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Handout

HPTLC-Bioluminescence-Coupling using *Vibrio fischeri*

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1 Effect-directed Analysis using HPTLC and luminescent *Vibrio fischeri* bacteria

Vera Baumgartner and Elisabeth Dytkiewitz

1.1 Characteristics

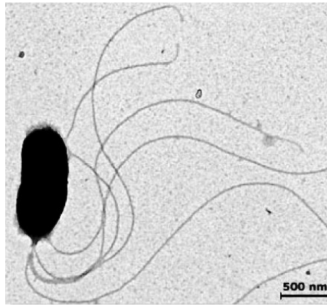

Fig. 1: Photo of *Vibrio fischeri*[1]

Vibrio fischeri is a gram-negative, comma-shaped rod with flagella (refer to Figure 1), which lives as plankton or in symbiosis in all seas.[5] It was discovered by Beijerinck in 1889 and is also known as *Aliivibrio fischeri*.[3] Its special characteristic is its ability to glow (refer to subsection 1.2).

Furthermore, the bacterium is robust, non-pathogenic and easy to cultivate, which makes it an ideal organism for the use in an analytical laboratory.

1.2 Generation of light

As all bioluminescent organisms, *Vibrio fischeri* uses a so-called *luciferin-luciferase-system* for the generation of light. Thereby, a substance, the *luciferin*, and oxigen are transformed by an enzyme, the *luciferase* into light and water. The structure of the luciferin depends on the organism.

The reaction is: $\text{Luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{LIGHT} + H_2O$

Vibrio fischeri uses riboflavin-5-phosphate, a reduced flavin mononucleotide ($FMNH_2$), as luciferin. The light emitted is cold light with at a wavelength of 490 nm.

To generate the light, energy in the form of 2 NAD(P)H and 1 ATP is necessary. The luminescence system is directly coupled to the respiratory chain and the ATP pool of the bacterium. Therefore, an inhibition of the luminescence indicates a disturbance of the metabolism.

Because the luminescence system is not essential, this energy can be saved in critical situations. That is why the bacteria luminize only when there are enough nutrients and oxygen, but no toxic substances. The light is not just switched on and off but can also be dimmed. This means, that the degree of the inhibition caused by a substance is analog to the toxicity on the bacterium.[5, 2, 4]

[1]Source: http://www.pnas.org/content/102/8/2673/F2.large.jpg (24.09.2007)

1.3 Application in the analytics

1.3.1 Present application

Vibrio fischeri is used in the field of (waste) water analytics for ecotoxicological tests since 1979.[1] In this test, an aliquot of the water to be tested is mixed with the bacteria and the glow is measured after a specific time (equipment *LUMIStox*, Hach-Lange).

A great disadvantage of this technique is, that the result is a sum parameter, because the resulting effect of all sample components is measured, regardless of weather single compontents have no effect, are enhancing or inhibiting.

1.3.2 HPTLC-Bioluminescence-Coupling

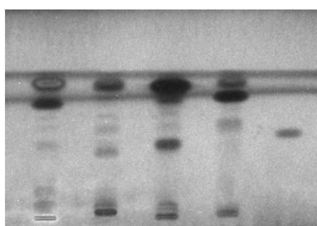

Fig. 2: Biodetection of an HPTLC plate

For the HPTLC-Bioluminescence-Coupling, the sample is applied on an HPTLC plate, and the compounds are separated using HPTLC. The developed HPTLC plate is dried to remove the solvents. After drying, the plate is dipped into the *Vibrio fischeri* solution (*Vibrio fischeri* is cultivated in a liquid media).

Then, a photograph of the wet plate is taken using the Bio-Luminizer device (CAMAG). The photo can then be evaluated qualitatively and (semi)quantitatively. Hence, specific information on the single components are obtained.

An example shows Figure 2.

1.4 Limitations and pitfalls of the *Vibrio fischeri* detection

Stationary phase: The plate coating has to be polar because of the wettability. Silica gel and LiChrospher coatings work well.

Mobile phase: Only solvents which are not toxic and evaporated without residues from the plate coat can be used. We used usually methanol, dichlormethan, tertiary butylmethylether and n-hexane.

Drying: The method is not suitable for thermolabile or volatile compounds, because after development, the HPTLC plate has to be carefully dried to evaporate all solvents properly, e. g. 60°C for 1 hour.

Degradation on the plate: For some substances, a degradation was observed on the HPTLC plate. This is not astonishing, the plate coating is quite active because of its big surface which is in contact with oxygen.

→ As for all analytical methods, it is necessary to assure the results by comparing them with other techniques. In the case of HPTLC this can be done e. g. by HPLC.

General References

[1] DIN EN ISO 11348-2: Water quality — Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) — Part 2: Method using liquid-dried bacteria (ISO 11348-2 : 1998), Dec. 1998.

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2 Examination of sunscreens as a motive for improvements of the method

Vera Baumgartner

2.1 Examination of sunscreens

The aim of the *Vibrio fischeri* detection is to find unknown (cytotoxic) substances which have been overlooked previously. The technique can help to close the gap between chemical-physical detection methods and toxicological relevance, because the information obtained from this bioactivity based analysis could help concentrate on those substances which need a (further) toxicological evaluation.

We tried to apply this in our first project for the examination of photodegradation products in sunscreens.[1] Sunscreen products are meant to protect people from damaging UVA and UVB radiation. However, in some formulations the UV filters they contain can react and form many photodegradation products. Their potential toxicity has not yet been investigated. In this study effect-specific analysis using HPTLC and detection with *Vibrio fischeri* has been used to evaluate the bioactivity of photodegradation products in sunscreens.

