HPTLC 2024

ABSTRACT BOOK

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Invited

T1: Fundamentals of HPTLC

HPTLC NOW in FRANCE, a review. Recent development of the Club de CCM and perspectives

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This year is the 27th year of the Club de CCM activity, with two national congresses every year, including at distance during the pandemic, and eight international ones until Bangkok end November 2018.

Since the joined ISSS symposium in Ljubjana, in June 2020, we had the chance in the Club de CCM to welcome interesting contributions.

This lecture aims to make a short review of the Club de CCM activity and to place it within the current analytical global trend. It will also be replacing these contributions in the perspective of which kind of results we got in the past.

What do we may reasonably expect from our future in HPTLC facing the other analytical methods, especially chromatographic ones. Will our future be bright or dark? Might be cyclical.

Metabolomics and HPTLC - unveiling the complex nature of herbal medicines

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Potential therapeutic leads from herbal medicines needs thorough study of the chemical profiling for quality control that will allow scientists studying natural products with therapeutic activity. Standard metabolic profile technique must be established in order to assure the quality of herbal medicine. Correlating the chemical profile with the biological activity is crucial in order to ensure a medicine's safe and effective application.

Metabolomics is currently employed to address this complexity, following the trends in systems biology in the biological sciences. The goal of metabolomics is to thoroughly profile every molecule that could be present in a biological sample at a specific moment in time. Understanding the metabolic changes as a consequence of many factors may be gained by comparing the metabolomes of samples collected from organisms under different circumstances or samples taken at different times. To ensure the quality of medicinal plants, only a few chemical markers are utilized at the moment. Single markers are typically insufficient to capture the diversity of phytometabolites, even in trace levels at ppb or even lower level, given the immense diversity of phytochemicals. Thus its common practice to employ non-targeted metabolomic approaches to address the difficulties in mixes (quality and quantity).

NMR and MS-based analytical systems are widely acknowledged as standard techniques for metabolomics research where the dynamic range is comparable to other omics approaches, but the overlapping of peaks limits the resolution, making it difficult to identify and quantify metabolites. LC, GC, and MS (/MS) are examples of separation-based metabolomics techniques that are used to solve this issue. This makes it possible to quantify every chemical found in the mixture in absolute terms. Since there is no correlation between concentration and signal size, no one analytical method can fully satisfy all the requirements for thorough profiling; instead, quantitative analysis needs calibration curves for each and every molecule.

HPTLC is a simple, quick, affordable, repeatable technology with excellent selectivity for secondary metabolites. When hyphenated to DAD or MS detectors, it is a valuable technique for metabolomics since it is similar to quick parallel analysis of a large number of samples, spanning a broad spectrum of metabolites and with reasonably good signal robustness. This turns HPTLC into a fully automated, highly repeatable analytical instrument that may compete with other chromatographic methods to improve sensitivity and easier identification for both qualitative and quantitative study of complex mixtures of chemicals. Data processing is necessary for HPTLC profiling applications in order to facilitate statistical analysis, including chemo metric techniques such multivariate data analysis.

With all the recent advancements, there are many opportunities to apply HPTLC in the study of herbal medicine research. It can be used to analyse complex mixtures, fractions, and pure compounds qualitatively and quantitatively; it can also be used to control the identity and biological activity of herbal medicines to better understand the secondary metabolites.

T2: Advances in Instrumentation

Orthogonal high-pressure electrochromatography – New planar separation technique

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Based on our previous research on orthogonal planar pressurised electrochromatography (OPPEC), we present the latest results on the development of this technique by our team. We present the next stages of work on improving the device. This work led to the creation of a new device, which in the latest version is equipped with a planar column, but not with a chromatographic plate as before. The device is equipped with a high-pressure pump generating hydrodynamic flow in one direction and a high-voltage DC power supply generating an electric field perpendicular to this flow. This is the first report of this device, so we called this new technique orthogonal high-pressure electrochromatography (OHPEC). The device can be used to separate mixtures of substances on a preparative scale and can operate for a much longer time than the previous OPPEC device. A characteristic feature of the device is the separation of substances on a preparative scale with continuous supply of the separated mixture and continuous collection of separated substances. The device can also work in analytical mode, which will be the subject of further research by our team.

Acknowledgements
This work was supported by the National Centre for Research and Development, Poland, Agreement No. TANGO2/341314/NCBR/2017

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T3: Multidimensional and Hyphenated Techniques

Development and application of HPTLC – Bioautography-DART-MS techniques in quality evaluation of Chinese herbal medicines

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Keywords: traditional medicines, TLC bioautography, TLC-DART-MS, quality evaluation

It is a challenge to identify diagnostic chemical markers with relevant bioactivity from the complex metrics of traditional medicines for the purpose of quality evaluation. Thin-layer chromatography (TLC)-bioautography technique combing TLC/HPTLC separation with in situ bioassay developed in our laboratories. Taking the advantage of quick, simplified and economic operation of TLC/HPTLC and high-throughput biological assays, a series of TLC-bioautographic methods have been developed for determining the free radical scavenging, hemolysis and active enzyme inhibitors of acetylcholine esterase, xanthine oxidase, a-glucosidase, lipase, and dipeptidyl peptidase 4 in medicinal herbs.

Features like simultaneous analysis of multiple samples, visualization of results, and convenient post-chromatographic derivatization by diverse chemical reagents make TLC/HPTLC- bioautographic one of the most effective methods in bioactivity-guided identification of herbal raw materials, extractives and finished products. Recently, the hyphenations of HPTLC-bioautography-DART-MS was utilized for rapid screening and identification of characteristic and bioactive components. Some of the analytical methods have been applied in the monographs in Chinese Pharmacopoeia.

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T6: Advances in Detection Methods

Advantages, drawbacks and obstacles in post-chromatographic derivatization of bioactive compounds in planar chromatography

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Keywords: detection reagent, derivatization, bioactive compounds, densitometry

Post-chromatographic derivatization is often an indispensable step in (HP)TLC analyses. Usually it increases the sensitivity and selectivity of the (HP)TLC methods and consequently decreases possible interferences of bioactive compounds. Researchers are still not aware that temperature, the time of derivatization and the time after derivatization have a big impact on the stability and intensity of the derivatized chromatographic zones. Detection reagents used for post-chromatographic detection are prepared as dipping or spraying reagents. Considerably more chemicals are used for dipping reagents than for spraying reagents. Although manual spraying is performed by a skilled user the results obtained are not comparable with those obtained with dipping, where detection reagents are more evenly distributed across the whole plate. However, a Derivatizer (CAMAG), a device that automatically sprays the detection reagent over the chromatographic plate, enables low consumption of chemicals and homogeneous distribution of the detection reagent over the entire plate. The Derivatizer also protects the user from reagent's aerosols as the plate is closed in a special chamber during spraying.

The lecture will present advantages, drawbacks and obstacles in post-chromatographic derivatization of bioactive compounds in planar chromatography. Comparison of detection limits for some phenolic compounds obtained before and after post-chromatographic detection with anisaldehyde, vanillin, 4-dimetilaminocinnaldehyde on different stationary phases will be presented. Stability of derivatized chromatographic zones for some phenolic compounds will also be presented. Obstacles and challenges related to optimizing spraying reagent and spraying parameters (nozzle, level) for the Derivatizer (CAMAG) to detect bioactive compounds in invasive alien plant species tree of heaven (*Alianthus altissima* (Mill.) Swingle) will also be discussed.

Acknowledgments
This study was supported by the Slovenian Research and Innovation Agency (ARIS; research core funding No. P1-0005 research project J4-50138, research project N1-0235 and "Young Researchers" program - Maja Bensa) and the National Research, Development and Innovation Office of Hungary (NKFIH; project SNN139496).

T7: Effect-directed Detection

The power of HPTLC combined with effect-directed assays for bio profiling of marine algae extracts

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Marine algae or seaweed are among the ocean's most valuable source of bioactive compounds. They are growing abundantly in harsh marine environments of high salinity, high oxygen concentrations and extreme sunlight. The lack of damage in their structural components indicates the presence of highly effective antioxidants and free radical scavengers as well as anti-inflammatory compounds.

This study examines the effect of solvent and microbial fermentation on the phytochemical composition and bioactivity in a number of marine algae extracts via HPTLC microchemical derivatization and HPTLC bioautography. Microchemical derivatization with anisaldehyde/sulfuric acid was used to assess complexity of the extracts, DPPH assay to estimate and detect antioxidant activity, and enzymatic bioassay was used to estimate and detect anti-inflammatory activity via a COX-1 enzyme inhibition assay. The red alga sample was selected for further characterisation due to the higher amounts of bioactive compounds present.

The potency of the extract with the highest COX-1 inhibition was evaluated, with the high-maximal inhibitory concentrations (IC_{50}) determined by an experimental HPTLC-based procedure, developed in our lab. The IC_{50} values for COX-1 enzyme inhibition, from both fermented and nonfermented extracts, were significantly lower when compared to IC_{50} for salicylic acid which was used as a reference standard.

Five chromatographic zones exhibited bioactivity, zones 1, 3, 5 and 7 with antioxidant activity and zones 5, 6 and 7 with anti-inflammatory activity. Compounds from bioactive zones were isolated using preparative TLC and flash chromatography, and further characterised using Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS).

High-performance thin-layer chromatography: The technique of choice for bioactive compounds detection

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Keywords: HPTLC, mass spectrometry, EDA

High-performance thin-layer chromatography (HPTLC) has proven to be the technique of choice for detecting and identifying bioactive molecules due to its unique features, such as the ability to perform post chromatographic derivatization including effect-directed analysis (EDA).

This presentation addresses some examples of HPTLC technique used to detect and identify bioactive molecules with positive and negative effects on human health. In the first case, a novel method to determine acrylamide will be presented. Acrylamide is an organic compound with low molecular weight, water-soluble, and high polarity. It is known for its neurotoxic, genotoxic properties and potential carcinogenesis. This new method is based on a derivatization reaction with a fluorescent coumarin reagent through a Michael addition. Critical reaction factors, i.e. temperature and time were optimized using a design of experiment to improve the efficiency of acrylamide derivatization.

For the second case the detection of bioactive molecules with therapeutic potential against chronic non-communicable diseases (CNCDs) and neurodegenerative diseases, particularly those with inhibitory activity over key enzymes such as acetylcholinesterase, alpha-glucosidase, and cyclooxygenase will be presented. CNCDs are pathologies characterized by long duration and slow progression. It is estimated that CNCDs are responsible for 41 million deaths per year, representing 74% of all deaths worldwide. In this context, the analysis of diverse matrices, including foods, plants, and microorganisms, will be presented, as well as the application of HPTLC/bioassay/MS to establish the role of gastrointestinal digestion as a novel source of bioactive molecules with impact over CNCDs.

Acknowledgments
Authors want to thank Chilean National Agency of Research and Development (ANID) for financial support, projects FONDECYT Nº1211803; 1201658, and 1200485, FONDEQUIP EQM210203 and EQY220018.

Bioprofiling of various cultivars of *Acinidia arguta*

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Keywords: *Actinidia arguta*, TLC, EDA

Kiwi (Actinidia), the fruits very popular on the European market, have long been used in the Traditional Chinese Medicine due to their pharmacological properties. Currently, they are known mainly for their flavor properties as natural antioxidants and as food additives [1]. Thanks to their enzymatic properties, they also serve to aid digestion.

Actinidia arguta, commonly known as mini kiwi, is a species relatively recently introduced to the market on a larger scale. The fruits of *A. arguta* can be treated as a healthy "super snack" not only because of health-promoting properties (antioxidant, antidiabetic, anticancer, antimicrobial and anti-inflammatory) but also because of small size and thin, edible peel [2].

Bioprofiling of ten various varieties of *A. arguta* was performed using thin layer chromatography combined with biological tests performed directly on a TLC plate followed by UV-VIS and LC-MS analysis. This effect-directed analysis enabled detection of active zones in both fruit and green shoot extracts indicating the presence of antioxidants and inhibitors of enzymes: glucosidase, acetylcholinesterase, butyrylcholinesterase, tyrosinase and lipase.

The green shoots are characterized by a high content of polyphenols, while the fruits contain a number of organic acids such as: ascorbic, citric, malic, oxalic, quinic and lactic. The first one mentioned exhibits the greatest biological activity.

Acknowledgments
Thanks to prof. Agnieszka Szopa from UJ Cracow, Poland, for providing plant materials.

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HPTLC-bioautograpic detection and identification of anti-*Neisseria gonorrhea* from Thai medicinal plants

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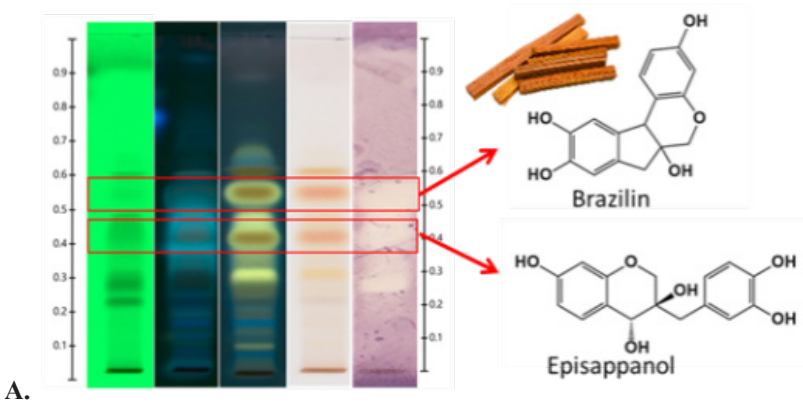
Keywords: anti-*Neisseria gonorrhea*, Thai herbs, active constituents, HPTLC-bioautograpy

Gonorrhea is a sexually transmitted disease caused by the obligate human pathogen *Neisseria gonorrhoeae*. *N. gonorrhoeae* is one of the 8 most frequently encountered pathogens in specimens collected from 65 different countries territories and areas (WHO 2020). Recently, *N. gonorrhoeae* strains with high-level resistance to azithromycin and the extended-spectrum cephalosporin emerged. Antimicrobial resistance against *N. gonorrhoeae* is a major problem for the treatment of the disease worldwide, and has been listed 2017 on the priority pathogens list for R&D of new antibiotics (priority 2: high) (WHO 2016). In this study, a technique of HPTLC-bioautography was developed to screen, detect and identify active extracts and constituents prepared from various Thai medicinal plants. Herbal extract samples were applied to a HPTLC plate and developed with suitable mobile phases. The potential inhibitory activities of the samples were assessed by culturing *N. gonorrhoeae* in GC agar base medium using a swabbing technique. The developed HPTLC plate was overlaid with GC agar and incubated for 24 hours. The plate was visualized by spraying with MTT reagent followed by 2-3 h incubation. Inhibition zones with white spots on a purple background indicate the positive anti-*N. gonorrhea* activity. The developed HPTLC bioautographic method was used to search for active anti-*N. gonorrhea* activity from 50 Thai herbal extracts. It was found that 10 out of the 50 samples showed good inhibitory activity. The positive bands were then identified for their structures by MS analysis as shown in Fig. 1.



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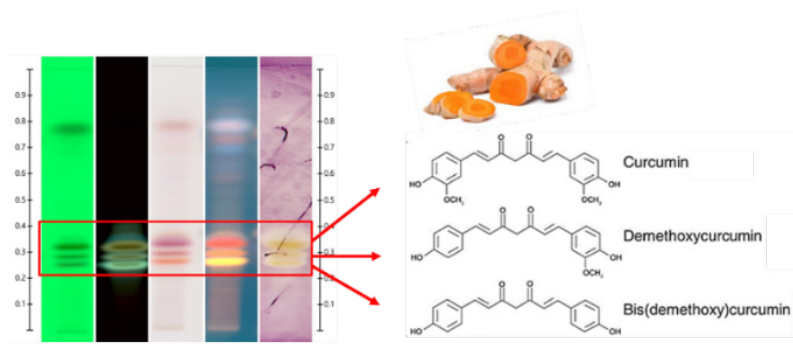

B.

Figure 1. TLC bioautography chromatograms show the anti-Neisseria gonorrhea activity of ethanol extracts of *Caesalpinia sappan* (A) and *Curcuma longa* L. (B).

Acknowledgments

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Previously unknown hazardous effects of food and cosmetics detected with innovative prioritization strategy

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Keywords: genotoxins, genotoxicants, endocrine disrupting compounds, cytotoxins

The rapid non-target planar bioprofiling of complex samples for hazardous compounds provides much more and, above all, new information compared to the status quo. The samples were analyzed as completely as possible, as almost no sample preparation was performed.
The investigated oils/fats [1, 2], cosmetics [3] and perfumes [4] contained genotoxins, *i.e.* (ep)oxidized fatty acid structures as well as mineral oil residues (MOSH/MOAH) and chlorinated paraffins [1–3]. Skin care products showed extreme genotoxic responses. In perfumes, endocrine disrupting, genotoxic, cytotoxic, neurotoxic and antibacterial compounds [4] were detected, exemplarily analyzed with several planar bioassays. Hence, a reassessment of the risk of topical application and oral intake of the tested products is required. No significant detoxification by simulated on-surface S9 liver metabolization was observed [2-4]. Effects were distinguished from opposite/cytotoxic responses due to planar separation followed by the on-surface bioassay [5, 6].
A new sustainable and portable 2LabsToGo system can be used for cost-effective analysis in routine. It takes 17 min per sample (of which 10% are manual operation), 0.5–1 € consumables per sample and 3600 € investment costs for the latest 2LabsToGo system [7, 8].

