removed very thoroughly from the TLC plate in order not to hinder the growth of the microorganism. Overlay media containing *C. albicans* or *Cryptococcus neoformans* and 1% phe-

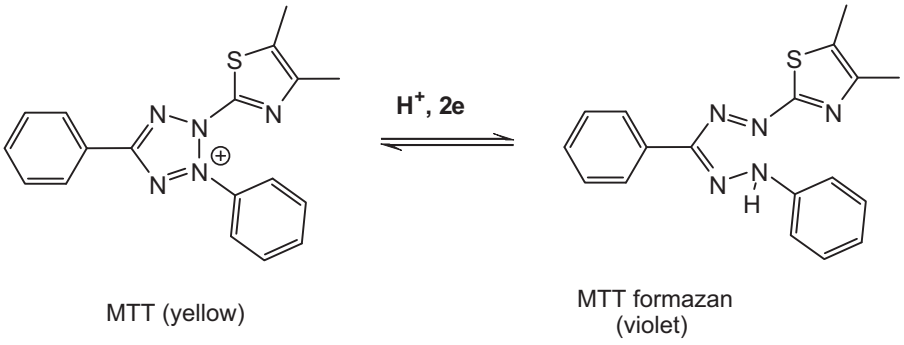

Fig. 2. Detection of microorganism dehydrogenase activity by reduction of tetrazolium salts.

nol red, were distributed over the TLC plates and the plates were incubated for 48 h at 37 °C. The bioautograms were sprayed with MTT and the inhibition zones noted. *Schinus molle* and *S. terebinthifolius* (Anacardiaceae) leaves had constituents which were active against *C. albicans*, while *Anacardium occidentale* (Anacardiaceae) leaves were active against *Cryptococcus neoformans* [30].

The antibacterial activity of isoflavonoid and sesquiterpenoid phytoalexins has been evaluated by an overlay method using *Pseudomonas syringae* pv. *phaseolicola*, with 2,3,5-triphenyl-tetrazolium chloride (TZC) as visualization reagent. Addition of glycerol to the overlay nutrient medium, as a carbon source, facilitated the reduction of TZC to pink colored formazans by bacterial dehydrogenases [31].

Antimicrobial compounds have been characterized in annatto (*Bixa orellana*, Bixaceae) seed extracts. These carotenoid-type substances are the main pigments of these natural coloring agents (Fig. 3). Their antibacterial activity was detected by overlaying TLC plates with agar containing *Staphylococcus aureus* and then spraying with the tetrazolium salt INT [32].

It is possible to spray the agar, mixed with microorganism, onto the TLC plate. Application of the plant-pathogenic fungus *Valsa ceratosperma* by this method allowed the localization of antifungal constituents in an extract of *Glycosmis pentaphylla* (Rutaceae) because after 60 h the spores themselves produced a dark-colored mycelial mat. Another feature of this method is the compatibility with reversed-phase TLC plates [33].

As an alternative to the use of tetrazolium salts, inhibitors of the plant pathogens *Erwinia carotovora* and *Erwinia herbicola* were revealed by incubation with esculin. The microorganisms hydrolyse aesculin to esculetin and under the conditions of the bioassay, the background of the TLC plate was a brown color. Zones of inhibition appeared as white spots on the brown background [34].

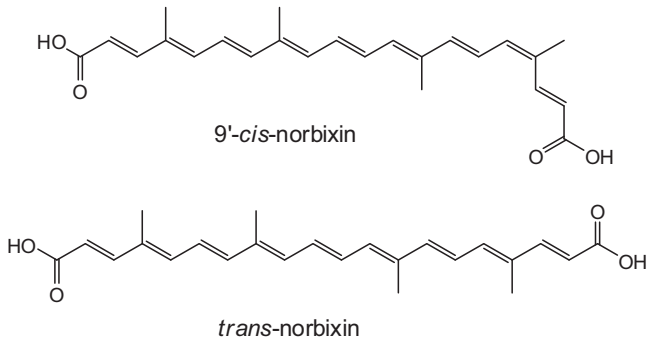

Fig. 3. Structures of antimicrobial compounds in annatto extracts.

3. Enzyme inhibition

Enzymes are important molecular targets for lead finding and hence drug discovery programmes often include enzyme targets in primary screening assays. These assays utilize partially purified or purified human enzymes and involve the measurement of product formation by a wide variety of methods, including radiometry, colorimetry and fluorimetry.

