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Short communication

An improved matrix deposition technique for thin layer chromatography coupled to MALDI-TOF mass spectrometry (TLC-MALDI)

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ABSTRACT

Analytical thin layer chromatography (TLC) is a simple yet powerful chromatographic technique that is widely used for the qualitative characterization of complex mixtures such as plant extracts. For their qualitative and visual characterisation, a large number of more or less specific colour reactions are at hand and numerous reference substances are available as well. However, the identification of extract components by colour and the comparison of retention times is not straightforward. In contrast, the coupling of TLC with MALDI-TOF mass spectrometry can enable the identification of components and contribute to the optimization of TLC protocols. One of the most important steps for a successful TLC-MALDI process is the deposition of a sufficient amount of matrix onto the TLC plate. Standard methods such as the dip-coating protocol have major drawbacks. Here we present an improved and robust procedure for matrix application by means of matrix lines. The practicability of the method was tested on plant extracts from *Agrostemma githago* L. and *Papaver somniferum* L. (opium).

1. Introduction

Thin layer chromatography (TLC) is a simple chromatographic method for the characterization of plant-derived extracts. In this context, TLC has a great significance within the European pharmacopoeia [1], which contains hundreds of monographs for medicinal plants and formulations thereof. With a minimum of analytical equipment, TLC provides a quick overview of the complexity of plant extracts. It was therefore obvious to couple such an easy-to-use yet powerful chromatographic method with state-of-the-art mass spectrometric techniques [2]. To do this, the separated compounds need to be extracted from the plate and transferred to the mass spectrometer. A commonly practised method is to scratch out/ extract the bands from the TLC plate [3], also reviewed in [4]. This requires UV-absorbing properties of the analyte in this spectral range, which is the case for substances with aromatic structures and/or conjugated double bonds such as flavonoids. Specifically for this purpose dedicated devices that enable the extraction of compounds directly from the TLC plate were introduced by the Swiss company CAMAG or Advion Interchim.

However, a huge number of natural products such as terpenoids do not contain corresponding chromophores and therefore do not possess UV-quenching properties, which makes a targeted extraction from the TLC plate impossible. Another issue is that the scratching/ extraction might affect the quality of the chromatogram of the extract constituents. These problems have been circumvented by the introduction of TLC-MALDI-TOF-MS, which enables direct measurement on the TLC plate [5]. However, since the introduction of TLC-MALDI in the 1990s, the number of publications has remained rather low. To this day, the search term "TLC MALDI" results in 31 entries in PubMed [6], dating back to 1998. There might be many reasons for this low publication rate. When we started our work with TLC-MALDI, we realised that the matrix application onto the TLC plate is one of the most critical factors for a successful analysis by TLC-MALDI. Another problem is the lack of quantitative data.

Apart from inorganic matrices, such as iron oxide magnetic nanoparticles for flavonoids [7], the most widely used matrices are small organic acids such as 2,5-dihydroxybenzoic acid (DHB). In some cases, the use of matrix can be avoided altogether due to the energy-absorbing character of the molecules of interest [8]. The matrix may be applied manually to each band [5,9,10] on the TLC plate. As mentioned above, this requires a UV-quenching activity at 254 nm of the molecules on the TLC plate or the usage of dyes such as primuline (Direct Yellow 59) as shown for lipids [5].