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T8: Food Quality and Safety

High-performance thin-layer chromatography – an useful technique for food authentication/adulteration

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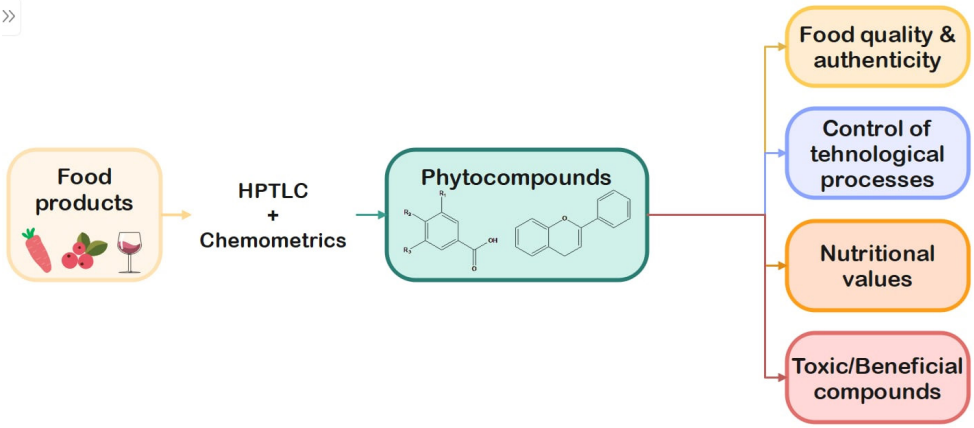
Keywords: HPTLC, food, authentication, polyphenolics, chemometry

The analysis of food products is important for the assessment of food quality and authenticity, the control of a technological process, the determination of nutritional values, and the detection of compounds which could exert beneficial or a toxic effects on human health. Techniques which are usually chosen in these purposes must provide accurate and reliable results, being relatively simple and inexpensive to perform.

Food products contain many classes of valuable compounds, which can also exert different biological activities, such as antioxidant and antimicrobial activity. Since foods contain important phytocompounds, they are often adulterated in economical purposes, not only by the botanical and geographical origin, but also by adding or subtracting of different substances which affect in a negative manner their quality.

High-performance thin-layer chromatography (HPTLC) is a widely used method that has increased the interest of researches in the las decades, being a fast and relatively inexpensive method of separating complex mixtures. With the high development of stationary phases and the possibility to be combined with accurate detection equipment and chemometrics, the technique is still increasing its uses in many research fields.

The aim of this paper is to overview the HPTLC methods developed by my research group, which are applied in the analysis of foods, in order to evaluate their composition and also to detect the adulteration. The focus will be on the bioactive compounds that are most abundant in foods of vegetable origin, i.e. phenolics, anthocyanins, etc. Moreover, a very important trend, such as the combination between HPTLC-image analysis and chemometrics will be presented.


Food products
HPTLC
+
Chemometrics
Phytocompounds
Food quality & authenticity
Control of tehnological processes
Nutritional values
Toxic/Beneficial compounds

Screening methods based on planar chromatography for modern food analysis

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Keywords: screening methods, high-performance thin-layer chromatography, planar solid-phase extraction (pSPE), food analysis, food fraud, food quality, food safety

Food safety has always been a crucial issue, and ensuring it is an important task for consumer protection. However, these days, ensuring safe and high-quality food is becoming increasingly challenging as the number of undesirable bioactive compounds in food that can cause adverse or even toxic effects in humans is growing. In addition, more and more such undesirable compounds are being discovered. Substances of concern include pesticides, mycotoxins, heavy metals, saturated and aromatic mineral oil hydrocarbons (MOSH and MOAH), chlorinated paraffins (CP), nonylphenols (NP) or phytotoxins such as pyrrolizidine alkaloids (PA) and tropane alkaloids (TA). For many residues and contaminants, maximum levels for certain foods are set. All these analytes also have to be determined in ever shorter times. Fast and easy-to-use screening methods are therefore becoming increasingly important in analyzing food ingredients. This applies in particular to the analysis of residues and contaminants, which can affect food safety.

The versatility and easy accessibility of planar chromatography provide an extremely interesting, highly effective, and efficient way of meeting all these analytical requirements. It saves time, is environmentally friendly, easy to perform, cost-effective, suitable for a wide range of analytical tasks, and can be used for all types of contaminants and any relevant food ingredients. Reliable screening methods for food and consumer good-relevant ingredients can be developed using high-performance thin-layer chromatography (HPTLC) as a modern and versatile method for cost-effective analysis and planar solid phase extraction (pSPE), which is based on HPTLC technology and is often coupled with mass spectrometry (MS). Examples from various areas for detecting food fraud, testing food quality, and ensuring food safety will be discussed.

T10: Botanicals and Traditional Medicines

HPTLC methods for quality control of single drugs and compound formulations of Indian system of medicine

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Keywords: HPTLC, Indian system of medicine, Unani, Ayurveda, bioautography

The global acceptability of herbal drugs and botanicals is the major concern in absence of their proper quality control analysis. The quality control analysis of herbal drugs involves various parameters including chromatographic and spectroscopic techniques. Now a days chromatographic hyphenated techniques are very efficiently used in quality control of herbal drugs and botanicals to analyse different markers responsible for ensuring correct identity as well as to establish the exact mechanism of their action. The HPTLC being an easy, economic, less time consuming and matrix friendly have played a larger role in quality control of herbal drugs and botanicals but proper and exact active metabolite based development of analytical method is essential.

Since, Indian traditional medicines are composed of different types of drugs and formulations including, solid, liquid, semi-solid, sugar based, mineral and animal origin drugs containing, single drug and or poly-herbal, herbo-minerals etc. HPTLC analytical methods have been developed for various single drugs and compound formulations in our laboratory for fingerprinting and quantification of metabolites of Ayurvedic and Unani drugs and formulations for their quality control. Further, TLC bioautographic methods have also been developed for some drugs to identify their bioactive metabolites. HPTLC methods have also been developed for distillate based formulations (Arqiyat) using their non polar solvent fractions.

Currently, HPTLC and MS based hyphenated techniques have been used to analyse targeted and untargeted metabolites and may solve the issues of quality control of traditional herbal drugs and botanicals in future.

T11: Natural Product Analysis

Utilising the full potential of HPTLC in analyses of bioactive compounds

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Keywords: HPTLC, densitometry, image analysis, mass spectrometry, effect-directed analysis

It seems that while experiencing the rapid development of various technologies, we are rapidly losing very important skills of observations and descriptions of the experiments performed so that everyone can repeat our experiments. Therefore, a lot of chemicals, other consumables, instruments' working hours and researchers' work are wasted daily all over the world. Reading scientific articles from different fields that include investigations performed by TLC or HPTLC shows that still nowadays there is a lack of knowledge about performing experiments correctly and/or describing experiments in such a way that other researchers can easily repeat them.

The aim of this lecture is to highlight some of the issues in development of methods based on HPTLC-densitometry, HPTLC-image analysis, HPTLC-MS^n and (HP)TLC–EDA (EDA: effect-directed analysis) for targeted and non-targeted analyses of antioxidants, antimicrobial compounds, enzyme inhibitors and other bioactive compounds in crude extracts of invasive alien plant species (Japanese knotweed, giant knotweed, Bohemian knotweed, tree of heaven) and food samples. Examples will include issues related to the stationary phase, pre-development of the plate, saturation of the developing chamber, drying conditions, derivatization conditions, documentation conditions, stability of the analytes, ion-suppression, etc. Optimization of all parameters is crucial in utilising the full potential of HPTLC in analyses of antioxidants, antimicrobial compounds, enzyme inhibitors and other bioactive compounds in plant and food samples.

Acknowledgments
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T18: Life Sciences

Lipidomic study of the incorporation of erucic acid in the biosynthesis of triacylglycerols during the maturation of Pennycress seeds (*Thlaspi arvense* L.)

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Keywords: *Thlaspi arvense*, seed, oil, TAG, erucic acid, lipidomics, LC-MS, HPTLC-MS

Thlaspi arvense (Pennycress) is an emerging raw material for biofuel production due to the high oil content of its seeds, enriched in erucic acid. Triglycerides (TAG) constitute 80-90% of the total lipids and are its main reservoir of erucic acid. We studied the mechanisms of incorporation of erucic acid into TAG, in function of the maturation of the Pennycress seed. We have carried out an exhaustive lipidomic study to analyze the glycerolipid content and the distribution of acyl groups during the different stages of seed maturation. Lipidomics is based on both LC-MS with triple quadrupole (QqQ) and HPTLC-MS with ion trap (IT). The quantitative profiles of the glycerolipid classes and their corresponding molecular species were obtained by LC-ESI (+)-MS/MS (QqQ) using Multiple Reaction Monitoring (MRM). In the case of HPTLC-MS (IT), the structural identification of the TAG molecular species was carried out by obtaining the MS, and selected ion MS/MS spectra directly from the separated band of TAG on the chromatographic plate, using an automatic interface based on band extraction/elution. This has made it possible to obtain detailed molecular information, enabling in some cases a positional analysis of the acyl chain in *sn*-2 in TAG. Specifically, it has been shown that 18:2 is in the *sn*-2 position in some of the TAG species abundant at all stages of maturation. Likewise, normalized HPTLC-ESI (+)-MS intensity profiles were also obtained, providing the relative abundance of TAG species. The profiles obtained by HPTLC-MS were correlated with the quantitative results obtained from LC-MS. Results from LC-MS and HPTLC were coherent and complementary. The results of both techniques confirm the incorporation of 22:1 to the TAG already in the initial stage of seed maturation (G), as well as the increase of 20:1, 22 :1 and 24:1 species as maturation increases.

This study was completed with an integrative transcriptomic analysis of gene expression to identify which genes of which TAG biosynthetic pathways in seed oil are acting at each maturation stage. Combined lipidomic and transcriptomic results point to a model in which

strong temporal coordination between pathways and isoforms in each pathway, both in expression and incorporation of the acyl group, contributes to the synthesis of erucic TAG in Pennycress [1].

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T21: Validation and Chemometrics

Green aspects of planar chromatography

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Keywords: HPTLC, green method, greenmetrics assestmant, TLC-electrochemical detection, chemometrics

The adoption of green analytical methods is revolutionizing research in food, pharmaceuticals, and forensics by significantly reducing the use of toxic solvents, energy, and reagents. High-performance thin-layer chromatography (HPTLC) has demonstrated its effectiveness as a green analytical tool in food analysis, enabling the detection of fraud in apricot juice and identification of pumpkin adulteration at levels as low as 2.5%[1]. The lecture emphasizes the use of Natural Deep Eutectic Solvents (NADES) as a cutting-edge green solvent for extracting phenolic compounds from various food and natural product samples. This innovative approach, coupled with planar chromatography, is recognized as an environmentally friendly method for quality control and has been positively assessed using the National Environmental Methods Index (NEMI), eco-scale scale, and AGREE software[2]. A comprehensive green approach involved the utilization of HPTLC, regression multivariate tools, and molecular modeling to identify anti-aging compounds from herbal samples. This pioneering method accurately identifies single antioxidants and skin anti-aging related enzyme inhibitors, effectively reducing environmental impact by avoiding conventional bioactive isolation techniques. HPTLC has proven to be an effective and sustainable analytical approach in forensic laboratories, combining pattern recognition techniques to distinguish between drug-type and fiber-type *Cannabis sativa* L. chemotypes based on cannabinoid profiles, thereby demonstrating its potential for extensive forensic applications[3]. Furthermore, a novel analytical approach integrating TLC with electrochemical detection was developed to detect separated bioactives from food matrices, as exemplified with phenolic compounds from apple peels. This eco-friendly approach allows for efficient bioactive detection with minimal solvent consumption, setting the stage for greener analytical practices. These advancements underscore the vital importance of green analytical methods in fostering sustainable research practices across diverse scientific disciplines, thereby facilitating enhanced bioactive compound analysis and environmental stewardship.and environmental stewardship.

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ORAL

T3: Multidimensional and Hyphenated Techniques

High-performance thin-layer chromatography coupled with direct analysis in real time high-resolution mass spectrometry for the analysis of enzyme inhibitory compounds

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Keywords: high-performance thin-layer chromatography, direct analysis in real time, high-resolution mass spectrometry, effect directed analysis, enzyme inhibitors

The majority of established planar enzyme inhibitory assays use spectrophotometric detection using chromogenic or fluorogenic substrates. However, also mass spectrometric detection has potential, as many enzyme substrates and the respectively formed products are ionizable and thus selectively detectable. The enzyme inhibitory compounds in samples can be detected both indirectly via the decrease in substrate and directly via the product formed. This is of particular interest when structural changes are minor, *i.e.* only one double bond is reduced enzymatically.

For such cases, hyphenated high-performance thin-layer chromatography coupled with direct analysis in real time high-resolution mass spectrometry (HPTLC-DART-HRMS) was studied and shown to be advantageous. For this a sufficiently high ionizability and thermal desorbability of the substrate/product from the silica layer was achieved. Moreover, the limited thermal desorption [1] of polar assay media and substances with higher molecular weights (such as enzymes) was advantageous and led to a reduction of background signals. These remained on the silica gel layer and did not enter the HRMS. Finally, occuring mass interferences were minimized by fragmentation in the collision cell. Depending on the substrate, detection limits down to the lower nanogram per band range were achieved.

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T7: Effect-directed Detection

The impact of external factors on the bioautography tests of α-glucosidase, acetylcholine esterase, and COX-2 inhibition in seedy banana extracts.

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Keywords: phenolic compounds, seedy banana, bioautography, enzyme inhibition essay

Seedy bananas, *Musa acuminata*, *Musa initirans,* and *Ensete glaucum*, are used as a traditional medicine, and the foundation of their pharmaceutical properties still needs to be understood. We are currently investigating if extracts of seedy banana show inhibitory activity on enzymes (acetylcholine esterase, α-glucosidase, COX-2) using HPTLC-bioautography, and on which compounds this pharmaceutical activity is based. For a reliable test for inhibiting compounds, enzyme activity must not be limited by other factors, and positive signals must not originate from chemical reactions. In our work, we noticed that these requirements are not necessarily met in the current protocols and present steps to identify these issues and how they can be overcome.

The activity of enzymes is pH-dependent, and a too acidic environment can reduce and even inhibit enzyme activity. For the separation of phenolic compounds in the extracts of seedy bananas, formic acid had to be added to the developing solvent, which had to be removed or neutralized completely for a valid inhibition test. In the acetylcholinesterase test, we observed a strong dependence in the observed enzyme inhibition on the neutralization protocol. The pH of the neutralizing buffer and the number of neutralization treatments are important for a successful determination.

In the enzyme tests, a dye is generated from naphthol, which is released by active enzyme, and Fast Blue B. The amount of Fast Blue B that is applied to the plate can affect the visibility of enzyme inhibition. The use of an excessive amount of Fast Blue B can lead to unwanted chemical reactions with analytes that also result in a stain. Therefore, the amounts of precursor and of plant extract extract need to be adjusted to be high enough to allow the detection of inhibiting compounds and to be low enough to avoid chemical reactions.

With the adjusted conditions, enzyme inhibitors were detected in the banana extracts. These were identified as stilbenes and flavonoids by LC-MS/MS and NMR.

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T8: Food Quality and Safety

Strategy to detect low levels of genotoxic chemicals in recycled plastic packaging using HPTLC

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Keywords: recycled plastic packaging, *in vitro* genotoxicity, planar chromatography coupled with bioassay, metabolic activation, fractionation

Virgin or recycled Food Contact Materials (FCMs) are perceived as sources of chemical non-food-related contamination, introducing safety uncertainties. Consequently, there has been an increasing demand to improve the hazard and risk assessment of FCMs. According to regulatory recommendations, the exclusion of mutagenicity in FCM is required. Therefore, the identification of mutagenic chemicals in FCMs is crucial to ensure food safety. According to a previous study using non-food grade recycling plastic materials (Mayrhofer et al., 2023), it was found that DNA-reactive contaminants may be formed during the recycling process. This publication highlights the need to identify the source of these critical contaminants to enable the future use of recycled plastic packaging in sensitive applications.