The use of a TLC support to screen for potential plant-derived enzyme inhibitors is a rapid method which is relatively free of disturbances due to solvent.

The main limitation of the use of enzymes is their availability (and price!). Spraying enzymes on TLC plates requires relatively large quantities – but certainly not as much as is needed for the continuous supply during analysis by HPLC on-line systems. The enzyme has generally to be sprayed in combination with a buffer to avoid reaction and/or degradation on the acidic silica gel surface.

3.1. Acetylcholinesterase inhibition

Some of the most recently introduced drugs for the management of Alzheimer's disease (AD) act by inhibiting acetylcholinesterase, thus correcting a deficiency in levels of the neurotransmitter acetylcholine. One of these drugs, galanthamine [35], is an alkaloid isolated from various species of the Amaryllidaceae family. However, the drugs administered at present only exhibit a slowing-down of the progression of AD and considerable effort is being devoted to finding more effective therapeuticals. Galanthamine originates from a plant source and so other plants are actively being screened and investigated for the presence of new inhibitors of acetylcholinesterase [36].

3.1.1. Detection by diazotization

An acetylcholinesterase (AChE) inhibition assay has been developed which is based on the reagents employed in a study of housefly esterases [37] and the enzymatic detection of pesticides [22,38] and chemical warfare agents [39]. In this bioassay, after the samples have migrated, the TLC plate is dried and then sprayed with enzyme (acetylcholinesterase or butyrylcholinesterase). The enzyme is allowed to incubate for 20 min at 37 °C and is then sprayed with a mixture of 1-naphthyl acetate and Fast Blue B Salt. After 1–2 min, the active components are seen as white spots on a purple background. In this assay, the enzyme reacts with 1-naphthyl acetate to produce 1-naphthol. This in turn undergoes a diazotization reaction with Fast Blue B salt, producing a purple azo dye. Enzyme inhibitors block the formation of 1-naphthol and hence no purple coloration is produced (Fig. 4). Detection limits for the known inhibitors physostigmine (eserine) and galanthamine are 1 ng and 10 ng, respectively [40]. This bioautographic method

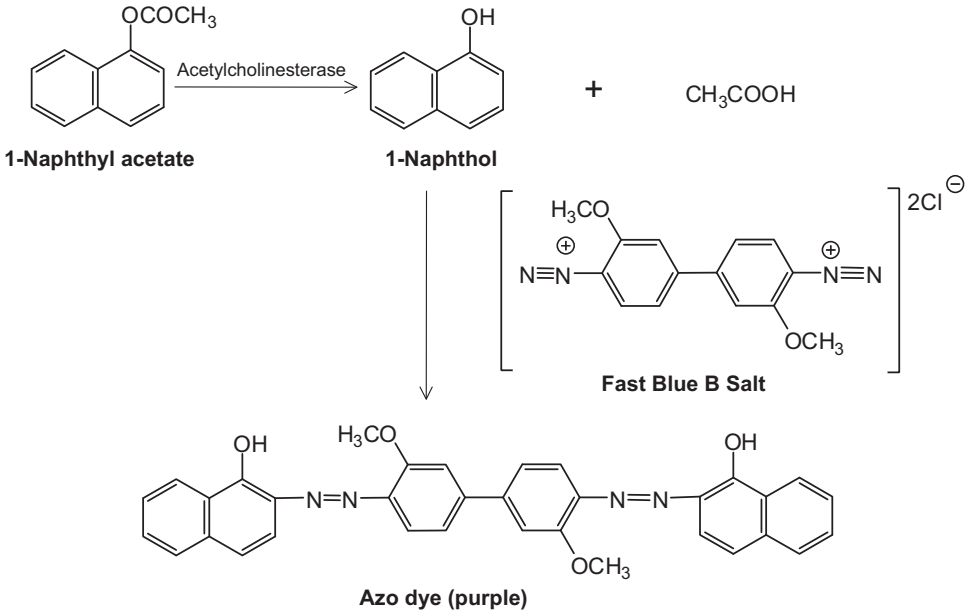

Fig. 4. Reaction of acetylcholinesterase with 1-naphthyl acetate to give 1-naphthol and subsequent formation of a purple azo dye with a diazonium salt.

provides an extremely rapid means for the screening of a large number of samples for AChE inhibition.

