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Review

Bioautography detection in thin-layer chromatography

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ABSTRACT

Bioautography is a microbial detection method hyphenated with planar chromatography techniques. It is based mainly on antimicrobial or antifungal properties of analyzed substances. The review discusses three versions of bioautography, i.e. contact, immersion and direct bioautography. The more concern is given to the last one. Many applications are quoted, not only for testing various groups of compounds, but also for investigating biochemical processes and factors influencing bacterial growth. Additionally, related methods, which can be included into direct bioautography, are discussed. The most promising among them seems to be TLC-bioluminescence screening.

Contents

1. Introduction

Bioautography belongs to microbiological screening methods commonly used for the detection of antimicrobial activity (Fig. 1). The screening can be defined as the first procedure, which is applied to an analyzed sample, in order to establish the presence or absence of given analytes [1]. Basically speaking, it is a simple measurement providing a "yes/no" response [2]. Quite often, screening methods give higher sensitivity than any other methods. Moreover, they are simple, cheap, time-saving and do not require sophisticated equipment. Bioautography screening methods are based on the biological activities, e.g. antibacterial, antifungal, antitumour, and antiprotozoae of the tested substances [3]. This detection method can be successfully combined with layer liquid chromatography techniques, such as thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), overpressured-layer chromatography (OPLC) and planar electrochromatography (PEC). In this review, the name TLC-bioautography is used mostly in its wide-ranging meaning concerning any planar technique linked to bioautography. In so-called direct bioautography, i.e. bioautography hyphenated directly with thin-layer chromatography (TLC-DB), both separation and microbial detection are performed on the same TLC plate. Generally, the method measures antibacterial properties of analyzed substances, i.e. changes in bacterial growth. However, other mechanisms of action can be considered, e.g. disturbing vital cell processes as it takes place when bioautography is performed using luminescent bacteria, in so-called TLC-bioluminescence method [4,5]. Both TLC-DB and TLC-bioluminescence enable searching for biological active substances in complicated mixtures and matrices, and can be included into effect-directed analysis (EDA), a new approach in environmental and hazard management based on biological response [6,7].

2. Microbiological screening methods

2.1. Diffusion methods

Diffusion methods are frequently used in testing antimicrobial susceptibility of pure substances, preferably polar than non-polar

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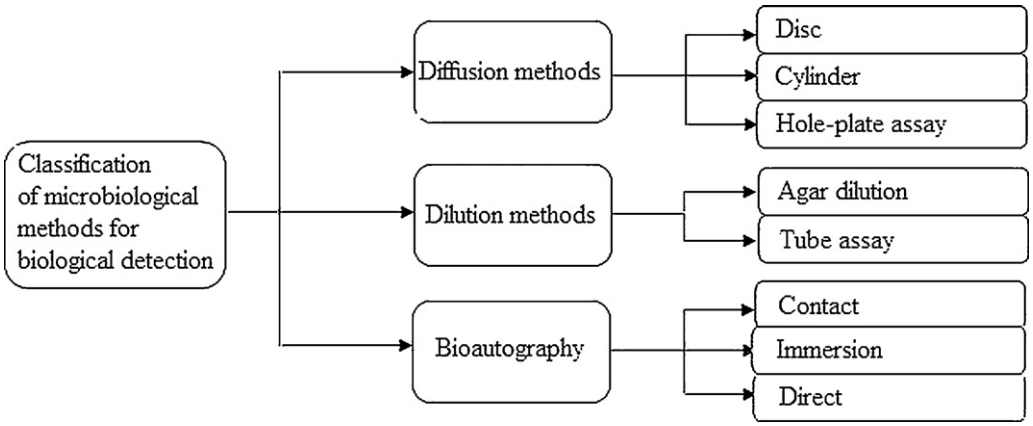

Fig. 1. The classification of microbiological methods for biological detection.

ones [8–10]. The disc method is the official one for quantitative detection of inhibitory substances in milk in the USA [11,12]. In this procedure, filter paper discs (about 6 mm diameter), containing the test compound, are placed on the agar surface previously inoculated with the test microorganisms (dipping a filter paper into a test compound solution should be avoided – it is advised to spot the substance on the disc surface). The antimicrobial agent diffuses into the agar and inhibits germination and growth of the tested microorganism. The Petri dishes are incubated and the zones of inhibition growth are measured. The similar procedure is carried out in E-test, where stripes are used instead of discs [13,14]. In the cylinder method, stainless steel or porcelain cylinders of uniform size (usually 8 mm × 6 mm × 10 mm) are placed on the inoculated agar surface of a Petri dish, and filled with samples and standards. After incubation, the cylinders are removed and the inhibition zones are measured. The cylinder method is the official one for quantitative detection of β-lactam residues [12,15,16]. In the hole-plate assay, a few millimeter diameter holes are cut in the inoculated agar surface and filled with the samples. The tested compound solution diffuses into agar medium causing growth inhibition of the microorganisms. The Petri dishes are left at room temperature, prior to incubation. Then, the zones of growth inhibition are measured [17]. The minimum inhibitory concentration (MIC) is determined visually, as the lowest test compound concentration, which causes recognizable zones of inhibition growth. However, diffusion methods are less suitable to determine the MIC values than dilution ones, because it is impossible to measure the amount of the test compound diffused into the agar medium (Fig. 2).

2.2. Dilution methods

The main advantage of dilution methods is possibility to estimate the concentration of the test compound in the agar medium or in the broth suspension; for this reason, they are commonly used for determination of MIC values [18]. The application range includes complex extracts, pure substances, and both polar and non polar samples. In the agar dilution procedure, various concentrations of the tested compound are mixed with a nutrient agar. The agar plates are inoculated and then incubated. The lowest concentration of the antimicrobial substance, at which no microorganism growth is detected, gives the MIC value. In the tube assay, various concentrations of the tested compound are mixed with bacterial suspension in series of tubes – the lowest concentration causing inhibition in microorganism growth corresponds to the MIC value. In the broth micro-dilution assay, the microorganisms are grown in the plate wells, to which various concentrations of the tested compound are added. The growth of the microorganisms is indicated by the presence of turbidity in the wells [19].

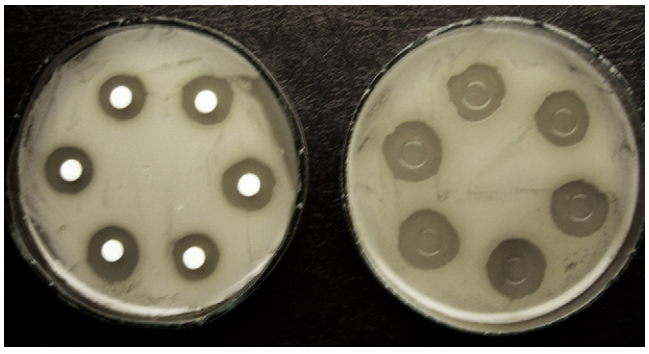

Fig. 2. Diffusion bioassays for flumequine standard solutions: agar disc (on the left) and agar cylinder (on the right) method. Test bacteria: *Bacillus subtilis*.