Many plant extracts contain pharmacologically relevant non-UV-

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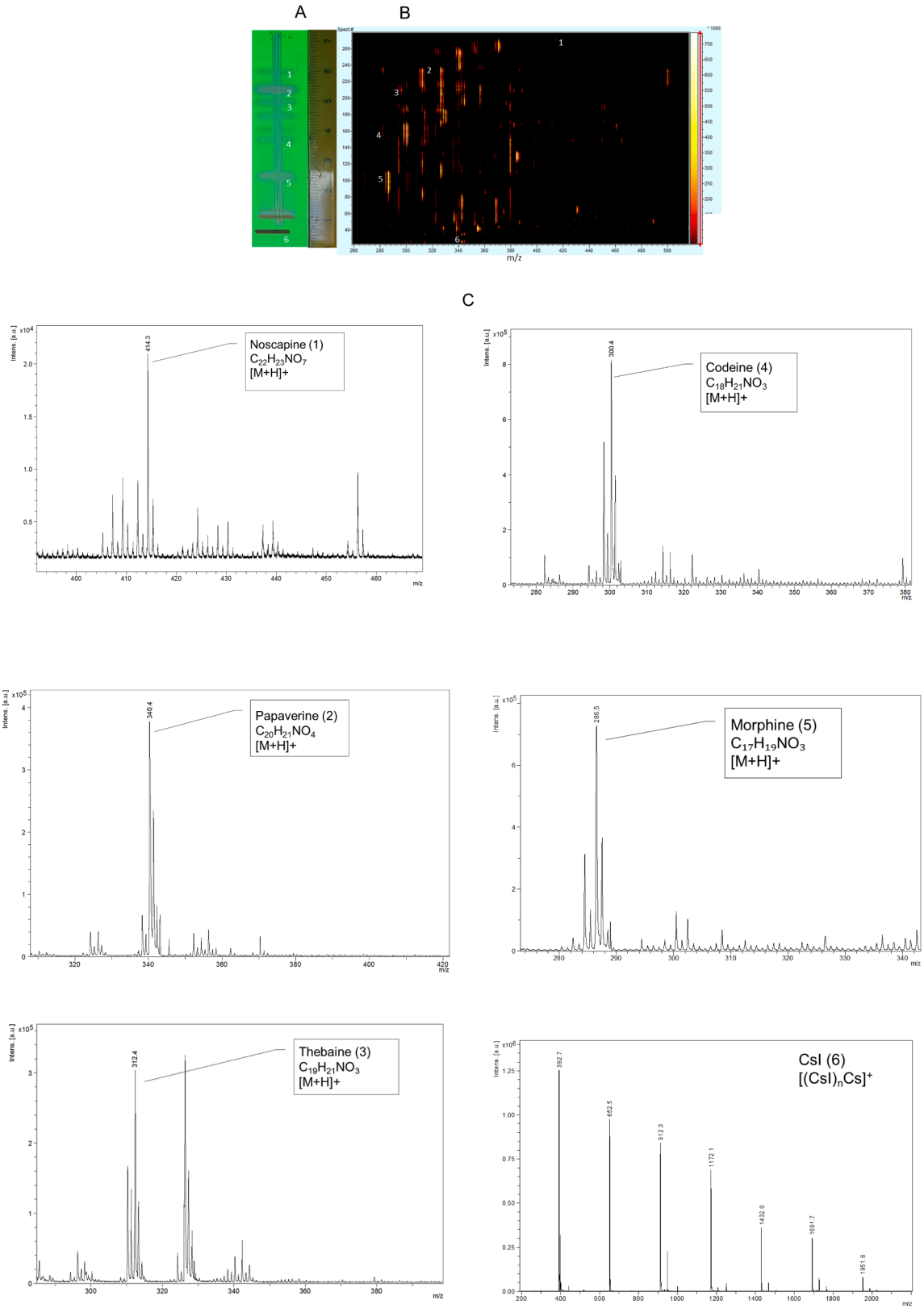

Fig. 1. TLC-MALDI of opium alkaloids.
(A) Alkaloids from a tincture of opium were separated on HPTLC Silica gel plates 60 F_{254}. Matrix lines were applied (HCCA) after air-drying the plate. Bands and matrix lines were visualized by fluorescence quenching at 254 nm. (B) Depiction of the TLC plate as a heat map, generated using TLC-MALDI (positive mode), where spect.# on the *y*-axis indicates the number of spectra that were acquired and represents the migration distance on the TLC plate (in A 0–7.5 cm). The compounds (numbered 1 to 5) could be assigned according to their position on the TLC plate, 6 represents the CsI standard. (C) Extracted MS spectra from B, representing five relevant alkaloids at the relevant locations; The number after the alkaloid indicates the position of the relevant alkaloid to fig. 1B, row 6: CsI-clusters.