In a modification of the above-mentioned method, the enzyme concentration is reduced from 6.7 U/ml to 1.0 U/ml. After spraying with enzyme, the plate is first incubated with 1-naphthyl acetate for 20 min and then sprayed with Fast Blue B Salt [41].

Another variant of the bioassay [42,43] uses 2.2 mM 2-naphthyl acetate in the TLC elution solvent and a 3 U/ml enzyme solution. Incubation with enzyme is for 10 min. This method supposedly gives a deeper violet background color and higher sensitivity of detection. However, the 2-naphthyl acetate is considerably more expensive than the 1-naphthyl acetate.

A microplate assay has been developed for the diazotization method [44]. This gives a quantitative result (such as IC_{50} values) but TLC assays, which are useful as qualitative methods, are in general more sensitive than microplate assays. However, there was a good correlation between the TLC bioautographic assay and the solution assay.

The original detection method by diazotization has been employed to screen Amaryllidaceae species for AChE inhibition [45]. Many of the 80% ethanol extracts tested produced white spots on the purple azo dye background. One of these, *Crinum x powellii*, was investigated, using a bioactivity-guided fractionation strategy. After solvent partition, silica gel column chromatography and semi-preparative HPLC on a C-18 column with acetonitrile-water 8:1 as eluent, the AChE-inhibitory compound was isolated and the structure determined as linoleic acid ethyl ester (Fig. 5). The alkaloid hippadine, a glycosylated benzyl alcohol derivative, calleryanin, and 4′-hydroxy-7-methoxyflavan were also isolated but these were inactive in the assay. It is unusual that the activity is due to the linoleic acid derivative because normally the active constituents of *Crinum* species are alkaloids. The parent acid, linoleic acid, was also an AChE inhibitor [45].

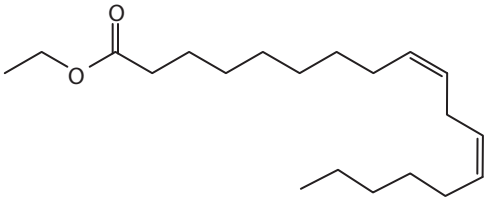

Fig. 5. Structure of linoleic acid ethyl ester isolated from *Crinum x powellii*.

3.1.2. Detection by the Ellman reaction

The Ellman method for the colorimetric determination of acetylcholinesterase activity was first described in 1961 (Fig. 6) [46]. Acetylthiocholine (ATCI) is cleaved by AChE to form thiocholine which in turn reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to give the yellow 5-thio-2-nitrobenzoate anion.

It has recently been adapted for the TLC screening of AChE inhibitors [47]. Standard development of samples is performed and after removal of solvent, a solution of DTNB and ATCI is sprayed on the plate, followed by enzyme. A pale yellow background forms within about 5 min. AChE inhibitors appear as white spots, which are generally more difficult to visualize than the diazo dye method above. False positive effects are possible with certain compounds, such as aldehydes [48].

3.2. α- and β-Glucosidase inhibition

Compounds which inhibit glucosidases are potentially useful as antidiabetic, anti-obesity, antiviral, antiadhesive, antibacterial or antimetastatic agents [49]. Inhibitors of α-glucosidase are of therapeutic interest in type 2 diabetes because they are able to slow down the release of glucose from oligosaccharides, lowering postprandial levels of glucose in diabetic patients [50]. The search for α-glucosidase inhibitors from nature led to the discovery of miglitol and acarbose, drugs currently used as therapeutics [51]. Furthermore, α-glucosidase is involved in the glucosylation process of viral membrane proteins responsible for cell adhesion. Consequently, α-glucosidase inhibitors are a potential source of antiviral compounds. One example is the polyhydroxy alkaloid castanospermine, which inhibits the growth of HIV *in vitro* [52].

