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DIPLOMARBEIT

Titel der Diplomarbeit

Detection of adulterated herbal medicines by Attenuated Total Reflectance (ATR) Fourier Transform Infrared (FTIR) spectroscopy: A simulation study.

Extraction solvent optimization for HPTLC fingerprinting

verfasst von

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Abstract

Background:

Quality control of herbal products represents a major concern in the pharmaceutical industry due to the huge variety of these species and their almost daily new scientific findings. The continuous development of innovative methods of adulteration is aggravating this dilemma in the quality control processes further. In order to identify a fingerprint for each plant, different analytical methods have been suggested such as infrared (IR) spectroscopy and high performance thin layer chromatography (HPTLC). One aim of this study was to investigate the sensitivity of ATR-FTIR spectroscopy for the detection of adulterated herbal medicines. The second part of my work was dedicated to the optimization of a solvent extraction protocol towards wide coverage of secondary metabolites for HPTLC fingerprinting.

Material and Method:

98 samples of different dried parts of medicinal plants (herbs, roots, leaf, bark, wood, flower) were measured by ATR-FTIR spectroscopy. The obtained data were evaluated using SIMCA (soft independent modeling of class analogies) in order to determine the detection limits for these putative adulterants when systematically mixing their spectra with a dataset comprising 157 batches of valerian root samples (*Radix Valerianae*). In the second part, a validated and commercially available HPTLC system was utilized to evaluate a series of ultrasound extraction experiments. The objective was to find the optimal ternary solvent composition consisting of methanol (MeOH), water (H_2O) and dichloromethane (CH_2Cl_2) for which the flavonoid fingerprint (mobile phase: ethyl acetate/acetic acid/formic acid/water 100:11:11:27. Derivatization: Natural product reagent) is most comprehensive. 6 different medicinal plants (Yarrow, Thyme, Senna, Atropa Belladonna, Liquorice and Hop) were analyzed for this purpose.

Results:

With regard to the chemometric analysis of the ATR-FTIR spectra we found that for this particular dataset the overall detection rates computed from leave-one-out cross-validation were 56.2%, 85.9% and 98.3% at 5%/10% and 20% contamination levels, respectively. Concerning my work on the extraction optimization, I found that a mixture of 40-50% Methanol, 30-50% Dichloromethane and 10-20% water was most efficient for the analysis of the flavonoid patterns in the 6 plant systems under investigation. Unfortunately, the chromatographic data could be evaluated by visual inspection only due to the lack of suitable software.

Conclusion:

According to our study the detection limit of ATR-FTIR does not meet the standard requirements of the institutional reference (Ph.Eur), which permits a maximum of 5% foreign materials for valerian root. Concerning the extraction optimization, a universal solvent could not be defined exactly by response surface methodologies as reported in similar studies involving different analytical platforms (mostly high performance liquid chromatography) but suggestions for near optimal solvent compositions with competitive performances compared to pure and 70% methanol could be made.

Zusammenfassung

Hintergrund:

Die Qualitätskontrolle pflanzlicher Produkte ist für die Pharmaindustrie von außerordentlichem Interesse, da hier eine riesige Vielfalt vorliegt und stetig neue Erkenntnisse gewonnen werden. Die fortlaufende Entwicklung neuer innovativer Verfälschungsmethoden stellt hierbei eine der größten Herausforderungen an die Qualitätskontrolle. Verschiedene analytische Verfahren, wie die Infrarot-Spektroskopie oder die Hochleistungsdünnschichtchromatographie (HPTLC), wurden bereits vorgestellt um jede Pflanze über einen sogenannten „Fingerprint" zu identifizieren. Ein Ziel meiner Arbeit war die Empfindlichkeit des Fourier-Transformations-Spektrometers in abgeschwächter Totalreflexion (ATR-FTIR) im Hinblick auf verunreinigte pflanzliche Arzneimittel zu testen. Der zweite Teil meiner Arbeit beschäftigt sich damit ein Extraktionsprotokoll zu erstellen um die Mehrheit sekundärer Metaboliten mittels HPTLC-Fingerprinting abzudecken.

Material und Methodik:

98 pflanzliche Proben unterschiedlicher Herkunft (Wurzel, Rinde, Blatt, Blüte, etc.) wurden mittels der ATR-FTIR gemessen. Um die Nachweisgrenze dieser vermeintlichen Verfälschungssubstanzen zu bestimmen wurden systematisch deren Spektra mit einem Datenset bestehend aus 157 Proben Baldrianwurzeln (*Radix Valerianae*) gemischt und anschließend statistisch mittels der SIMCA (soft independent modeling of class analogies)-Methode ausgewertet.

Im zweiten Teil der Arbeit wurde ein validiertes, kommerzielles HPTLC-Gerät eingesetzt um auf ultraschall-basierende Extraktionsexperimente zu evaluieren. Das Ziel war eine ternäre Lösungsmittelzusammensetzung zu bestimmen, bestehend aus Methanol (MeOH), Wasser (H_2O) und Dichlormethan (CH_2Cl_2), wobei sich hier der „Fingerprint" der Flavanoide als ideal präsentiert. Zu diesem Zweck wurden 6 verschiedene medizinische Pflanzen (Schafgarbe, Thymian, Senna, Tollkirsche, Süßholz, und Hopfen) untersucht.

Ergebnisse:

Die chemometrische Analyse der ATR-FTIR-Spektren zeigte eine Nachweisgrenze im Sinne der „leave-one-out"-Kreuzvalidierung von 56,2%, 85,9% und 98,3% bei einer Kontamination von 5%/10% und

20%. In Bezug auf die Extraktionsoptimierung hat sich eine Lösungsmittelzusammensetzung aus 40-50% Methanol, 30-50% Dichloromethan und 10-20% Wasser als effizienteste Mischung zur Analyse flavonoider Fingerprints in den 6 untersuchten Pflanzen herauskristallisiert. Leider konnten die chromatographisch gewonnenen Daten nur visuell beurteilt werden, da bisher noch keine entspechende Software entwickelt wurde.

Conclusio:

Die Ergebnisse unserer Studie belegen, dass die Nachweisgrenze der ATR-FTIR nicht die Standardanforderungen des Europäischen Arzneibuchs erfüllt, welches ein Maximum von 5% Fremdmaterial für Baldrianwurzeln zulässt.

In Hinblick auf die Extraktionsoptimierung konnte kein universelles Lösungsmittel definiert werden. Jedoch können Empfehlungen für eine nahezu optimale Lösungsmittelzusammensetzung abgegeben werden, vergleichbar mit purem und 70% Methanol.

Acknowledgement

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List of abbreviations

ATR	Attenuated total reflectance
ATS 4	Automatic TLC sampler
ADC2	Automatic development chamber
DCM	Dichloromethane
DOE	Design of experiment
DWP	Deep well plate
FT-IR	Fourier Transform Infrared
HDR	High dynamic range
HM	Herbal medicines
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
K2CO3	Potassium Carbonate
LOD	Limit of detection
MeOH	Methanol
NP	Natural product
PC	Principle component
PCA	Principle component analysis
PP	Polypropylene
p.a	Pro analysis
QC	Quality control
RPM	Rotation per minute
Rf	Retardation factor
SIMCA	Soft Independent Modeling of Class analogy
TE-DLATGS	Deuterated L-alanine doped triglycene sulphate
TLC	Thin-layer chromatography
UV-VIS	Ultraviolet-visible
ZnSe	Zinc Selenide

1 Introduction

Fingerprint searches are becoming very important for the classification and authentication of botanical species[1]. The fingerprint of a herbal medicine is considered as a unique chemical signature for its secondary metabolites[2]. Many analytical techniques such as HPLC (High Performance Liquid Chromatography), HPTLC (High Performance Thin Layer Chromatography), GC (Gas Chromatography), infrared (IR) and ultraviolet-visible (UV-VIS) spectroscopy can be applied as powerful tools for fingerprinting and Quality Control (QC) of herbal medicines[3].

