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BIOLUMINESCENCE DETECTION IN THIN LAYER CHROMATOGRAPHY

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ABSTRACT. The principles, techniques, advantages, and applications of toxicity screening of complex mixtures using bioluminescent bacteria such as *Vibrio fischeri* following separation on a thin layer chromatography or high performance thin layer chromatograpy plate are described. A commercial kit, Bioluminex, and detection device, BioLuminex, are available for the method.

Keywords: *thin layer chromatography, TLC, bioluminescence detection, Vibrio fisheri, Bioluminex, BioLuminizer*

INTRODUCTION

Physical, chemical, and biological or bioactivity-based methods are used to detect zones in thin layer chromatography (TLC) after development of the plate with the mobile phase [1]. Advantages of TLC combined with a bioassay are high specificity and reduced interference of the matrix, leading to less need for sample cleanup [2]; high sensitivity, with detection limits typically in the sub-ng to pg range; identification of separated toxic compounds, degradation products, and metabolites based on chromatographic retention (R_f values), physical detection, and specific biological activity related to the test system; and opportunity for quantitative analysis.

Biological detection methods for microbiologically active compounds have been classified as diffusion methods, dilution methods, and bioautography [3]. The principle of direct bioautography is that a suspension of a microorganism growing in a suitable medium is applied to a developed TLC plate after drying; incubation of the plate with the microbes at optimum humidity and temperature allows growth of the bacteria and, by use of a specific dye, live cells can be visualized because, e.g., dehydrogenases from living microorganisms convert a tetrazolium salt into intensely colored formazan [4]. This article will be limited to a description of TLC in combination with bioluminescence detection, in which antibacterial compounds are detected as dark zones against a luminescent layer background instead of as colorless zones against a colored layer

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background in bioautography. Bioluminescence-TLC is being increasingly used because of the commercial availability of a reagent kit, detection device, and supporting accessory instruments to standardize the method and provide the optimum results.

PRINCIPLES OF TLC-BIOLUMINESCENCE COUPLING

The technology for bioactivity screening by TLC-bioluminescence direct coupling was developed by Bayer AG (Leverkusen, Germany) and published in 1996 [5]. The method is based on the change in emission by luminescent microorganisms in the presence of bioactive substances, which provides an effective way to detect toxic compounds on a TLC plate. The combination of TLC separation technology with the biospecific sensing ability of living cells represents a novel screening tool for targeting bioactive compounds in complex samples. In the published study [5], the TLC-bioluminescence technique proved to be superior to a high performance column liquid chromatography (HPLC)-bioluminescence method.

After TLC separation and evaporation of the mobile phase, the plate is coated with a bioluminescent solution of the nonpathogenic marine organism *Vibrio fischeri* by dipping. *V. fischeri* emits light as a product of cellular respiration. As *V. fischeri* cells reach a critical cellular density, the bioluminescence catalyst, luciferase, is expressed. Luciferase, in the presence of oxygen, catalyzes an oxidation reaction that releases excess energy in the form of light. Toxic substances on the layer are selectively identified as dark zones (inhibited bacteria) on a luminescent background (viable bacteria). The degree of toxicity is proportional to the luminescence inhibition. The results occur within seconds and last until the plate dries. Results can be documented by imaging with a charge coupled device (CCD) camera inside of a dark box or by X-ray film. The detection result of bioluminescence is similar to classical fluorescence quenching detection in which compounds that contain aromatic rings or multiple bonds absorb 254 nm light from an ultraviolet (UV) source and appear as dark zones against a bright background when layers impregnated with a fluorescent phosphor (F_{254} layers) are used. Because a TLC plate is used only once, less purified samples can be tolerated compared with column chromatography, in which strongly retained impurities from a previous sample can be eluted and interfere with later samples injected onto the column.

HPTLC- BIOLUMINESCENCE WITH THE BIOLUMINIZER

In modern TLC-bioluminescence analysis, a high performance TLC (HPTLC) plate is used instead of a TLC plate in order to achieve greater resolution and speed. The sample is separated by HPTLC, and the plate is

subsequently immersed in the luminescent bacterial suspension (*V. fischeri*; Bioluminex assay kit from ChromaDex, Irvine, CA). After an incubation time for completion of the reaction, all zones with inhibitory or toxic effects appear black and those with stimulatory effect as lighter zones in the chromatograms on the plate, which is photographed by the Camag (Muttenz, Switzerland) BioLuminizer (Figure 1), a light-tight dark box imaging system employing a high resolution cooled CCD camera with 16 bit data acquisition carried out for a specified time period. The BioLuminizer was developed specifically to accumulate the light signal from HPTLC plates for bioluminescent-based effect-directed analysis (EDA); there is no lighting system in the BioLuminizer, and the image is only acquired from the luminescence zones on the plate. The camera is optimized for long exposure times and very little light (high efficiency, low readout noise, and low dark current due to active cooling). Digital chromatogram images produced with the BioLuminizer can be quantitatively evaluated by VideoScan software. The BioLuminizer has a holder that enables exact plate positioning (to 0.1 mm) and an optimized compartment that resists plate dryout and keeps the bacteria moist and luminescent for several hours, leading to predictable bioluminescence activity for consistent results. High quantum efficiency up to 65% allows short exposure times.

