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Review

Hyphenations in planar chromatography

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ABSTRACT

This review is focused on planar chromatography and especially on its most important subcategory high-performance thin-layer chromatography (HPTLC). The image-giving format of the open, planar stationary phase and the post-chromatographic evaporation of the mobile phase ease the performance of various kinds of hyphenations and even super-hyphenations. Examples in the field of natural product search, food and lipid analysis are demonstrated, which point out the hyphenation with effect-directed analysis (EDA) and mass spectrometry and illustrate the efficiency gain. Depending on the task at hand, hyphenations can readily be selected as required to reach the relevant information about the sample, and at the same time, information is obtained for many samples in parallel. The flexibility and the unrivalled features through the planar format valuably assist separation scientists.

Contents

1. Advantages of planar chromatography

Planar chromatography celebrated its 70th anniversary in 2008 [1]. It comprises all chromatographic techniques that use a planar open stationary phase present as or on a plane (layer) [2], i.e. thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), ultrathin-layer chromatography (UTLC), and preparative layer chromatography. Today, paper chromatography is hardly used anymore.

As the quality of data generated is closely related to the level of instrumentation, HPTLC is most important. HPTLC celebrated its 35th anniversary in 2010 [3] and stands for optimized coating material (lower particle size and narrower particle size distribution) in combination with the employment of advanced instrumentation for mostly all steps of the chromatographic process. The proper standardization of HPTLC methods allows reliable, precise and reproducible quantitative results. Using automated systems for its single steps, the labor time thereof is reduced to seconds per sample.

HPTLC is a complementary technique to HPLC using an orthogonal selectivity. Mostly, normal phase systems were employed for HPTLC versus reversed phase systems for HPLC. Although HPTLC has a lower separation power compared to HPLC and GC,

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nevertheless it offers several options for post-chromatographic improvement of the resolution, e.g., by use of selective derivatizations or electronic filters. These features highly compensate post-chromatographically for a lower previous separation power. Although being an offline method, HPTLC shows a sample throughput as high as ultra-rapid HPLC methods [4]. In contrast to HPLC or GC, the open planar chromatographic system might not be the best choice for volatile and sensitive (oxidation-prone, light-prone) samples. But for many other samples, HPTLC can be considered highly cost-effective as the open, planar, image-giving feature implies distinctive advantages like:

1. Sample preparation and chromatography: sample preparation can be kept simple and a complex matrix can remain at the single-use adsorbent without impact on proper quantification.
2. Dynamic sample application over a range of 10,000 (0.1 μL–1 mL) can ease analysis through omittance of an additional concentration or dilution step.
3. Sample components remained at the adsorbent are visible in the start region of the planar image. This additional information is not given by column chromatography, at which these components stick to the column head or in the GC liner and will not reach the detector.
4. Screening of samples is matrix-robust using area application [5].
5. Parallel chromatography allows a high sample throughput of up to 70 samples (applied spotwise on both plate sides) at one go. It is adaptive to the task at hand, and if required, speeded up to 1000 runs per 8-h day [4].
6. Matrix-rich samples do not change the adsorbent for successive samples, which can happen in column chromatography. Always the same fresh adsorbent is available for every sample side by side.
7. Chromatography of samples, especially when intended for direct comparison, is performed at the same time under utmost identical conditions, and not successively over a time period that can influence the property of the mobile phase, stationary phase and samples or the pressure/detector stability.
8. All components of a sample are stored on the plate, and if stable, available for later access or further evaluation (they are not in the waste bottle).
9. The planar image allows multiple detection and re-evaluation of the same sample in different ways, e.g., UV absorption and fluorescence measurement → recording of mass spectra → chemical derivatization → Vis absorption measurement [6].
10. A wealth of chemical reactions can be employed for derivatization, giving comprehensive information about unknowns or extended confirmation of findings.
11. Selective, almost specific derivatizations readily increase the resolution post-chromatographically making, *ab initio*, an extended sample preparation and a high separation power less important. They solve analytical tasks that are otherwise coped with universal detectors, which need a high separation power or extended sample preparation.
12. The change between different chromatographic systems is readily made.
13. The offline, flexible system is adaptive to the sample at hand: depending on the sample, the extent of HPTLC procedures can be selected as required leading to low costs.
14. Digital image evaluation is faster than conventional scanning and fulfilled by some mouse clicks.
15. The compatible method transfer to a method of lower separation power can be assured by the purity of mass spectra and remission spectra.
16. Improved capability of detection is possible through the use of, e.g., area application, automated multiple development (AMD2 system), spherical extra-thin plates or sensitive derivatizations.
17. Information about unknown samples can be gathered very fast due to the flexibility of the method.
18. Effect-directed analysis (EDA), which can readily be coupled with HPTLC, supplements target analysis by covering a more comprehensive range of potentially relevant compounds.
19. Through the open format, HPTLC x HPTLC can readily be performed, and hyphenations are not as costly and laborious as for other chromatographic approaches.

2. The challenges for super-hyphenations

Initially in 1980, the term hyphenation was advocated by Hirschfeld [7]. Generally, hyphenation comprises the different approaches to combine mainly spectrometers with chromatographic systems to get further information about the sample. As pointed out by Bogusz [8,9], the hyphen (-) of hyphenated methods, e.g., LC-MS or GC-MS, symbolizes this attempt of combination or coupling, which did not reach its stage of full maturity so far. But also the slash (/) is found for hyphenated methods at a maturate state, e.g., LC/MS or GC/MS [10], when the systems are regarded as validated, reliable and complete and the hyphenation is not noticed anymore.

Recently in 2007, the term *hypernation* was coined by Wilson and Brinkman [11] to place *all of the required* spectrometers into a single system so that all of the spectroscopic information is obtained in a single run. Hypernation, which might also be called super-hyphenation, represents a logical, rapid and efficient strategy for obtaining the most possible (relevant) information out of a single separation. The major problems associated with HPLC-based or GC-based hypernations are capital cost and strategies for dealing with the large amounts of data produced by such systems. The complexity of the instrumentation increases, which makes them difficult to operate in a routine way. A single eluent that is optimal for all detectors is difficult to obtain. Differences in sensitivity between spectroscopic techniques and spectrometers are challenging as well.