Screening methods for glucosidase inhibitors include spectrophotometry with *o*- or *p*-nitrophenyl glucopyranoside as substrate [53,54] or an agar plate method [55]. Both of these have limitations. With *p*-nitrophenyl-β-D-glucopyranoside, the enzyme glucosidase should cleave the sugar, to give a yellow background color due to formation of *p*-nitrophenol. However, this was only observed on the TLC plate when the pH of the buffer solution was

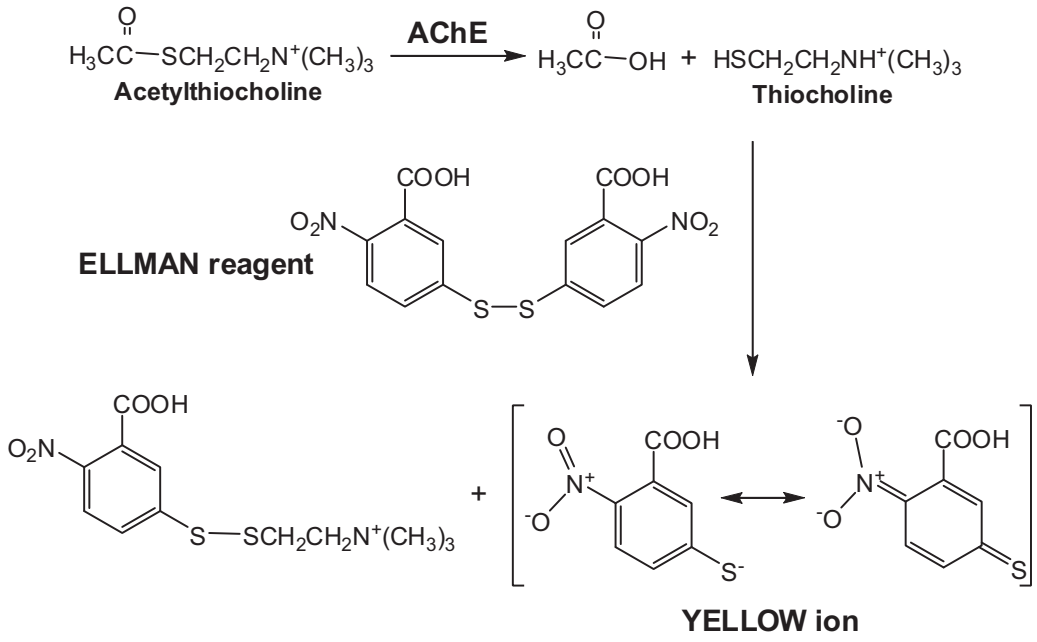

Fig. 6. Detection of acetylcholinesterase inhibition with the Ellman reagent.

adjusted to 7.5 and inhibition zones were poorly visible [56]. No yellow background color was observed in the corresponding test with *p*-nitrophenyl-α-D-glucopyranoside [56].

An alternative is a test which involves the cleavage of 2-naphthyl-α-D-glucopyranoside or 2-naphthyl-β-D-glucopyranoside by glucosidase inhibitors [56]. The 2-naphthol which is formed reacts with Fast Blue B salt, to give a purple-colored diazo dye (Fig. 7), as found in the TLC assay for the detection of acetylcholinesterase inhibitors [40].

For optimal results, α-D-glucosidase in pH 7.5 buffer is incubated with the sample on the TLC plate in a humid atmosphere for 60 min at room temperature, while β-D-glucosidase is incubated with the sample for 20 min at 37 °C. After incubation of the TLC plate, the purple background coloration is obtained by spraying with a mixture of naphthyl-glucopyranoside solution and Fast Blue Salt B, in a ratio of 1:1 for the α-D-glucosidase and in a ratio of 1:4 for the β-D-glucosidase. Activities of reference compounds (minimum amounts on TLC plates required to give visible white spots) were as follows: conduritol B epoxide (0.1 μg for α-, and β-D-glucosidase), castanospermine (0.05 μg for α-D-glucosidase) and miglitol (0.005 μg for α-D-glucosidase) [56].

A TLC method for the detection of β-glucosidase inhibitors which is based on the hydrolysis of esculin into esculetin (see section 2.3 and [34]) has been described. The esculetin reacts with