active compounds such as terpenoids, which cannot be detected by UV quenching. For this reason, for the analysis of these substances the MALDI matrix is usually spread over the entire plate. One of the most common methods for this type of matrix application is the dip-coating protocol [11,12], which is also favoured by Bruker [13] (this application note is available upon request), the market leader in the field of TLC-MALDI. While the dip-coating protocol is attractive due to its simplicity, the dried TLC plate simply needs to be immersed into a tank with matrix solution, this technique suffers from some decisive disadvantages. The silica layer can flake off from the aluminium backing during immersion, the analyte may spread upon the addition of the solvent to the TLC plate, compromising the quality of the chromatographic separation [14], and uneconomically high concentrations of matrix are used with several rounds of dipping. In a typical dip-coating protocol [15], 200 mg/ml DHB matrix solution is prepared, which means that several grams of matrix are required. The associated high costs might be prohibitive for many users. Costs can also be lowered by the use of lower-quality DHB such as for synthesis.

In this study, we propose a robust and cost-saving technique for applying the matrix to the TLC plate. This is achieved by the deposition of matrix lines, which are applied at right angles to the bands on the TLC plate. The method was employed for the analysis of different plant extracts / compounds from *Agrostemma githago* L. (corncockle), *Saponaria officinalis* L. (common soapwort) and *Papaver somniferum* L. (opium poppy).

2. Material and methods

2.1. Plant extracts

Seeds from *Agrostemma githago* L. were ground, defatted (hexane) and extracted by 90 % methanol. After vacuum distillation and lyophilisation, a dry extract was obtained. The whole procedure is described elsewhere [16]. Bisdesmosidic triterpene reference saponins (SO1861, SO1831, SO1730 and SO1700), isolated from the roots of *Saponaria officinalis* L., were produced by EXTRASYNTHESE, Lyon, France. A reference raw extract from *Saponaria officinalis* L. was generated by extracting the roots with 90 % methanol as described elsewhere [17]. Opium tincture Ph. Eur. from *Papaver somniferum* L. was obtained from Maros Arznei GmbH, Fürth, Germany.

2.2. Thin layer chromatography

HPTLC Silica gel plates 60 F_{254} MS-grade for MALDI, #151160 (Merck KGaA, Darmstadt, Germany) were used for TLC. Samples (5 or 10 µl) were applied by the Camag Linomat IV System (CAMAG Chemie-Erzeugnisse & Adsorptionstechnik AG, Muttenz, Switzerland). Nitrogen pressure was set to 5 bar; time/volume: 15 s/µl; band width was 10 mm. A solution of 10 µg/µl (90 % methanol) of *Agrostemma githago* L. dry extract and opium tincture (33 % ethanol) were sprayed onto the TLC plates. The reference saponins and the reference extract of *Saponaria officinalis* L. were applied after the development of the plate. For *Agrostemma githago* L. chloroform, methanol, deionized water, glacial acetic acid (6/4/1/0.5) was employed as TLC solvent for development. For opium tincture a mixture of acetone, toluene, ethanol, ammonia (26 %) (4.5/4.5/0.7/0.3) was used. Twin-trough chambers for TLC plates, 10 × 10 cm (CAMAG Chemie-Erzeugnisse & Adsorptionstechnik AG, Muttenz, Switzerland) were used for development. Following the development, plates were air-dried and fluorescent quenching at 254 nm was visualized under UV light.