Nowadays the field of QC faces major challenges due to dramatic expansion in usage of herbal medicine, the difficulties to prove herbal drugs authenticity and the detection of adulterations. Herbal drugs, unlike synthetic drugs, contain usually hundreds of complex secondary metabolites, which are in most cases unknown[4]. It is known that crude plant materials are chemically and naturally changeable due to many factors as harvest time, climate, geographical origin and storage conditions. In such cases fingerprint chromatogram/-spectrum can be successfully used to assess the quality of herbal drug preparations[3, 5].

In this work we determined fingerprints of herbal drugs by mainly two techniques, namely HPTLC and IR spectroscopy.

Detection of adulterated herbal medicines by Attenuated Total Reflectance (ATR) Fourier Transform Infrared (FTIR) spectroscopy

IR spectroscopy has been widely used for QC in the field of pharmaceutical, food and agricultural industry and is considered to be a clean, rapid, inexpensive and reproducible technique[6]. The wavelength's range from 1,500–900 cm^{-1} in an IR spectrum, as shown in figure 1.1, is named "fingerprint region" because the pattern of the bands is characteristic for the molecular composition and thus can identify even minor metabolites[7]. The technique of IR spectroscopy is based on the interaction of IR-light with molecules. It is known that molecules with functional groups, commonly organic compounds, absorb specific frequencies corresponding to certain wavelengths which are dependent on the molecular vibration of different molecules[8].

According to Schrader et al in ATR spectroscopy the measuring beam is reflected internally at the interface between an auxiliary medium and the sample. This auxiliary medium must be infrared

transparent and of high refractive index[9].“ The internal reflection elements are usually inert prisms made of diamond, silicon or zinc selenide (ZnSe). When total reflection occurs an evanescent wave is created which interacts with the sample resulting in attenuation of the IR beam of light that passed then to the detector in the IR spectrometer (Figure 1.2)[8, 10].

As IR fingerprints of herbal medicines become a point of interest in scientific research big emphasis has been put in developing an IR fingerprint database and a convenient chemometrics software[6].

Many studies have discussed the use of Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) in combination with chemometrics in QC of herbal medicines[11, 12] and their adulteration as well as in food industry[13-15].

On the other hand there are some publications debating the issue of validation of FTIR method concerning its sensitivity for detection of adulterated HM. According to pharmacopeia a certain limit of amounts of foreign substances is allowed in every crude drug, which should not be exceeded. A validated analytical method should be able to detect at least this limit. Adulteration of HM by replacing valuable ingredients with cheaper substitutes can result in major health hazards to consumers. There is no doubt that detection of adulteration of HM, either intentionally or accidentally, is a major concern in the field of QC[16-19].

ATR-FTIR spectroscopy allows qualitative and quantitative analysis of plant samples in solid (e.g.: powder, dried extracts) as well as in liquid forms (e.g.: plant’s extract, solutions), which represents an advantage over other spectroscopic and chromatographic techniques. Furthermore only insignificant or even no sample preparation leads to fast, simple and reproducible analysis[20]. However, taking all these positive characteristics of ATR-FTIR spectroscopy into account it is also crucial to evaluate the sensitivity of this useful instrument.

Chemometrics is defined as discipline that links analytical and measurement science using statistical, mathematical and other formal logical systems to extract the features of a substance from their chemical composition[21, 22]. It has found widthspread use in data-driven scientific fields where the number of observables exceeds the number of samples, which is becoming more and more the rule rather than the exception.

FTIR spectroscopy has been previously applied together with means of chemometrics in the discrimination of flowering plants depending on their different chemical profiles as well as in discrimination of counterfeit medicines[24, 25]. However, to the best of our knowledge systematic investigations that address the sensitivity of IR spectroscopy for the detection of adulterants are as yet missing.

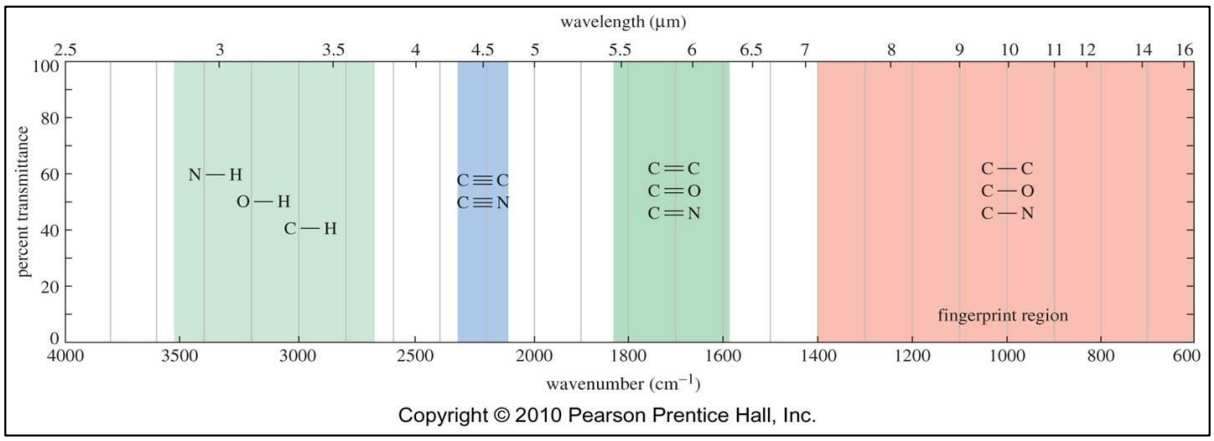


Figure 1.1: Functional group frequency and fingerprints region of an IR spectrum; wave number in cm^{-1}

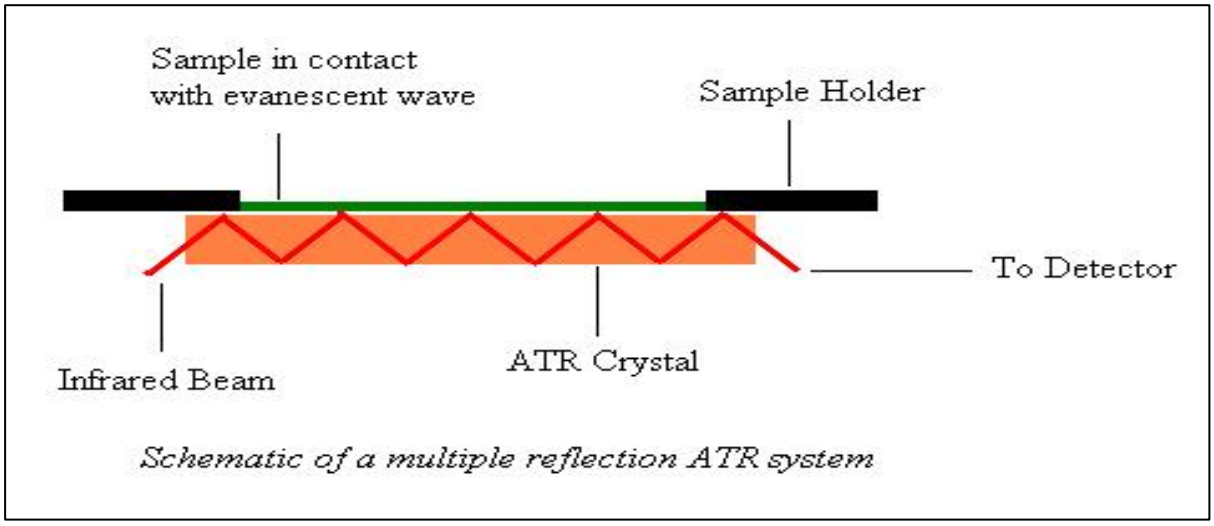


Figure 1.2: Schematic model of ATR system

Reference: Jigar Mistry, Graduate Research Student, Missouri S&T

Extraction solvent optimization for HPTLC fingerprinting

An effective extraction method and a chromatogram with clear separation of the detected secondary (2ry) metabolites are required for obtaining a informative fingerprints of herbal drugs that are useful to assess their quality[1]. Many recent papers have investigated various extraction methodologies[26, 27]. According to several publications a mixture of 3 solvents methanol/chloroform and/or acetone/water in different ratios are most effective in extracting both polar and nonpolar metabolites[28-31]. It is a fact that extraction protocols in the pharmacopoeia monographs are very heterogeneous and consequently leads us to the question: Can we meet the demand of an easy to-use-one-fits-all protocol, namely one solvent combination, that is useful to analyze a large number of herbal plants in terms of identity and quality?