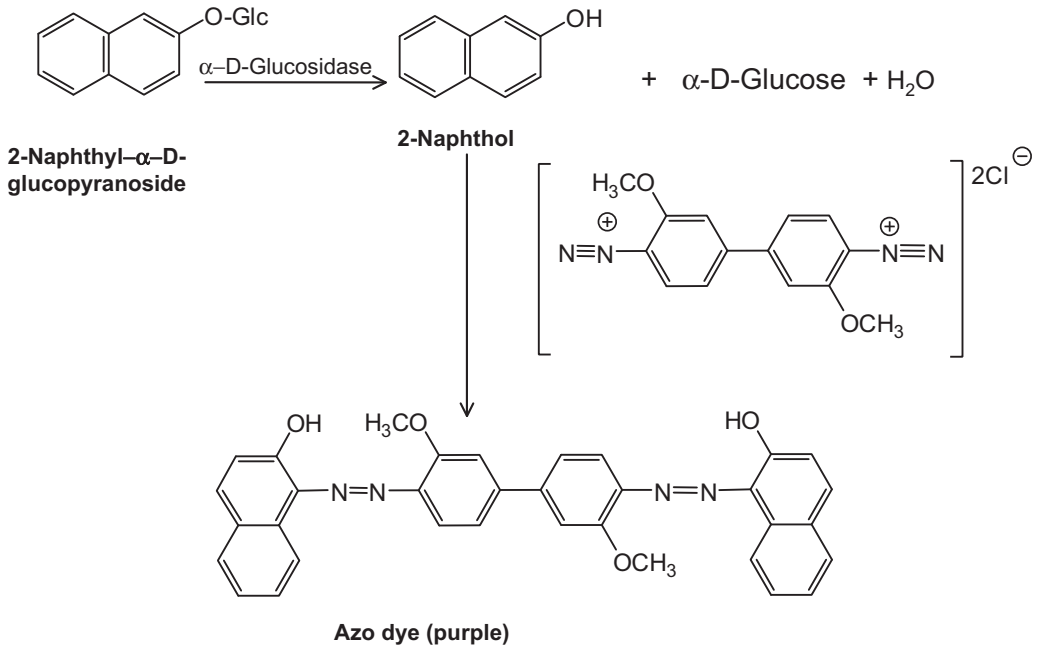

Fig. 7. Reaction of α-D-glucosidase with 2-naphthyl-α-D-glucopyranoside to give 2-naphthol and subsequent formation of a purple azo dye with a diazonium salt.

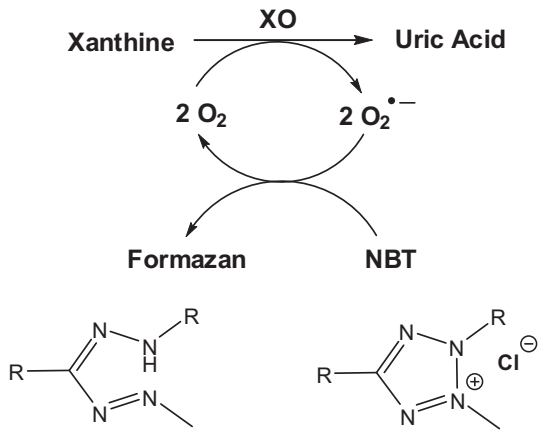

Fig. 8. Enzymatic oxidation of xanthine to uric acid with concurrent formation of purple formazan.

ferric chloride to provide a brown complex [57]. However, the method uses a natural product as enzymatic substrate, which may be difficult for the detection of inhibitors from plant extracts which contain other coumarin derivatives.

3.3. Xanthine oxidase inhibition

The enzyme xanthine oxidase (XO) catalyses the oxidation of hypoxanthine and xanthine to uric acid, producing superoxide radicals and hydrogen peroxide. The inhibition of XO diminishes oxidative stress and can thus have an effect on inflammation, arteriosclerosis, cancer, aging, etc. Inhibition of XO also has an application in the reduction of gout and kidney stones caused by hyperuricaemia. In order to investigate the XO inhibition on TLC plates, the enzyme is suspended in agar and distributed on the TLC plate (direct measurement of enzyme activity on the TLC plate is not possible). After solidification, the plate is immersed in a 3 mM solution of xanthine at 38 °C for 20 min in the dark. Enzymatic oxidation of xanthine produces superoxide radicals which reduce the pale yellow tetrazolium salt to a formazan and hence a purple background is obtained on the plate (Fig. 8). Allopurinol, an inhibitor of XO, is detected as white spots on the purple background. A detection limit of 5 ng was determined. A modification of the test (in which superoxide is generated chemically) allows the distinction between pure inhibitors of XO, such as allopurinol, and radical scavengers of the flavonoid type [58].

4. Antioxidant testing

There are literally thousands of publications which appear every year on the antioxidant properties of plant products. The great interest in this activity stems from epidemiological studies which show that a higher intake of antioxidant compounds is associated with a lower risk of mortality from cancer and coronary heart disease.