2.3. Bioautography

The procedure in bioautographic methods is similar to the one used in agar diffusion methods. The difference is that the tested compounds diffuse to inoculated agar medium from the chromatographic layer, which is adsorbent or paper [20,21]. In the contact bioautography, the TLC plate or paper chromatograms are placed on the inoculated agar surface for some minutes or hours to allow diffusion. Next, the plate is removed and the agar layer is incubated. The zones of inhibition growth appear in the places, where the antimicrobial compounds were in contact with the agar layer. In the immersion (agar-overlay) bioautography, the plate is first immersed in or cover with agar medium, which after solidification is seeded with the tested microorganisms and then incubated [22–24]. In order to enable better diffusion of the tested compound into the agar surface, the plates can stay at low temperature for a few hours before incubation. This method is a combination of contact and direct bioautography, because the antimicrobial compounds are transferred from the chromatogram to the agar medium, as in a contact method, but the agar layer remains onto the chromatogram surface during the incubation and visualization, as in direct bioautography.

Among the all bioautographic methods, the most widely applied is direct bioautography [3,25,26]. The principle of this method is that a developed TLC plate is dipped in a suspension of microorganisms growing in a proper broth and then incubated in a humid atmosphere. A silica surface of the TLC plate covered with the broth medium becomes a source of nutrients and enables growth of the microorganisms directly on it. However, in the places where antimicrobial agents were spotted, the inhibition zones of the microorganism growth are formed. Visualization of these zones is usually carried out using dehydrogenase activity-detecting

reagents; the most common are tetrazolium salts. The dehydrogenase of living microorganisms converts tetrazolium salt into intensely colored formazan. As a result, cream-white spots appear against a purple background on the TLC plate surface, pointing the presence of antibacterial agents.

3. Thin-layer chromatography-direct bioautography

The beginnings of coupling microbiological assay with planar chromatography date back to 1946, when Goodall and Levi [27] combined paper chromatography method (PC) with contact bioautography detection for the determination of the different penicillins. Fifteen years later, Fisher and Lautner [28], and Nicolaus et al. [29] introduced thin-layer chromatography (TLC) in the same field. The methods were described as simple, reproducible and highly sensitive. The first review of the application of bioautography in paper and thin-layer chromatography was presented in 1973 by Betina [25]. The author not only emphasized the advantages of the method, such as rapidity and versatility, but also pointed out the difficulties of quantitative interpretation of the obtained results. The influence of various factors, such as tested microorganisms, medium composition, pH, and solubility of the sample in the culture on the bioautographic detection was widely discussed by Rios et al. in a summary on screening methods for testing antimicrobial activity in natural products [30]. The authors concluded, that it is highly difficult to standardize these methods because of their diversity. Henceforth, other studies were done to estimate standardized parameters, which can influence the bioautographic detection. The broad review on various factors influencing bacterial growth, such as mobile phase and their additives, type of adsorbent, test microorganism, preconditioning of TLC plates, living conditions for test bacteria and post-chromatographic detection, as well as on bioautography methods was done by Botz et al. [26]. Many examples of various applications of TLC-bioautography can be found in the review article by Choma [3]. Morlock and Schwack, in the review about hyphenations in planar chromatography, give many examples on bioassays used for (HP)TLC-EDA [7]. They point to papers on bacterial assays with *Vibrio fischeri*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas savastanoi* [31–34] as well as to papers on bioautographic fungi assays [35–37]. The authors state, that the effect-directed analyses (EDAs), such as bioautographic assays, are especially suitable for selective detection while combining with chromatography.

3.1. New possibilities in TLC-DB

Tyihák et al. introduced a complex separation and detection system, called BioArena, which combines the advantages of overpressured layer chromatography (OPLC) or thin-layer chromatography (TLC) with those of bioautography [38]. Necrotrophic and/or biotrophic inoculum suspensions are used. According to the authors, BioArena is quite simple, inexpensive and reliable method. The big advantage of this system is also the possibility to modify the incubation time, e.g. shorter incubation time (1–2 h) allows usage of more sensitive biotrophic fungi spores in the special culture medium for bioautographic detection. BioArena can be used for investigating biochemical interactions between microorganisms and biologically active compounds (e.g. antibiotics, mycotoxins, and trans-resveratrol) in the adsorbent layer after chromatographic separation and for studying cell proliferation promoting and retarding processes. Especially, model reactions of endogenous formaldehyde (HCHO), a key molecule in biological systems particularly in cell proliferation, as well as of other small endogenous molecules (e.g. H_2O_2 and O_3) are given. BioArena enables visualization of the effect of HCHO-capturing species (e.g. L-arginine and glutathione) and its promoters (Cu^{2+} ions) [34,39–51] even for a week or more (Fig. 3). The attempt was also done to find relationship between chemical structure, hydrophobicity, biological activity of pesticides and HCHO and its products action [43]. The inhibition zones can be examined quantitatively by use of in situ densitometry to obtain calibration curve. BioArena enables also hyphenation with other spectroscopic method as MS, (FT)IR, FT-Raman or NMR [34].

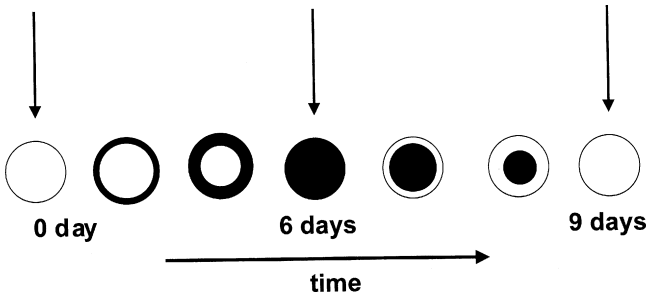

Fig. 3. Time-dependent change of inhibition zones of *trans*-resveratrol in BioArena after inoculation (with *Pseudomonas savastanoi* pv. *phaseolicola*), incubation and staining – schematic drawing.
Permission from Ref. [39].

About ten years ago, Merck developed the direct bioautography test called Chrom Biodip® antibiotics, for the detection of antibiotics separated by thin-layer chromatography. The test kit comprised *B. subtilis* spore suspension, nutrient medium and MTT detection reagent. The method involved the separation of substance mixtures on HPTLC silica gel 60 plates and subsequent visualization of antibiotic inhibitors by Chrom Biodip. Eymann and Hauck have summarized the possible applications of the test, e.g. for searching of new antibiotics, testing antibiotics in pharmaceutical preparations, control of food and feed, as well as detection of antibiotics in waste water [52]. Botz et al. stated that the incubation time of *B. subtilis*, proposed in instructions (4 h at 25 °C ± 3 °C or 2 h at 35 °C) is not sufficient to reach the log phase of the microorganisms, which resulted in less colored TLC plates after MTT detection [26]. After increasing the time of incubation from 2 to 4 h at 35 °C, the bacterial layer at TLC plates was more homogeneous and deeply colored giving sharp contrast between the inhibition zones and the background.