In this study, a new strategy was applied using flash purification system as a fractionation methodology combined with rapid biodetection monitoring p-UmuC bioassay (Debon et al. in 2022) and Ames-MPF genotoxicity screening method. Briefly, the extracts and fractions were directly applied onto a silica plate, subjected to chemical derivatization, and to p-UmuC bioassay allowing a rapid screening of the positive genotoxic fractions. The fractionation process allows the screening of genotoxic fractions to better target the chemical identification using biomonitored high-resolution mass spectrometry. This approach provides information regarding the genotoxic potential of the chemicals presents and help for their identification and toxicological characterization. These data demonstrate the feasibility of the integrated approach combining biomonitoring and chemical analysis to address the genotoxic potential of FCMs. However, there are still quite a few challenges to solve to better understand the genotoxicity measured and to relate them to mutagenicity.

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Combining fingerprint and techno-functional properties of E 472b emulsifiers added to aerosol whipping cream

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Keywords: food emulsifiers, densitometry, Partial Least Squares (PLS), fingerprint analysis, spectral similarity

E 472b emulsifiers are used in various foods, such as dairy products, bread, and ice cream, to adjust techno-functional properties [1]. They are defined as lactic acid esters of mono- and diacylglycerols from edible fats and oils [2]. The emulsifier composition depends on the oil base and synthesis conditions; thus, variations can be expected. It is well known that deviations in the emulsifier composition lead to different product properties, especially viscosity properties, shown, e.g., for E 471 emulsifiers [3,4]. Therefore, methods for characterizing the emulsifier composition are essential to ensure consistent high product quality and to avoid complaints.

The current study aimed to develop chemometric methods based on the densitometric fingerprint of the emulsifiers. 21 emulsifiers were analyzed using a modified high-performance thin-layer chromatography (HPTLC) method by Schuster et al. (2023) [5]. The concept of spectral similarity, typically used for database comparison in mass spectrometry, was successfully applied to the analysis of E 472b emulsifiers to compare different emulsifiers and different batches of the same emulsifier, thus, demonstrating the potential use for incoming goods inspection. Furthermore, time-dependent deviations in the emulsifier composition during storage at elevated temperatures, simulating transportation processes, were investigated. To combine the densitometric fingerprint with the techno-functional properties, four E 472b emulsifiers and four E 472b emulsifier products were selected for further experiments in model aerosol whipping cream. After standardized manufacturing of the aerosol whipping cream, storage stability, expressed as mean particle diameter and apparent viscosity, and foam stability, expressed as overrun, foam firmness, and drainage, were investigated. Based on the measured values, Partial Least Squares Regression (PLS) was used to estimate a model to predict these values based on the densitometric fingerprint.

To conclude, the study shows a comprehensive approach to analyzing and understanding the properties of E 472b emulsifiers in food products by HPTLC. Investigation of inter-batch deviations of the same emulsifier is enabled, highlighting a possible use for incoming goods inspection. In a first attempt, it was shown that techno-functional properties can be estimated based on the densitometric fingerprint by PLS. To further confirm the results, more emulsifier samples need to be investigated in the scope of a large-scale interlaboratory ring trial, which is beyond the scope and possibilities of the current study.

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T11: Natural Product Analysis

A validated method for identification and quantification of anthocyanins in different black rice (*Oryza sativa* L.) varieties using high-performance thin-layer chromatography

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Black rice (*Oryza sativa* L.) has gained prominence as a functional food due to its rich content of anthocyanins and polyphenols, offering potential health benefits. However, comprehensive research addressing the diverse anthocyanin compositions in black rice cultivars remains limited. This study aimed to quantify anthocyanin contents, specifically kuromanin, cyanidin-3-glucoside, peonidin-3-O-glucoside, peonidin-3-glucoside chloride, cyanidin-3-rutinoside chloride in 150 rice varieties sourced from the North Eastern Region of India using a robust High-Performance Thin-Layer Chromatography (HPTLC) method. Rice grains of varying colours-black, orange-reddish, and white-were subjected to methanol extraction under dark conditions through cold maceration for 72 hours. The resulting extracts underwent separation on HPTLC silica gel 60 F_{254} plates utilizing an optimized mobile phase of ethyl acetate, 2-butanol, formic acid, and water (9:6:3:3::v/v/v/v). Anthocyanins were detected and analysed via densitometry under white light illumination in transmission mode following development. Notably, anthocyanins were absent in grains of white and orange-reddish rice varieties, except for specific rice lines of Joha (JN 71, JN 83, JN 77, and JN 78) and all black rice variants. Among these, BR 15 exhibited the highest Kuromanin content (74.14 ± 0.82 µg/mg), while BR8 showcased the highest Peonidin-3-glucoside chloride concentration (27.59 ± 0.83 µg/mg). This comprehensive analysis provides detailed insights into the anthocyanin compositions of 15 significant black rice lines, offering crucial data for breeding programs targeting enhanced anthocyanin-rich cultivars and the development of functional foods.

Determination of sugar profile of Australian stingless bee honey by high-performance thin-layer chromatography

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Keywords: trehalulose, sugar, stingless bee honey, Australia, HPTLC

Stingless bee honey, known as sugarbag in Australia, has a history of traditional medicinal use among First Nations People. Despite its historical use, research on its physicochemical properties and bioactivities remains limited. Recent studies using ultra-performance liquid chromatography (UPLC-MS/MS) and NMR analysis have shown that trehalulose, not maltose as previously thought, is a primary sugar in this honey [1]. Trehalulose, a rare disaccharide and sucrose isomer, shows potential as a marker for authenticating stingless bee honeys and offers potential health benefits associated with its non-cariogenic and antioxidant properties, as well as its low insulinemic and glycemic index compared to sucrose.

To date, trehalulose in stingless bee honey has been identified and quantified using ultra-performance liquid chromatography (UPLC-MS/MS) and ion chromatography (IC-PAD). This study reports on an alternative, simple, and convenient approach using High-Performance Thin-Layer Chromatography (HPTLC) which allows not only to quantify trehalulose but also glucose, fructose, and sucrose in stingless bee honeys. The sugar profile of thirty-six Australian stingless bee honeys was determined using a validated method that employed silica gel 60 F254 HPTLC glass plates with 1-butanol–2-propanol–aqueous boric acid as mobile phase [2] and derivatisation with aniline–diphenylamine–phosphoric acid reagent.

The study found trehalulose concentrations in Australian stingless bee honeys ranging from 6.20 to 38.2 g/100 g, with fructose (7.79 to 33.4 g/100g) and glucose (3.36 to 26.8 g/100g) levels exhibiting a negative correlation with trehalulose. Interestingly, sucrose was below detectable limits, further supporting the hypothesis of trehalulose formation via an enzymatic reaction involving sucrose as the substrate [3].

The findings of this study demonstrate the consistent presence of trehalulose as one of the main sugars in all investigated Australian stingless bee honey samples, adding further weight to claims that this unusual sugar may serve as a potential marker compound for stingless bee honeys.

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T12: Pharmaceutical Applications

Planar chromatography (TLC/HPTLC) in the development and control of active pharmaceutical ingredients (APIs) and drug products (DPs)

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Keywords: TLC, HPTLC, analysis of APIs and DPs

Thin-Layer Chromatography (TLC) and its improved type High-Performance Thin-Layer Chromatography (HPTLC), known as fundamental chemical methods for separation of non volatile compounds in the complex mixture, are still very important tools in the pharmaceutical chemistry for APIs and DPs research. Several adventages, such as simple instrumentation and sample preparation, low cost and short time analyses give to those techniques significant role in the very early reasearch and development of drugs, also in the control strategy of the whole lifecycle of drug after marketing authorisation. TLC is still an official method for related substances in European Pharmacopoeia APIs monographs, and very often it is mandatory test for second identification of API in APIs or DPs monographs. TLC is used in the investigation of lipophilicity of newly synthesized drugs which determines its bioavailability. It can be used in force degradation studies of API and DP for fast prediction of degradation potential of API/DP and their stability. HPTLC hyphenated with some hybrid techniques for detection (e.g. tandem mass spectrometry (MS/MS), Fourier Transform Infrared spectroscopy (FTIR) etc.) is very useful for the very early identification of potential genotixic impurities with structural alerts, which could be of toxicological concern. TLC can be used in order to confirm some therapeutic effect (antioxidative, antifungal, antibacterial charscteristics) and to investigate drug metabolites in early pharmacokinetic studies. In the routine analysis, planar chromatography could be used in enantioseparation of hiral compounds, assay of API in APIs and DPs, determination of impurities in starting materials, intermediates , reagents of the synthesis of API, also to determine purity of API and DP. TLC is used as in process control in the synthesis of APIs in order to follow kinetic of reaction. Finally, after all, TLC, specially HPTLC, has high green chemistry potential as considerably less quantity of solvents are used compared to HPLC.

Analytical quality by design approach supported robust HPTLC method for assay of Metoprolol, Chlorthalidone, and Cilnidipine in tablet dosage forms

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Keywords: AQbD, Central Composite Design, HPTLC, Plackett-Burman Design

The simultaneous quantification of Metoprolol (MTP), Chlorthalidone (CTD), and Cilnidipine (CLD) in pharmaceutical formulations was achieved by the development and validation of a simple and rapid HPTLC approach using densitometric analysis. The Analytical Quality by Design strategy has been used to accomplish an adequate retardation factor, theoretical plate, and resolution between peaks by optimizing chromatographic variables such as mobile phase composition, chamber saturation time, and development distance. The Analytical Target Profile (ATP) and Critical Analytical Attributes (CAAs) for the analytical method were determined in this study. High-risk analytical conditions were distinguished through risk assessment studies and screened using Plackett-Burman Design and optimized using Central Composite Design to obtain the Method Operable Design Region (MODR), which gave the optimal mobile phase composition of hexane: ethyl acetate: methanol: ammonia (3.9:5:1.1:0.1 v/v/v/v), with silica gel 60 F_{254} plate as stationary phase, development distance of 80 mm, chamber saturation time of 20 min and detection wavelength at 230 nm. The results showed that the retardation factors for MTP, CTD, and CLD were 0.19±0.02, 0.49±0.02, and 0.82±0.02 respectively. The optimized method was validated according to ICH guideline Q2(R2), and statistical investigation revealed that the method is robust, specific, sensitive, accurate, and precise. The proposed HPTLC approach is a convenient and cost-effective method for routine quantitative estimation of MTP, CTD, and CLD containing marketed formulations.

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AQbD-based HPTLC method development for simultaneous quantification of alpha arbutin and niacinamide in nanocosmaceutical

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Keywords: AQbD, alpha arbutin, microemulsion, niacinamide

The purpose of this study was to develop a new failure mode effective analysis and design of experiment-based robust high-performance thin-layer chromatographic (HPTLC) method for separation and quantitation of alpha arbutin and niacinamide in a combined topical microemulsion formulation. Based on prior knowledge and experimentation, various risk factors were identified using the Ishikawa diagram. Risk assessment was done by assigning a criticality score to each failure mode based on prior experimental trials. Factors that have a score of more than sixty were further assessed and analyzed using the Placket-Burman screening design. From the factors studied, the volume of methanol, the volume of ethyl acetate, and plate activation time were found critical failure modes. These factors were further optimized using a central composite design to study and understand their effect on resolution between alpha arbutin and niacinamide. By implementation of design space, a control strategy was established for the development of the HPTLC method for simultaneous estimation of alpha arbutin and niacinamide. The optimized HPTLC method was validated according to International Conference on Harmonization (ICH) guideline Q2(R1). The results of the study clearly show that the analytical quality by design approach was successfully applied to optimize the HPTLC method. This developed and validated HPTLC method was successfully applied for the quantitative estimation of alpha arbutin and niacinamide in their combined topical microemulsion formulations.

Acknowledgment
The authors are grateful to Ramanbhai Patel College of Pharmacy and Charotar University of Science and Technology (CHARUSAT) for providing access to sophisticated instrumentation facilities.

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POSTER

T1: Fundamentals of HPTLC

Sustainable solvents in thin-layer chromatography: Evaluation of separation performance and environmental compatibility

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This scientific poster presents a comprehensive evaluation of the application of sustainable solvents in thin-layer chromatography (TLC), focusing on both their separation performance and environmental compatibility in comparison to conventional solvents. The study assesses the use of sustainable solvents, such as methanol, ethanol, isopropanol, acetone, as well as green alternatives for ethyl acetate and acetonitrile. The comparison is conducted using the AGREE (Assessment of Greenness through Reactions) methodology to provide a standardized and comprehensive sustainability assessment.

The research includes application examples demonstrating the benefits of these sustainable solvents. To ensure a reliable comparison of separation performance, a commercially available TLC standard mixture is employed. The results provide valuable insights into the potential of sustainable solvents for environmentally conscious thin-layer chromatography, representing a significant step in promoting environmentally friendly laboratory practices.

Effect of humidity on different fluorescent dyes during the analysis of MOSH/MOAH using pSPE-UV/FLD

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Keywords: MOSH, MOAH, pSPE

Mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) can contaminate foodstuffs through various means. These include migration out of packaging through the gas phase. According to the latest opinion of the European Food Safety Authority (EFSA), MOSH are regarded as less harmful to humans than previously reported. However, the three or more ring MOAH are still classified as probably carcinogenic [1]. Therefore, the analysis of MOSH/MOAH continues to be in demand. The analytical method of choice for MOSH and MOAH is on-line liquid chromatography-gas chromatography with flame ionization detection (LC-GC-FID) [2]. As a fast and more economical method, a planar solid phase extraction (pSPE) screening method was developed [3]. Using this method, many samples can be purified and analyzed simultaneously [3]. Detection on the plate for MOAH is carried out via absorption at UV 254 nm and for MOSH via fluorescence under UV light, which was obtained after impregnation with a fluorescent dye to gain a fluorescence signal [3]. Primulin was used for this purpose; however, the fluorescence activity strongly depended on humidity [3]. Fluorescence quenching occurred at relative humidity (RH) above 50% [3]. For this reason, alternative fluorescence dyes were investigated. Berberine and coralyne chloride showed similar intensities to primulin, but the plates exhibited more intensive background noise. Measurements at RH above 50% showed no fluorescence quenching for plates impregnated with berberine or coralyne chloride. Measurements of single standards, mix standards, and samples at different humidities with the different derivatization reagents demonstrated that berberine- and coralyne-impregnated plates could be used to determine MOSH without the limitation of humidity control.

Acknowledgments
The authors thank Merck, Darmstadt, Germany, for supplying plate material.

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Influence of relative humidity on the thin-layer chromatographic separation of E 472 emulsifiers

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Keywords: food emulsifiers, relative humidity (RH), E 472

In addition to the mobile and stationary phase, in high-performance thin-layer chromatography (HPTLC), the separation behavior of analytes is influenced by the relative humidity (RH) used to adjust plate activity [1,2]. This influence was investigated for the separation of different E 472 subgroups. E 472 emulsifiers refer to short-chained organic acid esters of mono- and diacylglycerols (MG/DG) from edible fats and oils [3]. Depending on the acid used (acetic acid, lactic acid, citric acid, tartaric acid, and mixtures of acetic and tartaric acid), the E 472 emulsifiers are divided into subgroups a-f, which also differ significantly in terms of the complexity of their composition [4].

The chromatographic conditions used were selected according to Schuster et al. [5]. A total of eight different saturated salt solutions and seven sulfuric acid solutions of varying concentrations (10-70% sulfuric acid) were used to investigate the range between 3 and 96% RH. Various options were explored for adjusting the RH, including development in the ADC2 (Automatic Developing Chamber 2) with a five-minute RH adjustment, chamber saturation for 15 minutes before development, and pre-conditioning of the plate for 15 minutes before development.

Acknowledgments
The authors thank Merck, Darmstadt, Germany, for supplying plate material.

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Establishment and application of a activity screening system for natural antibacterial ingredients based on HPTLC-bioactivity assay-MS technology

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Keywords: HPTLC-bioassay, *vibrio fischeri*, activity screening, TLC-MS

The proliferation of drug-resistant bacteria caused by the widespread use of antibiotics is a global threat. The development and use of new antibiotics are time-consuming and costly, and can lead to further resistance. Perillae folium is the dried leaf of *Perilla frutescens* (L.) Britt. The volatile oil from perillae folium has good antibacterial activity, but its composition is complex and there are significant differences between different varieties of Perillae folium.

High performance thin layer chromatography (HPTLC)-bioassay is a technique that combines HPTLC separation with bioactivity assay. If combined with modern separation and analysis techniques, it can achieve rapid discovery, separation, and identification of active compounds in complex systems such as traditional Chinese medicine.

This paper selected the antibacterial mode organism *Vibrio fischeri* as the research object and developed a HPTLC- bioassay method. A rapid screening system for antibacterial components was constructed using "HPTLC-bioassay-MS". 6 compounds with antibacterial activity including Perillaldehyde, Perillaketone, Eugenol, Elemicin, Hexahydrofarnesyl acetone and Sageone in volatile oil from perillae folium were screened out and identified through the system.