All these problems are much less challenging in HPTLC-based hypernations because of the open system that is (1) highly adaptive to different sensitivities, (2) cost-effective by modular instrumentation compared to the analytical status quo [12], (3) generating less data due to targeted access to points-of-care on the plate, and (4) directly accessible for the respective optimal solvent, because the eluent is evaporated after chromatography and does not impact the different detectors. The latter is extremely relevant for effect-directed detection with bioassays. The main difference is that after HPLC separation, the sample is normally in the waste, but in HPTLC still on the plate and available for gathering further information. This means that unknowns can be evaluated first and thereafter further information can be recorded just from regions/zones of interest whereas in HPLC the online system forces special pre-settings applied for every run independent on matrix interferences or negative findings. The flexible modular HPTLC instrumentation facilitates hyphenation required at any time for any project without any additional capital costs as like for a special hyphenated online system devoted solely to a single specified task.

For a single separation, multiple spectrometers were successively employed with ease, like UV, Vis, fluorescence detection (FLD) and FTIR or attenuated total reflection (ATR) followed by surface-enhanced Raman spectroscopy (SERS), MS or high resolu-

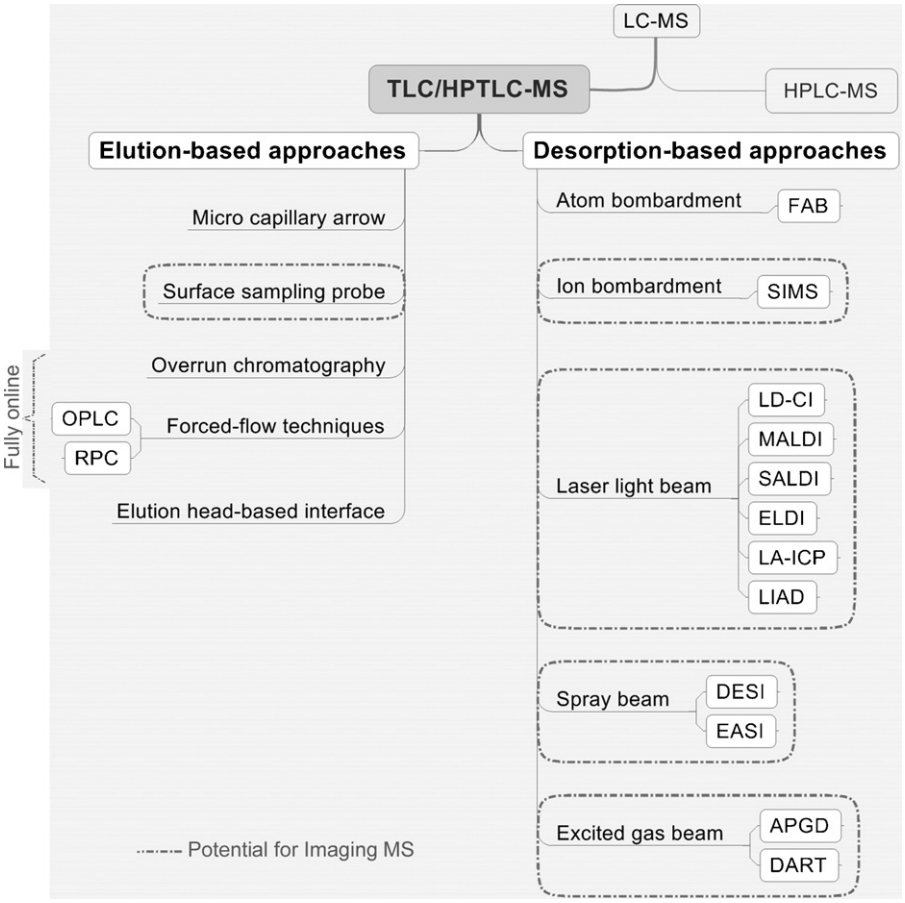


Fig. 1. Classification of the approaches for hyphenation of TLC/HPTLC with MS.

tion MS (HRMS). Existing combinations are:

- HPTLC-UV/Vis/FLD-MS [13,14],
- HPTLC-UV/Vis/FLD-bioactivity-HRMS [15],
- HPTLC-UV-FTIR [16,17],
- HPTLC-UV/Vis/FLD-FTIR ATR [18],
- TLC-Vis-SERS [12].

It is obvious that the image-giving format of the open, planar stationary phase eases the performance of various kinds of hyphenations and even super-hyphenations. The efficiency gain obtained can helpfully assist analysts. In the following sections, two hyphenations (with mass spectrometry and with bioassays) are exemplarily focused on the handling of samples given in the field of natural product search, food and lipid analysis.