The same and other shortcomings of the test were observed, independently, in our research group. We proposed increasing the incubation time (from 2 h at 35 °C to 4 h at 37 °C), as well as careful evaporation of the developing solvent by putting the plates, after TLC separation, to vacuum dessicator, for instance [53]. Although the Chrom Biodip is commercially unavailable now, many studies upon the residue determination of antibiotics using this test were published [33,53–58]. A semi-quantitative TLC-DB method for enrofloxacin and ciprofloxacin standards was established [53]. The obtained limit of detection equaled 0.01 ppm for both antibiotics, when 50 μl of antibiotic standard solutions was applied onto the TLC plate. This value was lower, than MRL values of these antibiotics established by the European Union for various species and matrices. It was shown that the size of inhibition zones depends on the applied volume of the antibiotic solution (the larger the volume applied, the larger the area obtained for the same amount of antibiotic in the spot). It was also proved that the relationship between the area of inhibition zone versus the logarithm of antimicrobial compound concentration is linear only for a narrow range of concentrations (one or two orders of magnitude), despite "the official bioautographic theory" [25,26,59,60]. For a wider range of concentrations, e.g. four orders of magnitude, exponential dependence fits better (Fig. 4).

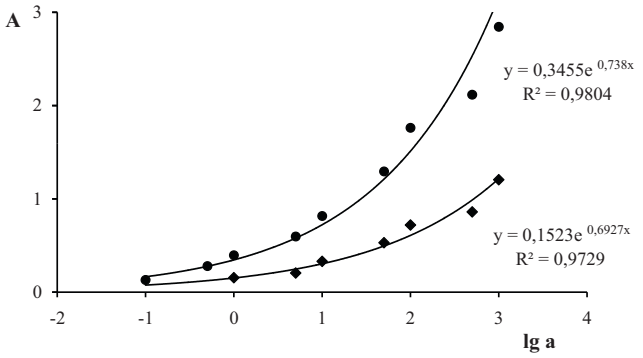

Fig. 4. Mean areas of inhibition zones (cm^2) plotted against logarithm of amounts of antibiotic standards (ng) applied in 10 μl volume. Rhombus for enrofloxacin, circle for ciprofloxacin. The plots are established on the basis of four bioautograms.

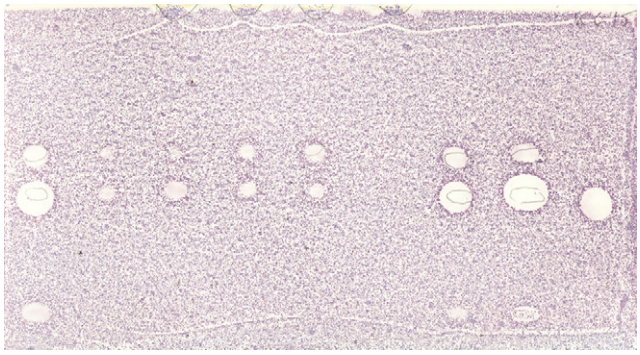

Fig. 5. TLC-DB of the eluates obtained from milk spiked at 0.05 ppm level. The amounts of ciprofloxacin and enrofloxacin in the eluates were 0.5 ppm each. Upper spots, enrofloxacin; lower spots, ciprofloxacin. From left to right: 1 ppm standard, 0.5 ppm standard, three eluates from three cartridges, blank, 1 ppm standard, two standards at 1 ppm (HPLC mobile phase and water instead of methanol). The volume spotted was 50 μl. Test bacteria: *Bacillus subtilis*.
Permission from Ref. [54].

The application of TLC-DB method for the screening of enrofloxacin and ciprofloxacin [54], flumequine [55] and cefacetrile [33] residues in milk was presented. The obtained LOD values were lower than established MRL values for the tested compounds. It was a clear proof that the TLC-DB method is applicable for the screening of food samples containing residues at their MRL level (Fig. 5). In the present, two new bioautographic tests, based on *B. subtilis* and on *E. coli* were prepared in our research group.

The advantages of bioautographic methods (including TLC-DB) for the rapid chemical and biological screening of plant extracts were described by Hostettmann et al. Once an activity has been located at the TLC plate, the sample can be analyzed by LC–MS to establish, whether known or new compounds and/or substance classes are involved (Fig. 6). Hostettmann's screening strategies concern detection of antibacterial and antifungal compounds [36,61–68] as well as radical scavengers and antioxidants [61,62,69].

The studies of antimicrobial activity of essential oils can be found in various works of Horváth et al. [70,71]. The authors investigated the phytochemical characteristics of essential oils, i.e. thyme, lavender, eucalyptus, spearmint and cinnamon, against *Xanthomonas*, *Pseudomonas* and *Staphylococcus* strains [72]. Among all the tested bacteria, *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *phaseolicola* seemed to be the most sensitive.

The quoted above and many other examples of TLC-bioautography (including TLC, HPTLC and OPLC analysis) are collected in Table 1.

3.2. Thin-layer chromatography-bioluminescence and other applications

TLC-bioluminescence can be considered as a variant of direct bioautography, although it is not based on the changes in bacterial growth but on the quenching bioluminescence of bacteria like *Photobacterium phosphoreum* or *V. fischeri* [3–7,31,102–104]. Besides naturally occurring bacteria, some genetically modified bacteria as *Acinetobacter* with incorporated bioluminescence gene can be applied [5,81,105]. The principle of the method is very similar to that of direct bioautography: the developed and dried