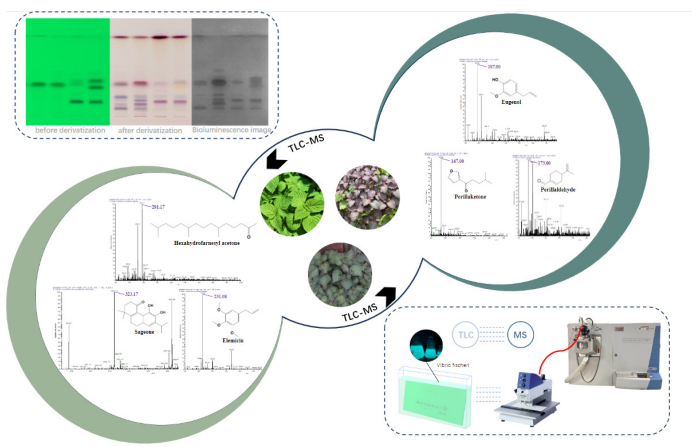

Figure 1 Establishment and application of a activity screening system for natural antibacterial ingredients based on HPTLC-bioassay-MS technology

T2: Advances in Instrumentation

Easy chemical derivatization of thin-layer plates *via* the gas phase

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Keywords: dynamic vapor sorption, infrared spectroscopy, organosilane, paraben, wettability

The use of reversed-phase stationary phases, i.e., silica that has been modified with organic substitutents, plays only a minor role in thin-layer chromatography, and opportunities for adjusted selectivity are therefore largely missed. We have developed a very facile approach to modify silica with organic substituents by derivatization *via* the gas phase [1]. Normal-phase silica plates are exposed to a volatile siloxane reagent in a closed container. To investigate the feasibility of the approach, methyltrimethoxysilane (MTMS) was used to introduce methyl groups onto the silica particles' surface.

The obtained modified plates were very hydrophobic, with a water contact angle of about 135°. They were further characterized by infrared spectroscopy and dynamic vapor sorption. The chromatographic performance of these plates was compared to commercial C18 plates and to plates that were non-covalently modified by impregnation with paraffin (Figure 1). Due to the intense hydrophobization, the gas-phase derivatized plates could not be used with pure water as the eluent. The height of a theoretical plate was comparable to that of a commercial C18-plate and better than that of a paraffin-coated plate. The gas-phase derivatized plates offered some selectivity to separate the isomeric analytes *n*-butylparaben and *iso*-butylparaben, which shows an additional regioselective separation mechanism that is not available on a C18-plate.

The presented derivatization approach can easily be extended to more complex organic modifiers, for example phenyl groups, to obtain additional interactions for improved separation.

Figures

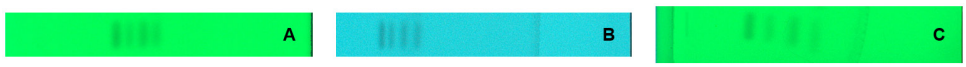

Figure 1. Separation of five parabens on a TLC plate derivatized with MTMS *via* the gas phase (A), a commercial C18-plate (B) and a plate impregnated with paraffin (C).

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2LabsToGo – HPTLC for everyone

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Keywords: HPTLC, Office Chromatography, open-source, portable

Office Chromatography (OC) aimed to offer a compact, cost-effective, and freely available miniaturized HPTLC system using office equipment [1–3]. The upcoming potential of open-source system engineering was exploited next [4–10]. The resulting 2LabsToGo system [11] uniquely combined a chemical and biological laboratory including all relevant processes. The latest version [12] newly offered an autosampler for sample application and a Nebulizer for the application of derivatization reagents and cell suspensions as a spray mist. Its energy consumption was reduced by operating using solar panels.

In the chemical laboratory of the 2LabsToGo system, many samples were applied on an adsorbent plate, simultaneously developed with a suitable mobile phase, if necessary, derivatized using the Nebulizer, and finally, detected at visible light, UVA, and UVC illumination using an integrated Raspberry Pi camera. The biological laboratory of the 2LabsToGo system offered the possibility of spraying cells onto the plate using the Nebulizer to perform planar bioassays. The required incubation took place in the self-developed Mini-Incubator. The Python-based software OCManager4 was controlled via a Raspberry Pi and operated via a browser. The mainly 3D-printed and thus customizable hardware was controlled by a Ramps board.

The functionality of the 2LabsToGo system has already been demonstrated in various applications, such as screening for lactose in lactose-free dairy products [12], screening for ergot alkaloids in rye [13], detection of bioactive compounds in smoked cigarette butts, estrogen-like substances in beer and wine, and genotoxic compounds in fat-containing food and cosmetic products. The prioritization strategy [15, 16] of the 2LabsToGo system allowed for proactive product safety.

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T3: Multidimensional and Hyphenated Techniques

Application of TLC-MS in qualitative analysis of traditional Chinese medicines

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Keywords: TLC-MS, *Sophora flavescens*, *Achyranthes bidentata*, qualitative identification

In the long historical usage of traditional Chinese herbal medicines, many different herbs with the same name have gradually emerged. Some of these herbs originated from the same species or even same genus, and while they had some similarities in their components, they also had differences. However, due to their similar appearance and identical names, they were easily confused or misused.

In this study, several commonly used traditional Chinese medicines were examined as examples. *Sophora flavescens* and *Sophora tonkinensis* are plants of the same genus, both of which had roots or rhizome used medicinally with similar effects; Some other species such as root of *Menispermum dauricum* (MD) and *Indigofera spp*. (IS) may be misused due to similar name. Another pair of species, *Achyranthes bidentata* and *Cyathula officinalis*, both from the Amaranthaceae family, had roots used for invigorating blood circulation to relieve menstrual pain and promoting diuresis to relieve stranguria. This study adopts a parallel comparative research approach, utilizing TLC-MS to quickly identify and distinguish the common and unique components in these pairs of herbs, providing an effective method for the rapid identification of such closely related species of traditional Chinese medicines. For SF and other species, multi-dimensional comparative research results showed that in addition to the well-known alkaloid components, a large number of differential flavonoid components with identification value were discovered existing in SF and ST. The structures of the target compounds were identified by MS/MS^2 fragmentations vis TLC-ESI-MS techniques, typical compounds were guided-isolated and structurally elucidated to validate the TLC-MS results. Lastly the appropriate components were chosen as quality-markers based on their content, specificity, measurability, and bioactivities based on literature findings, and corresponding qualitative or quantitative methods were established for enhancing their quality control standards.

Figures

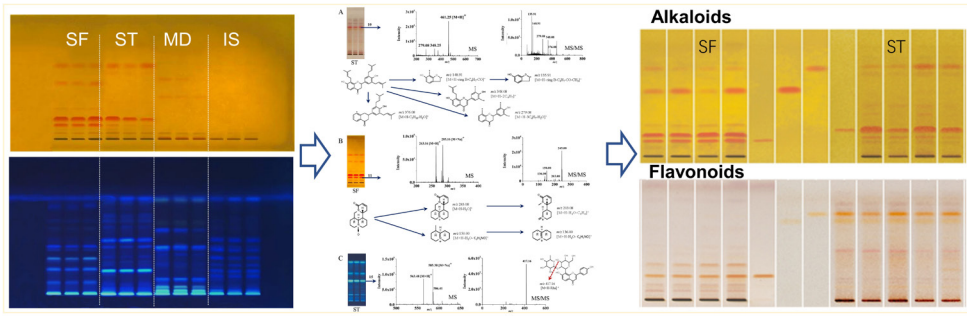

Figure 1 TLC-MS analysis of root of *Sophora flavescens* and confusable varieties

TLC-MS bioautography a high throughput hyphenated technique for detection and identification of acetylcholinesterase inhibitory metabolites from traditional formulations and *In vivo* UPLC-MS pattern recognition studies

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Keywords: HPTLC bioautography, mass spectroscopy, acetylcholinesterase inhibtory metabolites, metabolomics, pattern recognition analysis

Traditional formulations are widely utilised worldwide for their holistic approach to health and wellness with minimum adverse effects. Itrifals have been used for centuries as a part of the Unani system of medicine for several diseases without any supporting scientific evidence. *Itrifal Muqawwi Dimagh* (IMD) and *Itrifal Sana* (IS) are two polyherbal formulations specifically formulated to support and strengthen brain function. The present study aims to detect and identify the bioactive metabolites responsible for acetylcholinesterase inhibitory activity by using a high-throughput screening approach of thin layer chromatography-mass spectrometry based bioautography (TLC-MS bioautography) and a to perform comprehensive metabolic profiling was before and after oral administration to rats using UPLC-MS at intervals of 0, 1, 2, 4, 6, 8, and 12 hours to determine the fate of metabolites in blood. The authentication of individual constituents of the formulations were done using powder microscopy. Hydroalcoholic extract, ethylacetate fraction and hydrolysed ethylacetate fraction of hydroalcoholic extract of the formulations were extensively prepared for comprehensive phytochemical analysis. *In-vitro* acetylcholinesterase (AChE) inhibitory activity was determined using Ellman's method. TLC-MS bioautography was employed to detect and identify the AChE inhibitory bioactives within the formulation targeting cholinergic deficiency. The *in-vitro* detection of AChE inhibitory activity by Ellman's method suggested the IC_{50} values of IMD and IS to be 135.00 ± 0.44 µg/mL, and 222.96 ± 0.31 respectively, as compared to the reference drug, galanthamine, with an IC_{50} of 24.00 ± 0.46 µg/mL. The study revealed the presence of scopoletin, tannic acid, ellagic acid, and catechin as potential bioactive AChE metabolites in IMD and rosmarinic acid, apigenin and catechin as bioactives in IS. Metabolomic profiling using UPLC-MS revealed clear metabolite pattern in extract and in blood after its oral administration. Hence, based on our findings, it can be concluded that these traditonal formulations have great potential to overcome cholinergic deficiency and can be used for neuroprotection and other neurological disorders after successful *in vivo*, pharmacokinetic and toxicity validation.

Acknowledgments
Central Council for Research in Unani Medicine, Ministry of AYUSH, Government of India.

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TLC-MS bioautography for detection and identification of acetylcholinesterase inhibitory metabolites from traditional Unani formulations and *In vivo* UPLC-MS pattern recognition studies

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Application of TLC-MS technology in the identification and analysis of precious traditional Chinese medicines

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Keywords: TLC-MS, precious traditional Chinese medicines, quality control

Drug standards serve as guidelines for controlling the quality of drugs, with thin layer chromatography (TLC) emerging as the preferred method for identifying traditional Chinese medicines (TCMs) owing to its intuitiveness, visualization, and exclusivity. Approximately 94% of the TCM varieties included in the Pharmacopoeia of the People's Republic of China (ChP, 2020) utilized TLC for identification methods. The chemical reference substance (CRS) applied in these standards plays a key role in enhancing the quality control standards of TCMs and ensuring the safety and efficacy of the medications. However, there are still numerous TCMs lacking effective CRS. The conventional methods for chemical separation and purification were often time-consuming and labor-intensive. The combination of TLC and mass spectrometry (MS), including the elution-based TLC-MS interface and the emerging ambient ionization mass spectrometry (AIMS) technologies, offers innovative approaches for the discovery and identification of characteristic chemicals, thereby enhancing the precision and efficiency of TCM quality control.

Given the significant market demand but the scarcity of production for precious TCMs, these TCMs command premium prices, resulting in prevalent issues of counterfeiting and uneven quality. Consequently, robust quality control measures for these herbal medicines have emerged as a critical challenge, with the identification of quality markers being paramount to addressing this issue. In recent years, our research group has established series of TLC-MS methods for the qualitative analysis of characteristic components in precious and exquisite TCMs. These methods leverages the strengths of thin-layer chromatography for separation, coupled with mass spectrometry's rapid identification capabilities. By employing the elution-based TLC-MS interface, we successfully identified baimuxinal as the characteristic component of *Aquilaria sinensis* (Lour.) Gilg using TLC. Subsequently, this developed TLC method of *A. sinensis* will be included in the new version of TLC Atlas of Traditional Chinese Medicines in Pharmacopoeia of the People's Republic of China (2020). Additionally, we developed a laser ablation-assisted direct analysis in real-time mass spectrometry (LA-DART-MS) platform, which has been applied to the compositional analysis of precious and exquisite TCMs, particularly Dalbergiae Odoriferae Lignum and Dendrobii Caulis. Utilizing this technique, we analyzed Dalbergiae Odoriferae Lignum using pinpointed sativanone as its quality marker, thereby addressing the lack of CRS. Furthermore, our method enabled us to detect four characteristic bibenzyl components (tristin, moscatil, gigantol, and erianin) in Dendrobii Caulis of different origins and species, which were typical multi-source species that can be distinguished by TLC. Notably, dendrobium phenol was present in all Dendrobium species, while moscatil served as a distinguishing marker for *D. Chrysotoxum.* The above findings indicated that TLC-MS has a promising future in the field of analysis of natural medicines, with significant implications for enhancing quality standards and assurance in the TCM industry.

T5: Image Analysis

New approach for the in-vitro evaluation of antioxidant potential of medicinal plants extracts using thin-layer chromatography assisted by image analysis

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Keywords: image analysis, RP-stationary phases, simulated substrates interaction, antioxidant potential, medicinal plant extracts

Due to its simplicity, flexibility and accessibility, thin layer chromatography assisted by image analysis method (HPTLC-IA) was applied in different assays for antioxidant activity evaluation [1-4]. According to literature, there are two principal chromatographic approaches that can be used for the evaluation of antioxidant activity. In the first one, *typical planar chromatographic assay* (HPTLC) [5], developed plates are sprayed or immersed with/in DPPH$^{\bullet}$ radical solution and the active antiradical constituents are quantified based on the integrated area of the chromatographic spot. In the second approach, micro-HPTLC assay [2], total antioxidant activity is evaluated by quantitative analysis of radical DPPH$^{\bullet}$ and DPPH-H molecules after reaction with the samples and separated from the interfering compounds. In this study a new chromatographic approach involving the typical planar chromatographic assay with 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS^{*+}) radical and image analysis procedure (HPTLC-IA method) is proposed for the first time for *in-vitro* evaluation of the substrate-interaction effect on the antioxidant potential of medicinal plants extracts. HPTLC plates with high and moderate polarity (modified RP-8, RP-18, CN and Diol respectively silicagel plates) were chosen for the chromatographic investigations in order to provide additional selectivity and simulate the binding substrates able to block different active groups of plant extract constituents and allow the evaluation of radical scavenging activity in simulated physiological conditions. Different image analysis procedures were applied for accurate determination of antioxidant potential of a large set of 44 medicinal plants extracts after interaction of separated constituents with stationary phases of various polarity. According to the obtained results, the antioxidant potential evaluated on different stationary phases decreased after the interaction of separated compounds with CN, RP-8 and RP-18 phases respectively. Developed HPTLC-IA method can be successfully used as efficient tool for rapid *in-vitro* evaluation of antioxidant potential of medicinal plants extracts after the possible interaction of active compounds with constituents from physiological environment.

Acknowledgments
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Characterization of some pepper hydroalcoholic extracts by using thin layer chromatography coupled with image analysis

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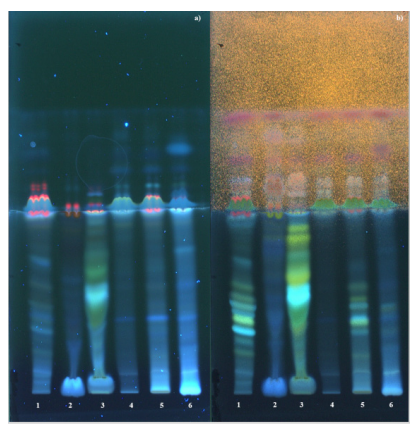
Keywords: pepper, hydroalcoholic extracts, TLC-image analysis, fingerprints

In this study hydroalcoholic extracts of white, black, red, green, long and Sichuan pepper were analysed by thin layer chromatography coupled with image analysis. The chromatograms were obtained on HPTLC Silica gel 60 F_{254} plates using monodimensional double development. The first development was performed over a distance of 8 cm with ethyl acetate - formic acid - acetic acid - water (100:11:11:20, v/v). The second development was carried out with toluene - ethyl acetate (93:7, v/v) over a distance of 15 cm. After the first development the visualization was achieved by fluorescence quenching at 254nm and in fluorescence at 366nm. After the second development, the plate was sprayed with NTS on the lower part (0-9cm) and with anisaldehyde on the upper part (9-15cm). Visualization was achieved in fluorescence at 366nm for the lower part and day light for the upper part respectively. The chromatographic fingerprints were obtained using the TLC-Analyzer program on different pure color channels (red, green and blue) and on the neutral channel (grey). Utilizarea canalului verde pentru procesarea imaginii a evidentiat cea mai buna discriminare a celor sase amprente obtinute dupa prima si a doua developare si vizualizare prin fluorescenta la 366 nm. The best discrimination between the six fingerprints was achievd on the green channel after the first and second development with fluorescence visualization at 366nm. Blue channel revealed the best rezults when second development and derivatization were performed.