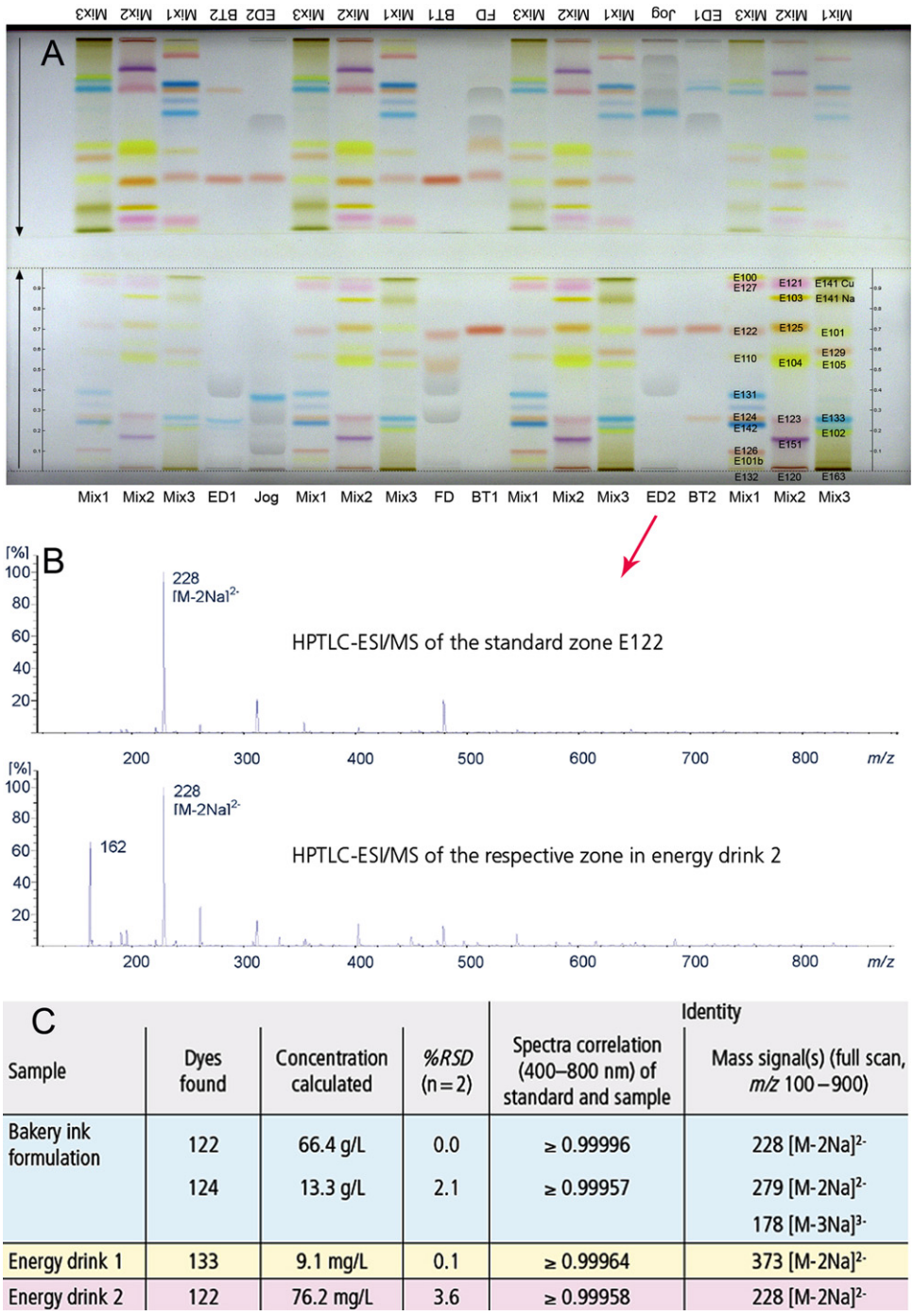
3. Hyphenation with MS

Hyphenation of an open planar system with MS required more effort than that with column-derived techniques. For example, the incompatibility between the static planar, open chromatogram and the dynamic, stable liquid sample introduction into the MS was challenging when coupling TLC/HPTLC with electrospray ionization (ESI)-MS, atmospheric pressure chemical ionization (APCI)-MS or atmospheric pressure photo ionization (APPI)-MS. Reviews about TLC-MS were regularly reported by Busch et al. [19–22] or Wilson et al. [23–25], but not until the last decade, it has attracted interest because of several successful approaches. The invention of ion sources working under ambient conditions and atmospheric pressure enormously eased the introduction of a planar object. The different TLC/HPTLC-MS approaches (Fig. 1) can be grouped into elution-based and desorption-based techniques, which are discussed in detail elsewhere [26]. Concerning terminology, we now have to think about LC-MS, because LC comprises all chromatographic techniques employing a liquid mobile phase, including TLC and HPTLC. Therefore, LC-MS does not only represent HPLC-MS, anymore. Peculiarities of the hyphenation HPTLC-MS are illustrated by the following two examples.

3.1. HPTLC-UV/Vis/FLD-ESI/MS for analysis of food dyes

The first hyphenated example is given in the field of food analysis. Mostly, TLC is used to just identify the dyes found in food followed by HPLC for quantification. This analytical approach is wondering, because already with a single HPTLC run all relevant analytical questions are answered. This is demonstrated for quantification of the most important 25 water-soluble dyes in various food samples by HPTLC-UV/Vis/FLD-ESI/MS (Fig. 2) [13]. As particular advantages can be considered that

1. up to 36 samples (diluted, degassed or centrifuged and applied as areas to allow high matrix-load) can be screened at one go for 25 water-soluble dyes using UV/Vis/FLD images (Fig. 2A),
2. still on the same plate, positive findings were quantified by digital evaluation, and
3. depending on the sample, confirmation of the positive findings was performed by Vis spectra library search or recording of mass spectra (Fig. 2B and C).



C

Sample	Dyes found	Concentration calculated	%RSD (n = 2)	Identity: Spectra correlation (400–800 nm) of standard and sample	Identity: Mass signal(s) (full scan, m/z 100–900)
Bakery ink formulation	122	66.4 g/L	0.0	≥ 0.99996	228 $[M-2Na]^{2-}$
	124	13.3 g/L	2.1	≥ 0.99957	279 $[M-2Na]^{2-}$
					178 $[M-3Na]^{3-}$
Energy drink 1	133	9.1 mg/L	0.1	≥ 0.99964	373 $[M-2Na]^{2-}$
Energy drink 2	122	76.2 mg/L	3.6	≥ 0.99958	228 $[M-2Na]^{2-}$

Fig. 2. HPTLC-UV/Vis/FLD-ESI/MS for analysis of food dyes: (A) screening of 36 samples in parallel (energy drink (ED), bakery ink formulation (BT), yoghurt (Jog), fruit drink (FD), exemplarily the visible image is shown), (B) in case of need, confirmation of selected samples (exemplarily shown by MS), and (C) all information obtained from a single HPTLC separation.