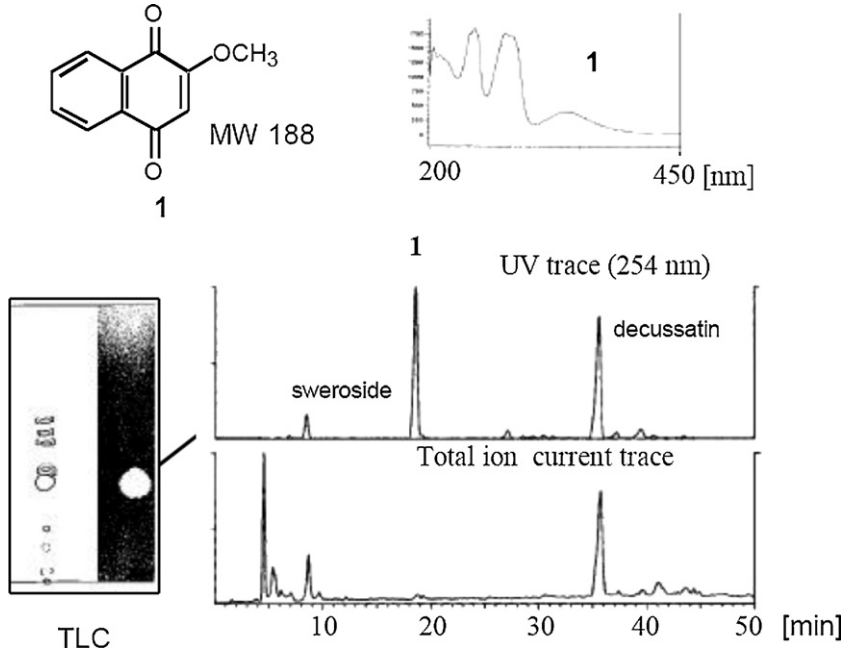

Fig. 6. TLC bioautography (*C. cucumerinum*) and HPLC–UV-MS analysis of *Swertia calycina* (*Gentianaceae*).
Permission from Ref. [61].

Table 1
TLC-direct bioautography and TLC-bioluminescence: examples of analysis.

Biological activity	Substance	Bacteria/fungi	Planar chromatography technique	References
Antibacterial	Cefacetrile	*Bacillus subtilis*	TLC	[33]
	Ciprofloxacin, enrofloxacin	*Bacillus subtilis*	TLC, HPTLC	[53]
	Ciprofloxacin, enrofloxacin	*Bacillus subtilis*	TLC	[54]
	Ciprofloxacin, enrofloxacin	*Bacillus subtilis*	TLC	[56]
	Flumequine	*Bacillus subtilis*	TLC	[55]
	Flumequine	*Bacillus subtilis*	TLC, HPTLC	[58]
	Flumequine, doxycycline	*Bacillus subtilis*	TLC	[57]
	Sulfonamides (sulfaguanidine, sulfapyridine, sulfathiazole, sulfadiazine, sulfamethoxazole) and amphotericin	*Serracia marcescens*, *Bacillus subtilis*	TLC	[73]
	Vitamin B_{12} from the short-necked clam (*Ruditapes philippinarum*) extract	*Escherichia coli*	TLC	[74]
	Trans-resveratrol	*Pseudomonas savastanol* pv. *phaseolicola*	OPLC	[39]
	Trans-resveratrol	*Bacillus subtilis, Pseudomonas savastanol* pv. *phaseolicola*	OPLC, TLC	[40]
	Trans-resveratrol, red and white wines extract	*Pseudomonas savastanoi* pv. *phaseolicola*	OPLC	[45]
	Trans-resveratrol, Pinot noir red wine extract	*Pseudomonas savastanoi* pv. *phaseolicola*	OPLC	[75]
	Aflatoxins B_1	*Pseudomonas savastanoi* pv. *phaseolicola*	TLC	[46]
	Aflatoxins B_1, B_2, G_1, G_2	*Pseudomonas savastanoi* pv. *phaseolicola*	OPLC	[42]
	Aflatoxins B_1, B_2, G_1, G_2	*Pseudomonas savastanoi* pv. *phaseolicola*	OPLC	[76]
	Aflatoxins B_1, B_2, G_1, G_2	*Pseudomonas savastanoi* pv. *phaseolicola*	OPLC	[77]
	Chelidonium majus L. Alkaloids	*Pseudomonas savastanoi* pv. *phaseolicola*	TLC	[41]
	Hypericum brasiliense polyphenols	*Bacillus subtilis*	TLC	[66]
	Thymus phenols	*Erwinia amylovora*, *Erwinia carotovora subsp. carotovora*, *Erwinia carotovora subsp. atroseptica*	TLC	[71]
	Essential oils from: *Thymus vulgaris* L., *Thymus serpyllum* L., *Thymus x citriodorus* (Pers.) Schreb., and *Thymus x citriodorus* "Archer's Gold"	*Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae* pv. *phaseolicola*	TLC	[70]
	Essential oils of thyme (*Thymus vulgaris* L.), lavender (*Lavandulaangustifolia* Mill.), eucalyptus (*Eucalytus globulus* Labill.), spearmint (*Mentha spicata* L.) and cinnamon (*Cinnamomum zeylanicum* Presl.)	*Pseudomonas syringae* pv. *phaseolicola* (Burkholder),*Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye, *Staphylococcus epidermidis*, *S. saprophyticus*, *S. aureus*	TLC	[72]
	Essential oils from: leaves of *Piper cernuum*, *P. diospyrifolium*, *P. crassinervium*, *P. solmsianum*, *P. umbelata* and fruits of *P. cernuum* and *P. diospyrifolium*	*C. sphaerospermum* (Penzig), *C. cladosporioides* (Fresen) de Vries	TLC	[78]
	Artemisia annua, *Artemisia dracunculus*, *Eucalyptus globulus Humulus lupus*, *Mentha longifolia* essential oils	*Enterobacter cloacae*, *Humulus lupulus*	TLC	[79]
	Origanum onites essential oils	*Bacillus subtilis*	OPLC	[80]
	Essential oils from: *Tanacetum argenteum* (*Lam.*) *Willd.* ssp. *argenteum*, *T. densum* (Lab.) *Schultz Bip.* ssp. *amani Heywood*	*Bacillus subtilis*, *B. cereus*, *Vibrio fischeri*	TLC	[81]
	Essential oils from: *Tanacetum argyrophyllum* (*C. Koch*) *Tvzel* var. *argyrophyllum*	*Bacillus subtilis, B. cereus, Vibrio fischeri*	TLC	[82]
	Essential oils from: *Tanacetum parthenium* (L.) *Schultz Bip.*	*Bacillus subtilis*, *Staphylococcus aureus*, *Vibrio fischeri*	TLC	[83]
	Matricaria recutita extracts	*Pseudomonas syringae* pv. *maculicola*	TLC	[84]
	Cordia gilletii De Wild (*Boraginaceae*) root	*Staphylococcus aureus*, *Vibrio fischeri*	TLC	[85]
	Rhizome/root and leaves from: *Actaea racemosa* L. (Ranunculaceae), *A. pachypoda* Ell. (*Ranunculaceae*) and *A. podocarpa* DC. (*Ranunculaceae*)	*Vibrio fischeri*	HPTLC	[86]
	Shiitake mushroom (*Lentinus edodes*)	*Micrococcus luteus*	TLC	[87]
	Pesticides	*Pseudomonas savastonoi* pv. *phaseolicola*	TLC	[43]
	Cholinesterase	*Vibrio fischeri*	HPTLC	[4]
	Human urine; garlic and curry extracts	*Vibrio fischeri*	TLC	[5]
	Sunscreens in cosmetic products	*Vibrio fischeri*	HPTLC	[88]
	Water	*Vibrio fischeri*	HPTLC	[89]
	Water	*Vibrio fischeri*	HPTLC	[90]
	Sewage effluent, surface water, drinking water	*Vibrio fischeri*	HPTLC	[91]
	Industrial wastewater	*Vibrio fischeri*	TLC	[92]
	Marine sponge (*Hymeniacidon perleve*)	*Staphylococcus aureus*	TLC	[23]
	Marine sponge (*Dysidea avara*)	*Vibrio fischeri*	HPTLC	[93]
Antibacterial and antifungal	Extracts from: *Rhododendron* (*Ericaceae*), *Leonurus* (*Lamiaceae*), *Phlomis* (*Lamiaceae*), *Morina* (*Morinaceae*), *Asperula* (*Rubiaceae*), *Putoria* (*Rubiaceae*), *Wendlandia* (*Rubiaceae*), *Scrophularia* (*Scrophulariaceae*), *Urtica* (*Urticaceae*)	Bacteria: *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*; and yeast: *Candida albicans*	TLC	[22]

