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# Thin-layer chromatography coupled with biological detection to screen natural mixtures for potential drug leads☆

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ABSTRACT

This review article summarizes the use of thin-layer chromatography coupled with biodetection for discovery of new drug leads. All the currently used TLC-based techniques were described, including detection and identification of antimicrobials, antioxidants and enzyme inhibitors. Thin-layer chromatography coupled with biological detection can be considered as a high-throughput, inexpensive and reliable procedure for screening plant extracts for the presence of potential drugs. Apart from well known examples of using TLC-biautography the article delivers information on most recent solutions developed with the use of different bioassays. A still-growing collection of newly developed approaches clearly shows TLC has become an attractive alternative to other preliminary assays, commonly used in phytochemistry laboratories for fishing experiments.



**Contents**



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## 1. Introduction

Preliminary examination of biological activity of new compounds is often a necessary step prior to performing any in vivo pharmacological studies. High-throughput techniques are commonly used for screening libraries of newly synthesized organic compounds to fish out the most promising drug candidates. Preliminary screening of biological properties also plays an important role in searching for active secondary metabolites present in natural products.

Natural product extracts can be considered as an invaluable source of biologically active secondary metabolites. Apart from obtaining qualitative and quantitative data, various analytical methods, enabling bioactive detection can be used to fish out compounds exerting desired pharmacological effect. Different analytical techniques coupled with biological detection have become an indispensable tool in the effect-oriented analysis focused on detection, identification and isolation of structurally new and active components. Taking into account the complexity of screened samples as well as their numbers, the applied techniques should be high-throughput, simple, fast and relatively inexpensive. Spectrophotometric and chromatographic methods seem to meet the majority or even all of the abovementioned criteria. They are commonly applied for fast screening of natural samples for compounds exerting the following activities: antioxidant, antimicrobial and inhibitory toward selected enzymes.

It should be emphasized, that among chromatographic techniques coupled with biodetection, thin-layer chromatography (TLC) is especially useful for screening experiments. TLC has many advantages which are crucial for biodetection, just to mention the following ones: it is the only chromatographic method enabling presentation of results as a picture-like image; many samples can be compared side by side; all samples on the plate are analyzed under the same conditions, which is impossible to perform in case of sequential mode of HPLC; rapid results are obtained, as many samples are analyzed at the same time, what leads to solvent savings; many factors can be changed during chromatographic process, that influence the resolution of analyzed compounds; detection can be repeated several times, without and with derivatization; complicated clean-up procedures are not needed, that usually are indispensable in case of HPLC, to avoid column contamination; sample preparation step does not have to be modified even if one wants to focus on different substance classes present in the extract. In case of biodetection the conditions of the process can be easily optimized in terms of temperature, humidity, and time. Another factor—time interval between reaction and detection which significantly influences bioresponse can easily be adjusted. However, one of the greatest drawbacks of TLC-based effect-directed analysis is its rather low separation capacity when compared to HPLC. The observed zones of desired activity can be a mixture of different compounds and biological effect is due to the interaction between the metabolites. Therefore, it should be stressed that TLC effect-directed analysis is only a preliminary tool which is useful in identifying new drug candidates. The obtained data should not be overestimated and exaggeratedly interpreted (Cieśla and Kowalska, 2013).

There are several book chapters (Botz et al., 2001; Cieśla and Kowalska, 2013; Tyihak et al., 2008; Waksmundzka-Hajnos et al., 2014) and review articles (Cheng and Wu, 2013; Choma and Grzelak, 2011; Cieśla, 2012; Marston, 2011; Tyihak et al., 2012) that summarize the use of TLC-bioautography assays. This review presents an up-date as well as most recent applications of all known TLC-bioautography tests used to screen plant extracts for the presence of potential drugs.

## 2. Antioxidant assays

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify (reduce by appropriate enzymes) the reactive byproducts. Loss of balance in the normal red-ox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, especially proteins, lipids, and DNA. However, some reactive oxygen species act also as cellular messengers in red-ox signaling. ROS are used by the immune system as a way to eliminate pathogens (Valko et al., 2007).

According to several hypotheses, oxidative stress is considered to be responsible for development of numerous human diseases such as: cancer, neurodegenerative processes, atherosclerosis, heart failure, myocardial infarction and still others (Valko et al., 2007).

Consumption of foods rich in chain-breaking antioxidants has been found supportive for the antioxidant enzymes effectively defending cells from free radicals. A lot of papers have been published reporting on the use of different analytical techniques to search for natural antioxidants (Cieśla and Kowalska, 2013). One of the simplest and most commonly applied approaches is the application of spectrophotometric measurements with the use of relatively stable free radicals. All the techniques utilizing relatively stable free radicals are usually included into the group of methods with biological detection. In case of antioxidant assays this classification is based on the biological effect free radicals have on living organisms not on the character of derivatizing agents.

Spectrophotometric measurements have one serious drawback as far as drug discovery is considered: the observed activity is a response of all metabolites present in the extract. Therefore, separation based techniques (HPLC and TLC) followed by biodetection are recommended for fishing for new antioxidants. In case of HPLC the eluate is derivatized post-column with relatively stable nitrogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH*) or 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) $ABTS^{*+}$ (Bandoniene and Murkovic, 2002). Apart from the use of relatively stable free radicals, electrochemical detectors are applied for the analysis of antioxidants in various matrices, by means of HPLC. These methods are based on oxidation of antioxidants on the inert electrodes. The produced current is directly related to the concentration of compounds of interest (Sochor et al., 2013).

TLC coupled with biodetection has been found to be especially useful in detection and identification of natural antioxidants. In this approach components of natural mixtures are first separated in the adsorbent bed of a TLC plate and subsequently dipped or sprayed with DPPH* or $ABTS^{*+}$ solutions. Another step, which may lead to identification of biologically active metabolites is coupling TLC with MS detection, using for example a special interface enabling transfer of separated compounds directly from the adsorbent layer to a mass spectrometer (Cheng et al., 2011).

### 2.1. TLC-DPPH* test

DPPH* was proposed for chemical detection of antioxidants by Blois (1958), who discovered that the relatively stable radical reacts quantitatively with for example cystein, ascorbic acid or tocopherol. Due to delocalization of its odd electron over the molecule, DPPH* does not dimerize and possesses absorption maximum at 517 nm (Molyneux, 2004). Upon reduction the characteristic violet color of DPPH* solution changes into yellow caused by the presence of picric acid group (Fig. 1).

TLC coupled with DPPH* staining was first proposed by Glavind and Holmer (1967) who investigated free radical scavenging



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