Is the food dye labeling correct? Is the limit value adhered to? Is there any unauthorized dye present? If yes, which one? All the relevant information (Fig. 2C) to solve the analytical questions mentioned before was obtained from the same sample (run), and even more, for many food samples in parallel. Depending on the task at hand, hyphenations were selected as required, from visual inspection of the plate to quantification of selected samples and to recording of absorbance or mass spectra from zones of interest. The analytical extent is adjusted to the sample at hand reducing the analytical steps to a minimum, thus saving costs, time and resources.

3.2. HPTLC-UV/Vis/FLD-MALDI/TOF MS for analysis of phospholipids

The second hyphenated example is given in the field of phospholipids' analysis. Phospholipids can selectively be detected by derivatization, e.g., with primuline reagent. The analysis tolerates a high matrix-load and the ease of changing the chromatographic selectivity is treasured as well as the separation in a normal phase system according to functional groups. Moreover, structural assignment of the compounds found can be assured by matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)

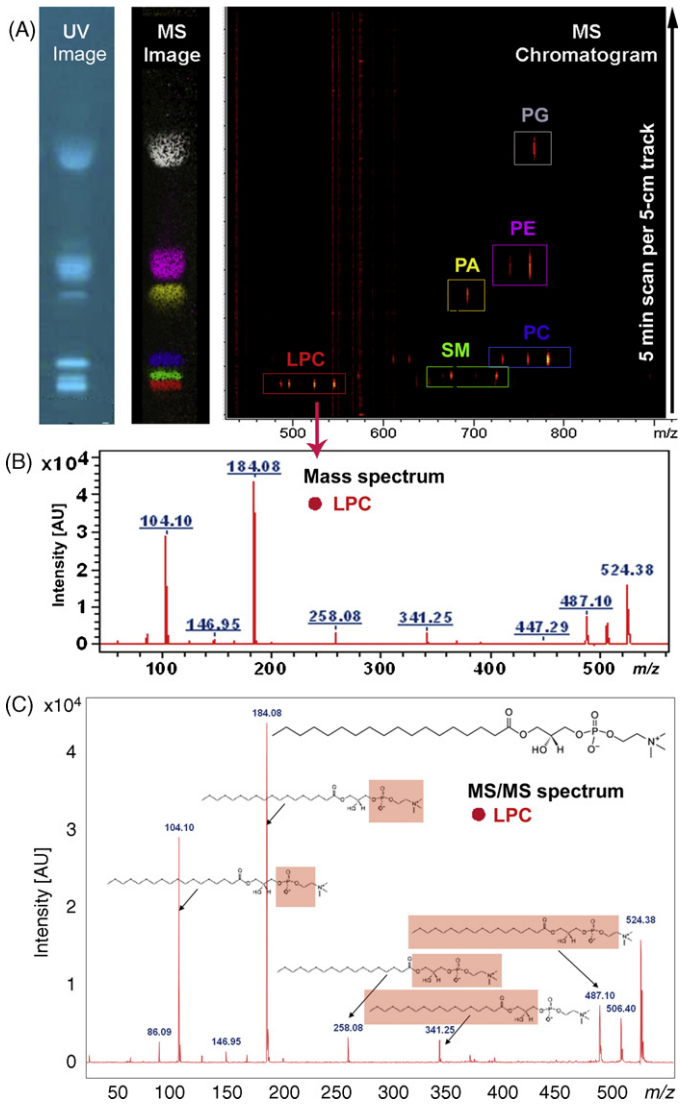


Fig. 3. HPTLC-UV/Vis/FLD-MALDI/TOF MS for analysis of phospholipids: (A) image after derivatization with primuline (left), resulting MS image (middle) and MS chromatogram (right); (B) mass spectrum exemplarily shown for lysophosphatidylcholine (LPC) as well as (C) MS/MS spectrum with signal assignments.

MS directly on the plate. Hence, often HPTLC is the method of choice in this analytical field. Peculiarities of hyphenated HPTLC-UV/Vis/FLD-MALDI/TOF MS (Fig. 3, [27,28]) are:

1. Parallel screening of samples is followed, in case of need, by HPTLC-MALDI/TOF MS imaging of selected tracks or zones.
2. The resulting MS image looked alike the image after derivatization with primuline, however, it allows to distinguish over-lapping lipids in the ion density image (Fig. 3A). A faster access provides the MS scan along the chromatographic track (5 min/track) depicting the density plot of migration distance versus *m*/*z* ions along with the respective mass spectra (Fig. 3B).
3. On particular bands, MS/MS experiments give further information to assign lipids with regard to their respective sn1/sn2 substituents and their head groups (Fig. 3C).

Hence, all relevant information is obtained by a single separation for several samples in parallel. Even detailed MS/MS information was obtained to clear up the structure from zones of interest.

3.3. Advantages of HPTLC-MS

Owing to the offline system and to the local fixation (storage) of the separated substance zones on the adsorbent, antecedent evaluation is allowed. Thus, depending on the sample and analytical task, TLC/HPTLC procedures can be selected as required. Expensive mass spectrometric recording of background, matrix or unknown samples with negative findings can be reduced to a minimum. This unique advantage is common for all TLC/HPTLC-MS approaches, but not for fully online techniques (see bracket in Fig. 1). Such ability to pre-screen samples and, still on the same plate (run), to evaluate them to the degree necessary is a unique advantage of HPTLC as substances are stored on the adsorbent and still available for further steps. Other chromatographic techniques, such as HPLC/UV prior to HPLC/MS, also allow pre-screening using lower cost technologies before confirmatory analysis of relevant samples, but require an additional chromatographic run for MS recording then.

