



Natural Product Analysis by TLC-Bioluminescence

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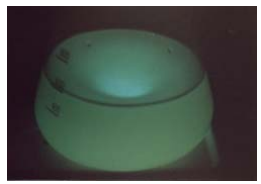
BACKGROUND

The screening of complex mixtures for toxins and adulterants or potential biological activity is often expensive and time consuming. To identify the active or toxic constituents in such mixtures requires the tedious isolation of single components followed by assays of their biological effects. Alternatively, standard rapid-screening tests only establish the overall toxicity of a mixture disregarding the identification of the active culprit. Additionally when these materials are tested in their mixture condition there is a high risk of artifacts or false data due to interferences and interactions with other components in the mixture. Optimally, a rapid screening assay which analyzes activity of the individual components of a mixture in an economical and efficient fashion would be of great value. The Bioluminex™ (ChromaDex™, Irvine, CA) assay, which is based on direct bioautography, is one such technique that can rapidly analyze a variety of complex mixtures.

The Bioluminex™ assay couples the separation power of high-performance thin-layer chromatography (HP-TLC) with the biosensor properties of bioluminescent microorganisms.¹⁻⁵ This technology provides a characteristic chemical and biological toxicity profile or "fingerprint" for each mixture analyzed and can be used to identify potentially bioactive compounds or adulterants. Additionally, the profile can be used to help support material identity. This TLC-bioluminescence assay offers several advantages over standard toxicity screening assays. First, activity is assigned to single components of a mixture thereby identifying the active constituent and eliminating interferences from other analytes. Secondly, the sample medium is evaporated from the TLC plate prior to introducing the biosensor organism allowing for the analysis of non-aqueous samples. Thirdly, the need for a secondary visualization agent is eliminated by employing a variety of bioluminescent organisms as the primary detection reagent. Fourthly, this economical assay can simultaneously analyze up to 20 samples with the organism toxicity results produced within 2 minutes. Lastly, this TLC-bioluminescence assay has been designed to be kit compatible, providing a rapid and inexpensive analysis for many complex samples.

The Bioluminex™ assay uses the naturally bioluminescent marine bacterium *Vibrio fischeri* (Beijerinck 1889) Lehmann and Neumann 1896, which has been well characterized and used in standardized and validated ecotoxicity assays for over 25 years.⁶ The bioluminescent bacteria, *V. fischeri*, emits light as a by-product of cellular respiration. As these bacteria cells reach a critical cellular density the lux operon expresses the enzyme luciferase. Luciferase, in the presence of oxygen, catalyzes an oxidation reaction that releases excess free energy in the form of blue-green light (Image 1). This reaction is directly proportional to microbial metabolism. Therefore, any compound that interferes with this process decreases luminescence. Thus a reduction in light emission is a measure of toxicity towards *V. fischeri* and can be selectively viewed and quantitated directly on the HPTLC plate.

Image 1. Luminescing *Vibrio fischeri*

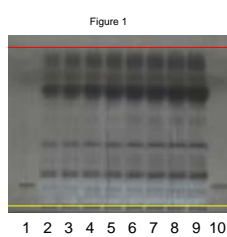
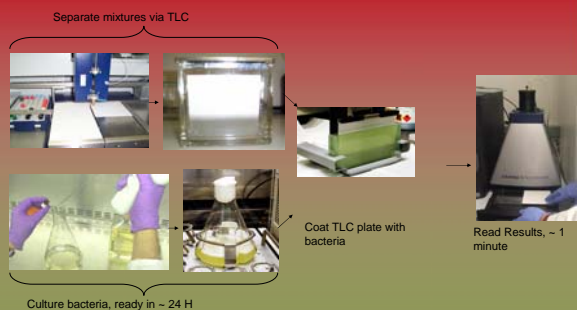


TECHNOLOGY
INVENTED
BY BAYER

BIOLUMINEX™ ASSAY OVERVIEW (Image 2)

Complex mixtures are first separated by HP-TLC. After compound separation, the mobile phase is evaporated and the matrix plate is coated with a broth of bioluminescent bacteria employing a simple dipping procedure. The broth can be used to dip multiple plates and be buffered to use with mobile phases containing acids or bases. Results occur within seconds and last until the plate dries in approximately 30 minutes, or greater with the use of the Bioluminizer (CAMAG, Switzerland) which optimizes the plate compartment for prolonged bacterial activity. Results identify single compounds, which inhibit luminescence resulting in dark zones (quenched bioluminescence) on a luminescent background where the bacteria remain viable. Data can be documented by direct contact of photographic film such as x-ray and Polaroid film or indirectly, such as with a cooled CCD (charge-coupled device) camera. Additionally this technology has been developed into a kit format.