HPTLC is an advanced automated planar chromatography technique, which depends on separation of substances physically between a stationary and a mobile phase. The mobile phase moves over the stationary phase in certain velocity and particular direction according to its polarity and affinity. HPTLC is the optimum unification of technology, science and standardized methodology which offers many advantages such as high sensitivity, visual presentation of the results, equivalent analysis of samples, fast and simple operation, cost effectiveness [2].

The HPTLC instrument of CAMAG, a leading company in instrumental planar chromatography, is advantageous because all main steps such as sample application, plate development and evaluation can be performed separately in time and place. However full automation is not possible yet. The software (vision CATS) manages all the steps of HPTLC including sample application, documentation of images and assessment of chromatograms under white and UV light[32].`

Nowadays HPTLC has been involved in many important fields such as QC of pharmaceuticals, cosmetics, food and the detection of adulteration in herbal drugs. Nevertheless some limitations of HPTLC have to be taken in consideration such as the lower separation power than HPLC (usually one order of magnitude). Furthermore a full automation is not a practicable option and thus it is an open system where plates are exposed to environmental factors such as air, light, temperature and humidity[2]. In addition to that it has to be mentioned that this method depends on the user´s subjective visual impression and interpretation of the results.

Aims

IR: In attempt to evaluate the sensitivity of ATR-FTIR spectrometer as a QC tool in detection of even minimum amounts of adulterants - in other words to define the detection limit of FTIR-ATR spectroscopy - 98 samples of different dried herbal plants in addition to 157 Valerian root samples were measured by using ATR-FTIR spectroscopy. The resulting data were analyzed with the help of chemometrics (SIMCA) whereby the spectra of the valerian samples were adulterated virtually in the computer by the 98 plant samples at different concentrations.

HPTLC: Study the influence of solvent mixtures with different polarities (methanol, water and dichloromethane) in different ratios (24 varieties) on the analysis of the flavonoid pattern of different herbal medicines.

Detection of adulterated herbal medicines by Attenuated Total Reflectance (ATR) Fourier Transform Infrared (FTIR) spectroscopy

2 Material and Method

2.1 Material

98 samples (table 2.1) of different herbal drugs in powder form (75µm mesh) were kindly provided by the Department of Pharmacognosy (University of Pharmacy/Vienna). The 98 plants are listed in the Appendix (table 6.1).

2.2 Method

2.2.1 Preparation of samples

Samples from previously grinded stocks were collected in small-labeled sachets without any further preparation from the depot of the Department of Pharmacognosy. Then they were directly placed on the crystal surface of ATR-FTIR spectrometer and measured as triplicates.

2.2.2 Spectra acquisition (ATR FTIR SPECTROSCOPY)

Spectra were collected using a Bruker Tensor 27 FTIR spectrometer (Bruker, Germany), as seen in figure 2.1, with ZnSe crystal cell ATR (PIKE, USA) equipped with a deuterated L-alanine doped triglycene sulphate (TE-DLATGS) detector. Spectra were measured at room temperature, at resolution of 4cm^{-1}, 16 scans for each sample in a wavenumber range of 4000cm^{-1} to 600cm^{-1}. A background spectrum was scanned before the analysis of each sample to cancel the effect of air components in the spectrum. After each measurement the crystal was wiped off with lab soft tissue wetted by alcohol. Collected spectral data were subjected to a baseline correction (rubber band method) and vector normalization by software named Opus 5.5. The final analysis was accomplished by in-house software using Matlab 2014b (Mathworks, Natick MA, USA).

Figure 2.1: FT-IR Spectrometer - TENSOR 27 from Bruker Optics/Germany[35]

2.2.3 Statistical analysis of data

Multivariate statistical approaches deal with the analysis of large datasets with several variables. Reduction of the dimensionality via excluding irrelevant dimensions is considered to be the main purpose of the multivariate analysis making the interpretation of data much easier.

In this work Principle Component Analysis (PCA) and Soft Independent Modeling of Class analogy (SIMCA) were performed using in-house software. PCA (unsupervised classification method) is a characteristic dimension reduction method, which is considered to be the basic ground of the multivariate data analysis. The main application of PCA is lowering the number of variables and determination of similarities among them, identification of outliers and presentation of data in less dimensional space[22, 36]. The concept of PCA is to detect the principle components (PCs), which are new variables that include most of the variability in the multivariate data matrix and consequently facilitate the presentation of information with fewer variables than in the first data[36]. The first principle component explains the most variance in the data, the second principle which is orthogonal to the first PC explains the highest remaining variance element and so on[37].

SIMCA (supervised classification method) is a pattern recognition method based on PCA[38] and a common tool of class modeling that is used in chemometrics mainly to obtain a robust model of a multivariate data group. SIMCA assesses the analysis of differences or resemblance among number of spectra (classes), making a definite confidence zone around each class[39]. That is the reason why it represents an independent modeling and it is soft because samples can belong to no class, one class or multiple classes simultaneously.

First, all spectra (n=157) were imported to MATLAB and then divided into 156 training samples and 1 testing sample for leave-one-out cross-validation. PCA was used to build a model for the training samples (n= 156). The test sample or left out spectrum was then synthetically adulterated with each of the 98 adulterant spectra in different concentrations ranging from 0% to 50% in order to detect which concentration is associated with a distance to the model (DModX) beyond the confidence region (critical distance). This procedure was replicated for every adulterant and concentration until every valerian spectrum had been left out from the modeling process once (figure 2.2). To put it in other words, 157 Valerian roots spectra were subsequently adulterated with each of the 98 spectra in 8 different concentrations (0-50%) one after the other.