Direct comparisons between the HPTLC/TLC-MS approaches are difficult to make because the approaches differ in ion sources and analyzers, in the substances selected, and on the fact, whether the whole system was optimized. However, some differences are evident:

- For desorption techniques, sputtering and conduction into the MS must be constructed very effective to guarantee the respective sensitivity and reliability because only an aliquot of the zone size is hit on its very surface. Thus, often automation and internal standards were used for desorption techniques. Moreover, sensitivities can depend on the retardation factor (hR_F-value) of a substance besides other factors of influence as reported for MALDI [29] or desorption electrospray ionization (DESI) [30,31].
- Contrarily, most elution-based techniques have the ability to elute and transfer the whole substance zone inclusive its depth profile into the MS. Exceptions are the micro capillary arrow and the surface sampling probe. Due to the closed connection and transfer to the MS, elution head-based techniques were shown to be reliable also without internal standards and automation [32,33].
- Most elution-based approaches as well as DESI, direct analysis in real time (DART), atmospheric pressure glow discharge (APGD) and electrospray-assisted laser desorption/ionization (ELDI) do not require any plate pre-treatment. With these ion sources, MS spectra are obtained directly from the plate within seconds. The spectra provide mostly the de-/protonated molecule and/or potential adducts making interpretation easy. This is advantageous over fast atom bombardment (FAB), MALDI, surface assisted laser desorption/ionization (SALDI), or secondary ion mass spectrometry (SIMS), where a strict plate pre-treatment protocol over several steps is requisite and the interpretation of the mass spectra might be more challenging.
- By nature, thermal desorption techniques are limited to lower mass species amenable to vaporization and thermally stable compounds. Several approaches are not commercially available so far. New ion sources working under ambient conditions are not yet *status quo* in the most MS laboratories and must be bought specifically for TLC/HPTLC-MS, which might be hindered by an expensive price. These circumstances lessen the impact of the new development. On the other side, having a forced-flow technique in the laboratory or the CAMAG TLC-MS Interface, coupling is readily feasible with any HPLC-MS system.
- It depends on the analytical question whether point-of-care recording just of zones of interest or imaging of a whole track is required. HPTLC-MS can be employed highly targeted, just in zones of interest, at a reasonable storage of data and in the shortest possible time period. Thus point-of-care recording is the most time saving and the most cost effective regarding equipment and

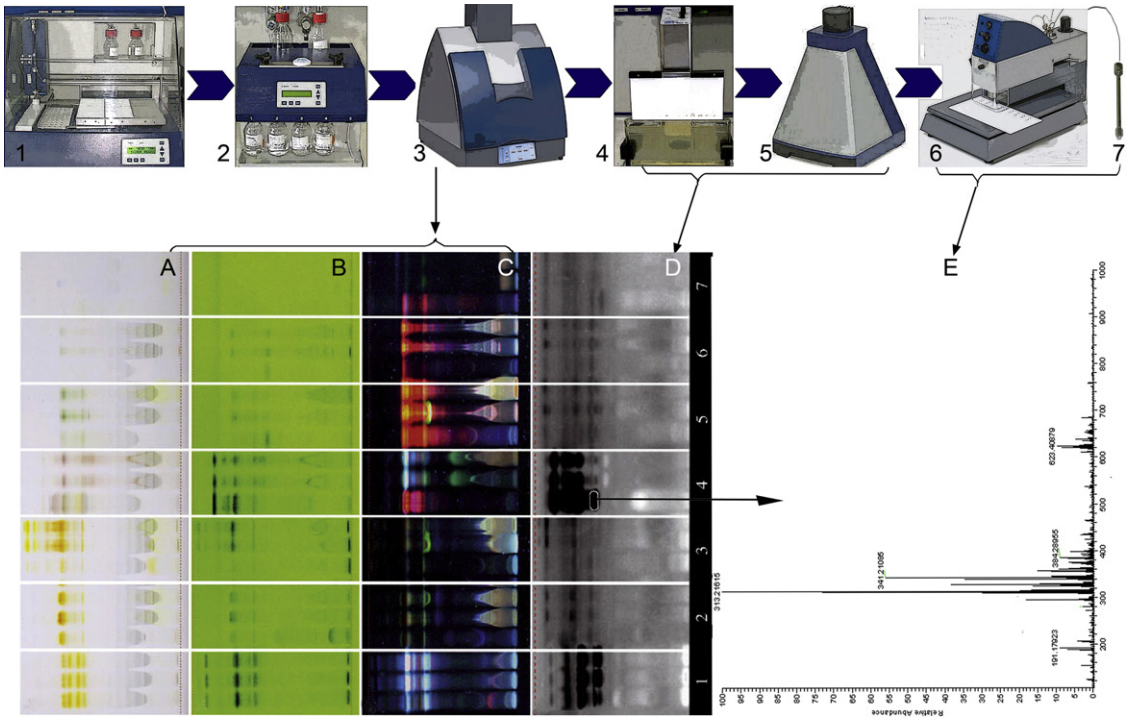


Fig. 4. HPTLC-UV/Vis/FLD-bioactivity-HPLC-HRMS hyphenation for many samples in parallel (21 samples: seven different sponges grown in three different media each): The protocol is based on the following automated steps: (1) application of complex samples, (2) matrix-robust gradient development over a wide polarity range of many samples in parallel, (3) plate documentation at Vis/UV/FLD (A–C), (4) dipping in the bioassay, followed by (5) imaging of bioactivity (D), and after evaluation (6) recording of HRMS spectra of relevant substance zones (E), whereby (7) integration of a HPLC column in the interface outlet line enables a second, orthogonal selectivity before MS recording.

operation. In case of in situ scanning of a whole track, the surface sampling probe and various desorption techniques (Fig. 1, dashed areas) show this ability under the precondition of a high spatial resolution and a sufficient sensitivity in the low ng/zone range.

The feasibility to obtain mass spectra free of contamination from zones of interest on a HPTLC plate within 1 min or even seconds highly contributes to the progress of planar chromatography. It took a long time, but the contamination-prone *scrape off and elute* is past!