2.3. Matrix lines

DHB (2,5-Dihydroxybenzoic acid 99 %, #10127943) was purchased from Thermo Scientific Chemicals, Darmstadt, Germany, HCCA (α-Cyano-4-hydroxycinnamic acid ≥98 %, #C2020) was obtained from Sigma-Aldrich, Taufkirchen, Germany. Following optimization of the method, solutions of DHB (20 mg/ ml) and of HCCA (40 mg/ ml) each in deionized water/ acetonitrile (1:1), 0.1 % trifluoroacetic acid (TFA) were prepared. The matrix lines were applied perpendicular to the developed bands using the CAMAG Linomat IV System; nitrogen supply was set to 1 bar; time/ volume: 6 s/µl; volume matrix/ path length: 1 µl/ mm. Two matrix lines were applied at a parallel distance of ~0.5 mm to each other. Caesium iodide (5 µL) 50 mg/ml in tetrahydrofuran (THF) (Sigma-Aldrich, Taufkirchen, Germany, #203033) was applied (without any matrix) on the TLC plate after application of the matrix lines. The plate was stored in an evacuated desiccator until MALDI measurement.

2.4. TLC-MALDI

The TLC plate was mounted on an MTP-TLC-Adapter (#8255595) and inserted into a MALDI-TOF/TOF mass spectrometer (UltrafleXtreme, Bruker Daltonics GmbH & Co. KG, Bremen, Germany). FlexControl version 3.4, TLCMaldi version 1.1.7.0 and flexAnalysis version 3.4 were used as acquisition/ analysis software. In a typical experiment, a TLC lane was measured in auto-mode in 0.2 mm steps (X-step), the number of Y-spots for summation was 9 at a distance of 0.33 mm steps (Y-step), resulting in a total of 9000 laser shots/ TLC spot in reflector mode. Laser intensity was set to 60 %, reflector voltage to 4.8 × 1972 V, the Smartbeam laser was set to ultra, and the measurement was performed in positive (opium) or negative (triterpenes) mode, respectively.

3. Results and discussion

3.1. TLC-MALDI of alkaloids from Papaver somniferum L.

It is obvious that the application of matrix is one of the most critical steps in TLC-MALDI. In our first experiments with TLC-MALDI, we followed the recommended dip-coating protocol (version 1.6, Bruker). It quickly became apparent that this protocol was not well suited for performing TLC-MALDI. The matrix concentration is high (200 mg/ml) and the amount of matrix needed is also high, since a 50 ml glass dipping chamber is recommended for dipping and the solution is single use. The dipping and drying process sometimes blurred the TLC separation, especially since both steps are recommended to be performed twice. It also happened that the TLC layer peeled off after dipping. Our aim was therefore to devise a robust and cost-efficient method for matrix deposition. For this reason, we used a matrix solution that was 10 times less concentrated. Initially, we used the Chromajet DS20 system (Bionis, Houdan, France), a dedicated automated TLC spraying device. However, at a matrix concentration of 20 mg/ml it was not feasible to apply enough matrix to the TLC plate, consequently no signal could be acquired by MALDI. Another disadvantage of methods covering the whole TLC plate with matrix is the fact that the TLC bands are no longer visible on the TLC plate, as the fluorescence quenching at 254 nm is lost. When thinking about this problem, we came up with the idea of applying matrix lines perpendicular to the TLC lanes. At the intersection of TLC compound bands with the MALDI matrix line one would expect to obtain a compound-specific MS signal, while the remainder of the band would still be available for other purposes such as TLC densitometry or recovery of the substance. To test this principle we chromatographed opium tincture and used HCCA as matrix (Fig. 1A). Indeed we were able to apply enough matrix, while maintaining the band separation and the fluorescence quenching properties at 254 nm (Fig. 1A). As shown in Fig. 1A, alkaloids from the opium tincture remained visible after applying the HCCA matrix as lines perpendicular to the TLC bands. Thus, after measuring the TLC plate by MALDI, individual spots on the corresponding heat map (Fig. 1B) could be matched with the position of the bands on the TLC plate (Fig. 1A), which means that a correlation can be established also with the TLC-Retention Factor (R*f*). Based on the molecular ion peaks noscapine (*m/z* 414), papaverine (*m/z* 340), thebaine