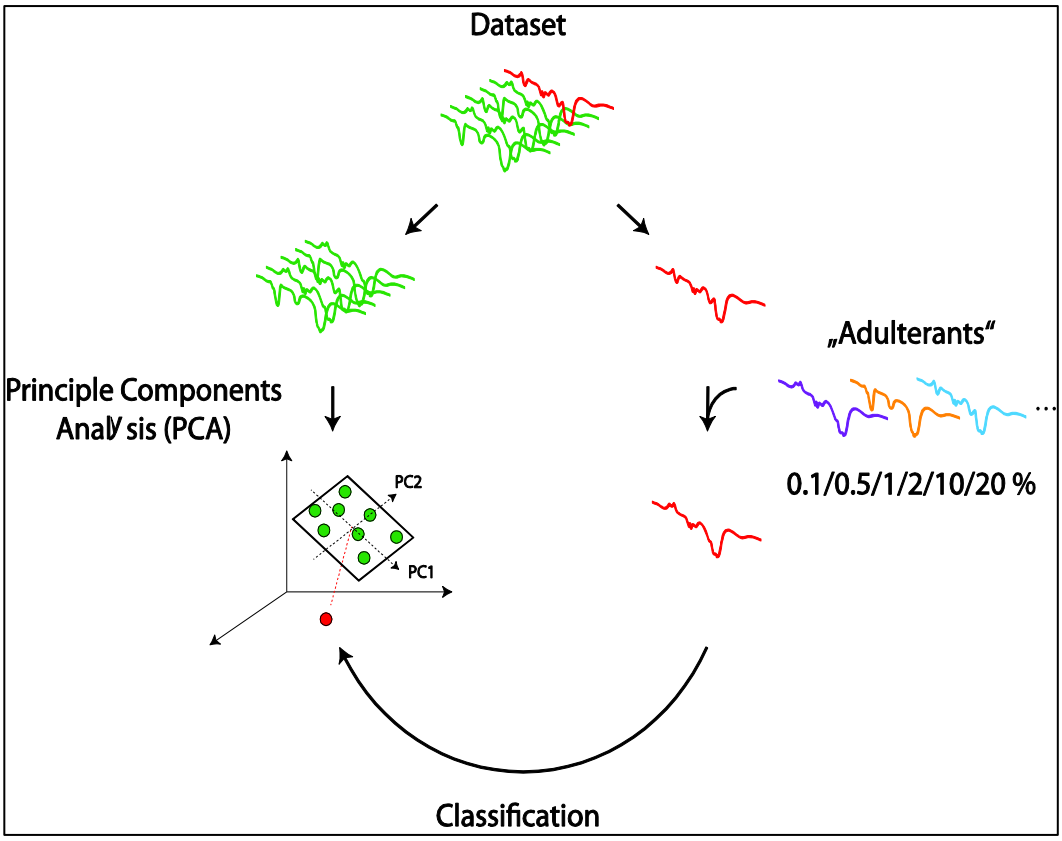


Figure 2.2: Schematic illustration of data analysis via PCA and SIMCA

Reference: Msc. Ramin Nikzad, Department of Pharmacognosy/University of Vienna

3 Results

Class membership of the test samples can be predicted according to their distance from the class boundary (critical region). An adulterated test sample which lies above the class boundary can be considered as successfully detected by the method.

Figure 3.1 shows the overall results from the simulation including all 98 putative adulterants. Our data indicate that contaminations below 5% are hardly detectable by this method. Only 56.2% of all test samples were correctly predicted as being adulterated using the 90%-confidence limit. 85.9% and 98.3% were correctly identified at 10% and 20% contamination levels. Comprehensive detection (i.e. 100%) was achieved at 40% and 50% contamination. Our data further indicated that the detection rate (i.e. the probability for detection) is dependent on the similarity between sample and adulterant spectra (figure 3.2).

According to the British Pharmacopeia 2007 not more of 5% of stem bases and not more than 2% of other foreign matters are permitted in valerian roots. All in all, these results suggest that ATR-FTIR in combination with the here presented chemometric technique (SIMCA) lacks sufficient sensitivity to meet current quality control standards.

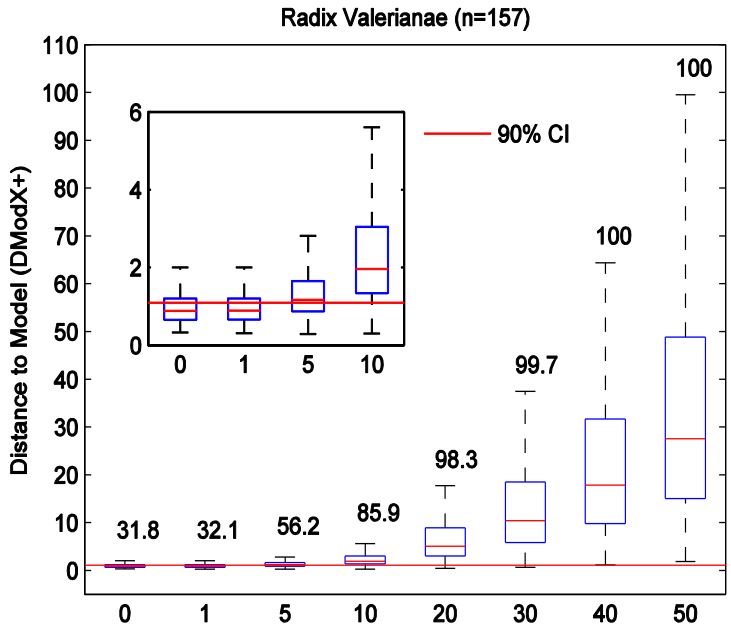


Figure 3.1: Detection frequency of adulterated samples. X-axis: Adulterant level (%). Y-axis: Augmented distance to the model plane taking into consideration the orthogonal distance and scores space distance

Reference: Msc. Ramin Nikzad, Department of Pharmacognosy/University of Vienna

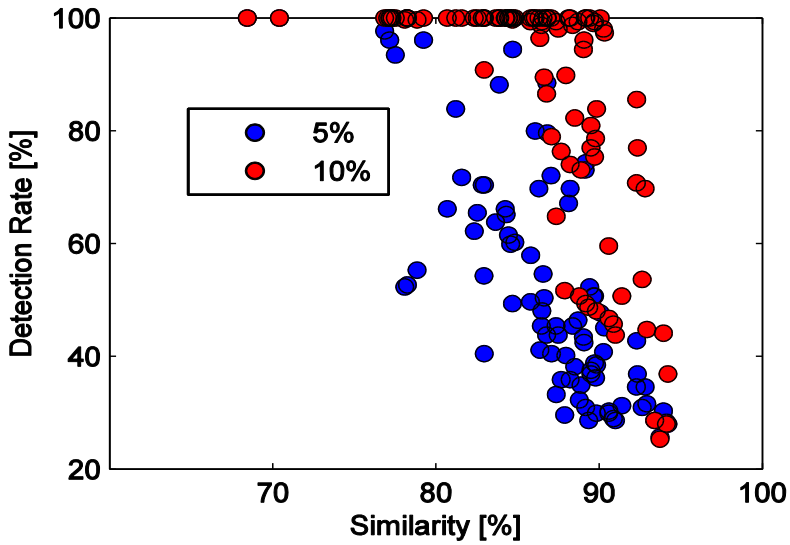


Figure 3.2: Relation between detection rate (%) and similarity between sample and adulterants at 5% (blue) and 10% (red) contamination level for all 98 putative adulterants used in this study. The similarity is expressed as Pearson's correlation x 100

Reference: Msc. Ramin Nikzad, Department of Pharmacognosy/University of Vienna

4 Discussion

Nowadays spectroscopic tools are becoming routine procedure in QC of crude drugs. FTIR spectroscopy, when combined with chemometrics represents a powerful tool for authentication of medicinal plants and detection of adulterants because it is simple, fast (measurements done within minutes), accurate, non-invasive, environment friendly (acquire no sample preparation), and can be classified as a fingerprinting technique[17, 39].

This chapter presents a major concern in QC, namely the adulteration of medicinal plants. Adulteration can be done through the partial or total substitution of the original plant with foreign materials or plant´s analogues making the adulterant detection more difficult[19].

In this work we tried to determine the detection limit of adulterants of ATR-FTIR technique, which is a very important parameter of analytical method validation and development[16].

According to the Association of Analytical Communities (AOAC) the limit of detection is defined as the least content of the analyte, which can be measured with acceptable statistical certainty[16].

Practically we measured 157 samples of Valerian root in addition to 98 samples of different medicinal plants using ATR-FTIR technique. Then valerian spectra were virtually adulterated by the 98 plants spectra in different concentrations using well established chemometric techniques (PCA & SIMCA).

Valerian roots are commonly adulterated with other valerian species, which do not possess biological activity. Consequently, these circumstances make their detection more challenging because of high similarity between originals and adulterants[40].

Our results suggest acceptable probability to detect adulterants in a non-targeted way (i.e. if the spectrum of the adulterant is *a prior* unknown) when present at concentrations of 10% or more. However only 56% of the adulterated samples with 5% concentration were detected. This unsatisfying outcome is not accepted by most of the guidelines concerning QC protocols and pharmacopeias since a maximum of 5% foreign material is allowed in medicinal plants. Furthermore, 32% of non-adulterated valerian spectra went beyond the critical region (false positives), which can be attributed to human errors during experimental work or to inappropriateness of the chemometric technique used in this study. In this context further investigation using alternative statistical methods would be of great value.