4. Hyphenation with bioassays

4.1. Scope of effect-directed analysis (EDA)

Almost 50 million substances are registered in Chemical Abstracts Service (CAS), and it is not overrated when about 100,000 chemicals are estimated to be in daily use [34]. All these chemicals could be potential contaminants. To be honest, the trend of multi-methods, which cover even of up to 500 substances, will not reach the relevant scope. For example, food is nowadays coming from all over the world and worldwide over 1000 pesticides are relevant. However, a mean number of 200–300 pesticides has reached in the daily routine of food analysis. What about the other 70–80% pesticides that might be in? Additionally, the relevant metabolites and breakdown products during food processing are not covered comprehensively and most of them are not in the analytical focus, even not known. However, also the opposite approach of *detecting everything* is limited. A recent review about the analysis of complex, poorly defined mixtures by LC-ESI-FT-ion cyclotron resonance (ICR)-MS illustrated what effort it takes to find out most of the compounds of a natural organic matter sample containing several thousands of substances [35]. Also comprehensive methods were treated as key tools for future food safety [36]. Which database will cope with identification of all those peaks? Are high-sophisticated, hyphenated, comprehensive online methods really the key for routine food safety? Do they offer a fast, matrix-tolerant, cost-efficient and rugged throughput? Is it reasonable to subject *every* sample to a detailed track of its history? Quite another option for handling of such samples would be to focus just on the relevant samples and compounds therein, e.g., harmful substances detected by a bioassay. This strategy would dramatically decline the number of substances to identify as well as the data flow.

This so-called EDA, in which a bioassay used for selective detection is the key element, leads to more concrete answers, especially when combined with chromatography [37–39]. It supplements target analysis by covering a more comprehensive range of potentially relevant compounds, importance of which is triggered by the growing global threat to ecosystems. All substances, which are generating a distinct effect, are detected in complex mixtures. This implies that, apart from the analytes in focus, unknown metabolites, side products, process contaminants, degradation products, adulterants, migration products, or residues are also detected. Thus, additional relevant compounds not necessarily detected using standard techniques are discovered. These compounds might be also important, but have not been in the analytical focus so far. HPTLC-EDA allows the localization of the activity even in a complex matrix and therefore permits a target-directed isolation of the active constituents. Tedious single compound screening is avoided.

4.2. Ease of HPTLC-EDA

EDA can readily be coupled with HPTLC. Matrix interferences are reduced by the high specificity of bioassays, which compensates to a certain extent the lack in high separation power. Screening of samples and tracking of relevant constituents therein can be performed on the same plate with less effort and data handling. The

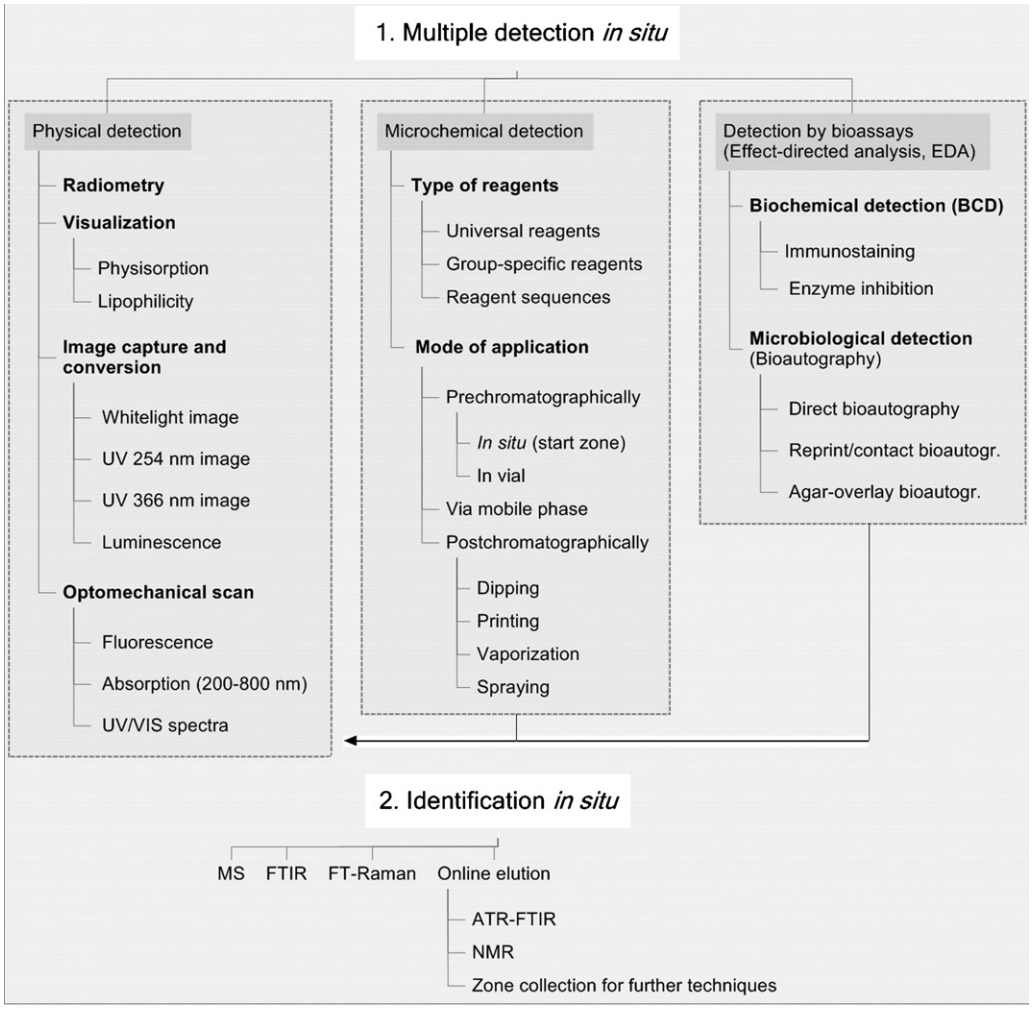


Fig. 5. Potential for hyphenations: multiple detection and identification options for many samples in parallel with a single chromatographic HPTLC separation.

open planar format of HPTLC is perfect for coupling with bioassay. Firstly, it offers a chromatographic separation in contrast to other high-throughput systems. Secondly, it is matrix-tolerant and sample preparation can be kept simple which allows a more comprehensive view on the sample because the sample is less tailored to subsequent analysis. After chromatography, all samples were readily coupled with the respective bioassay at one go and the substances indicated were directly transferred to the HRMS. Just *one* HPTLC run is required to identify relevant compounds in many different samples and to obtain their sum or even structure formulae, depending on the analyzer used. Also parallel information about several response mechanisms can readily be obtained for samples in a single run, e.g., the repeated application of five samples can be subjected to five different bioassays after chromatography and plate cut.