Table 1 (*Continued*)

Biological activity	Substance	Bacteria/fungi	Planar chromatography technique	References
Antifungal	Iprodione (Rovral®), Mepronil (Basak®), *Glycosmis pentaphylla* root	*Valsa ceratosperma*	TLC	[63]
	Brazilian medicinal and fruit bearing plants	*Candida albicans*, *Cryptococcus neoformans*	TLC	[24]
	Tropical plants	*Candida albicans*, *Cladosporium cucumerinum*	TLC	[36]
	Amphotericin B	*Candida albicans*, *Saccharomyces cerevisiae*	TLC	[94]
	Angelica sinensis (*Dong Quai*)	*Colletotrichum* spiecies	TLC, OPLC	[95]
	Garcinia atroviridis fruits (acid esters)	*Cladosporium herbarum*	TLC	[96]
	Gladiolus dalenii van Geel (*Iridaceae*) bulb extracts	*Aspergillus niger*	TLC	[97]
	Erythrina vogelii root	*Cladosporium cucumerinum*	TLC	[98]
	Essential oils from: *Tibetan Junipers Juniperus saltuaria*, *J. squamata var. fargesii* (*Cuperssaceae*)	*Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*	TLC	[99]
	Scaligeria tripartita essential oils	*Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*	OPLC	[100]
	Thuja orientalis L. essential oils	*Alternaria alternata*	TLC	[101]

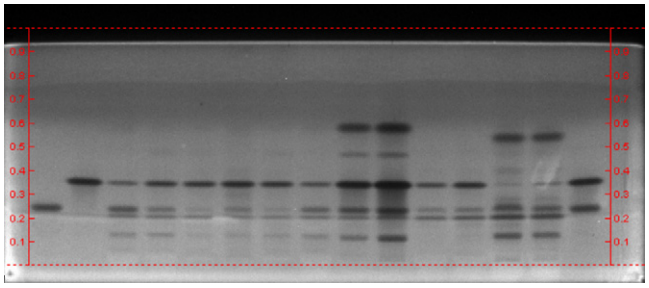

Fig. 7. Berberine alkaloids by the Bioluminex assay. Permission from Ref. [111].

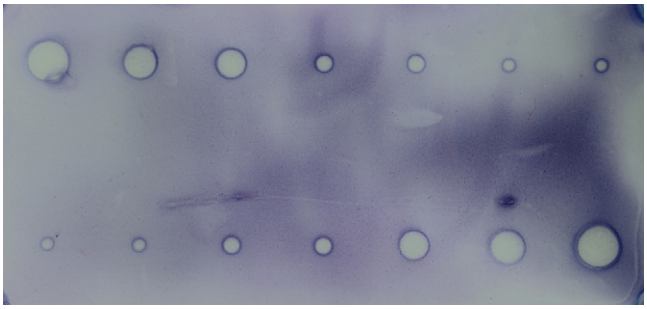

Fig. 8. TLC-DB of flumequine standards, applied at 10 μl volume on $Si60F_{254}$ plate. The amounts of flumequine per spot are as follows: first track: 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 (μg); second track: 0.005, 0.01, 0.05, 0.1, 0.5, 1.0 (μg), respectively. Test bacteria: *Escherichia coli*.

(HP)TLC plate is immersed in suspension of luminescent bacteria and after a short incubation, bioluminescence is measured using CCD camera or luminograph video imaging system [5]. Toxic substances, like pesticides, antibiotics, aflatoxins, etc., disturb vital cell processes of bacteria, giving dark spots on luminescent background of the plate. Detection limits for these compounds are in the picogram range. There are commercially available tests for bioluminescence produced by ChromaDex and Camag [106,107]. The method is relatively new (it has been used for the about 15 years). However, there is an increasing interest in this type of assay, especially for analysis of toxins, plant and sponges extracts, as well as in purity control of pharmaceuticals, chemicals and water [81–83,86,89,90,92,108–111] (Fig. 7).

Thin-layer chromatography is frequently hyphenated with other bioassays, which are generally based on the inhibition/stimulation of growth or activity of test organism, which can be, besides bacteria, yeast cells, mold spores, cell organelles (e.g. chloroplasts) or enzymes [112,113]. Enzyme inhibition test seems to be the most common among the above mentioned methods and allows detection and quantitative analysis of toxic substances in water, soil, air and food samples. The (HP)TLC plate is sprayed or dipped sequentially with enzyme solution and substrate (sometimes also dye) to give spots different in color from the background. The most popular enzymatic assay is acetylcholinesterase inhibition test, usually based on the Ellman reaction [114,115]. Other enzymatic tests use glucosidase or xanthine oxidase inhibition [116–118]. Estrogenic activity can be evaluated by the YES assay [119]. In the presence of estrogenic substances, yeast cells, growing directly on the (HP)TLC plate, produce β-galactosidase enzyme. Following incubation, estrogenic substances are detected by spraying with a chromegenic or fluorogenic substrate. Antioxidant and radical scavenging activities can be tested using β-carotene, DPPH (2,2-diphenyl-1-picrylhydrazyl) or ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) reagents [61,62,81–83,120,121]. When the (HP)TLC plate is sprayed with β-carotene solution, orange zones on a cream-white background indicate the presence of antioxidants. In case of DPPH, yellow spots on a purple background are observed in places of radical scavengers. The DPPH reagent can be replaced by ABTS, which gives pink or colorless spots on a green background. Similar to conventional bioautography, both TLC-bioluminescence and the methods described above can be coupled with MS, IR or NMR techniques to obtain full information about the structure and biological activity of the compounds under investigation [61,62,81–83,92,93,102,105,122].