Figure 1. Bioluminizer detection system for bioactive compounds.
Photograph supplied by Camag, Muttenz, Switzerland

EDA combining HPTLC with direct bioluminescence is a practical alternative to complex target analysis and provides a fast, low cost means of demonstrating biological activity in cases where many unknown substances are present, such as environmental samples. The time and effort required to examine structures or isolate substances can be restricted to a few relevant substances. By using HPTLC to separate the test sample into individual substances, the possibility of antagonistic effects or interferences leading to false results that can occur with classic cuvet tests (DIN 38412 L34) are eliminated. Detection levels are typically in the picomole range. Inhibition

values can be determined by special software for quantification of luminescence inhibition. In addition to bioluminescent bacteria, HPTLC-EDA has been proven effective for other types of bioassays, such as acetylcholinesterase inhibition, penicillin, and *Bacillus subtilis*, as well as genotoxicity tests such as umu (*Salmonella thyphimurium*) and yeast estrogen screen (YES).

The plate is typically dipped for 2 s into the luminescent bacteria in the Bioluminex assay. Instead of manual dipping, this step can be carried out more reproducibly using the Camag Chromatogram Immersion Device or Desaga TLC Dip-Fix (Desaga products are supplied by Sarstedt, Inc., Newton, NC) [6], which offer uniform vertical speed of immersion and withdrawal for selectable time periods. The dipping devices are also useful for prewashing the HPTLC plates to remove layer impurities before sample application. For highest resolution, samples can be applied bandwise with an automated instrument such as the Camag Linomat 5, Camag Automatic TLC Sampler, or Desaga HPTLC-Applicator AS 30 [7]. Ascending chromatogram development is usually carried out in a traditional rectangular glass chamber (N-chamber) or Camag twin trough chamber with a wedge shaped ridge along the bottom [8], but improved reproducibility can be obtained by using the Camag Automatic Developing Chamber ADC2 or Desaga TLC-MAT automatic chamber [6], which control chromatographic conditions such as saturation, layer activity, and drying. The highest resolution separations can be performed by automated multiple development (AMD) with the Camag AMD2 using a mobile phase gradient of decreasing elution strength over increasingly longer development distances with drying between steps [9]. Chromatograms can be photographed under white light (colored zones detected), 254 nm (fluorescence quenched zones), or 366 nm UV light (fluorescent zones) using a Desaga Digital Documentation System DD 50, ChromaDoc-IT Imaging System (Analtech, Newark, DE), or Camag Digistore-2 Documentation System (now replaced by the TLC Visualizer) [6]. The zones on plates can be quantified based on their scan areas compared to standard zones measured with a slit scanning densitometer (Camag TLC Scanner 3 or Desaga CD 60) [6, 10] or a diode array scanner (Tidas TLC 2010; J&M, Aalen, Germany), or by using image analysis software available with a Camag videodensitometer, the three documentation systems mentioned above, or a flatbed scanner densitometer (Chromimage; AR2i, Le Plessis Robinson, France). These accessory instruments improve the methodology and complement the analytical information generated by the BioLuminizer.

APPLICATIONS OF TLC- BIOLUMINESCENCE ANALYSIS

Application areas suggested by ChromaDex on its website [11] are natural biological and chemical toxins, chemical contaminants and pollutants, identification of potential biological activity (antimicrobiological screening), raw

material identity testing, simultaneous chemical and biological profiling, simultaneous parallel processing of up to 22 samples, and qualitative and quantitative analysis. Specific applications offered on the ChromaDex website are mycotoxin in corn; strychnine in infant formula; carbaryl insecticide in wine; fingerprint of *Capsicum annum* in cayenne; and steroid, As(III), and the herbicide metolachlor in tap water. For these applications, images of chromatograms are presented by ChromaDex.