Figures



.Chromatograms obtained after the second developement (a) fluorescence at 366nm; (b) after spraying, fluorescence 366nm; 1-green pepper; 2-red pepper; 3-Sichuan pepper; 4-white pepper; 5-black pepper; 6-long pepper.

T7: Effect-directed Detection

Discovery of novel antibacterial clerodane diterpenes from the roots of giant goldenrod (*Solidago gigantea* Ait.) by combining HPTLC with bioassays

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Keywords: phytochemistry, effect-directed analysis, direct bioautography, antibacterials, isolation

Plants are widely recognized for their rich reservoir of secondary metabolites that possess a wide range of beneficial biological activities and various chemical structures. *Solidago gigantea* Ait., commonly known as giant goldenrod, is a plant indigenous to North America but has spread as an invasive species in certain parts of Europe. Despite its potential, the bioactive compounds found in this plant have not been thoroughly investigated.

This work aimed to detect, characterize, isolate, and identify the bioactive constituents in the ethanol extract obtained from the roots of *S. gigantea*. High-performance thin-layer chromatography coupled with direct bioautography (HPTLC-DB) was utilized for non-targeted, effect-directed screening of antibacterial components using Gram-positive *Bacillus subtilis* as a test microorganism. Subsequent preparative flash column chromatography and semipreparative high-performance liquid chromatography (RP-HPLC) were employed for bioassay-guided fractionation, purification, and isolation procedure supervised by HPTLC-DB. The structures of the molecules were elucidated using one- and two-dimensional nuclear magnetic resonance spectroscopy (NMR) and high-resolution tandem mass spectrometry (HRMS/MS).

Seven new *cis*- and *trans*-clerodane diterpenes were isolated from the roots of *S. gigantea* exhibiting a wide range of structural motifs including furan, butenolide (lactone), five-membered cyclic hemiacetal, and isobutyrate. The epimers of a rare, highly oxygenated, tetrasubstituted tetrahydrofuran ring along with angelate moieties in unusual positions were revealed. Their antibacterial activity against Gram-positive *B. subtilis* bacteria was confirmed by direct bioautography. The corresponding IC_{50} values will be determined by *in vitro* microdilution assays. Future research will explore their antimicrobial activity against phytopathogenic bacteria (e.g. *Rhodococcus fascians*) and fungi (e.g. *Fusarium avenaceum*), along with their enzyme inhibitory activities (such as lipase, acetylcholinesterase, α-glucosidase). The isolated compounds can serve as a starting point for the synthesis of more potent antibacterial agents.

Acknowledgments
This study was supported by the National Research, Development and Innovation Office of Hungary (K128921 and SNN139496).

HPTLC analysis of vegetable oils: Impact of heating temperature and meal composition on genotoxic substance formation

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Keywords: lipid, unsaturated fatty acid, oxidation, high-performance thin-layer chromatography

Unsaturated fatty acids prevalent in commonly consumed vegetable oils are particularly susceptible to oxidation. Some oxidation products are known to be genotoxic. Recently, genotoxic signals in vegetable oils have been detected by hyphenated HPTLC, tentatively assigned to (ep)oxidized fatty acids [1]. This study aimed to investigate the influence of heating temperature and meal composition on the formation of genotoxic substances in oil-rich potato meals. Homemade French fries (fried 120–200°C) and diverse potato dishes (homemade and convenience products) were analyzed using the planar SOS-Umu-C bioassay. Neither frying temperature nor extended oil heating (1 h) had a significant influence on the genotoxic response. However, differences were evident between various oil-rich potato meals, suggesting other food components than vegetable oil may influence genotoxic compound formation. Further research is necessary to identify these genotoxic compounds and develop strategies to minimize their formation during food preparation.

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Estrogen-like substances in milk with pYES bioassay

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Keywords: milk, estrogen, pYES, bioassay

Estrogen-active substances are suspected of causing developmental and reproductive disorders, as well as being involved in the development of breast cancer. Milk is an important source for the intake of estrogen-active substances like estrone and estradiol because their lipophilic structure enables them to cross the blood-milk barrier. Their concentration is in the nanogram-per-liter-range and varies depending on the age, physiological condition and a possible pregnancy of the cows.

In the literature, the radioimmunoassay plays an important role in the analysis of estrogens in milk. Alternatively, the analysis can also be performed using high performance liquid chromatography or gas chromatography coupled with tandem mass spectrometry. This study aimed to demonstrate the analysis of estrogen-active substances using high-performance-thin layer-chromatography (HPTLC) coupled with the planar yeast estrogen screen (pYES) bioassay, which was never done before. In comparison to commonly used methods HPTLC is characterized by a higher sample throughput and lower solvent consumption. Another advantage is the detection by pYES bioassay as a non-target analytical method in which estrogenic substances can be detected down to the low picogram-per-band-range.

The analysis of estrogen-active compounds is carried out after a liquid-liquid extraction, in which the steroid hormones pass into the organic phase. After evaporation to dryness and reconstitution, the extract can be used directly for HPTLC analysis. In contrast to the microtiter plate assay, it is not necessary to defat the milk sample. As a result, these matrix components are not neglected in the analysis and working time can be saved, which represents progress. The chromatographic separation of the dominant milk fat, which is coextracted with the steroid hormones estrogens from the lipids, poses a particular challenge. Apart from the expected signals caused by estrogens such as estrone and estradiol, there is a pronounced signal from milk fat. The results indicate a significantly greater estrogenic potential of milk fat compared to the steroid hormones in milk.

Separation of bioactive compounds from inedible *Rubroboletus satanas* using dual-eluting flash chromatography and HPTLC detection with diverse bioautography methods

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Keywords: antimicrobials, antioxidants, direct-bioautography, flash chromatography, HPTLC

Diverse chromatographic techniques and coupled cell-based assay were applied to separate and identify bioactive compounds from the inedible mushroom *Rubroboletus satanas* (*Boletaceae*)s. Starting with a complex matrix, a novel step-wise gradient normal-phase flash chromatography method with two different solvent systems (1. *n*-hexane-ethyl acetate, 2. acetonitrile-water) was developed and utilized, enhancing the resolution and yield of bioactive fractions. In the first step of this flash-chromatography-based procedure, the weaker eluent system (*n*-hexane-ethyl acetate) achieved separation and collection of the antimicrobials. Meanwhile, antioxidants with different physicochemical principles remain on the column, so the separation of antimicrobials from antioxidants also occurred. The applied silica flash column was drained and dried before using the second eluent system that separated and collected numerous water-soluble antioxidant constituents.

Following the separation, high-performance thin-layer chromatography (HPTLC) hyphenations served to detect and preliminarily identify the bioactive compounds. HPTLC, coupled with various direct bioautography methods, facilitates the determination of the retardation factor (hR_F) of bioactive zones on the chromatographic plate. The direct bioautography methods include antimicrobial (both bacterial and fungal), antioxidant, and enzyme inhibition assays, which provide a comprehensive profile of the biological activities of the separated compounds.

In this study, this method of flash chromatography allowed the efficient separation and detailed bioactivity profiling of the compounds found in *R. satanas*. The results highlighted several minor compounds with significant bioactivities, which will be further subjected to spectrometric and spectroscopic analyses for structural elucidation.

The identification of these bioactive compounds would contribute to the expansion of chemical and mycological knowledge on *R. satanas* and would open potential areas for their application in pharmacy, agriculture, and biotechnology. This study highlights the importance of the combination of advanced chromatographic techniques with biological assays in mycological research, providing a solid base to discover novel bioactive compounds.

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Comparative screening of submerged cultured mycelia and fruiting bodies for bioactive secondary metabolites using HPTLC

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Keywords: bioactive secondary metabolites, high-performance thin-layer chromatography, basidiomycota

Because of a rising world population, the supply of alternative protein sources and the use of sidestreams is crucial for the food of the future. To open up new ways of food production, the use of biomass with a limited nutritional value for humans, like sidestreams of the food industry, is a promising approach. Those sidestreams often cotain for example high amounts of indigestible carbohydrates and lignin.

Basidiomycetes are ideal tools for converting these high-molecular biopolymer compounds, as they have complex enzyme systems that enable the fungi to break them down and to convert them into valuable products for human consumption (Bouws et al., 2008). The fruiting bodies are commonly known and a lot of the basidiomycetes are edible. The largest part, the mycelium of the fungi actually grows below the surface. The mycelium can be cultivated in submerged or in surface cultures. Because of the regulations of the EU especially the novel food regulation, it can not be selled for human consumption without novel food application. To simplify novel food approval, the different fungal mycelium culture variants were compared with the corresponding fruiting bodies.

The different samples were analyzed and compared using HPTLC coupled to various bioassays (Schreiner et al., 2021). For antibacterial activity or cytotoxicity testing, *Bacillus subtilis* served as the model organism for Gram-positive bacteria and *Aliivibrio fischeri* for Gram-negative bacteria (Jamshidi-Aidji, Morlock, 2016, International Standard Organization, 2007). The samples were also studied for possible endocrine, genotoxic and neurotoxic effects.

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Characterization of bioactive tree of heaven components

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Keywords: *Ailanthus altissima*, TLC–*Bacillus subtilis*, TLC–lipase assay, TLC–HRMS/MS, flash chromatography, NMR

Tree of heaven (*Ailanthus altissima*) is an invasive plant species almost in whole Europe, including Hungary. The tree is notified mainly for its aggressive spreading and damaging effects on natural habitats and urban areas. Due to its therapeutic effects it has been used in Chinese medicine since ancient times to treat diarrhea and other digestive problems. Although its beneficial effects, and the active ingredients of the plant are still not fully understood.

A cost and time effective approach for screening and purifying of potentially relevant bioactive compounds from natural products is the effect-directed analysis (EDA), in which a bioassay as a key element is used for selective detection [1-3]. EDA can readily be coupled with high-performance thin-layer chromatography (HPTLC) that is a high-throughput and matrix-tolerant technique. Further HPTLC hyphenations, such as HPTLC–UV/Vis/FLD–HRMS/MS enables the characterization of the components present in the inhibition zones. Bioassay-guided isolation process monitored by HPTLC–bioassays can provide the pure compounds responsible for the desired effect, which isolates can be identified by NMR [2].

In this study, the *Ailanthus altissima* (tree of heaven) bioactive components responsible for lipase inhibitory effect and antibacterial activities against *B. subtilis* were explored and identified by the use of TLC–bioassays, TLC–HRMS/MS (TLC–TLC-MS Interface–orbitrap), effect-directed isolation, and NMR. It was revealed that *A. altissima* is rich in antibacterial compounds, e.g. fatty acids, fatty acid derivatives, terpenoids, and alkaloids.

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Developed planar Ames assay for fast detection of mutagens

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Keywords: planar bioassay, *Salmonella* Typhimurium, complex mixtures

In 2022, approximately 19.9 million new cancer cases were reported globally among both sexes, with an estimated mortality rate of around 9.7 million deaths. It is therefore important to develop a rapid method to identify the maximum number of mutagenic substances in complex sample matrices. A new planar Ames assay was developed on the HPTLC plate silica gel 60 to assign individual mutagens in complex mixtures owed to the separation. It was based on the current *in vitro* Ames assay in the microplate format which, however, was found to be not sufficiently sensitive and specific.

The co-culture of *Salmonella* Typhimurium strains TA98 and TA100 were adjusted in the optical density and applied on the chromatogram. Various positive controls were tested, whereby 4-Nitrochinoline-*N*-oxide was selected as positive control. The assay incubation time was reduced from 48 h to 5 h. The newly developed planar Ames assay was applied to perfume analysis, but no mutagens were revealed due to the mentioned lack in sensitivity, which was not improved by just transfering the *in vitro* format into the planar format. Hence, further research is required using different substates to improve the capability of detection.

T8: Food Quality and Safety

Scanning of chicoric acid in different parts of *Cichorium intybus* by a new HPTLC method

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Keywords: HPTLC, chicory acid, *Cichorium intybus*

Cichorium intybus is a very widespread plant and well known since ancient times both for its healing properties and for its use as coffee. *Cichorium intybus* is part of the *Asteraceae* family and is very widespread in Romania in lowland and mountain areas, along roads and rivers.

The main classes of compounds present in chicory are polyphenols, terpenes, flavonoids, caffeic acid derivatives, sugars, volatile oils, alkaloids, coumarins, hydroxycoumarins, vitamins, which give the plant beneficial effects such as: cleansing the body of toxins, stimulating digestion, a insulin secretion causing a decrease in blood sugar [1,2]. Chicoric acid is one of the most important constituents of the *Cichorium intybus* and has antioxidant, antiviral, anticarcinogenic, antibacterial activity [3].

The aim of the work was to check if the amount of chicoric acid varies depending on the part of the plant. For this purpose, after picking, the parts of the plant, namely flowers, leaves, root and whole plant were separated, dried in the dark and grounded. Extractions were carried out by maceration in 70% ethanol, ultrasound-assisted extraction in 70% ethanol and enzymatic extraction with cellulase (3% of plant material) in 55% ethanol. In each case, the ratio between vegetable material and solvent was 1:10. The analysis of chicoric acid in the prepared extracts was carried out by an HPTLC method using the optimized conditions: mobile phase – ethyl acetate : formic acid : acetic acid : water 8:0.2:2:1.8, v/v/v/v and stationary phase - silicagel F_{254}. Both the standard chicoric acid solution (1 mg/mL) and the plant extracts (5 µL from flowers and leaves extracts and 10 µL from root and whole plant extracts) were applied in 8 mm strips and the plates were developed in a presaturated chromatographic chamber with the mobile phase for 30 min. The documentation was performed in UV light at 366 nm and the image of the plates was analyzed using ImageJ software. The quantitation of chicoric acid in each extract was made on the basis of the calibration curves obtained by integrating the areas of the spots in three color channels: green, blue and the original plate. From the calculated values for LOD and LOQ, it can be seen that the most sensitive determination is the one in the green channel (LOD = 179 ng/spot and LOQ = 591 ng/spot). From the values obtained for the chicoric acid content, it can be seen that the highest amount is found in the leaves, but its concentration in the extracts depends on the extraction method used, the most effective method being enzymatic extraction. The smallest amount of chicory acid is found in the root.

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Planar solid phase extraction for chlorinated paraffin screening – irradiation chamber for standardized derivatization

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Keywords: chlorinated paraffins, planar solid phase extraction, UV-C irradiation, benzidine derivatives

Our study focused on the chlorinated paraffin (CP) screening by planar solid phase extraction (pSPE). Based on previous work [1], we investigated the derivatization process of PCA with benzidine derivatives and UV-C irradiation on planar thin-layers to ensure robust and reproducible quantification. We aimed to construct a simple to-handle irradiation chamber after applying *o*-tolidine and tetramethylbenzidine (TMB) as derivatization reagents. The following study investigated the stability of the derivatization solutions, the use of different concentrations, and the optimization of the irradiation time. The method shows robustness regarding signal intensity at various irradiation times and concentrations of used standards. The analysis applied to PCAs with chain lengths between C_{10} and C_{23}, emphasizing the method's versatility. It could be shown that the derivatization reaction is independent of the chain length and only of the degree of chlorination of the used standards.
In addition to the valuable result for PCA screening and the irradiation with UV-C radiation, such a chamber can also be helpful for other applications in the field of HPTLC whenever uniform irradiation of the thin-layer is necessary as the chamber can also be operated with other light sources.

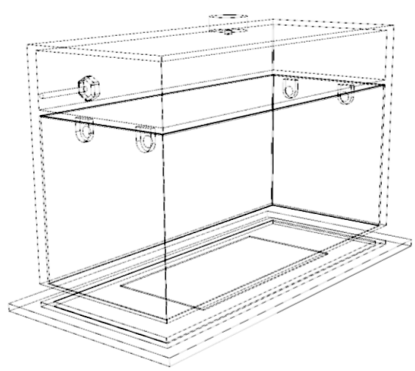

Fig. 1 Schematic Illustration of the irradiation chamber

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Method development for the analysis of genotoxic, cytotoxic and estrogenic substances in chocolate utilizing HPTLC

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Keywords: chocolate, MOSH, MOAH, genotoxic substances, cytotoxic substances, estrogenic substances, High-performance thin-layer chromatography, HPTLC

Chocolate is a popular food; however, it is often contaminated with mineral oil-saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH). The source of these contaminants is mainly migration from recycled packaging or, even before that, during cocoa bean harvesting, through usage of mineral oil-coated jute bags for storage. These contaminants exhibit genotoxic, cytotoxic, and estrogenic effects. The current analysis is mostly carried out using HPLC-HPLC-FID or GCxGC-MS with complex sample preparation. Furthermore, HPTLC-GC-FLD methods have been presented that can be used to analyze samples with complex matrices. However, this coupled method requires extensive sample preparation (saponification and solid-phase extraction).