Concerns about sufficient separation power of HPTLC and potential coelution with regard to complex mixtures are defanged using (1) comprehensive HPTLC and/or for extraction of the relevant zones (2) the TLC-MS interface, in which a short HPLC column is attached in the outlet capillary line. Thus potentially coeluted zones or bioassay media can be separated from the analyte.

4.3. Types of bioassays used for HPTLC-EDA

Coupling microbiological assays with planar chromatography (bioautography) has a long tradition [40]. Bioautography plays a key role for screening of complex, mainly botanical samples for specific natural constituents. Several microbiological assays were established carried out directly on the plate. Bacterial assays were reported for detection of, e.g., (1) bioactive compounds in complex samples by *Vibrio fischeri* (current survey in [41]), (2) vitamin B12 in food by *Escherichia coli* 215 [42,43], and (3) antibiotic compounds in environmental and food samples by *Bacillus subtilis* [44–49] or in natural products by *Pseudomonas savastanoi* [50,51].

Apart from bacterial assays, fungi assays were used for detection of antifungal compounds by *Candida albicans*, *Trichophyton rubrum*, *Cryptococcus neoformans*, *Cladosporium cucumerinum* or *Aspergillus niger* [52–56]. But for example, also the alga *Pseudokirchneriella subcapitata* was used for detection of algicides [52]. Besides those bioassays, especially customized bacteria strains or genetically engineered luminescent or specified microorganisms, so-called reporter gene assays, show strong effects. One might be genetically engineered yeast cells containing the human estrogen receptor DNA sequence (YES, yeast estrogen screen) for detection of estrogens and endocrine disrupting compounds [57], which was recently used, however as microtiter plate test, for analysis of endocrine disrupting compounds in mineral water [58].

But also biochemical detections (BCD) are apparently increasingly grouped under bioassays, although enzymes are neither a living organism nor an isolated organelle. They can be carried out highly selective and sensitive down to the pg/zone range, such as the esterase inhibition by anti-cholinesterase compounds like organophosphates, thiophosphates or carbamates [59–63], and immuno-staining of gangliosides [64–66], glycosphingolipids

[67,68] or antiphospholipid antibodies [69–71]. Enzymes used by gel entrapment as agar overlay were also rapid and simple to use, e.g., for screening for glucosidase inhibitors present in a complex matrix [72]. Glucosidase activity was detected using esculin as the substrate to produce esculetin, which reacts with ferric ion to form a brown complex.

Bioassays on the plate can readily be adapted to other analytical fields. The further transfer of bioassays, especially reporter genes assays, from the Petri dish, microtiter plate or cuvette to the HPTLC plate will be interesting topics of future research. The hyphenation of HPTLC with a bioassay and HRMS is illustrated in the following example.

4.4. HPTLC-UV/Vis/FLD-bioactivity-HPLC-HRMS for analysis of sponges

Hyphenations like HPTLC-UV/Vis/FLD-bioactivity-HPLC-HRMS (Fig. 4) clear up several tasks with a single separation. For example in natural products search,

1. up to 30 sponges (just lyophilized, dissolved and centrifuged) were simultaneously screened for natural bioactive secondary products (UV/Vis/FLD/EDA images),
2. high producers of bioactive products were identified,
3. information about the range of bioactive products produced by the sponge was given, and
4. the sum formula was obtained from bioactive zones of interest.

All this information was reached by a single matrix-robust HPTLC separation for up to 30 sponges in parallel [15,73]. The saying "The whole solution on a single plate!" might point this out. As nowadays the current analysis in this analytical field is performed in a multi-step column-based protocol, in which the cuvette test (sum parameter) with *V. fischeri* is a *universal* standard test for any bioactivity of the sample, the efficiency of a HPTLC-based hyphenation is evident:

- For fractionation, the combination of different methods (solid phase extraction, gel permeation chromatography, preparative HPLC) can be skipped as HPTLC is highly matrix-tolerant.
- Isolation and purification of bioactive fractions, again followed by bioactivity testing of the purified compounds, can be skipped as the matrix-tolerant HPTLC can immediately be combined with the bioassay (organic solvent used for chromatography is evaporated).
- Just bioactive zones found are directly eluted from the HPTLC plate and online transferred into the HRMS within seconds.

HPTLC coupled with bioactivity-based detection by *V. fischeri* bacteria was first reported in 1996 [74]. It is a quick tool to figure out bioactive compounds in complex samples, to screen for new bioactive natural products or to study structure-bioactivity relationships. It can be grouped into direct bioautographic methods (Fig. 5), however, it immediately indicates the desired effect. The rapid response is given due to the intrinsic bioluminescence of the bacteria, working without need for growth and use of specific coloration substrates. The non-pathogenic, facultative anaerobic, Gram negative *V. fischeri* bacteria emit greenish light as a product of cellular respiration at a critical cellular density. Luciferase, the bioluminescence catalyst, is expressed and catalyzes an oxidation reaction that releases excess energy in the form of light. The luminescent image can be captured within a minute and easily be followed-up to monitor time-dependent changes. In case of bioactivity, single compounds are selectively and sensitively identified as darkened (inhibited bacteria) or enhanced (stimulated vitality of the bacteria) zones visible on the luminescent plate background (viable bacteria). The bioassay can be integrated in a short-term protocol based on automated steps: (1) application of complex samples, (2) gradient development over a wide polarity range of many samples in parallel, (3) plate documentation at UV/Vis/FLD, (4) dipping the developed HPTLC plate in the bioassay, (5) imaging of bioactivity observed in the dark, e.g., with Bioluminizer of CAMAG, and after evaluation (6) recording of HRMS spectra of relevant substance zones, whereby (7) the integration of a HPLC column in the interface outlet line enables a second, orthogonal selectivity before MS recording (Fig. 4).