Applications of the Bioluminex assay in a published article by scientists at the ChromaDex laboratories [12] include detection of the illicit bulking agent melamine in pet food; fingerprint comparison of fresh versus cured tobacco [methanol extraction of tobacco products and silica gel TLC with chloroform-methanol-ammonium hydroxide (9:1:0.05) mobile phase]; screening for compounds with biological activity in methanol extracts of Asian red yeast rice [chloroform-ethyl formate-formic acid-methanol (5:5:2:2) mobile phase]; structure-activity relationship (SAR) analysis of the structurally similar compounds quercetin, isoquercetin, and quercetin-3-rutinoside; and detection of ochratoxin in canned corn, aflatoxin B1 in honey, digoxin in milk, benzopyrine in celery seed, capsaicin in cayenne pepper, strychnine or monofluoroacetic acid in various drinks, domoic acid in soda, and patulin in apple juice.

Applications of TLC-bioluminescence described on the Bayer website [13] are toxicity profiling of wastewater over a 15 day period, analysis of natural products extracts, detection of tea seed oil residual, quantification of toxicity by toxicity equivalents based on calibration with 4-nitrophenol, United States Pharmacopeia (USP) method for *Matricaria chamomilla* using bioluminescence and anisaldehyde/366 nm detection, analysis of kava kava, and toxicity in process water.

Specific applications for the HPTLC-BioLuminizer method offered on the Camag website [14] are the following: analysis of wastewater containing X-ray contrast media for oxidation products produced by irradiation with UV light; toxicity screening for environmental applications; risk assessment and monitoring of drinking water, wastewater, and natural attenuation processes; detection of toxins and chemical adulterants in foodstuffs, beverages, and cosmetics; identification of biological activity in natural product extracts; determination of pesticides, heavy metals, organic pollutants, pharmaceuticals, and mycotoxins in a variety of complex matrices; and bioactivity-based analysis of irradiated sunscreens.

The determination of berberine containing drugs in methanol extracts of powdered raw material is illustrated in Figure 2 and 3. Additional compounds are detected after HPTLC by bioluminescence compared to fluorescence under 366 nm UV light.

Luminographic detection of toxins and pollutants in raw water and drinking water was performed with the Bioluminex-Bioluminizer system [15]. Analytes were recovered from water samples using Isolute ENV+ solid phase

extraction (SPE) with methanol eluent, 20 x 10 cm silica gel 60F_{254} HPTLC plates, application of samples as bands with an Automatic TLC Sampler, and chromatography with an AMD2 using a 25 step gradient based on acetonitrile-formic acid-dichloromethane with a maximum developing distance of 80 mm.

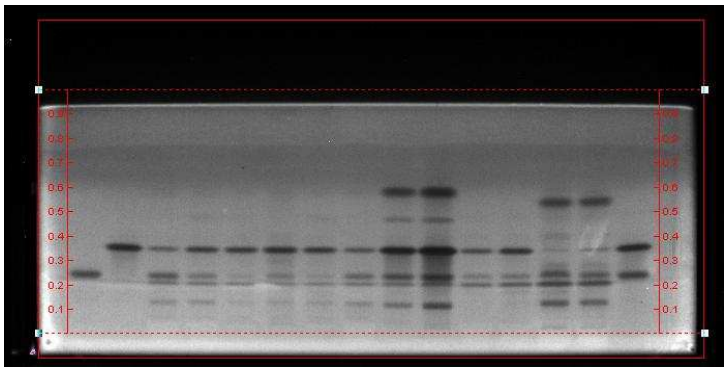

Figure 2. Detection of berberine alkaloids by their green to yellow fluorescence under 366 nm UV light using a Digistore-2 Documentation System and by the BioLuminex assay. Lane numbers: 1, palmatine; 2, berberine; 3-8, Mahonia sp.; 9-10, Coptis chinensis; 11-12, Phellodendron chinensis; 13-14, tinospora sp.; 15, palmatine plus berberine. Chromatographic conditions: HPTLC silica gel 60F254 Merck plate, toluene-ethyl acetate-methanol-isopropanol-water (60:30:20:15:3) mobile phase, 1 uL test and standard solutions applied as 8 mm bands, development in a twin trough chamber saturated with ammonia vapor. Photograph supplied by Camag, Muttenz, Switzerland