The most relevant are candidates with adverse effects among thousands of mineral oil compounds, which can be present in a complex sample. Therefore, toxicological analysis via tedious bioassay-guided fractionation and performance of the bioassays must be followed; however, it faces solubility issues in the polar assay medium. In this study, a high-performance thin-layer chromatography method was developed for the analysis of chocolate that does not require pre-concentration of the sample, clean-up, or extensive purification. The development of this method followed the principles of Green Chemistry. It avoids toxic solvents and reduces the volume of solvents used as much as possible. The combination of chromatographic HPTLC separation and toxicological assays allowed for the screening of genotoxic, cytotoxic, and estrogenic substances in approximately 50 different chocolates. The developed method is comparable to existing analytical methods, but requires significantly less time and effort and is comparatively more sustainable. This makes it highly suitable for routine chocolate analysis.

Coupling of planar solid-phase extraction with mass spectrometry (pSPE–MS) for the analysis of pyrrolizidine alkaloids in tea

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Keywords: pyrrolizidine alkaloids, tea, planar solid-phase extraction (pSPE), food contaminants

Tea is the second most widely consumed beverage in the world after water. In addition to its pleasant taste, tea is known to be beneficial to helath due to its content of polyphenols, amino acids and antioxidants. However, contamination with natural toxins such as pyrrolizidine alkaloids (PA) and their N-oxides (PA-N-oxides (PANO)) can also occur, posing a risk to human health [1]. PA/PANO are a group of hepatotoxic and carcinogenic phytochemicals that can contaminate food and feed during harvest. In recent years, the occurrence of PA/PANO in food has increasingly become the focus of public attention and PA/PANO findings of concern (especially in herbal teas) have been reported at regular intervals [2].

In December 2020, the European Union set maximum levels for PA/PANO in certain foods (including tea) in Regulation (EU) 2020/2040 [3].

Due to the toxicity and the frequently occurring contamination of various herbal teas with PA/PANO as well as the recently established maximum levels, the developement of rapid analytical determination methods is of great importance. Several methods for analysis of PA/PANO in various matrices are described in the literature [4]. Due to the very complex composition of the tea matrix, suitable clean-up methods are of crucial importance in order to obtain reliable and valid results.

Based on this knowledge, an analytical method for PA/PANO in tea was developed using planar solid-phase extraction coupled with mass spectrometry (pSPE–MS) after sample preparation. For the coupling of pSPE with MS, a visual marker was first sought that elutes with the PA/PANO in the target zone and makes it visible. Various dyes were tested as visual markers. Using the visual marker (Sudan red I), various elution conditions (solvent, time and flow rate) were investigated using selected PA/PANO and tea extracts. The elution of the analytes from the target zone was perfomed using TLC–MS Interface 2. Mixtures of acetonitril or methanol with water and different concentrations of formic acid or ammonium formate were tested as eluents. The success of the elution was assessed on the basis of the sensitivity for PA/PANO and matrix reduction.

Acknowledgments
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T10: Botanicals and Traditional Medicines

A rapid method for differentiating two botanical sources of Sinapis Semen by high-performance thin-layer chromatography

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Keywords: *Sinapis alba*, *Brassica juncea,* chemical analysis

Sinapis Semen is the dried ripe seed of *Sinapis alba* or *Brassica juncea* (Chinese Pharmacopoeia, 2020 Edition). The former is known as "White Sinapis Semen". The latter is known as "Yellow Sinapis Semen". In Chinese herbal medicine, it is commonly used to relief dyspnea and cough by eliminating cold phlegm, reduce nodulation and relief pains by removing the obstruction of collaterals. Traditionally, the two botanical sources of Sinapis Semen are believed to have similar therapeutic actions and they are used interchangeably. However, recent studies showed that the two herbs were different in chemical composition and toxicity. Since the two drugs have similsr appearance, it is important to distinguish them by chemical analysis.

A method of high-performance thin-layer chromatography was developed to distingish the two herbs. The powdered samples of *Sinapis alba* and *Brassica juncea* were extracted by sonication with 50% ethanol. The extracts were were separated by a developing solvent system composed of acetone, ethyl acetate, formic acid and water (10:7:2:1, v/v) on a HPTLC silica gel F254 plate. After the development, the plate was dried in air and examined under UV light (254 nm). The TLC chromatograms showed that *Sinapis alba* and *Brassica juncea* were having different pattern of bands. These results indicate that the method can be applied to distinguish *Sinapis alba* and *Brassica juncea* rapidly.

Authentication and differentiation of *Croton caudatus* and *Mallotus repandus* by HPTLC fingerprints

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Keywords: type your, keywords here, separated, by commas

Muscular soreness is a common disease which caused from occupation or bad posture. The patients usually take non-steroidal anti-inflammatory agents (NSAIDs) for symptomatic relief but these drugs are costly and are often associated with side effects. Thai herbal medicines is an alternative to use to relieve pain because do not cause side effects and are better to be used for long-term treatment. In Thailand, many herbs are used for pain relief. Among them, Kho Khlan (Thai name) which normally refer to *Mallotus repandus* is one of the most popular herbs found in traditional drugs and is mentioned in the National List in Essential Medicine of Thailand. However, the same plant name can also be *Croton caudatus.* Both species are has records for pain relieving function, anti-inflammatory, antioxidant and antimicrobial. Moreover, another species, *Anamirta cocculus* is also often referred as Kho Khlan and its seed contain picrotoxin that is a strong poison causing side effects such as spasms and twitching. The use of *A. cocculus* is now abandoned because of safety concerns associated with it. These lead to confusion in the identification of species for use. Thus, this study aims to develop HPTLC methods to differentiate Kho Khlan and test their products labeled as Kho Klan available in the market. The six raw materials from pharmacy stores indicated the same chemical profile but they were totally different from both authentic plants. This suggests that Kho Klan that are available in the market are not *M. repandus* and *C. caudatus*. Moreover, two products labeled as Kho Klan did not show the characteristic zone of both species. It might be wrong species used in the products. These results indicated that HPTLC chemical fingerprint obtained can efficiently use as a tool to differentiate confused of Kho Klan species and confirmation the quality of raw materials and products.

Acknowledgments
PMU-C of the Office of the National Higher Education Science Research and Innovation Policy Council.

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Simultaneous detection of carbohydrate digestive enzyme inhibitors of Piperaceae widely used in Thailand using HPTLC/MS/MS

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Keywords: *piper*, diabetes, α-amylase, α-glucosidase

Diabetes mellitus (DM) affects approximately 537 million people worldwide, with projections indicating an increase to 643 million by 2030. Type 2 diabetes arises from the body's inefficient utilization of insulin. Postprandial hyperglycemia can be mitigated through the inhibition of carbohydrate-degrading enzymes. α-Amylase initiates starch hydrolysis, subsequently broken down to glucose by α-glucosidases. Therefore, inhibiting α-amylase and α-glucosidase actions to delay starch hydrolysis is crucial in managing postprandial glucose levels and hyperglycemia [1].

Piper, the largest genus in the family Piperaceae, comprises nineteen species documented in Thailand. Indigenous Thai communities utilize *piper* plants as a dietary staple. This study aims to investigate active compounds exhibiting inhibitory activity against carbohydrate-hydrolyzing enzymes, specifically α-amylase and α-glucosidase. Fifteen samples from five species, collected from diverse regions in Thailand, were investigated for enzyme inhibitory activities. The technique was carried out on silica gel GF_{254} HPTLC plates using a mobile phase composed of *n*-hexane: ethyl acetate: formic acid: methanol (60:30:5:5). After separation and drying, the plate was sprayed with an α-amylase solution and red starch as a substrate, and the plate was incubated, and the active compounds on α-amylase were detected as colorless zones over the pink background. For α-glucosidase inhibitory detection, the plate was sprayed with an α-glucosidase solution and 2-naphthyl-α-*D*-glucopyranoside as a substrate [2], with inhibitors appearing as colourless zones over a purple background. The clear inhibitory bands were observed and identified using HPTLC/MS/MS/NMR techniques.

Conclusively, caffeic acid, myricetin, genistein, piperine, and eugenol were isolated and identified as α-amylase and glucosidase inhibitors. Myricetin, notably active in *Piper sarmentosum* roots, exhibited the highest inhibitory activities against both enzymes, consistent with in silico predictions for pancreatic α-amylase and α-glucosidase. Caffeic acid and eugenol were identified in *Piper betel*. In summary, this study developed an α-amylase and α-glucosidase HPTLC-bioautography method to elucidate inhibitors from *Piper* herbs.

Acknowledgments
PMU-C of the Office of the National Higher Education Science Research and Innovation Policy Council.

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2D-TLC–bioautography–HRMS-based identification of antibacterial compounds of *Myrtus communis* L.

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Keywords: *Myrtus communis*, TLC–direct bioautography, antibacterial activity, *Bacillus subtilis*, TLC–HRMS/MS

Myrtus communis L. (common myrtle) belonging to Myrtaceae family is an evergreen plant grown mainly in the Mediterranean region [1]. Its essential oil is especially known for potent antibacterial properties, demonstrating efficacy against wide range of bacterial strains [2]. *Bacillus subtilis*, commonly found in soil and human gastrointestinal flora, is a Gram-positive bacterium that is typically benign to humans but might shift to opportunistic pathogenesis in certain circumstances [3]. The development of effective phytochemicals with minimal side effects and the application of less time consuming and cost effective screening methods for antibacterial agents are critical in ongoing fight against diseases.

(HP)TLC is a fast and useful chromatographic method for identification of compounds. Moreover, it serves opportunity to combine with different methods and techniques, including direct *in situ* bioassays as well as mass spectrometry. (HP)TLC–direct bioautography serves as a screening tool, offering insights into the chromatographic behavior of bioactive components.

In this study, hydroalcoholic *M. communis* leaf extract was analysed for detecting the compounds with antibacterial activity against *B. subtilis* by TLC–direct bioautography. Two-dimensional TLC was performed to achieve the best chromatographic separation and to detect the minor bioactive compounds. TLC silica gel 60 F_{254} plate was utilized as stationary phase and the following developing solvent systems toluene-ethyl acetate-methanol (6:3:1, *V*/*V*/*V*) and in orthogonal direction toluene-isopropyl acetate-acetic acid (90:10:2, *V*/*V*/*V*) were used respectively. Later, TLC bioatogram was coupled with mass spectrometry using an elution head-based interface (TLC–*B. subtilis*–TLC-MS Interface–orbitrap). For the characterization of the bioactive compounds HRMS and HRMS/MS were recorded. As a result, targeted characterization of the compounds showing antibacterial activity against *B. subtilis* were identified such as Myrtucommulone A and nor-semimyrtucommulone.

Acknowledgments
This study was partially supported by the National Research, Development and Innovation Office of Hungary (NKFIH; project SNN139496).

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HPTLC fingerprint of *Mentha spicata* (Lamiaceae) and the antioxidant activity

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Keywords: Mentha spicata, HPTLC fingerprint, antioxidant, botanical, functional food

Mentha spicata (Spearmint) extracts and essential oils have been investigated for different health benefits such as antioxidant, anticancer, antiparasitic, antimicrobial, and antidiabetic effects. In vitro and in vivo studies have reported positive effects as a result of bioactive compounds present. This study aimed to demonstrate the HPTLC fingerprint and the antioxidant activity using DPPH radical scavenging assay.

The HPTLC fingerprint was demonstrated using ethyl acetate:formic acid:acetic acid:water (100:11:11:27). Two spots on the extract chromatogram matched rutin and chlorogenic acid used as standards. Antioxidant activity was determined by DPPH free radical scavenging assay using gallic acid as standard at a concentration range of 100 - 0.915 µg/ml and a concentration range of 500 - 0.98 µg/ml for *M. spicata* extract. The IC_{50} value for gallic acid standard was 8.05 ± 0.22µg/ml and 79.16 ± 0.5 µg/ml for *M. spicata.* The values show that the antioxidant activity is very strong for gallic acid and strong for *Mentha spicata.* The activity in addition to the nutritive content of the leaf suggests the potential use of the plant as a functional food when taken as a tea infusion.

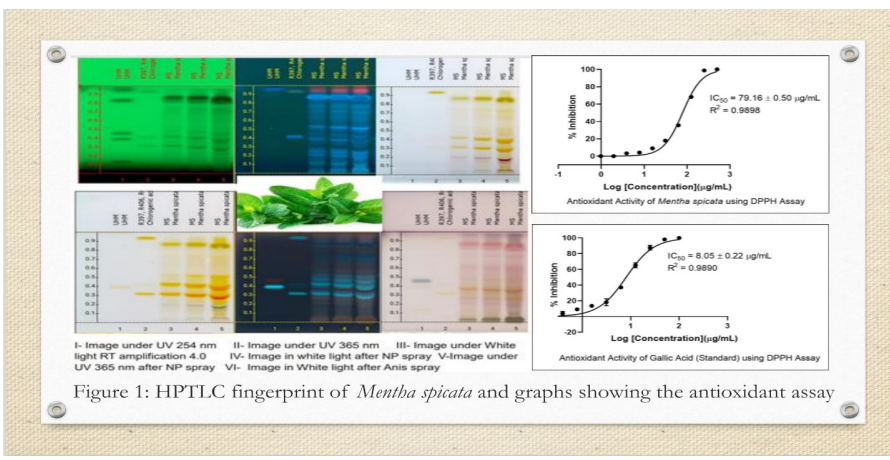

Figure 1: HPTLC fingerprint of *Mentha spicata* and graphs showing the antioxidant assay

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T11: Natural Product Analysis

Bioassay-guided detection, identification and assessment of antibacterial and anti-inflammatory compounds from olive tree flower extracts by HPTLC

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Keywords: olive flower, fermentation assisted extraction, COX-1 inhibition, HPTLC protocol for IC_{50}

Olive trees are one of the most widely cultivated fruit trees worlwide. The chemical compositions and biological activities of olive tree fruit and leaves have been extensively studied for their nutritional and health-promoting properties. However, not much information is known about olive flowers. Thus, the aim of this study was to analyse bioactive compounds in olive flower extracts and the effect of fermentation-assisted extraction on phenolic content and antioxidant activity. For that purpose High-performance thin-layer chromatography (HPTLC) was hyphenated with the bioassay-guided detection and spectroscopic identification of bioactive compounds. Enzymatic and bacterial *in situ* bioassays were used to detect COX-1 enzyme inhibition and antibacterial activity. Multiple zones of antibacterial activity and one zone of COX-1 inhibition were detected in both, non-fermented and fermented, extracts. An HPTLC-based experimental protocol was develop for the assessment of the relative potency of the extracts in inhibiting COX-1 enzyme and antibacterial activity via high-maximal inhibitory concentrations (IC_{50}). Strong antibacterial activities against *Enterococcus faecalis* were detected in chromatographic zones 4 and 7 (IC_{50} = 57–58 µg for zone 4 and IC_{50} = 157–167 µg for zone 7) compared to ampicillin (IC_{50} = 495 µg). The COX-1 inhibition by the extract (IC_{50} = 76–98 µg) was almoust 10 times higer compared to that of salicylic acid (IC_{50} = 557 µg). The eluates from bioactive HPTLC zones were further analysed by FTIR NMR, and LC-MS spectroscopy. Multiple zones of antibacterial activity were associated with the presence of triterpenoid acids, while COX-1 inhibition was related to the presence of long-chain fatty acids.

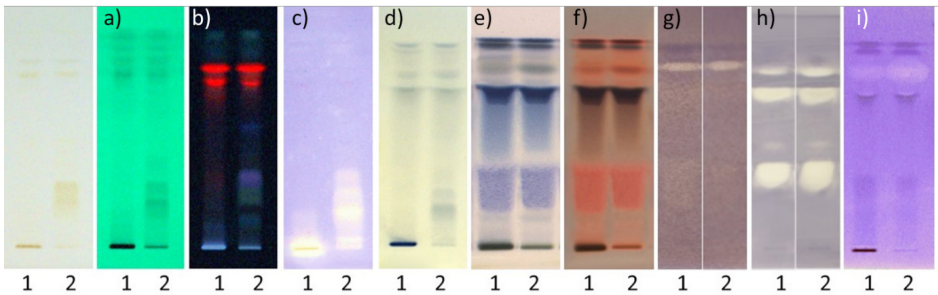

Figure. HPTLC fingerprints of olive flower nonfermented (left) and fermented (right) ethyl acetate extract a) UV 254 nm; b) UV 366 nm; c) DPPH• assay d) $FeCl_3$; e) anisaldehyde-sulfuric acid (ASA), white light; f) ASA, UV 366 nm; g) COX-1 assay; h) MTT assay. Mobile phase: *n*-hexane - ethyl acetate - acetic acid (15:9:1). Track 1; track 2, fermentation assisted ethyl acetate extract.