Extraction solvent optimization for HPTLC fingerprinting

2 Material and Method

2.1 Material

For the experiments of extraction optimization samples of 6 different medicinal plants were used including Yarrow, Thyme, Senna, Liquorice, Hips and Belladonna. Each of the selected plants contains different secondary metabolites such as alkaloids, saponins, essential oils, anthraquinones and flavonoids. The samples were selected from different parts of the plants.

All this samples were obtained from the depot of the Department of Pharmacogonosy. Samples were mainly produced from the manufacturer Kottas (Vienna, AT).

Plant Name	Family	Main Secondary metabolite	Part of plant	Use
Yarrow *Millefolii herba*	*Asteraceae*	Flavonoids,volatile oils,sesquiterpenes	Herb	Anorexia Dyspeptic disorders of GIT[41]
Thyme *Thymi herba*	*Lamiaceae*	Essential oils	Herb	Bronchitis Whooping cough Infections of upper respiratory tract[41]
Belladonna *Atropa belladonna*	*Solanacae*	Alkaloids	Leaf	Anticholinergic effect: Parkinson, Peptic-ulcer[42]
Senna *Sennae folium*	*Caesalpiniaceae*	Dianthronglycosides Anthraquinones	Leaf	Short term Constipation[41]
Hops *Lupuli flos*	*Cannabaceae*	Resinous substances, bitter acids and volatile oils	Flower	Sedative in Agitation, Anxiety and Insomnia[41]
Licorice *Liquiritiae radix*	*Fabaceae*	Triterpensaponins	Root	Gastritis Coughing and bronchitis[41]

Table 2.1: Extraction optimization; name of used plants, their origin, main secondary metabolites and medical use

2.2 Method

2.2.1 Preparation of samples, references (dilution series) and reagents

Samples were freshly grinded to fine powder with a particle size of 25µm. 150mg of the powdered samples were transferred to a 96 deep well plate and then mixed with 1.2 ml solvent according to a specific scheme illustrated in table 2.2. Samples were well mixed and sonicated for 10 minutes, then mixed again and sonicated for additional 10 minutes. The extracts were then centrifuged at a maximum speed of 4400 rotations per minute (RPM) at room temperature for 10 minutes. Supernatants were transferred into a clean deep well plate and centrifuged again (maximum speed 4400 RPM at room temperature). Finally supernatants were pipetted off in HPLC vials with inlet and subjected to HPTLC analysis.

For the HPTLC process one standard mobile phase and one derivatizing agent were used in all analyses. The mobile phase was freshly prepared prior analyses and consisted of a mixture of ethyl acetate, formic acid, acetic acid and water in ratios (100:11:11:27). All plates were derivatized using a natural product (NP) reagent that was prepared by dissolving 1g Diphenylborinic acid in 200ml ethyl acetate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	1	2	3	4	5	6	7	8	9	10	11	12
D	13	14	15	16	17	18	19	20	21	22	23	24
E	1	2	3	4	5	6	7	8	9	10	11	12
F	13	14	15	16	17	18	19	20	21	22	23	24
G	1	2	3	4	5	6	7	8	9	10	11	12
H	13	14	15	16	17	18	19	20	21	22	23	24

Table 2.2: pipetting scheme of a 96 DWP: each number in this scheme represents an ID number for the solvents combinations

Dilution series:

For comparison and statistical normalization of the chromatograms across different HPTLC plates a dilution series of a botanical reference material was prepared. To this end for each of the 6 herbal medicines 0.5g of powdered sample was mixed with 25ml 70%Methanol in a glass tube. The extract was sonicated for 10 minutes, mixed by using a spatula, and sonicated for another 10 minutes.

After a centrifugation step (10 min, 3000 rpm) samples were transferred into Schott bottles using round filter paper. The dilution series (table 2.3) was prepared in Eppendorf tubes. Finally the diluted extracts were filled in labeled HPLC vials with inlet. Solvents, chemicals and instruments, which were used in the experiment, are listed subsequently in tables 2.4 and 2.5.

200%	100%	85%	70%	55%	40%	20%
Extract (4µl)	Extract (2µl)	850µl Extract +150µl 70% MeOH (2µl)	700µl Extract + 300µl 70% MeOH (2µl)	550µl Extract + 450µl 70% MeOH (2µl)	400µl Extract + 600µl 70% MeOH (2µl)	200µl Extract + 800µl 70% MeOH (2µl)

Table 2.3: Dilution series preparation. Application volumes used for HPTLC analyses are indicated in parentheses

Name	Manufacturer	Quality
Water	In-house demineralization	
Methanol	J.T.Baker	HPLC Gradient Grade
Dichloromethane	AnalaR Norma Pur	HPLC Gradient Grade
Ethyl acetate	J.T:Baker	p.a.
Formic acid	Gatt-Koller	p.a.
Acetic acid	Gatt-Koller	p.a.
2-Aminoethyl diphenylborinate, Natural Product (NP)	Roth	p.a.

Table 2.4: Solvents and chemicals (p.a.: pro analysis)

Name	Manufacturer
Automatic TLC Sampler 4 (ATS 4)	CAMAG,Switzerland
Twin Trough Chamber 20cm×10cm	CAMAG,Switzerland
Automatic Developing Chamber (ADC 2)	CAMAG,Switzerland
Chromatogram Immersion Device	CAMAG,Switzerland
TLC Visualizer	CAMAG,Switzerland
Filter paper for saturation	CAMAG,Switzerland
Mill ZM 100	Retsch,Germany
Balance MA BA 200	Sartorius,Germany
Analytical Balance KB BA 100	Sartorius,Germany
Ultrasound bath Transsonic T 460	Elma,Germany
Centrifuge labofuge 400	Heraeus,Germany
Centrifuge Multifuge1S-R	Heraeus,Germany

Table 2.5: Instruments

Extraction solvent preparation:

We prepared combinations of the 3 solvents methanol (MeOH), water (H_2O), and dichloromethane (DCM) in different ratios. Miscibility of the three solvent mixtures in different proportions was tested before used and mixtures that were not miscible were excluded from the optimization (figure 2.1). Miscible mixtures are listed in the table 2.6.

100 ml stocks of the 24 solvent mixtures were prepared and kept in Schott bottles at room temperature.

Solvent ID	MeOH	H_2O	DCM
1	100	0	0
2	0	100	0
3	0	0	100
4	40	0	60
5	40	10	50
6	40	60	0
7	50	0	50
8	50	10	40
9	50	20	30
10	50	50	0
11	60	0	40
12	60	10	30
13	60	20	20
14	60	30	10
15	60	40	0
16	70	0	30
17	70	10	20
18	70	20	10
19	70	30	0
20	80	0	20
21	80	10	10
22	80	20	0
23	90	0	10
24	90	10	0

Table 2.6: List of solvents and their composition in %

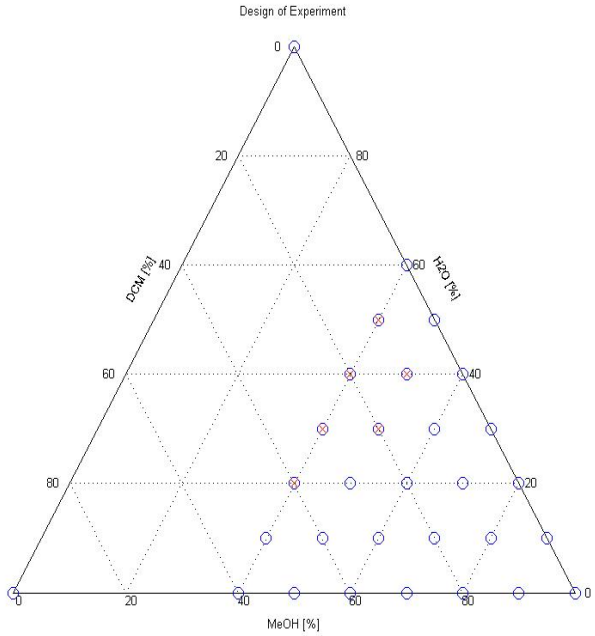


Figure 2.1: Design of Experiment for MeOH, DCM, and H2O. Immiscible mixtures are indicated with red crosses

Reference: Msc. Ramin Nikzad, Department of Pharmacognosy/University of Vienna

2.2.2 Data acquisition

Samples were analyzed using high performance thin-layer chromatography HPTLC. HPTLC is a technique, which separates the ingredients in the plant´s extracts according to their affinities between a stationary phase (HPTLC plates of silica gel on a glass plate 60F 254 from Merck; plate format: 20cm×10cm) and a mobile phase (ethyl acetate, acetic acid, formic acid and water in 100:11:11:27).