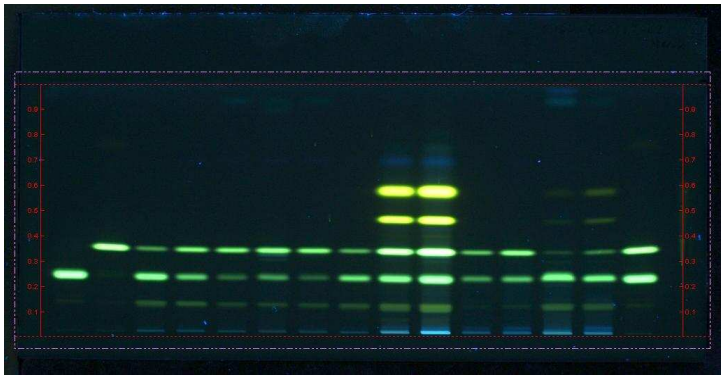

Figure 3. HPTLC bioluminescence image of the same plate as in Figure 2. Note the different numbers of zones in the chromatograms detected in some of the lanes by the two methods to provide complementary information on the samples. Photograph supplied by Camag, Muttenz, Switzerland

Similar methods were used to compare the bioactivity of several types of sports field granules and of expressway wastewater and landfill leachate [16]. In this study, the AMD gradient had 13 steps based on methanol (containing 5% ammonia), dichloromethane, and *n*-hexane.

HPTLC was coupled with bioluminescence and mass spectrometry (MS) for bioactivity based analysis of secondary metabolites in marine sponges [17]. Lyophilized and ground sponge tissue was extracted overnight with methanol, and the extract was evaporated and redissolved for analysis. HPTLC silica gel $60F_{254}$ plates and bandwise application of samples using an Automatic TLC Sampler were used. A 15 step gradient, with 3 mm increments, based on methanol, dichloromethane, and *n*-hexane was used for development. Optional derivatization with sulfuric acid reagent and Bioluminex detection with *V. fischeri* were done using a Chromatogram Immersion Device III, and documentation was with a DigiStore 2. Zones of interest were identified by online Fourier transform (FT) MS.

For analysis of sunscreens irradiated with either artificial light or sunlight, samples were applied with a Linomat 5 onto a LiChrospher Si $60F_{254}$ HPTLC plate; separated by AMD (six steps with diisopropyl ether-*n*-hexane, drying time between steps 2-3 min, migration distance 50 mm); documented under 254 and 366 nm UV light; scanned at 200-400 nm with a TLC Scanner 3 and WinCats software; and detected with *V. fischeri*. Zones of special interest were scratched out of the layer and reanalyzed by HPLC with a diode array detector and HPLC/MS [18]. Bioluminescence detection allowed location of zones of compounds undetected by physical or chemical methods and identified bioactive photodegradation products that need further toxicological evaluation.

Adulteration of black cohosh (Actaea racemosa) was determined by HPTLC-bioluminescence performed as follows: sonication extraction of powdered plant material with methanol, application of samples with an Automated TLC Sampler 4 as bands to a silica gel $60F_{254}$ HPTLC plate, plate development with toluene-ethyl formate-formic acid (5:3:3) in a saturated ridged bottom glass chamber, zone detection under 254 and 366 nm UV light and by spraying with sulfuric acid-anisaldehyde reagent and heating (125^{o}C, 5 min), and bioactivity detection with the Bioluminex/BioLuminizer system. Yellow cohosh and other common adulterants were readily differentiated from black cohosh [19].

Additional biologically active compounds and samples that have been analyzed by TLC-*V. fischeri* bioluminescence coupling include 3,5-dichlorophenol [20]; 4-nitrophenol in urine, and garlic and curry food ingredients [21]; the cytostatic agent avarone, with subsequent confirmation by MS [22]; toxic methylmercury and the banned pesticide *o*,*p*-dichlorodiphenyldichloroethane (DDD) [23]; organic compounds in tannery effluent and molasses wastewaters

(AMD-TLC fractionation) [24]; and pentachlorophenol (graph of inhibition versus concentration was nearly linear in the range of 20-80 ng with densitometer and videodensitometer measurement) [25].

CONCLUSIONS

TLC or HPTLC combined with bioluminescence detection is an important method for effect directed analysis of bioactive zones on the layer. Bioluminescent bacteria can be used, or other types of bioassays can be carried out [26]. A system incorporating *V. fischeri*, HPTLC, and detection with a CCD camera in a dark box (BioLuminizer) is available from Camag. Various accessory HPTLC instruments can be incorporated to obtain improved results and additional analytical information. Key advantages are that tedious single compound screening is avoided, rapid response time and fast results, no false results due to antagonistic effects/interferences, detection in the picomole range, high sample throughput due to parallel analysis of multiple samples on one plate, low cost, and possibility of further investigation of bioactive zones, e.g., by MS. Use of online HPTLC/MS to obtain information about bioactive unknowns is now greatly facilitated by the availability of a commercial interface that can be connected to any HPLC-coupled mass spectrometer and gives direct extraction of zones from the plate into the ion source [27].

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