Finally, it is worthwhile to mention about papers concerning optimization of bacteria growth conditions. Apart from the previously cited papers by Botz, Betina, Rios or Eymann [25,26,30,52], there are interesting works by Nagy and co-workers related to the problem. They studied optimal life conditions for Gram-positive bacteria, *B. subtilis*, used in bioautographic TLC detection [123]. The authors found that the plate incubation time, previously reported as 18 h [52], was too long, and proposed 4.8 h at 37 °C as a more appropriate value. The similar studies were done for Gram-negative bacteria, *E. coli* [124], and after optimization process the incubation time was shortened to 3 h, instead of recommended overnight incubation [125]. The optimization procedure was also done for fungus, *Candida albicans* [35]. The viability of the tested microorganisms, i.e. bacteria or fungus, on the TLC plates was measured using bioluminescent ATP assay. As it was mentioned earlier we optimized and prepared two bioautographic tests based on *B. subtilis* and *E. coli*. The sensitivity of the method seems to be pretty good, however the data are not published yet (Fig. 8).

4. Conclusions

Thin-layer chromatography-bioautography enjoys its great come-back now. The success of TLC with microbial detection is connected mainly with growing interest in an effect-directed analysis. The presence of increasing number of the known and unknown pollutants in the environment imposes the necessity for searching potentially harmful substances in various matrices. TLC-bioautography, including TLC-bioluminescence, accomplishes this task successfully. The bioassay, together with spectroscopic methods, provides full information about both bioactivity and the structure of the analytes.