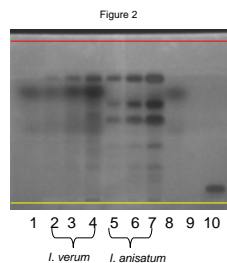
Image 2. Bioluminex Assay Overview Scheme



"Fingerprint" Profiling Analysis

Fadogia agrestis Schweinf. ex Hiern (Rubiaceae) is an African shrub, whose stem is indigenously used to treat erectile dysfunction. Despite its growing popularity in the United States, at this time only one chemical analysis study has been published.⁷ The investigation presented here (Figure 1) displays the TLC-bioluminescence fingerprint of *F. agrestis* stem extract. This fingerprint simultaneously provides a chemical and biological profile of the unknown and potential biologically active components. This assay can then be used for bioassay guided fractionation of the compounds of interest.

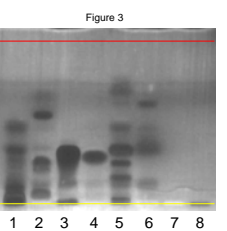
Figure 1. TLC-bioluminescence image of increasing concentrations (22.2 µg -74.0 µg) of *F. agrestis* stem extract (0.5 g, 5 mL CH₂OH) in tracks 2-10. Tracks 1 & 10 contain rutin (3.0 µg). Chromatogram was developed with chloroform/ acetic acid/ methanol/ water (64:32:12:8, v/v/v/v).



Species Differentiation of Natural Products

The Bioluminex™ assay can be used for species differentiation. In this example, *Illicium verum* Hook. f (star anise) extracts are distinguished from the similar, more toxic species, *Illicium anisatum* Hook (Japanese star anise). Additionally, this assay could be used to detect adulteration, such as spiking of the toxic *I. anisatum* into the edible *I. verum* product.

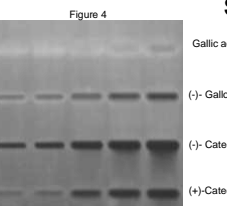
Figure 2. TLC-bioluminescence image of increasing concentrations (2.7, 5.4, and 10.8 µg) of *I. verum* extract in tracks 2-4 and *I. anisatum* extract in tracks 5-7; 1.2 µg of *trans*-anethole standard in tracks 1 and 8; 4.0 µg of Bioluminex™ positive control in track 10 (track 9 is a matrix blank). Chromatogram was developed with toluene/ ethyl acetate/ formic acid (9:1:0.9, v/v/v).



Identification of Antimicrobial Compounds

Sources of new antimicrobial compounds could be determined using the Bioluminex™ assay. This example shows the activity of a variety of essential oils with reported antibacterial properties. Compounds that inhibit the luminescence of the *V. fischeri* bacteria are detected as dark bands on a luminescent background.

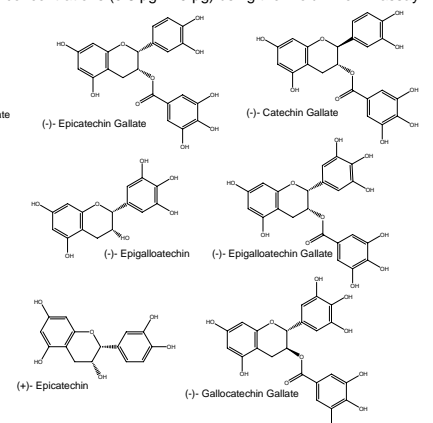
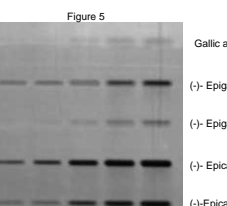
Figure 3. TLC-bioluminescence image of oils of *Cinnamomum camphora* (21.0 µg, track 1), *Myristica fragrans* (16.0 µg, track 2), *Syzygium aromaticum* (1.0 µg, track 3), *Eucalyptus globulus* (100.0 µg, track 4), *Citrus limon* (85.0 µg, track 5), and *Rosemarinus officianalis* (24.0 µg, track 6); 4.0 µg of Bioluminex™ positive control in track 8 (track 7 is a matrix blank). Chromatogram was developed with toluene/ ethyl acetate (9.5:0.5, v/v/v) and analyzed using the Bioluminex™ assay.



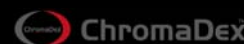
Structure Activity Relationships (SAR)

SAR studies were conducted to determine the effects toward *V. fischeri* of adding a gallic acid to catechin and the catechin derivatives, epicatechin and epigallocatechin. These data (Figure 4 & 5) indicate that the addition of a gallic acid ester to the catechin base structure increases *V. fischeri* inhibition.

Figure 4. TLC-bioluminescence images of catechin, epicatechin, epigallocatechin and their corresponding gallate derivatives. Compounds were analyzed at increasing concentrations (0.8 µg - 4.0 µg) using the Bioluminex™ assay.



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