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HPTLC based approach for assay-guided evaluation of potential biological activities and iridoid content of *Lamium album* extract

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Keywords: iridoids, HPTLC, *Lamium album*, biological activities

Throughout history, humans have employed plants and their extracts as remedies for various ailments. These extracts contain high levels of bioactive compounds, responsible for specific physiological functions in living organisms. The main purpose of this study is to collect data on the iridoid content and certain biological activities of *Lamium album*, a widely dispersed but relatively understudied plant.

Lamium album is a perennial herbaceous plant traditionally known as the "white dead nettle" or "non-stinging nettle" that belongs to the *Lamiacea* family, in the *Lamium* genus. What draws particular attention to this plant is the presence of a significant amount of iridoids, which are characterized by a pyranoid skeleton often referred to as the iridan skeleton, in its composition [1]. The ethanolic extracts were prepared by ultrasound-assisted extraction (UAE), the solid-solvent ratio used was 1:10 (w/v). For each sample the extraction was achieved through three successive extractions and the obtained phases were then combined. The solvent was completely evaporated and the resulting solid was resuspended successively in solvents with different polarity [2]. Regarding the investigation for the presence of iridoids normal phase – HPTLC, silica gel 60 F254 20 × 10 cm plates were used. Following appropriate separation through optimization of the mobile phase (ethyl acetate–methanol–hexane (7.5:1:0.5 v/v/v)), the chromatographic plates were treated with the Ehrlich reagent, which selectively interacts with these class of chemical compounds [3]. The results revealed considerable differences between the obtained extracts after fractionation hence the presence of iridoids of different polarities in the samples was shown. As an open chromatographic system, HPTLC is compatible with (bio)chemical assays, since its mobile phase can be easily removed after plate development and prior to the bioassay. Combining effect-directed assays and biochemical derivatizations enables screening of multifunctional phytocompounds in highly complex samples, such as plant extracts. For evaluating the chosen biological activities, various assay-guided HPTLC methods were used that share a common principle: after HPTLC separation, the plate is treated with an appropriate detection system (chemical reagent, enzymatic reaction). In the areas where target compounds are present, a specific interaction or effect occurs that can be visualized. This allows a direct correlation between the separated chemical components and their specific properties or activities [4].

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Characterization of bioactive tree of heaven components

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Keywords: *Ailanthus altissima*, direct bioautography, effect-directed analysis, TLC–HRMS/MS, flash chromatography

Tree of heaven (*Ailanthus altissima* (Mill.) Swingle) is native in the Far East, but has become a successful invader in many temperate regions of the world, like in Hungary and Slovenia. In the traditional Asian medicine the various plant parts (e.g. root bark, stem, leaves, and samara) are used for the treatment of colds, fever, bleeding, epilepsy, asthma, endoparasites, diarrhea and gastrointestinal diseases. In this study we aimed the screening, characterization, and identification of the antibacterial ingredients of the bark of the tree trunk.

High-performance thin-layer chromatography ((HP)TLC) combined with different methods and techniques, including direct *in situ* bioassays and mass spectrometry, provides an affordable, high-throughput, and time effective tool for non-targeted detection and highly targeted characterization of the bioactive components of complex matrices. The fast (HP) TLC–effect-directed analyses (EDA) are suitable also for biomonitoring the fractionation and purification steps of a bioassay guided isolation.

Antibacterial bark components were revealed by TLC-*Bacillus subtilis* assay, which were characterized by chemical reagents, and TLC–UV/Vis/FLD–HRMS/MS. For MS experiments, the online combination of an UHPLC, an elution head based TLC-MS Interface, and hybrid quadrupole-orbitrap mass spectrometer equipped with a heated electrospray ionization probe was utilized. Isolation of the selected active compounds were performed by flash chromatography.

The different parts of the bark contained different antibacterial compounds that were identified by NMR as alkaloid, fatty acid, fatty acid derivative, or dicarboxylic acid.

Acknowledgments
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Optimization of primuline spraying reagent and spraying parameters for detection of bioactive compounds in tree of heaven extracts

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Keywords: primuline detection reagent, derivatization, bioactive compounds, tree of heaven

Tree of heaven (*Alianthus altissima* (Mill.) Swingle) a deciduous dioecious plant native to China and Taiwan is an invasive alien plant species in Europe and North America, and present a growing threat to biodiversity also in Slovenia and Hungary. Fruits called samara grow in clusters and are spread by wind over long distances. In each cluster can be hundreds of seeds. Tree of heaven is also spread vegetatively by roots on short distances. Although different bioactive compounds with antimicrobial, antioxidative, cytotoxic and insecticide activity were isolated from tree of heaven, many bioactive compounds remain unknown.

The aim of this work was to investigate the potential of HPTLC analysis of crude extracts of tree of heaven before and after post-chromatographic derivatization with primuline and anisaldehyde detection reagents. The most common detection reagents (e.g. anisaldehyde reagent) can easily be transferred to the Derivatizer (CAMAG), but some of them cannot be. One of such reagents is primuline detection reagent, which is usually prepared by dissolving primuline in acetone:water (4:1, v/v). However, this reagent could not directly be applied to the Derivatizer. Therefore, the aim of the study was 1) to find the optimal solvent for primuline detection reagent to be appropriate for the Derivatizer, 2) to select the optimal color-coded nozzle and 3) spraying mode for detection of bioactive compounds in extracts prepared from different parts (leaves, petioles, young shoots, bark, inflorescence, young fruits) of tree of heaven collected in Slovenia and Hungary. Light blue fluorescent zones detected on the dark blue background of the plate can further be analyzed by mass spectrometry as primuline does not covalently bond to lipids and their derivatives.

Acknowledgments
This study was supported by the Slovenian Research and Innovation Agency (ARIS; research core funding No. P1-0005, research project N1-0235) and the National Research, Development and Innovation Office of Hungary (NKFIH; project SNN139496).

Application of the HPTLC technique to detect plants with the ability to inhibit α-Amylase – A study on edible flower extracts

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Keywords: α-amylase inhibition, diabetes, edible flowers, HPTLC, phytochemicals

Type 2 diabetes is one of the most widespread metabolic diseases in the world and poses a serious health risk. A basic strategy used in the treatment of hyperglycaemia involves the use of amylolytic enzyme inhibitors. Due to the undesirable side effects of synthetic inhibitors of these enzymes, there is a constant search for compounds with similar properties from natural sources. Commonly, spectrophotometric methods are employed to detect natural inhibitors by quantifying reducing sugars released from starch substrates in the presence of enzymes and inhibitors.

The aim of the work was to modify this typical method and improve it by using the chromatographic technique. The modification involved changing the detection method of starch decomposition products, incorporating their separation using High-Performance Thin-Layer Chromatography (HPTLC). This approach, combined with densitometric measurements, enabled the quantification of plate-separated starch degradation products incubated with α-amylase in the absence or presence of an inhibitor.

Verification of the proposed analytical procedure was carried out on samples of 70% ethanolic extracts from various edible flowers. Among tested samples *Rosa rugosa*, *Armeria martima*, *Tagetes tenuifolia* and *Chaenomeles superba* extracts demonstrated the highest α-amylase inhibitory activity. This method also facilitated a comparison of the plant-derived inhibitors with the commercially used inhibitor, acarbose, based on IC50 parameters.

The results of this study indicate that the proposed methodological approach, unlike typical spectrophotometric tests, enables quantitative tracking of all enzymatic decomposition products formed from the substrate, not just their total content. Additionally, the use of modern equipment solutions for the high-efficiency separation process, along with the method of visualization and evaluation of the obtained chromatograms, provides detailed quantitative data. The automation of most steps of the procedure and the ability to analyse multiple samples simultaneously supports the use of this approach as a screening method.

Effect directed detection of bioactive compounds in leaf extracts from *Salvia* species with high-performance thin-layer chromatography fingerprint analysis

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Keywords: AChE inhibitors, α-Amylase, antimicrobial, Salvia species

Extracts of two Salvia species, *Salvia officinalis* (common sage) and *Salvia apiana* (white sage) were screened for phytoconstituents with the ability to act as antidiabetic, cognitive enhancing, or antimicrobial agents, by hyphenation of high-performance thin-layer chromatography with enzymatic and microbial effect directed assays. Two bioactive zones with α-amylase inhibition (zone 1 and zone 2), 3 zones for acetylcholinesterase inhibition (zones 3, 4 and 5), and two zones for antimicrobial activity (zones 4 and 5) were detected. The compounds from the five bioactive zones were provisionally characterised by comparing their RF values with coeluted standards and this was confirmed with the ATR-FTIR spectra of eluted bioactive zones. The ATR-FTIR spectra of two zones with α-amylase inhibition, indicated that flavonoids and phenolic acids were responsible for α-amylase inhibition. Multiple zones of acetylcholinesterase inhibition were related to the presence of phenolic abietane diterpenoids and triterpenoid acids. The presence of abietane diterpenoids and triterpenoid acids was also found responsible for the mild antimicrobial activity. Flash chromatography was used to isolate sufficient amounts of bioactive compounds for further characterisation via NMR and MS spectroscopy. Five compounds were assigned to the bioactive zones: cirsimaritin (zone 1), a caffeic acid polymer (zone 2), 16-hydroxyrosmanol (zone 3), 16-hydroxycarnosic acid (zone 4), oleanolic and ursolic acids (zone 5).

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Bioassays guided detection isolation and identification of bioactive compounds from *Ficus carica L.* leaf extracts

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Keywords: *Ficus carica L.* leaf extracts, bioassays guided identification, antiinflammatory, antimicrobial, HPTLC based protocol for potency evaluation

This study compares effects of different solvent systems and fermentation on the phytochemical composition of leaf extracts from *Ficus carica* Linn. The aim was to detect and identify bioactive compounds that are responsible for acetylcholinesterase (AChE), α-amylase and cyclooxygenase-1 (COX-1) enzyme inhibition, and compounds with antimicrobial activity. Bioactive zones in chromatograms were detected by combining High-performance thin-layer chromatography (HPTLC) with enzymatic and biological assays. A new experimental protocol for measuring the relative half-maximum inhibitory concentration (IC_{50}) was designed to evaluate the potency of the extracts compared to the potency of known inhibitors. Although the IC_{50} of the fig leaf extract for α-amylase and AChE inhibition were significantly higher when compared to the IC_{50} values for acarbose and donepezil, the COX-1 inhibition by the extract (IC_{50} = 627 µg) was comparable to that of salicylic acid (IC_{50} = 557 µg), and antimicrobial activity of the extract against *Enterococcus faecalis* (IC_{50} = 375–511 µg) was similar to ampicillin (IC_{50} = 495 µg). Four chromatographic zones exhibited bioactivity. Compounds from detected bioactive zones were inisially identified by comparing the band positions to coeluted standards, and by Fourier transform infrared (FTIR) spectra from eluted zones. Flash chromatography was used to fractionate larger amount of extract and fractions rich in bioactive compounds were further characterisation with nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) analysis.

The main bioactive compaunds were identified as umbelliferon, furocoumarins (psoralen and bergapten), fatty acids and pentacyclic triterpenoids (calotropenyl acetate or lupeol).

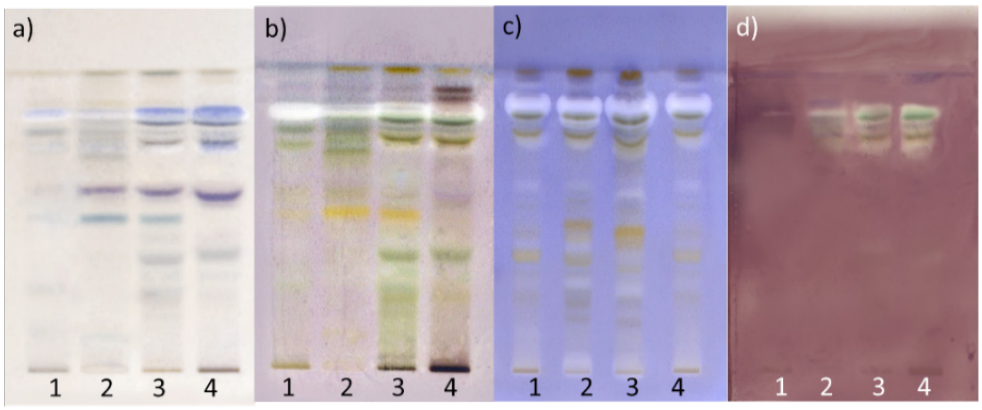

Figure: Chromatograms with bioactive zones in the *Ficus carica* L. leaf extracts in a) α-amylase assay; b) acetylcholinesterase assay; c) COX-1 anti-inflammatory assay; and d) MTT antimicrobial assay against *E. faecalis*. Track 1, methanol; track 2, ethanol; track 3, ethyl acetate; track 4, ethyl acetate extract from fermented plant material.

Monitoring of downstream processing of rutinosidase from Tartary buckwheat extract by TLC-DPPH• assay

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Keywords: thin-layer chromatography, rutin-degrading enzyme, 2,2-Diphenyl-1-picrylhydrazyl, rutin, quercetin

Rutinosidase (EC 3.2.1.168) catalyses the transformation of rutin to quercetin and rutinose (1). Control of rutinosidase *in situ* is crucial in the Tartary buckwheat (*Fagopyrum tataricum*) industrial processing focused on producing food and beverages where rutin must be maintained (2). Also, this enzyme has an application at the rutinose production (3) and organic synthesis (4). Nonetheless, a critical aspect is to establish Tartary buckwheat extract recovery conditions for rutinosidase before the enzyme purification. This research aimed to develop a monitoring, rapid and simultaneous assay of the rutinosidase by the accumulation of rutin after TLC-DPPH• assay method. Tartary buckwheat (*F. tataricum* var. Madawaska) seeds were obtained from the seed bank of the National Agricultural and Food Centre, Research Institute of Plant Production Piestany (Piestany, Slovakia). The TLC-DPPH• assay applies to initial velocity of enzymatic activity detection, which is proportional to time. This protocol evaluates the effect of solid-liquid ratio (pH 4.50/30 min) for rutinosidase extraction from Tartary buckwheat flour. Another evaluated effect was the contact time for the recovery of the rutinosidase activity (5, 10 15, 20, 25, 30 min). As the extract volume increased, enzymatic activity additionally escalated. This method has become a better alternative to HPLC use since it is cheaper and it can be compared to results obtained via HPLC technique. This work shows that for a rutinosidase detection study, it is advisable to develop and optimize a TLC procedure coupled with the antioxidant activity assay with DPPH• for enzymatic activity of obtained rutinosidase from Tartary buckwheat extract.

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The *Mycobacterium bovis* BCG substrain Pasteur cells contain a diverse array of lipids

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Keywords: lipids, high-performance thin-layer chromatography, MALDI-TOF Mass Spectrometry, *Mycobacterium bovis* BCG

Mycobacterium bovis BCG (Bacillus Calmette-Guérin) is a widely used vaccine strain that contains live attenuated mycobacteria and is used for both the prevention and prophylaxis of tuberculosis (TB) and the immunotherapy of bladder cancer. Among the various substrains, BCG Pasteur is extensively studied due to its stable genetic structure and broad application in research [1,2].

Mycobacteria are known for their high lipid content, which constitutes 40% of their dry mass, and their cell walls can contain up to 60% lipids. The outer layer of the mycomembrane consists of various lipid molecules, such as trehalose-containing glycolipids, trehalose mono- and dimycolate (TMM and TDM), and species-specific lipids like phthiocerol dimycocerosate (PDIM), phenolic glycolipids (PGL), sulfo-glycolipids (SGL), glycopeptidolipids (GLP), and mycolic acids. Lipids play a key role in pathogenicity, modulation of the immune response, and the overall biology of mycobacteria [3-5].

BCG encompasses a range of substrains that exhibit genetic and biochemical differences. It is un-known whether and how these differences affect the efficacy of BCG. Compared to other BCG strains, early strains do not synthesize phthiocerol dimycocerosates (PDIM) and phenolic glycolipids (PGL), two lipid virulence factors. Various studies indicate that the loss of PDIMs/PGLs reduces the virulence and protective efficacy of BCG [5].

The lipid composition of the late BCG Pasteur strain could offer valuable insights into its mechanisms of action and potential differences in immune response compared to other substrains. This study aims to characterize some of the lipids of the *M. bovis* BCG Pasteur substrain using High-Performance Thin-Layer Chromato–graphy (HPTLC) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS). The lipid extracts of *M. bovis* BCG include, among others, phenolic glycolipids, glycerol monomycolate (GroMM), phospholipids, and phosphatidylinositol mannosides (PIMs) and mycolic acids. Additionally, further studies will be conducted to examine the antigenic properties of isolated individual lipids.

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HPTLC method for screening of plant petals tyrosinase inhibitors

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Keywords: HPTLC, bioautography, tyrosinase inhibition assay, cosmetics, petals

Skin disorders are common among large demographics and are generally considered undesirable. Problems such as hyperpigmentation, solar lentigo, melasma, and other unaesthetic skin manifestations arise from the excessive activity of the enzyme tyrosinase[1]. Given the negative associations of existing tyrosinase inhibitor-based treatments, such as hydroquinone, there is a constant need to discover new inhibitors from natural sources leading to the development of green and natural cosmetics.

