Rapid Screening of Complex Mixtures using TLC-Bioluminescence

Sheryl M. Verbitski, Gerald T. Gourdin, Larissa M. Ikenouye, James D. McChesney
ChromaDex Analytics Inc., 2830 Wilderness Place, Boulder, CO 80301 USA

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, Santa Ana, CA) assay, which is based on direct bioautography is one such technique that can rapidly analyze a variety of complex mixtures such as such as foodstuffs (Figure 1), waste water (Figure 2), beverages (Figure 3), and dietary supplements (Figure 4).

The Bioluminex™ assay couples the separation power of thin layer chromatography (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 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 *V. 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] As *V. fischeri* cells reach a crucial cellular density their *lux* operon expresses the reaction catalyst luciferase. In the presence of O2 and luciferase, a NADH (nicotinamide adenine dinucleotide)-reduced riboflavin phosphate (FMNH2) and a long chain fatty aldehyde are oxidized. The resulting interaction forms an excited yet highly stable intermediate, which decays slowly, resulting in the release of excess free energy in the form of a blue-green light (490 nm). The observed bioluminescence reflects the metabolic status of the cell and will decrease for cells exposed to toxic substances. Thus a reduction in light emission is a measure of toxicity towards *V. fischeri* and can be selectively viewed and quantitated directly on the TLC or HPTLC plate. Typical limits of detection for toxic substances are in the picomol range.

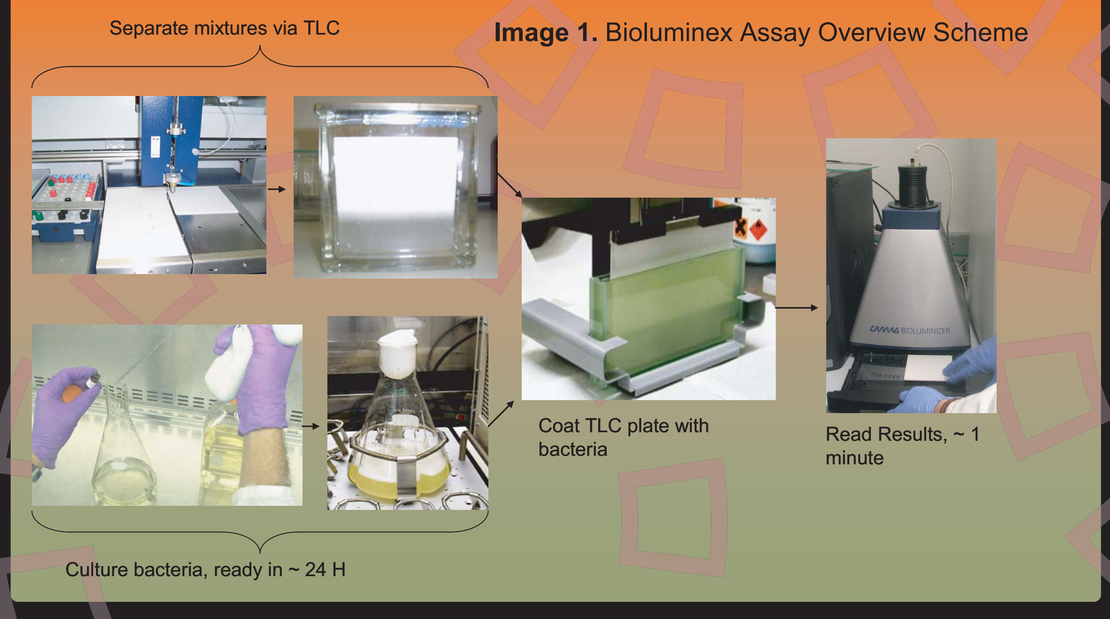

Image 1. Bioluminex Assay Overview Scheme

BIOLUMINEX ASSAY OVERVIEW (Image 1)

In the TLC-bioluminescence assay complex mixtures are first separated by TLC or HPTLC (high performance thin layer chromatography). 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 that do not fully evaporate during the drying phase. Results occur within seconds and last until the plate dries, 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, video imaging, a Polaroid documentation system or a 35 mm camera. Additionally this technology has been developed into a kit format (Image 2.)