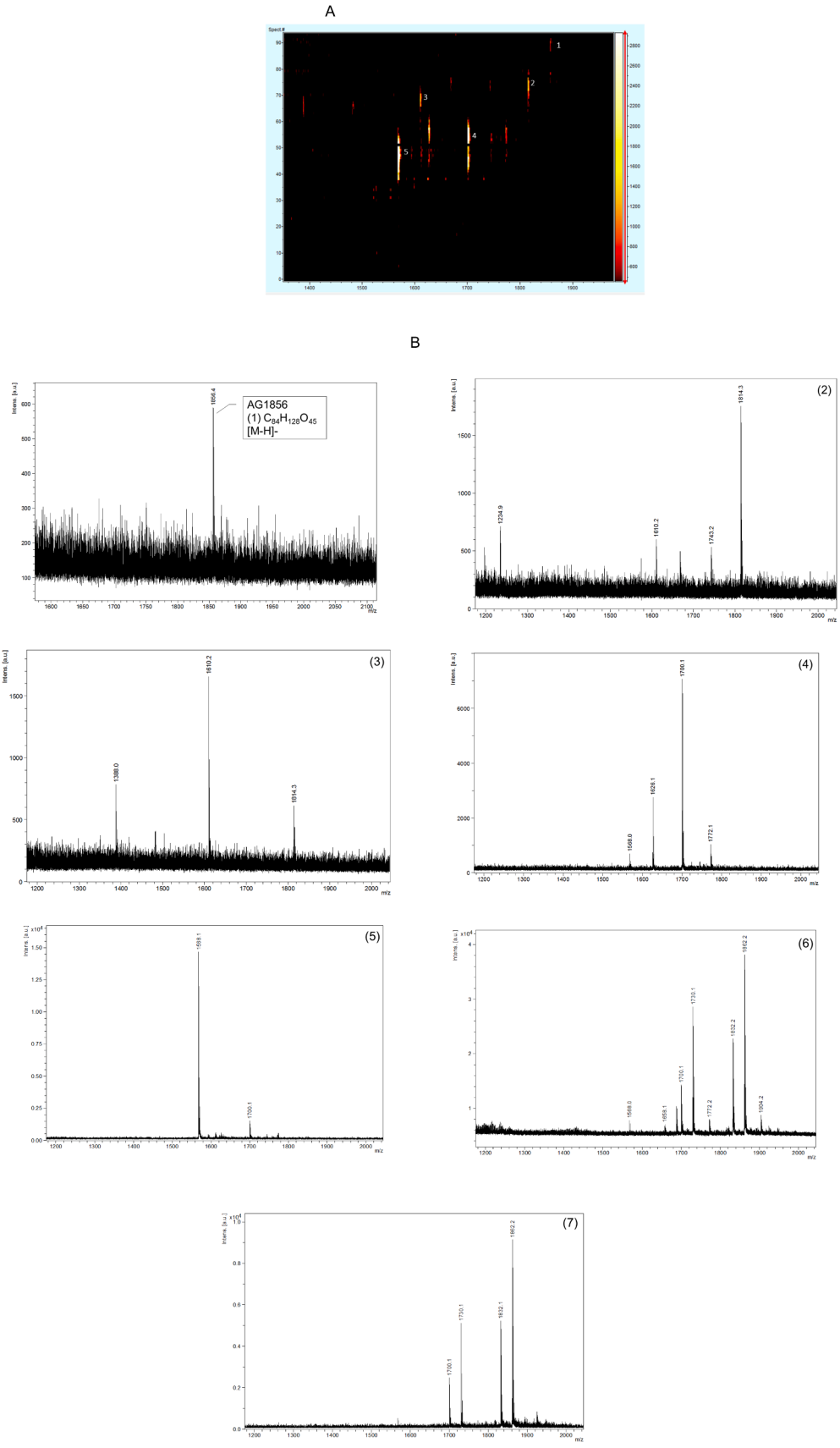

Fig. 2. TLC-MALDI of extract from *Agrostemma githago* L.
(A) Heat map representation of the separated (TLC) raw extract. (B) Different bisdesmosidic triterpene saponins were detected. The number in the MS-spectra indicates the position of the relevant triterpene saponin in Fig. 2A. Compound AG1856 (upper left) is a minor compound in *Agrostemma githago* L. (≤0.01 %) and has

recently been structurally characterized [16]. The last two rows (6 + 7) represent the reference extract of *Saponaria officinalis* L. (6) and commercial reference saponins (7) from *Saponaria officinalis* L. Both were applied after the TLC separation.