So far coupling of HPTLC with bioluminescence detection was employed, e.g., for analysis of

- photodegradation products in sunscreens [75],
- black cohosh (*Actaea racemosa*) extracts with regard to adulteration [76],
- secondary metabolites in marine sponges [15,73,77],
- waste probes, urine and extracts of natural substances [74,78],
- sports field granules, expressway wastewater and landfill leachate [79],
- chloridazone metabolites in water [80].

A recent survey comprised further applications [41]. Detectabilities (lowest observed effect level) are down to the picomol range depending on the response of the substance. Single bioactive compounds can be identified in contrast to standard bioassays in the cuvette (e.g., Microtox®) or microtiter plate, with which just the overall activity of a complex sample can be measured and the identification of the active compounds required the protracted, tedious isolation of single components confirmed by the bioassay again. Further coupling of HPTLC-bioactivity-based detection with HRMS [15,73,77], provides an economic alliance for identification of new bioactive compounds in complex mixtures. Therefore, mass analyzers of high resolution and soft fragmentation are required to obtain elemental compositions and hints with regard to the structure. Unfortunately, the hyphenation HPTLC-bioluminescence was patented [81–83] alike for HPLC, which do hamper its progress.

4.5. Advantages of HPTLC-EDA

Key advantages of the open planar format help in coupling with effect-directed detection:

- In contrast to other high-throughput systems, HPTLC offers a *chromatographic separation*. This avoids false results due to interferences or antagonistic or synergistic effects, which can occur using the cuvette [84] or microtiter plate tests.
- Due to the matrix-robustness of HPTLC (using area application) and the single use of the stationary phase, less need for tedious sample cleanup is required. Sample preparation can be kept simple and does not interfere with the chromatographic separation in HPTLC, but would be enriched in a HPLC column.
- Based on reduced sample preparation, HPTLC allows a more comprehensive view on the sample, which helps in profound search for new compounds.
- Organic solvents, which readily cause inactivation of enzymes or death of living organisms, are readily evaporated before biodetection.
- HPTLC proved to be a rugged method compared to HPLC [74] at low costs, fast results, and rapid response times, especially for bioactivity.
- HPTLC reduces the number of substances, to be analyzed in a complex mixture, to a reasonable scale and helps in *effective* decisions, like which substances are relevant for further analysis and toxicological evaluation.

- It shows high detectabilities with detection limits typically in the sub-ng to pg-range. The image can be used for quantification using digital image evaluation systems.
- For structure elucidation of relevant zones, hyphenation with HRMS, NMR, ATR-FTIR [18], static nanospray, direct inlet electron impact (EI)-MS and MALDI, etc. can be employed highly targeted. Co-eluting substances can be separated attaching a short HPLC column in the outlet capillary line of the TLC-MS interface.
- For more complex samples, HPTLC × HPTLC can readily be employed.

Laborious attempts to couple bioassays with a flowing system [85] and finally with HPLC [74,86–89] have successfully been reported, although various difficulties are intrinsic:

- The online system is limited in the throughput:
  - o Sample analysis is performed subsequently, not in parallel.
  - o Analysis time is limited by the successive online assay, even in the age of rapid separations.
  - o A forced stop at a small link stops the whole online system.
  - o Thorough care during operation must be warranted because the growth of any biofilms has to be prevented in the whole online system.
- The scope of analysis is limited:
  - o Separation of samples is restricted to physiological HPLC eluent compositions.
  - o High salt concentrations for enzymes do interfere with online MS detection.
  - o Only fast enzymatic reaction times can be realized to avoid dramatic peak broadening and a reasonable analysis time.
- The operating costs are comparably high:
  - o Background and matrix is recorded by MS generating much higher costs and data flow compared to HPTLC, in which the bioassay is evaluated first.
  - o Sample preparation is increased because is has to be adjusted to the requirements of HPLC (matrix may not remain on the column).
- The quality of the results can be limited:
  - o Time-depending effects on the bioassays that are essential for the result cannot be studied in the flow-system [85], e.g., for *V. Fischeri* contact times of 5 min, 15 min, 30 min with the sample components are important according to ISO 11348.
  - o The limit of detection (LOD) was much higher, e.g., by a factor of 5500 for analysis of paraoxon (LOD of 7.4 ng/peak by HPLC [86] versus 1.3 pg/zone by HPTLC [62,90]).

Apart from flow-systems, another approach was the use of microtiter plates for parking of HPLC fractions in duplicate [91], one 96-well plate for the bioassay [92] and another one for later transfer of suspect fractions to HPLC-HRMS. Although many disadvantages of a flow-system are circumvented by this approach, a second run and analysis is relevant. Open, offline, planar HPTLC does not have all these disadvantages. Hence, the sound knowledge of the boundaries between the analytical methods makes it evident: the most efficient way of coupling bioassays with chromatography is via HPTLC. There is great potential for this hyphenation, which is still at its infancy as HPTLC has been overlooked in analytical chemistry for decades. Hence, progress in HPTLC would imply progress in EDA without any high-cost instrumental effort.

5. Conclusions

Nowadays, HPLC-MS seems to be used as panacea method thereby impeding the chance for other methods to solve it differently, possibly in a more simple way. Also miniaturization does not always downscale the analytical process. One has to be careful in applying all technical possibilities, just for the sake of their beauty. The more sophisticated an analytical system is, the higher the costs are, when the sophisticated system is forced to stop and the whole analysis chain is interrupted due to troubles at a small link. Hence, irrespective of the prevailing trend, the most appropriate scale for each task has to be chosen. This requires a sound knowledge of the boundaries between the analytical methods and their pros and cons.

This review pointed out the ease of hyphenations given by the planar, open format and advised HPTLC as highly effective complementary and orthogonal method to HPLC and GC. Information is obtained for many samples in parallel. The decisive advantages of HPTLC-based hyphenations valuably assist researcher and depending on the task at hand, hyphenations can readily be selected as required. To make analysts familiar with this technique, HPTLC knowledge should be adequately trained and taught in analytical chemistry courses. This would help in forming profound researchers and qualified decision makers.

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