Ochratoxin A Detection in Spiked Corn

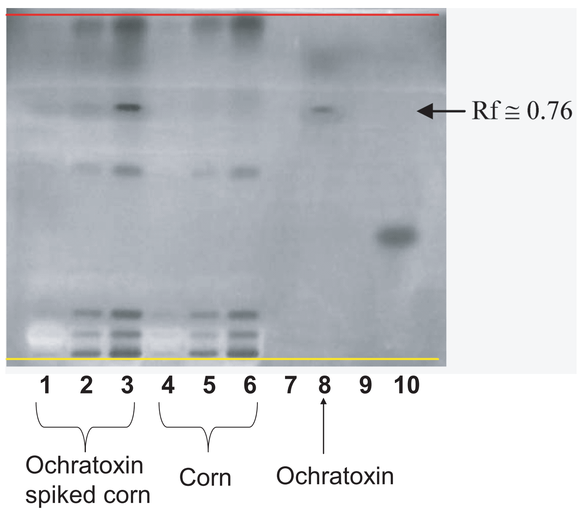

Figure 1. TLC-bioluminescence image of increasing concentrations (0.5 µg analyte, 1.5 µg analyte, and 3.0 µg analyte) of ochratoxin A spiked corn extract (1 g freeze-dried corn, 10 mL CH3OH) in tracks 1-3, respectively; equivalent amounts of unspiked corn extract in tracks 4-6; 2 µg of ochratoxin A standard in track 8; 4 µg and 8 µg of Bioluminex™ negative and positive controls in tracks 9 and 10, respectively (track 7 is a blank). Chromatogram was developed with ethyl acetate: methanol: formic acid: water (50:2:5:3, v/v/v) and analyzed using the Bioluminex™ assay

Simultaneous Detection of a Steroid, Pesticide, and Heavy Metal in H_2O

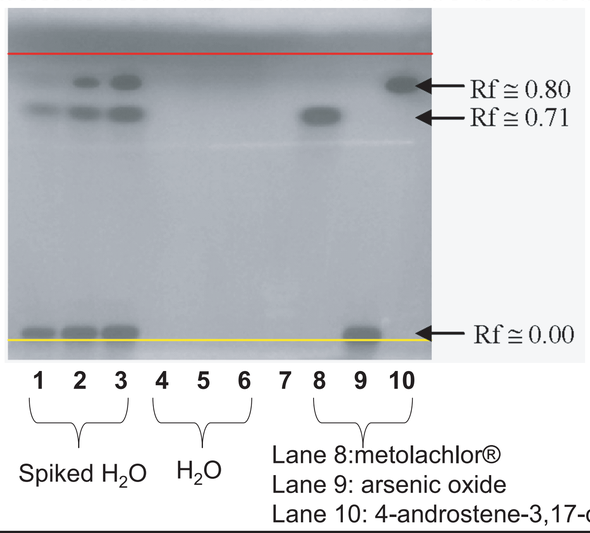

Figure 2. TLC-bioluminescence image of increasing concentrations (0.6 µg, 1.1 µg, and 1.6 µg of each analyte per lane) of 4-androstene-3,17-dione, arsenic oxide, and metolachlor® spiked tap water in tracks 1, 2, and 3, respectively. Corresponding application volume (8 µL, 14 µL, and 20 µL) of unadulterated water in tracks 4, 5, and 6, and 2 µg of each analyte, 4-androstene-3,17-dione, arsenic oxide, and metolachlor in tracks 8, 9, and 10, respectively. Chromatogram was developed with toluene: ethyl acetate: formic acid: water (4:8:1.1:0.2, v/v/v/v) and analyzed using the Bioluminex™ assay.