The analysis of one plant (extracted with 24 different solvent combinations) was analyzed on 3 separate plates on which the first 7 positions were reserved for the samples of the dilution series. The remaining 8 positions were reserved for the extracts samples in ascending order of content of water, because we also wanted to understand the influence of water content in extractions solvents with respect to the efficiency of extraction.

A clean plate image was taken under white light by CAMAG´s visualizer prior analyses. Then 15 samples were applied on each plate as 8mm bands by spray on technique using the auto sampler (ATS 4). The samples were applied 3.4mm apart from each other, 8mm apart from the lower edge and 20mm from both the right and the left edge to the center point of the first and last bands. Volumes of 2µl from each sample and from dilution series were applied. The development was done in the automatic development chamber (ADC 2). For each plate 35ml mobile phase, consisting of 25ml for saturation and 10ml for development, were freshly prepared and filled in the 2 inlets. Then the filter paper was placed in the backside of the twin trough chamber. The plate was placed and the operation proceeded in the following stages: drying of the plate; humidity establishment to 43% relative humidity with 800ml potassium carbonate (K_2CO_3) solvent for 10 minutes; saturation of the chamber by mobile phase for 20 minutes; plate development up to migration distance of 70 mm from the lower edge and finally drying for 5 minutes. Steps of development are illustrated in figure 2.2.

Afterwards a second picture of the developed plate was taken under UV 254nm and UV 366 nm light whereby the picture quality was enhanced by high dynamic range (HDR) mode. Subsequently the plate was dipped in natural product reagent for the derivatization using CAMAG Chromatogram Immersion Device (dipping speed 5, dipping time 0) and dried under the fume hood for 5 minutes.

Finally a third picture was taken under UV 366nm in HDR mode. Steps of HPTLC operation are summarized in figure 2.3.

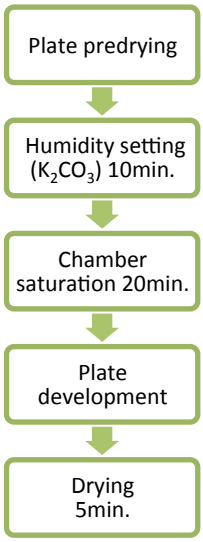


Figure 2.2: HPTLC: steps of development

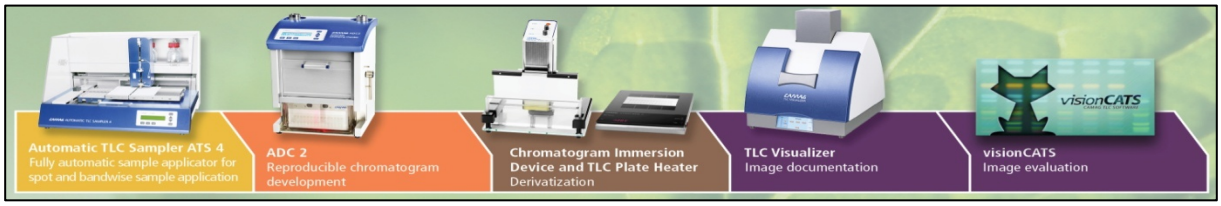


Figure 2.3: Steps of a HPTLC system

Reference: www.camag.com

3 Results

3.1 Preconditions of the main Experiment Extraction Optimization

A number of experiments were performed before the main extraction optimization experiments to fulfill the requirements of the work and improve practical conditions of principal experiment. We tried a 96 DWP as extraction bowel, which is unusual for such experiments. Therefore we needed to test the application to evaluate their suitability for the practical work. Experiments included dilution series, reproducibility tests of 96 DWP, comparison between extraction yields in DWP and in Eppendorf tubes and finally exhaustive extraction. Used solvents and reagents as well as instruments are listed in tables 3.1.1 and 3.1.2

3.1.1 Dilution series

Goal of the analysis: To investigate if retention index shifts are dependent on concentration**.**

Material and Method:

A freshly milled 0.5g powder of *Herba Millefolii* was mixed with 5ml of each of these 3 solvents (40% Methanol, 40% Methanol/10% Dichloromethane, 60% Methanol/10% Dichloromethane). Extracts were mixed and sonicated for 10 minutes, then centrifuged for 10 minutes with maximum speed (4400RPM) and filtered on cotton into 3 labeled falcon tubes. In 12 labeled falcon tubes (1:2-1:4-1:8-1:16) 500µl Methanol were added and then supernatants of each solvent were used to prepare dilution series whereby 500µl of supernatant were pipetted off in falcon tube with label 1:2, well mixed and afterwards 500µl were transferred into tube with label 1:4 and so on until the last diluted sample resulted in a concentration of 1:16.

After the preparation of the dilution series of 3 solvent mixtures, extracts were filled in HPLC vials with inlets in order to carry out a HPTLC analysis.

2 analysis were performed:

First analysis: Detection of Polar compounds:

Mobile phase: 1-Butanol: Acetic Acid: Water (7:2:1)

For Derivatization: freshly prepared Anisaldehyde reagent.

The reagent was prepared by carefully adding 10ml sulfuric acid to an ice-cooled mixture of 170 ml Methanol and 20ml Acetic acid. 1ml Anisaldehyde was added to this solution. The plate is immersed in the reagent for 1s, then heated at 100 °C for 2 – 5 minutes

Results:

Retention index shifts appear clearly in samples of concentrations of 25 and 50% shown in figure 3.1.1.1. These shifts are probably due to overloading of the stationary support.

Considerable blurring and tailing did occur and are most likely due to the slight acidic character of phenolic compounds in the sample, which tend to deprotonate. Better results could eventually be obtained when using formic acid in the mobile phase to keep substances protonated. Appearance of secondary fronts might be due to contamination of the mobile phase or the derivatizing agent.

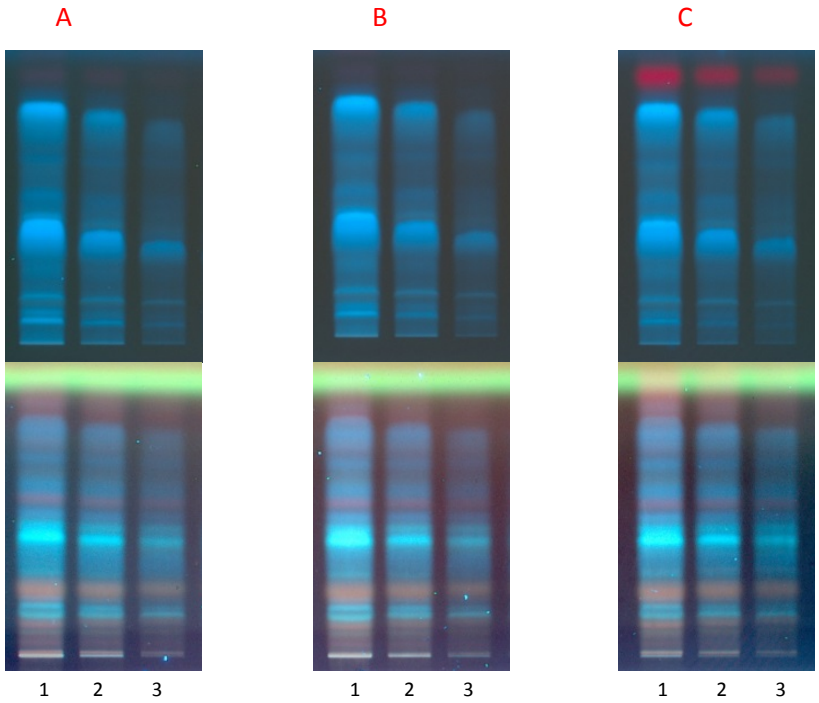


Figure 3.1.1.1: Extraction optimization; HPTLC picture of polar compounds; dilution series; A: samples extracted with 40% MeOH before (above) and after (below) derivatization; B: samples extracted with 40% MeOH/10% DCM before (above) and after (below) derivatization; C: samples extracted with 60% MeOH/10% DCM before (above) and after (below) derivatization; tracks (1-3): concentrations of dilution series: track1: 100%, track2: 50%, track3: 25%