Plant metabolites, especially those from medicinal herbs, rich in phenolic acids and flavonoids, are concentrated in leaves and flowers but remain largely unexplored in flower petals. Many studies have focused on examining the roots, leaves, or fruits[2], while limited research has been conducted exclusively on flower petals of certain species. Considering that the petals of Serbian cultivated plants are generally unexplored, our study aimed to examine the phenolic profile and anti-pigmentation potential of 17 methanolic extracts of petals originating from Serbia.

High-performance Thin Layer Chromatography (HPTLC) as sophisticated, simple, green, and cost-effective technique, enables rapid screening analyses[3]. HPTLC combined with enzymatic detection using tyrosinase, enabling fast and effective detection of single inhibitors from natural extracts. Ethyl acetate/toluene/formic acid = 10/8/3 (*v*/*v*/*v*) was selected for the optimal separation of phenolic compounds. To the best of our knowledge, for the first time, petals were analyzed using tyrosinase inhibition bioautographic assay. Additionally, to identify classes of tyrosinase inhibitor compounds, phenolic (NP reagent derivatization) and terpenoid profiles (*p*-anisaldehyde derivatization) of the petal extracts were determined. Additionally, the MTT colorimetric assay for the viability of human keratinocytes (HaCaT cells) in the presence of these petal extracts was determined.

Bioautography results revealed the highest number of inhibitory zones within the red chestnut sample, while the most intense inhibitory zones were observed in lilac and jasmine extracts. Identified inhibitors included the terpenoid oleanolic acid, as well as compounds at hR_F 85, 73, and 22. Cytotoxicity results showed that petal extracts at a concentration of 5 µg/mL were completely non-cytotoxic, except for marigold and snapdragon, which showed a significant reduction in cell viability.

Acknowledgments
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Detection of hemolytic components in Polygala Radix by HPTLC bioassay combined with two-dimensional chromatography and mass spectrometry

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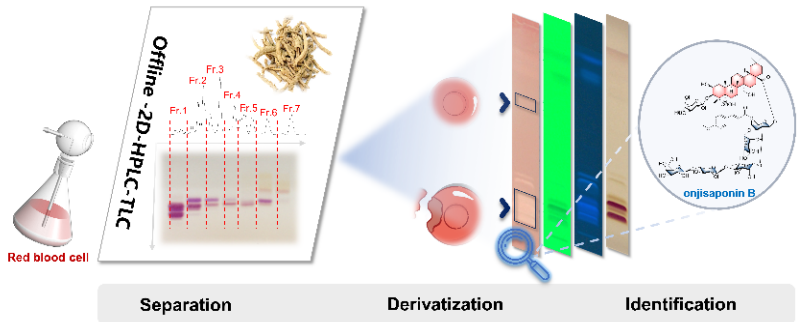
Keywords: HPTLC, bioassay, hemolysis, 2D-HPLC-TLC separation, TLC-MS

Hemolysis is the destruction or rupture of red blood cells, leading to the release of hemoglobin and other intracellular contents into the surrounding fluid. This process can be occurred naturally in the body or be induced by various factors such as drug toxicity or physical damage. Polygalae Radix (PR), originated from the dried root of *Polygala tenuifolia* Willd or *Polygala sibirica* L., is a commonly used traditional Chinese medicine which has been applied in over 800 prescriptions due to its wide range of pharmacological effects including neuroprotection, cognitive improvement, antidepressant, expectorant and antitussive, and anti-inflammatory properties. However, recent clinical data indicated that the improper use of PR may cause a side effect of acute hemolysis.

To clarify which specific components in PR lead to hemolysis effect, we conducted a series of studies based on combination of thin-layer chromatography (TLC), bioassay, 2D HPLC-TLC separation, and TLC-ESI-MS techniques. Hemolysis TLC-bioassay were conducted as follows: fresh rat blood cells were collected and immersed on the surface of the TLC plate, which had been developed with a suitable mobile phase and then dried. Components with hemolytic properties appeared as white spots against a pale blood-red background, thus revealing their presence. For separation of complex constituents of PR, a preparative -liquid-chromatography was used to acquire first-dimensional eluting-fractions, then TLC was followed to get the second-dimensional separation chromatogram. After detecting by above TLC-bioassay, the target zones were locked and transferred to TLC-ESI-MS to identify the compounds within the zones, and the fluorescence and color features of the target compounds on TLC were used as an auxiliary method for identification. Lastly, the structures of the target compounds were identified based on a self-built database according to the MS/MS fragments. As a result, over 40 saponins with different potential hemolytic effects have been identified in PR, and their structure-hemolysis effect relationship has been discussed. This method combines the TLC-bioassay, 2D-HPLC-TLC separation and TLC-MS techniques, using advantages of reverse phase chromatography and normal-phase chromatography, allowing for bidirectional separation in both lateral and longitudinal directions for each sample segment, greatly increased the separation and identification efficiency.

Figure



Scheme 1. Detection of hemolytic components in Polygalae Radix by TLC bioassay combined with two-dimensional chromatography and mass spectrometry

T12: Pharmaceutical Applications

Analysis of some sympathomimetic compounds by thin-layer chromatography based on their antioxidant activity

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Keywords: sympathomimetic compounds, thin layer chromatography, antioxidant activity, image analysis

Sympathomimetic compounds are drugs that mimic or enhance the effects of the sympathetic nervous system, which typically bind to and activate adrenergic receptors, leading to increased heart rate, dilation of airways, etc., being often used in medical treatments such as bronchodilators for asthma, decongestants, appetite suppression, pupillary dilatation and some types of blood pressure medications [1]. Besides their known therapeutic effects, these drugs are also of interest due to their possible neuroprotective effects that have been associated with the antioxidant activity that some of these compounds seem to exert [2]. The aims of this study are to evaluate the antioxidant activity of four sympathomimetic compounds namely salbutamol, terbutaline, norepinephrine and epinephrine using 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical by spectrophotometric measurements and to develop a thin layer chromatographic – image analysis (TLC-IA) method for simultaneous analysis of these compounds. The results obtained showed that among all tested compounds, epinephrine possess the highest antioxidant activity, and also that the color of adducts formed with $ABTS^{\bullet+}$ are orange, in case of terbutaline, and violet for salbutamol. Simultaneous separation of analyzed compounds was obtained on Silica gel $60F_{254}$ TLC plates using a mixture of ethyl acetate: methanol: water: formic acid 7:2:1:0.1 (v/v/v/v) as mobile phase. Taking advantage of the antioxidant properties of the analyzed compounds, the chromatographic plates were documented both in UV light, at 254 nm, and in white light, after immersing the plates in $ABTS^{\bullet+}$ solution, thus significantly improving their detection limits and their determination limits. The proposed method can be successfully applied in order to quickly and efficiently determine these drugs.

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Separation and determination of *Z* isomer and *N* desmethyltamoxifen by thin-layer chromatography

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Keywords: tamoxifen, TLC, the separation of geometric isomers, antineoplastics

Tamoxifen (2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-*N,N*-dimethylethanamine was developed as an antiestrogenic agent and as a selective estrogen receptor modulator (SERM). It is indicated as adjuvant therapy in the treatment of axillary node–negative or –positive breast cancer following partial or full mastectomy. Only *Z* isomer possess antagonist activity in others (e.g., breast and uterus) and agonist activity in certain tissues (e.g., bone and cardiovascular). In pharmaceutical dosage forms is always present as an impurity *N*-desmethyltamoksifen, which represents the major active metabolite of tamoxifen. The aim is this study is optimization of chromatographic conditions for the separation of *Z* isomer of tamoxifen and *N*-desmethyltamoxifen by thin layer chromatography and their determination in pharmaceutical dosage forms. The study of chromatographic conditions were used: the reference standards tamoxifen citrate and *N*-desmethyltamoxifen (La Chema, Czech Republic), chromatographic plates Silica gel GF254 (Merck, Germany), methanol, diisopropylether, ethyl acetate, cyclohexane, toluene and triethylamine (Merck, Germany). The optimal conditions for the separation and determination of Z isomer and *N*-desmethyltamoxifen achieved with a mobile phase composition: toluene / cyclohexane / triethylamine 6.5 / 2.5 / 2.0 (*v / v / v*). The satisfactory separation and resolution ($\alpha > 1$ and $Rs > 1.2$) between *N*-desmethyltamoxifen and *Z* isomer, has been achieved with applied TLC method.(Fig.1). Identification and quantification of impurity in the investigated tamoxifen tablets were performed. The *N*-desmethyltamoxifen was present in an amount of less than LOQ (15 ng). On the basis of these results it can be concluded that the TLC method is a fast and efficient method for testing the purity of tamoxifen in the bulk and pharmaceutical dosage forms.

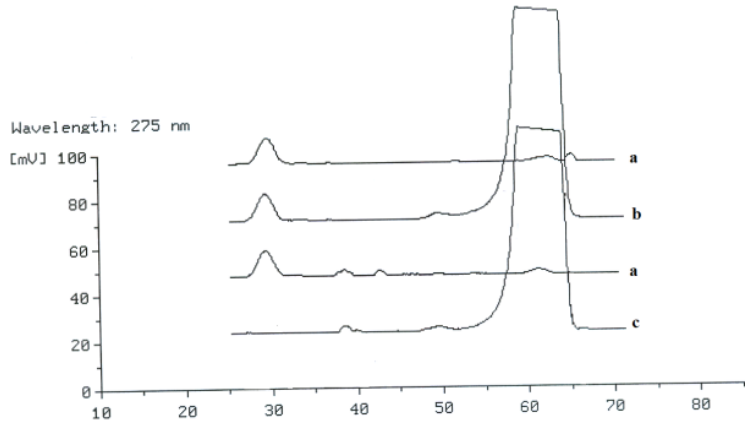

Figure 1. Densitogram of determination of N-demethyltamoxifen in tamoxifen tablets (a- impurity of N demethyltamoxifen, b- Tamoxifen tablets burdened with impurity standard, c- Tamoxifen tablets)

Acknowledgments

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Synthesis, characterization, and application of NADES as mobile phases in thin-layer chromatography

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Keywords: Natural Deep Eutectic Solvents (NADES), mobile phase, Thin Layer Chromatography (TLC), Attenuated Total Reflectance (ATR), drug quality control

NADES are a type of Deep Eutectic Solvents (DESs), i.e. a mixture of naturally occurring substances such as sugars, organic acids, primary and secondary plant metabolites, which have the ability to act as hydrogen bond donors and acceptors, resulting in a mixture with a lower melting point than that of the individual components. Due to their food grade property, biodegradability and biocompatibility, NADES represent a promising alternative that addresses the growing awareness of the need for environmentally friendly drug quality control due to its enormous environmental footprint. The aim of the study was the synthesis and characterization of NADES and the investigation of their potential application as mobile phases in TLC. Six NADES solvents were synthesized: glycerol: citric acid (1:3), glycerol:lactic acid (1:3), choline chloride:glycerol (1:2), choline chloride:lactic acid (1:2), choline chloride:ethylene glycol:lactic acid (1:2:1), citric acid:glycerol (3:1). In addition, the viscosity was adjusted by adding 40% w/w acetone, 40% w/w ethanol or 10% w/w water and the resulting systems were characterized by ATR. The addition of acetone or ethanol up to 40 %w/w or water up to 10 %w/w had no effect on the eutectic mixture, but led to a significant decrease in viscosity compared to the originally synthesized mixture. The synthesized and characterized NADES were used as mobile phases in TLC to analyze retention behavior of a selected group of analytes. The most commonly used analgetics were selected for the study: acetylsalicylic acid and its impurity salicylic acid, diclofenac and its four impurities, paracetamol, caffeine and ibuprofen. In addition, five steroids (betamethasone propionate, dexamethasone sodium phosphate, estradiol valerate, ethynilestradiol and spironolactone) and four benzodiazepines (diazepam, flurazepam, medazepam and nirtazepam) were included in the study. All solutions were prepared in methanol at a concentration of 1 mg/mL and 4 µL of each solution were spotted onto the plate. Separations were performed on SiO 60 RP-18 $F_{254}S$ TLC plates and developing chamber was thermostatted to approximately 40°C to further control viscosity. After development, the plates were dried at room temperature before being placed in a UV cabinet to examine thin-layer chromatograms under UV light of 254 nm in the absence of ambient light. All synthesized NADES were found to interfere with the visualization of salicylic acid, acetylsalicylic acid, ibuprofen, estradiol valerate and ethynilestradiol. For salicylic acid, acetylsalicylic acid, estradiol valerate and ethynilestradiol, the spots were quite faint, while the ibuprofen spot was not observed at all. Paracetamol and benzodiazepines produced streaks rather than spots, probably due to the pH around 5.0 of

the synthesized NADES. The presence of water in the synthesized NADES significantly affected the appearance of the spots and resulted in tailing, especially in the case of diclofenac and its impurities. Although the initial results have shown that NADES are promising mobile phases in TLC systems, the specific interactions between NADES and the analytes, as well as the underlying separation mechanisms need to be further investigated. Obviously, a particular challenge will be to achieve adequate retention/separation of ionizable and polar analytes.

T18: Life Sciences

Separation of polar and neutral lipids by high performance thin-layer chromatography

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Keywords: HPTLC, lipid separation, normal phase

Recent advancements in thin layer chromatography (TLC) methodology have significantly enhanced its resolution capabilities. Particularly, through the introduction of new equipment that allow precise control of critical parameters, such as humidity, during plate development. These innovations have made TLC a more reproducible and reliable technique. High performance TLC (HPTLC) has emerged as an efficient and rapid tool for analyzing various metabolites, namely lipids. Although mass spectrometry (MS) has largely replaced lipid analysis techniques over the past few decades due to its comprehensive lipidome profiling capabilities, it typically lacks the speed of TLC. HPTLC remains advantageous due to its lower cost, ease of use, simpler data interpretation, and its ability to be coupled with MS for detailed lipid species identification.

TLC analysis of polar and neutral lipids is commonly done separately. In this study, using CAMAG equipment and building on previously published elution methods, we have developed a protocol that allows the separation of both polar and non-polar lipids on a single normal phase plate. This method has been successfully applied to various mammal cell lines and yeast.

T21: Validation and Chemometrics

Development and validation of HPTLC method for quantitative determination of Formoterol fumarate and Fluticasone propionate

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Keywords: Formoterol fumarate, Fluticasone propionate, HPTLC, validation

The present research work aims to develop a robust high-performance thin-layer chromatography (HPTLC) method for the quantitative determination of Formoterol fumarate and Fluticasone propionate in pharmaceutical formulations. The quantitative estimation of Formoterol fumarate and Fluticasone propionate was carried out using optimized chromatographic conditions on aluminum-backed precoated silica gel plates 60 F_{254} using a blend of Hexane: Isopropyl alcohol: Methanol: Ammonia in the ratio of 8: 2: 0.3: 0.1 %v/v as mobile phase. Densitometric evaluation of separated compounds was done at 289 nm. The Rf value was found to be 0.25 and 0.79 for Formoterol fumarate and Fluticasone propionate, respectively. The optimized method was validated according to ICH Q2(R2) guidelines and found specific, accurate, precise, and robust. The validated HPTLC method was successfully applied for routine quality control analysis of Formoterol fumarate and Fluticasone propionate in in-house tablet formulations.

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Application of AQbD approach to design and develop robust HPTLC method for determination of Dapagliflozin and Teneligliptin

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The present research work aims to develop robust high-performance thin-layer chromatography (HPTLC) method for the quantitative determination of Dapagliflozin and Teneligliptin in *in-house* tablet formulations using the Analytical Quality by Design (AQbD) concept. Critical risk factors (parameters) and critical quality attributes (CQAs) were identified based on prior knowledge and results of preliminary experimental trials with the help of the Ishikawa diagram for the development of the proposed HPTLC method. Risk ranking of critical risk factors (volume of mobile phase components, development distance, the volume of injection, plate activation time, plate activation temperature, sample bandwidth, chamber saturation time, dimensions of development chamber, size of the plate, etc.) was performed using the risk assessment tool and preliminary trails. Based on risk ranking, factors were identified and selected for further screening. Plackett–Burman screening design was used to screen the critical risk factors considering their significant effect on dependent variables (retardation factor, resolution, number of theoretical plates, and tailing factor). The central composite design was used to optimize the screened risk factors, define the design space, and propose a control strategy to obtain reproducible results. The quantitative estimation of Dapagliflozin and Teneligliptin was carried out using optimized chromatographic conditions on an aluminum plate precoated with silica gel plates 60 F254 using a blend of methanol, ethyl acetate, and acetic acid as mobile phase. The optimized method was validated according to ICH Q2(R2) guidelines and found specific, accurate, precise, and robust. The validated HPTLC method was successfully applied for routine quality control analysis of Dapagliflozin and Teneligliptin in in-house tablet formulations.

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