Strychnine Detection in Infant Formula

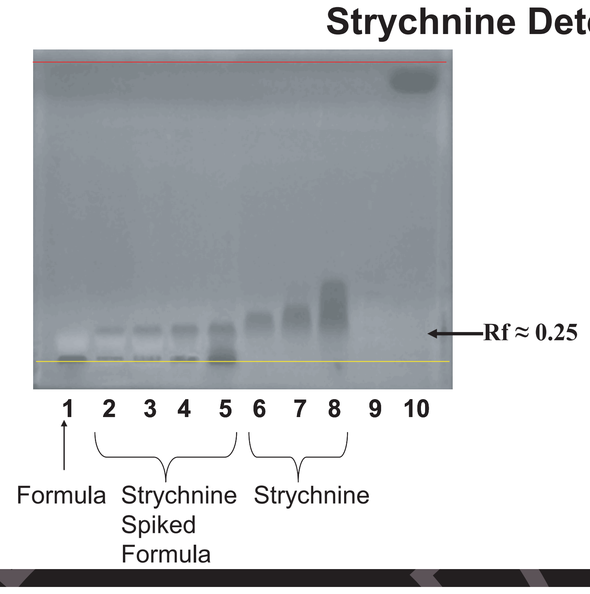

Figure 3. TLC-bioluminescence image of increasing concentrations (5 µg, 10 µg, 20 µg and 50 µg) of strychine spiked infant formula in tracks 2, 3, 4, and 5, respectively. Increasing concentrations of the strychnine standard (10 µg, 20 µg and 50 µg) are in tracks 6, 7, and 8, respectively and 20 µl of infant formula was applied in track 1. The Bioluminex™ negative and positive controls are in tracks 9 and 10, respectively. Chromatogram was developed with toluene: ethyl acetate: formic acid (5:3:2 v/v/v/v) and analyzed using the Bioluminex™ assay.

Unknown Adulterant Detection in *Actaea racemosa* (Black Cohosh)

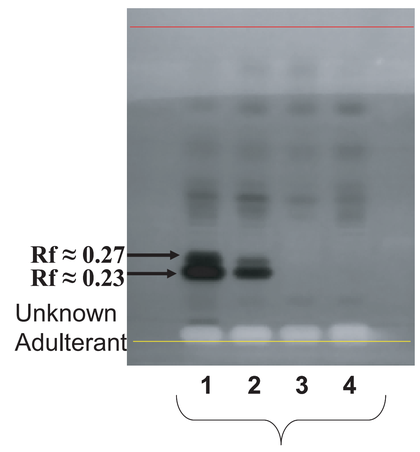

Figure 4. TLC-bioluminescence image of 4 authenticated samples of *Actaea racemosa* (black cohosh (10 µg extracts) in tracks 1-4. Samples in tracks 3 & 4 display a characteristic Bioluminex fingerprint of *A. racemosa*; where else in tracks 1 & 3 unidentified adulterate(s) are detected. Chromatogram was developed with toluene: ethyl formate: formic acid: water (5:3:2, v/v/v) and analyzed using the Bioluminex™ assay.

Image 2. Bioluminex Kit

1. Verbitski, S. M.; McChesney, J. D.; Gourdin, G. T.; Ikenouye, L. M. The Development of Methodology Involving the Coupling of Bioluminescent Bacteria to Thin-Layer Chromatography for Detecting
2. Active Components in a Variety of Complex Mixtures. U.S. **Patent Pending** *January 2006*.
3. Weisemann, C.; Kreiss, W.; Rast, H.-G.; Eberz, G. Analytical Method for Investigating Mixtures of Toxic Compounds. **European Patent** *1998*; EP 0 588 139 B1.
4. Eberz, G.; Rast, H. G.; Burger, K.; Kreiss, W.; Weisemann, C., Bioactivity Screening by Chromatography Bioluminescence Coupling. **Chromatographia** *1996*, 43, 5-9.
5. Becvar, J. E.; Becvar, L. E. Luminous Bacteria and Methods for the Isolation, Identification, and Quantitiation of Toxicants. **U.S. Patent** 2000; 6,017,722.
6. Becvar, J. E.; Becvar, L., E. Kit for the Isolation, Identification, and Quantitation of Toxicants. **U. S. Patent** 2002; 6,340,572.
7. Use of Luminescent Bacteria for Determining Toxicity in Aquatic Environments. In *Aquatic Toxicity, ASTM STP 667*, Markings, L. L.; Kimerle, R. A., Eds. American Society for Testing and Materials: Philadelphia, PA, 1979; pp 98-106.

ChromaDex™
2952 S. Daimler • Santa Ana, CA 92705 • Tel. (949) 419-0288
Fax. (949) 419-0294
www.chromadex.com

www.bioluminex.com