Second analysis: Detection of Flavonoids:

Mobile phase: Ethyl Acetate: Acetic Acid: Formic Acid: Water (100:11:11:27)

For Derivatization: freshly prepared Natural product reagent (NP) by dissolving 1g Diphenylborinic acid in 200ml Ethyl acetate (dipping speed: 5, dipping time: 0).

Results:

Retention index shifts: when another mobile phase was used these shifts disappeared and the corresponding zones had nearly the same R_f values.

Secondary fronts: No secondary fronts are observed even though the same vials were used indicating that there was an issue either with some mobile phase constituents or with the derivatization reagent in the first plate (figure 3.1.1.2).

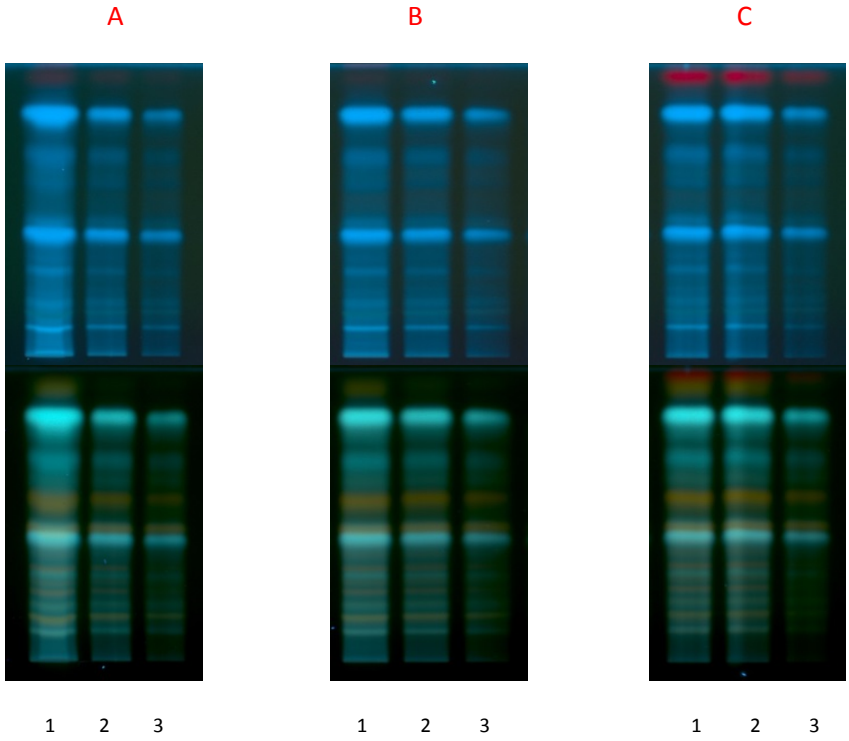


Figure 3.1.1.2: Extraction optimization; HPTLC picture of flavonoids; dilution series; A: samples extracted with 40% MeOH before (above) and after (below) derivatization; B: samples extracted with 40% MeOH/10% DCM before (above) and after (below) derivatization; C: samples extracted with 60% MeOH/10% DCM before (above) and after (below) derivatization; tracks (1-3): concentrations of dilution series: track1: 100%, track2: 50%, track3: 25%

3.1.2 High throughput extraction of H. Millefolii - First Step (Reproducibility of DWP)

Goal of the Experiment: Investigate the efficacy (reproducibility) of 96 deep well plates for ultrasound extraction by determination of the dried extract´s weights.

Material and Method:

20g of *Herba Millefolii* were freshly milled (to 25 µm particle size).

Each pore of 96 DWP (1) was filled with 150mg powder using a sensitive digital scale. The plate was then covered with a silicon lid and kept at room temperature. After that, extraction solvents (100% methanol, 70% methanol/30% dichloromethane, 40% methanol/10% dichloromethane) were prepared in a separate DWP (2) as shown in table 3.1.2.1. At this step methanol was applied using multi-channel pipette and Dichloromethane was applied using a dispenser pipette. 1200µl of the extraction solvents were added to the herb powder in DWP (1) using a multi-channel pipette (200µl 8-channel pipette from CAPP equipped with 300µl OneTouch filter tips - Biozym). Afterwards the plate was closed with silicon lid, placed, mixed and placed again in an ultrasound bath for 10

minutes. Samples were then centrifuged for 10 minutes with 4400 RPM. 500µl supernatants were then carefully transferred to previously tarred Eppendorf tubes (64 labeled Eppendorf tubes which were placed in the desiccator overnight and then weighed) and allowed to evaporate overnight (about 16 hours) using the GeneVac (very low boiling point mixture). Extracts, selected for the drying process, were in columns numbered with 3, 4, 5, 8, 9, 10, 11, and 12 in DWP (8×8). The next day dried extracts were weighed and yields were calculated in an excel sheet.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500
B	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450
C	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750
D	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450
E	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500
F	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750	600 150 750
G	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450	1050 450
H	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500

Table 3.1.2.1: the table represents a DWP; MeOH in black, DCM in red, water in blue; extraction optimization; DWP reproducibility; pipetting scheme in µl. Rows A, E, H: 100% MeOH; Rows B, D, G: 70% MeOH/ 30% DCM; Rows C, F: 40% MeOH/10% DCM

Results:

As shown in figures 3.1.2.1-3 extract yield was roughly the same in samples extracted by 100% Methanol and 70% MeOH/30% Dichloromethane (between 0.005 and 0.008mg) but rose dramatically in samples extracted with 40% Methanol/10% Dichloromethane because of its higher water content (between 0.009 and 0.011 mg).

Reproducibility of 96-Deep well plate is considered to be acceptable and there is no general tendency that extraction efficiency in the middle of the plate (e.g. well D8) is different from the edge (e.g. well A12) except of individual spikes, which occurred maybe due to improper mixing of samples or unintentional human error.

Manual shaking of the plate was not effective because of leakage of the lid. As a result we decided to use a multichannel pipette for mixing samples in the further experiments.