First, biodetection was performed on pure standard solutions of the UV filters. UV filters with molecular weight >400 had no bioactivity; these included all newer UV filters (not in use before 1998). Five commercially available sunscreens with different UV filter combinations were then analyzed. They were irradiated on microscope slides withartificial light and natural sunlight and on the skin with natural sunlight. The bioactivity which can be indicative of (cyto)toxic effects of the photodegradation products was higher than that of the corresponding UV filter.

In comparison of HPLC-DAD and LC-MS with detection with *Vibrio fischeri*, a high signal in chemical-physical detection did not always correspond to high bioactivity, and vice versa. It was shown that biodetection with *Vibrio fischeri* was a suitable method for examination of photodegradation products in sunscreens, making this bioassay a useful addition to conventional analytical methods.

2.2 Quantitative Evaluation

The evaluation of the photo, which is the result of the *Vibrio fischeri* detection, was usually done visually and, hence, qualitatively. Currently, available image evaluation programs showed to be unsuitable for quantitative evaluation because of insufficient background correction and/or tedious procedures. Furthermore, special corrections like a horizontal background correction and the recalculation of the sigmoid dose response relationship of the bacteria's reaction are needed. Available programs could not fulfill these requirements.

Therefore, a method was developed by using existing common or freeware programs with which the proper corrections could be accomplished. Steps were the selection of the regions of interest from the HPTLC image, the conversion of the image file into a text file, followed by

the main calculation in the spreadsheet program Microsoft Excel. For calculation, adapted versions of the cuvette test calculations were used. As Excel does not contain the necessary integration tools, two export methods were included. The first method allowed for a routine evaluation of chromatograms giving peak height and area as parameters. The second method enables an in-depth evaluation of chromatograms using an HPLC software leading to parameters like, e.g., signal-to-noise ratio, peak asymmetry, or peak width. Results obtained with the method were convincing. With a macro bundle, the calculations were not very time consuming and could be applied for routine use.[3],[2]

2.3 New application technique of *Vibrio fischeri* bacteria on the HPTLC plate

Currently, the application of the *Vibrio fischeri* bacteria is done by dipping, which is a very common technique for the derivatization of HPTLC plates.

But experience with the *Vibrio fischeri* detection showed, that dipping is not always practicable, especially not, when polar samples are applied. These zones tend to bleed and show a tail. Currently, we are working on another application technique based on rolling, which seems to be very promising.

2.4 Conclusion:

We could show in our work, that biodetection in an analytical laboratory is applicable; it works and is reproducible.
Further achievements were made in the field of an effective quantitative evaluation and an improved application.

→ The *Vibrio fischeri* biodetection is ready for use.

References

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3 Plasticizers: HPTLC-MS and *Vibrio fischeri*? Two complementary techniques to target critical substances

Elisabeth Dytkiewitz

3.1 General information

In food industry, plastic foils of polyvinyl chloride (PVC) are used to package meat and cheese as well as for wrapping fresh vegetables. To achieve PVC foils of suitable flexibility, good thermal resistance or slow material aging, a wide variety of additives is needed in high percentages. Beside plasticizers, also antioxidants, UV stabilizers and lubricants are added to the PVC. Nearly all have in common, that they are not bonded to the polymer, thereby being potentially free for migration into packaged foods. According to the European legislation, migrating constituents must not endanger the human health.[1]

Therefore, migrating studies with food simulating solvents must be performed to check the compliant composition. Hence, the foils were treated with so-called food simulating solutions like water, ethanol or olive oil to achieve an adequate migration effect.[2] Due to the different physicochemical properties of the additives, various analytical methods are required to analyze the extracts.[3]

3.2 Application of *Vibrio fischeri* for PVC additives

By using effect-directed analysis we brought the bioactivity of additives into focus. Substances with a toxicological potential could be exposed. After separation of several plastic foil extracts we were able to perform a detection with bioluminescent *Vibrio fischeri* bacteria. Some of the substances inhibited the luminescence, others even enhanced it. The strength of effect was a further information.

Not having standard solutions for every additive, the identification was made by coupling HPTLC with mass spectrometry via the TLC-MS Interface. The spots were eluted with ethanol and detected by an ESI-MS system within seconds. Sodium adducts appeared, which was caused by the saline bacteria suspension remaining on the HPTLC plate surface.

To enable the identification by a low resolution mass spectrometer, the assigned signal had to be improved. Exact masses and a molecular formula were obtained by calculation with the MassWorks (Cerno) software. Thus, substances with toxicological potential were identified.

3.3 Extraction of plastic foils by the TLC-MS Interface

For screening extractable additives from PVC plastic foils, we placed the plastic foil on the back of a TLC aluminium foil. After tightening the elution head of the TLC-MS Interface, a mass spectrum was recorded directly from the foil surface. In this way, the main components were identified. A further summary of our work can be found in CBS 105.[4]

References

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[2] Attachment 10 of §11 BedarfsgegenständeVO (*Ordinance on Commodities*) as amended in september 23, 2009

[3] Silva A. S. et al.. Compilation of analytical methods and guidelines for the determination of selected model migrants from plastic packaging. *Trends in Food Science Technology*, 17:535–546, 2006

[4] CAMAG Bibliography Service, No. 105, published by CAMAG Switzerland, *(in print)*