Simple and rapid tests are needed for antioxidant and radical scavenging properties of the many samples which have to be screened.

4.1. Inhibition of bleaching of β-carotene

Completely dried TLC plates are sprayed with a 0.05% solution of β-carotene in chloroform. They can be left at room temperature for 12 h until decoloration of the background or they can be placed under 366 nm UV light. Active compounds remain as yellow-orange spots on a white background [59].

4.2. Inhibition of bleaching of β-carotene induced by autooxidation of linoleic acid

Dry TLC plates are sprayed with a mixture of linoleic acid in ethanol and β-carotene in chloroform. After exposing the plate to sunlight, antioxidant activity is shown by the presence of orange spots on a white background [60].

5. Free radical scavenging activity

5.1. DPPH test

The stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) [61] has an absorption maximum at 517 nm, which decreases upon reduction through reaction with a radical scavenger. The corresponding color change can thus be observed in a TLC bioassay [62]. The TLC plate with samples is developed with the elution solvent

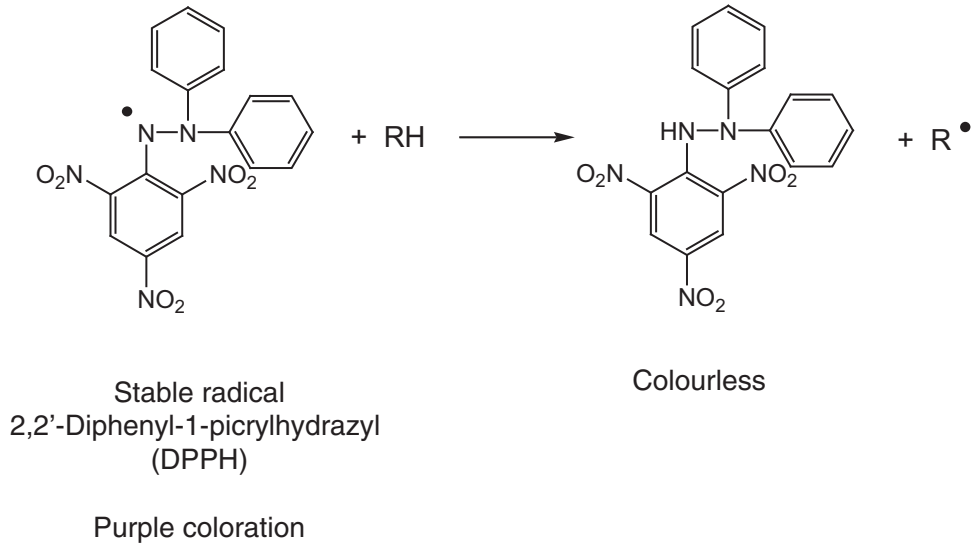

Fig. 9. Reaction of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) with radical scavengers.

and dried. It is then sprayed with a solution of 0.2% (DPPH) in methanol. The plate is examined in daylight after 30 min. Active (free-radical scavenging) compounds appear as yellow-white spots against a purple background (Fig. 9).

A so-called "dot-blot" test has been used to investigate the radical scavenging capacity of foodstuffs. In this method, samples are deposited on a TLC plate without migration and then the plate is dipped in a solution of DPPH. The intensity of the yellow color is measured with a chromameter [63]. This has the disadvantage of not separating the samples in each spot but the test does give comparative values and it was employed to check the radical scavenging capacities of teas, wines, apple and spinach [63].

5.2. ABTS test

Determination of free radicals is also possible with the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic) acid radical (ABTS) [64] and this method can be extended to TLC plates. Layers stained with ABTS show a green background and radical scavengers give colorless or pink spots. However, DPPH is more stable on the plates and gives a more homogeneous coloring of spots [62].

6. Conclusions

Despite the wide scale use of sophisticated HPLC and GC techniques, on-line HPLC bioassays and hyphenated methods, TLC is still proving its worth as a simple, inexpensive and robust means for the chemical and biological screening of plant extracts, with subsequent activity-guided isolation of natural products [65]. The potential for increased use of biological methods for sensitive and selective TLC detections is very great. Although different bioassays are successfully run on TLC, there is plenty of scope for adding to the number of bioautographic methods, especially with enzymes. TLC-bioassay in combination with the newly-introduced TLC/MS hyphenated system [66] will increase the power of TLC in natural products, phytochemistry, food and toxin analysis.

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