(*m/z* 312), codeine (*m/z* 300) and morphine (*m/z* 286) were identified (Fig. 1C). Considering the rather apolar eluent (acetone, toluene, ethanol), the migration order of compounds on the TLC plate is plausible, with morphine being the compound with the lowest R*f* value. This is due to the deprotonation of the phenolic hydroxyl group at the alkaline pH (ammonia, 26 %). The method is therefore also well suited to determine the dependence of the migration properties of compounds in extracts on the polarity of the mobile phase and thus facilitate structure-migration studies on TLC plates. As the matrix lines can be sprayed onto the TLC plate in virtually any geometry, the method is applicable for the optimization of 2D-TLC protocols. CsI is well suited for calibration purposes on TLC plates (Fig. 1C, 6), as it forms clusters both in positive mode as $[(CsI)_nCs]^+$ and in negative mode as $[(CsI)_nI]^-$ without the need for a matrix [18].

3.2. TLC-MALDI of triterpenes from Agrostemma githago L. and Saponaria officinalis L.

After applying our method to alkaloids from opium, we additionally analysed extracts from *Agrostemma githago* L., that contain a mixture of complex bisdesmosidic saponins. Based on the molecular ions (Fig. 2A/B), we were able to detect a number of these saponins, which are also described in the literature [16,19]. Beyond an assignment of the molecular identity based on molecular mass alone, MALDI-MS in case of doubt also allows this assignment to be confirmed by the detection of specific molecular fragments in MS/MS experiments. High-quality MALDI spectra with good resolution were obtained with the matrix line technique (Fig. 2B), however, on a critical note, we have observed that the mass accuracy is sometimes suboptimal and the recorded mass may vary slightly from plate to plate.

We hypothesize that this might have to do with the thickness of the silica layer, a corresponding unevenness of the surface after application of the matrix lines and a reduced conductivity of the silica TLC plate. Proper conductivity is essential for TLC-MALDI (reviewed in [14]). However, for the applicability of the proposed method these effects are not critical, since the aim rather is to give an overview of the complexity of extracts and to provide information about the position and migration profile (see above) of individual compounds on the TLC plate. Due to the thinness of the matrix lines applied by this technique—in contrast to plate-covering methods—the major part of the TLC bands is still accessible and even compounds such as triterpene saponins, whose detection on the TLC plate depends on UV quenching at 254 nm, might be directly obtained by scratching from the plate. The proposed technique is thus very helpful to optimize methods for the isolation of compounds via preparative TLC.

The fact that different MALDI matrices might be applied in parallel as lines perpendicular to the TLC bands provides a cost-saving approach to identify a suitable matrix on TLC plates for MALDI measurement. Depending on the bandwidth of the matrix lines, which is adjustable, a high number of matrices can be used in one analysis.

4. Conclusions

An improved, simple and low-cost method for applying the matrix in TLC-MALDI is presented. Matrix lines (DHB/HCCA) were applied perpendicular to the TLC bands. The method was used to analyze plant-derived complex mixtures from *Papaver somniferum* L. (opium alkaloids) and *Agrostemma githago* L. (triterpenes). The method enables the application of matrix, without obscuring the separation of the bands. This facilitates follow-up analyses such as TLC densitometry, the isolation of compounds directly from the TLC plate, an inexpensive identification of suitable matrices and the investigation of the dependence of the migration profile of compounds on the mobile phase.

CRediT authorship contribution statement

Alexander Weng: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **René de Vaumas:** Writing – review & editing, Resources. **Christoph Weise:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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