References

[1] M.M.L. Aerts, A.C. Hogenboom, U.A.Th. Brinkman, J. Chromatogr. B 667 (1995) 1.
[2] R. Muñoz-Olivas, Trends Anal. Chem. 23 (2004) 203.
[3] I.M. Choma, LC–GC 18 (2005) 482.
[4] Ch. Weins, H. Jork, J. Chromatogr. A 750 (1996) 403.
[5] G. Eberz, H.G. Rast, K. Burger, W. Kreiss, C. Weisemann, Chromatography 43 (1996) 5.
[6] W. Brack, Anal. Bioanal. Chem. 377 (2003) 397.
[7] G. Morlock, W. Schwack, J. Chromatogr. A 1217 (2010) 6600.
[8] Ch.D. Steward, S.A. Stocker, J.M. Swenson, C.M. O'Hara, J.R. Edwards, R.P. Gaynes, J.E. McGowan Jr., F.C. Tenover, J. Clin. Microbiol. 37 (1999) 544.
[9] J.L. Burns, L. Saiman, S. Whittier, D. Larone, J. Krzewinski, Z. Liu, S.A. Marshall, R.N. Jones, J. Clin. Microbiol. 38 (2000) 1818.
[10] M. Janssen, J.C. Scheffer, A.B. Svendsen, Planta Med. 53 (1987) 395.
[11] Association of Official Analytical Chemists, J. Assoc. Off. Anal. Chem. 65 (1982) 466.
[12] L.J. Maturin, FDA Bacteriological Analytical Manual, Revision A, 8th ed., 1998 (Chapter 20A).
[13] L. Kelly, M.R. Jacobs, P.C. Appelbaum, J. Clin. Microbiol. 37 (1999) 3296.
[14] T.A. Davies, L.M. Kelly, M.R. Jacobs, P.C. Appelbaum, J. Clin. Microbiol. 38 (2000) 1444.
[15] Code of Federal Regulations, Title 21, Sec. 436.105, U.S. Government Printing Office, Washington, DC, 1976.
[16] Association of Official Analytical Chemists, Official Methods of Analysis, 14th ed., secs 16.163, AOAC, Arlington, VA, 1984, pp. 42.299.
[17] A. Shitandi, K. Gathoni, Food Control 16 (2005) 227.
[18] J.D. Paxton, in: K. Hostettmann (Ed.), Methods in Plant Biochemistry – Assays for Bioactivity, vol. 6, Academic Press, London, 1991, p. 33.
[19] L. Otvos, M. Cudic, in: G.B. Fields (Ed.), Peptide Characterization and Application Protocols (Methods in Molecular Biology), Part II, vol. 386, Humana Press, Totowa, NJ, 2007, p. 309 (Chapter 12).
[20] E. Meyers, D. Smith, J. Chromatogr. 14 (1964) 129.
[21] G.H. Wagman, J.V. Bailey, J. Chromatogr. 41 (1969) 263.
[22] D. Tasdemir, A.A. Donmez, I. Calis, P. Ruedi, Pharm. Biol. 42 (2004) 374.
[23] L. Zheng, H. Chen, X. Han, W. Lin, X. Yan, World J. Microbiol. Biotechnol. 21 (2005) 201.
[24] G. Schmourlo, R.R. Mendonca-Filho, C.S. Alviano, S.S. Costa, J. Ethnopharmacol. 96 (2005) 563.
[25] V. Betina, J. Chromatogr. 78 (1973) 41.
[26] L. Botz, S. Nagy, B. Kocsis, in: Sz. Nyiredy (Ed.), Planar Chromatography, Springer, Budapest, 2001, p. 489.
[27] R.R. Goodall, A.A. Levi, Nature 158 (1946) 675.
[28] R. Fischer, H. Lautner, Arch. Pharm. 294 (1961) 1.
[29] B.J.R. Nicolaus, C. Coronelli, A. Binaghi, Farmaco Ed. Prat. 16 (1961) 349.
[30] J.L. Rios, M.C. Recio, A. Villar, J. Ethnopharmacol. 23 (1988) 127.
[31] J. Sherma, Studia Universitatis Babeş-Bolyai, Chemia 2 (2009) 5.
[32] M. Nishioka, F. Kanosue, E. Miyamoto, Y. Yabuta, F. Watanabe, J. Liq. Chromatogr. Relat. Technol. 32 (2009) 1175.
[33] I.M. Choma, C. Kowalski, R. Lodkowski, A. Burmańczuk, I. Komaniecka, J. Liq. Chromatogr. Relat. Technol. 31 (2008) 1903.
[34] Á.M. Móricz, N. Adanyi, E. Horváth, P.G. Ott, E. Tyihák, J. Planar Chromatogr. 21 (2008) 417.
[35] S. Nagy, B. Kocsis, T. Koszegi, L. Botz, JPC – J. Planar Chromatogr. – Modern TLC 20 (2007) 385.
[36] K. Hostettmann, O. Potterat, ACS Symp. Ser. 658 (1997) 14.
[37] U. Baumann, C. Brunner, E. Pletscher, N. Tobler, Umweltwiss. Schadst.-Forsch. 15 (2003) 163.
[38] E. Tyihák, L. Botz, S. Nagy, B. Kocsis, E. Mincsovics, in: Sz. Nyiredy (Ed.), Proc. Intern. Symp. on Planar Separations, Planar Chromatography, Res. Inst. Med. Plants, Budakalász, 2001, p. 3.
[39] E. Tyihák, P. Ott, Á.M. Móricz, G. Kátay, Z. Király-Véghely, J. Planar Chromatogr. 17 (2004) 84.
[40] E. Tyihák, L. Botz, P. Ott, S. Nagy, B. Kocsis, Z. Király-Véghely, E. Mincsovics, Chem. Anal. 48 (2003) 543.
[41] Á. Sárkozi, Á.M. Móricz, P.G. Ott, E. Tyihák, Á. Kéry, J. Planar Chromatogr. 19 (2006) 267.
[42] Á. Móricz, K.H. Otta, P. Ott, E. Tyihák, J. Planar Chromatogr. 16 (2003) 417.
[43] M. Janicka, E. Tyihak, A.M. Moricz, B. Ościk-Mendyk, JPC – J. Planar Chromatogr. – Modern TLC 21 (2008) 161.
[44] E. Tyihák, Á.M. Móricz, P.G. Ott, M.L. Hajnos, K. Głowniak, JPC – J. Planar Chromatogr. – Modern TLC 21 (2008) 331.
[45] Z. Király-Véghely, Á.M. Móricz, P.G. Ott, G. Katay, I. Belai, E. Tyihák, J. Liq. Chromatogr. Relat. Technol. 32 (2009) 1259.
[46] Á.M. Móricz, E. Horváth, P.G. Ott, E. Tyihák, J. Raman Spectrosc. 39 (2008) 1332.
[47] G. Katay, P.G. Ott, E. Katay, D. Magyar, E. Tyihák, Biomed. Chromatogr. 23 (2009) 412.
[48] E. Tyihák, Á.M. Móricz, P.G. Ott, JPC – J. Planar Chromatogr. – Modern TLC 21 (2008) 77.
[49] E. Tyihák, E. Mincsovics, G. Katay, Z. Király-Véghely, Á.M. Móricz, P.G. Ott, JPC – J. Planar Chromatogr. – Modern TLC 21 (2008) 15.
[50] E. Tyihák, Á.M. Móricz, P.G. Ott, G. Katay, Z. Király-Véghely, JPC – J. Planar Chromatogr. – Modern TLC 18 (2005) 67.
[51] E. Tyihák, G. Kátay, M.A. Móricz, G.P. Ott, Z. Király-Véghely, Acta Pharm. Hung. 77 (2007) 53.
[52] R. Eymann, H.E. Hauck, in: Sz. Nyiredy (Ed.), Proc. Intern. Symp. On Planar Separations, Planar Chromatography, Res. Inst. Med. Plants, Budakalász, 2000, p. 67.
[53] I.M. Choma, A. Choma, I. Komaniecka, K. Pilorz, K. Staszczuk, J. Liq. Chromatogr. Relat. Technol. 27 (2004) 2071.
[54] I.M. Choma, J. Planar Chromatogr. 19 (2006) 104.
[55] I.M. Choma, J. Liq. Chromatogr. Relat. Technol. 29 (2006) 2083.
[56] I.M. Choma, I. Komaniecka, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 2467.
[57] I.M. Choma, A. Choma, K. Staszczuk, JPC – J. Planar Chromatogr. – Modern TLC 15 (2002) 187.
[58] I.M. Choma, A. Choma, K. Staszczuk, J. Liq. Chromatogr. Relat. Technol. 25 (2003) 1579.
[59] J. Kádár Pauncz, I. Harsányi, J. Chromatogr. 195 (1980) 251.
[60] A. Ramirez, R. Gutierrez, G. Diaz, C. Gonzalez, N. Perez, S. Vega, M. Noa, J. Chromatogr. B 784 (2003) 315.
[61] K. Hostettmann, Ch. Terreaux, A. Marston, O. Potterat, J. Planar Chromatogr. 10 (1997) 251.
[62] K. Hostettmann, IUPAC 1999, Invited lecture at International Conference on Biodiversity and Bioresources – Conservation and Utilization, Phuket, Thailand, 1997, www.iupac.org/symposia/proceedings/phuket97/hostettmann.html.
[63] N. Islam, S.A. Parveen, N. Nakazawa, A. Marston, K. Hostettmann, Pharm. Biol. 41 (2003) 637.
[64] K. Hostettmann, A. Marston, in: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, vol. 17, Elsevier, Amsterdam, 1990, p. 405.
[65] L. Rahalison, M. Hamburger, K. Hostettmann, M. Monod, E. Frenk, Phytochem. Anal. 2 (1991) 199.
[66] L. Rocha, A. Marston, O. Potterat, M.A.C. Kaplan, H. Stoeckli-Evans, K. Hostettmann, Phytochemistry 40 (1995) 1447.
[67] S. Rodriguez, J.L. Wolfender, E. Hakizamungu, K. Hostettmann, Planta Med. 61 (1995) 362.
[68] C. Terreaux, M. Maillard, K. Hostettmann, G. Lodi, E. Hakizamungu, Phytochem. Anal. 5 (1994) 233.
[69] M. Cuendet, K. Hostettmann, O. Potterat, W. Dyatmiko, Helv. Chim. Acta 80 (1997) 1144.
[70] G. Horváth, L.Gy. Szabó, E. Lemberkovics, L. Botz, B. Kocsis, J. Planar Chromatogr. 17 (2004) 300.
[71] G. Horváth, B. Kocsis, L. Botz, J. Németh, L.G. Szabó, 7th Hungarian Congress on Plant Physiology, Acta Biologica Szegediensis, vol. 46, 2002, p. 145.
[72] G. Horváth, N. Jámbor, A. Végh, A. Böszörményi, E. Lemberkovics, É. Héthelyi, K. Kovács, B. Kocsis, Flavour Fragr. J. 25 (2010) 178.
[73] L. Williams, O. Bergersen, J. Planar Chromatogr. 14 (2001) 318.
[74] K. Ueta, M. Nishioka, Y. Yabuta, J. Liq. Chromatogr. Relat. Technol. 33 (2010) 972.
[75] E. Mincsovics, Gy. Kátay, P.G. Ott, Zs. Király-Véghely, Á.M. Móricz, E. Tyihák, Chromatographia (Suppl. 62) (2005) S51.
[76] Á.M. Móricz, P.G. Ott, K.H. Otta, E. Tyihák, in: Sz. Nyiredy (Ed.), Proc. Intern. Symp. On Planar Separations, Planar Chromatography, Res. Inst. Med. Plants, Budakalász, 2003, p. 319.
[77] A. Moricz, K.H. Otta, P. Ott, E. Tyihak, JPC – J. Planar Chromatogr. – Modern TLC 16 (2003) 423.
[78] A. de, A. Morandim, A.R. Pin, N.A.S. Pietro, A.C. Alecio, M.J. Kato, C.M. Young, J.E. de Oliveira, M. Furlan, Afr. J. Biotechnol. 9 (2010) 6135.
[79] A.R. Shahverdi, F. Abdolpour, H.R. Monsef-Esfahani, H. Farsam, J. Chromatogr. B 850 (2007) 528.
[80] E. Mincsovics, N. Tabanca, Á.M. Móricz, D.E. Wedge, E. Tyihák, JPC – J. Planar Chromatogr. – Modern TLC 23 (2010) 225.
[81] K. Polatoglu, F. Demirci, B. Demirci, N. Goren, K. Baser, J. Oleo Sci. 59 (2010) 177.
[82] K. Polatoglu, F. Demirci, B. Demirci, N. Goren, K. Baser, J. Oleo Sci. 59 (2010) 307.
[83] K. Polatoglu, F. Demirci, B. Demirci, N. Goren, K. Baser, J. Oleo Sci. 59 (2010) 361.
[84] Á.M. Móricz, E. Tyihák, P.G. Ott, JPC – J. Planar Chromatogr. – Modern TLC 23 (2010) 180.
[85] P.N. Okusa, C. Stevigny, M. Devleeschouwer, P. Duez, JPC – J. Planar Chromatogr. – Modern TLC 23 (2010) 245.

