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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.

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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]).

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