[86] S. Verbitski, G. Gourdin, L. Ikenouye, J. McChesney, JAOAC Int. 91 (2008) 268.
[87] V. Molnar, F. Billes, E. Tyihák, P.G. Ott, JPC – J. Planar Chromatogr. – Modern TLC 21 (2008) 423.
[88] V. Baumgarten, Diploma thesis, University of Hohenheim, 2007.
[89] W. Schultz, W. Seitz, S. Weiss, W. Weber, M. Bohm, D. Flottmann, JPC – J. Planar Chromatogr. – Modern TLC 21 (2008) 427.
[90] W. Weber, W. Seitz, A. Aichinger, R. Albert, Camag Bibliography Service 95 (2005) 2.
[91] G. Morlock, L. Schuele, S. Grashorn, J. Chromatogr. A 1218 (2011) 2745.
[92] T. Reemstma, A. Putschew, M. Jekel, Waste Manage. 19 (1999) 181.
[93] A. Kloppel, W. Grasse, F. Breummer, G. Morlock, JPC – J. Planar Chromatogr. – Modern TLC 21 (2008) 431.
[94] A. Fittler, B. Kocsis, Z. Matus, L. Botz, JPC – J. Planar Chromatogr. – Modern TLC 23 (2010) 18.
[95] N. Tabanca, D.E. Wedge, X.N. Wang, B. Demirci, K.H.C. Baser, L.G. Zhou, S.J. Cutler, Nat. Prod. Comm. 3 (2008) 1073.
[96] M.M. Mackeen, A.M. Ali, N.Hj. Lajis, K. Kawazu, H. Kikuzaki, N. Nakatani, Z. Naturforsch. 57c (2002) 291.
[97] J.A. Odhiambo, G.M. Siboe, C.W. Lukhoba, S.F. Dossaji, Afr. J. Trad. Complement. Altern. Med. 7 (2010) 53.
[98] E.F. Queiroz, J.L. Wolfender, K.K. Atindehou, D. Traore, K. Hostettmann, J. Chromatogr. A 974 (2002) 123.
[99] D.E. Wedge, N. Tabanca, B.J. Sampson, C. Werle, B. Demirci, K.H.C. Baser, P. Nan, J. Duan, Z.J. Liu, Nat. Prod. Comm. 4 (2009) 123.
[100] N. Tabanca, B. Demirci, K.H.C. Baser, E. Mincsovics, S.I. Khan, M.R. Jacob, D.E. Wedge, J. Chromatogr. B 850 (2007) 221.
[101] S. Guleria, A. Kumar, A.K. Tiku, Z. Naturforsch. C: J. Biosci. 63 (2008) 211.
[102] Ch. Weins, JPC – J. Planar Chromatogr. – Modern TLC 21 (2008) 405.
[103] J. Sherma, in: J. Cazes (Ed.), Encyclopedia of Chromatography, 3rd ed., Taylor & Francis, New York, 2009.
[104] G. Morlock, W. Schwack, LC–GC Europe 21 (2008) 366.
[105] D. Abd-El-Halem, S. Zaki, J. Microbiol. Biotechnol. 16 (2006) 1706.
[106] Bioluminex, www.bioluminex.com.
[107] BioLuminizer, www.camag.com/v/products/evaluation/bioluminizer.html.
[108] Ch. Weins, PhD thesis, Universität Basel, Switzerland, 2006.
[109] V. Baumgartner, W. Schwack, J. Liq. Chromatogr. Rel. Technol. 33 (2010) 980.
[110] S. Verbitski, G. Gourdin, L. Ikenouye, J. McChesney, Am. Biotechnol. Lab. September (2006) 40.
[111] Application notes, Camag laboratory, F-34 1/22/2007.
[112] R. Akkad, W. Schwack, JPC – J. Planar Chromatogr. – Modern TLC 21 (2008) 411.
[113] C.F. Poole, J. Chromatogr. A 1000 (2003) 963.
[114] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, Biochem. Pharmacol. 7 (1961) 88.
[115] I.K. Rhee, R.M. van Rijn, R. Verpoorte, Phytochem. Anal. 14 (2003) 127.
[116] T. Mroczek, J. Mazurek, Anal. Chim. Acta 633 (2009) 188.
[117] A. Marston, J. Kissling, K. Hostettmann, Phytochem. Anal. 13 (2002) 51.
[118] I.A. Ramallo, S.A. Zacchino, R.L.E. Furlan, Phytochem. Anal. 17 (2006) 15.
[119] M. Müller, C. Dausend, C. Weins, F. Frimmel, Chromatography 60 (2004) 207.
[120] V. Kamath, P.S. Rajini, Food Chem. 103 (2007) 428.
[121] S. Rout, R. Banerjee, Bioresour. Technol. 98 (2007) 3159.
[122] E. Tyihák, Á.M. Móricz, P.G. Ott, in: M. Waksmundzka-Hajnos, J. Sherma, T. Kowalska (Eds.), Thin Layer Chromatography in Phytochemistry, CRC Press, Boca Raton, FL, 2008, p. 193.
[123] S. Nagy, B. Kocsis, T. Koszegi, L. Botz, J. Planar Chromatogr. 15 (2002) 132.
[124] S. Nagy, T. Koszegi, L. Botz, B. Kocsis, J. Planar Chromatogr. 16 (2003) 121.
[125] M. Hamburger, G. Cordell, J. Nat. Prod. 50 (1987) 19.