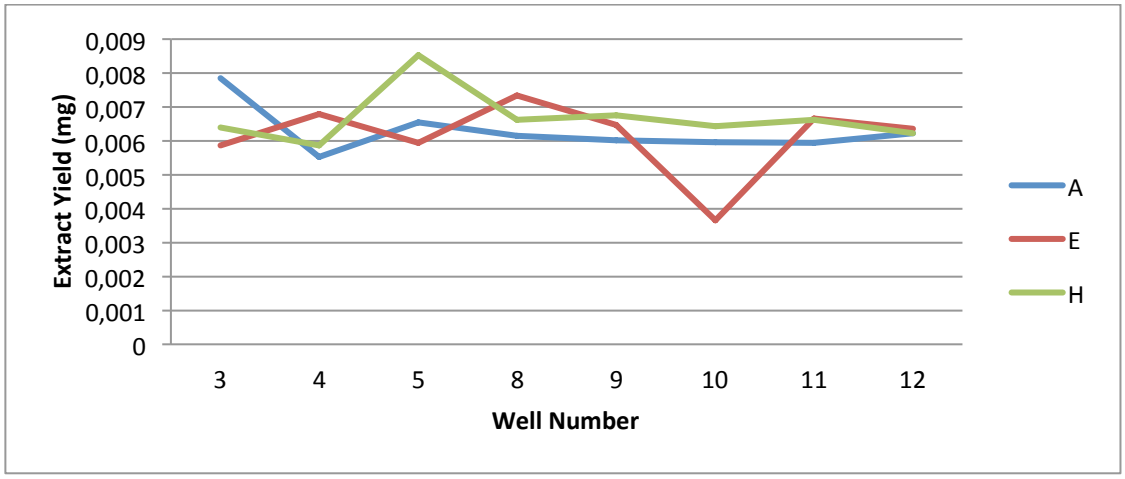


Figure 3.1.2.1: Extraction optimization; DWP reproducibility; Y-axis: yield of H. Millefolii extracted with 100% MeOH; X-axis: well number; A, E, H: rows of DWP

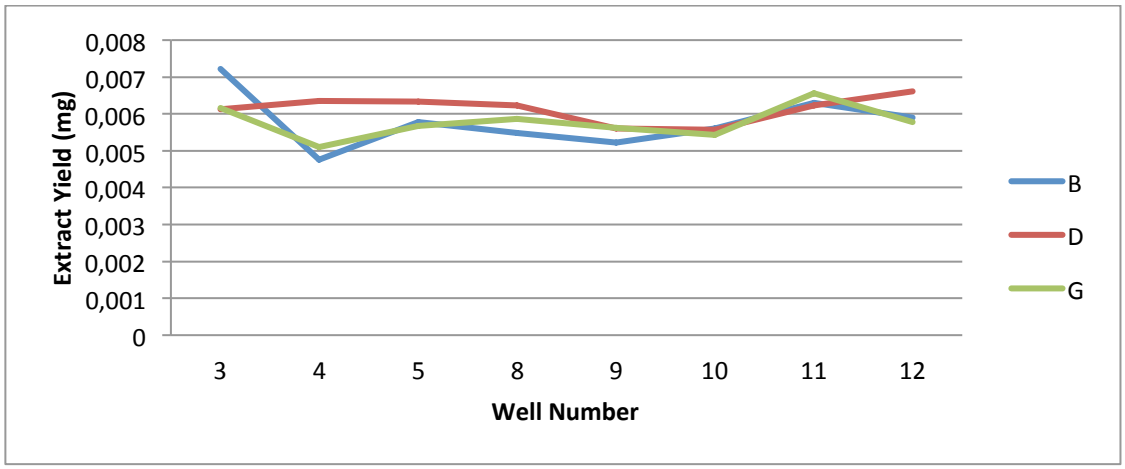


Figure 3.1.2.2: Extraction optimization; DWP reproducibility; Y-axis: yield of H. Millefolii extracted with 70% MeOH/30% DCM; X-axis: well number; B, D, G: rows of DWP

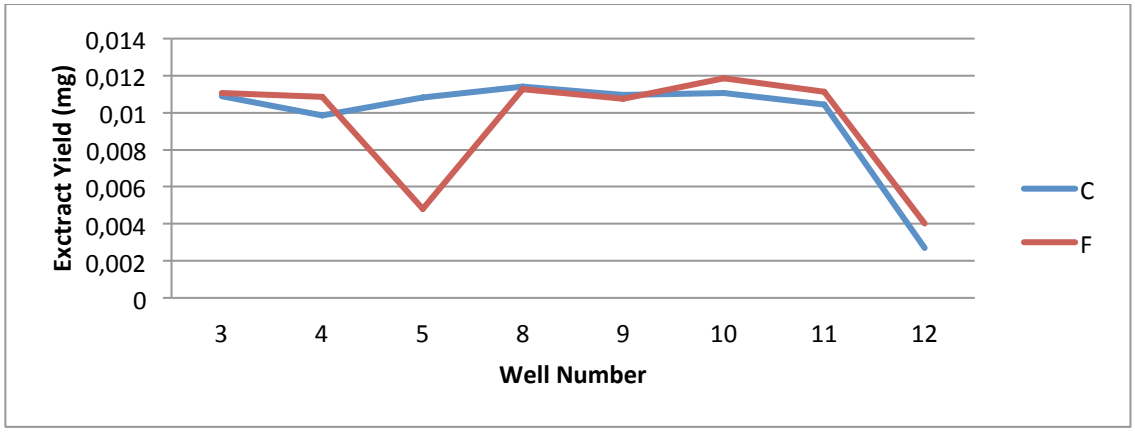


Figure 3.1.2.3: Extraction optimization; DWP reproducibility; Y-axis: yield of H. Millefolii extracted with 40% MeOH/10% DCM; X-axis: well number; C, F: rows of DWP

96 DEEP WELL PLATE (DWP):

DWP were selected for this experiment because it is possible to prepare large number of samples and to work time efficiently.

Eppendorf Deep Well plates are manufactured from pure polypropylene (PP), which is highly resistant to chemicals, mechanical stress and temperature extremes. DWPs also are centrifugation stable and are specially designed in terms of rounded edges and conical bottoms so that liquids collect in the well´s center leading to an acceleration of the mixing and pipetting processes. Alphanumeric labels at the border of the plate help to find samples faster and facilitate the application of pipetting scheme.

DWPs are provided with lids to minimize sample evaporation. However, they were not effectively sealed and during extraction in the ultrasound bath some samples have been leaked. DWPs and their lids were not easy to clean and sometimes pigmentation from samples were not removable[43].

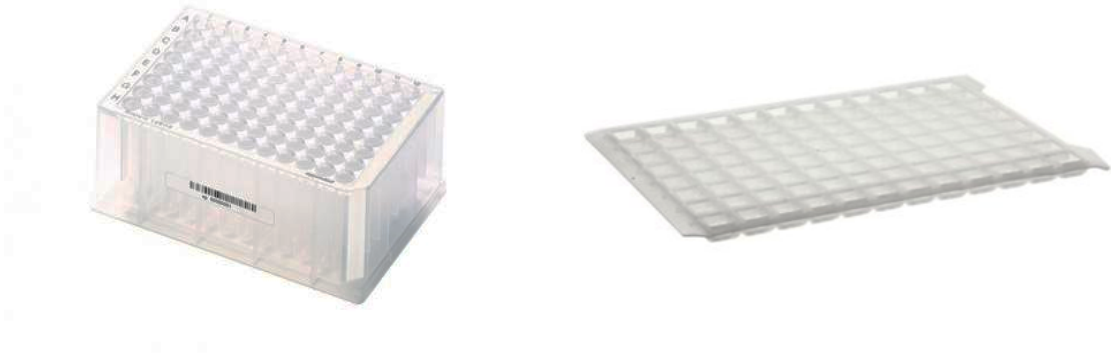


Figure 3.1.2.4: left: 96 DWP; right: silicon lid of 96 DWP[43]

3.1.3 High throughput extraction of H. Millefolii - second step (reproducibility of DWP)

Goal of the analysis: to compare the dried extracts´ weights in DWP and Eppendorf tubes in order to prove the reproducibility of 96DWP

Material and Method:

Same procedure was repeated but this time directly in previously tarred Eppendorf tubes and the results were compared with those of the DWPs:

20g *Herba Millefolii* were freshly milled to a particle size of 25µm. 15 Eppendorf tubes were then divided into 3 groups and labeled with the 3 extraction solvents: 100% Methanol, 70% Methanol/30% Dichloromethane and 40% Methanol/10% DCM. Afterwards tubes were weighed data were written down.

Another 15 labeled Eppendorf tubes were then filled with 150mg powder using a sensitive digital scale. 1200µl of each extraction solvent (100% Methanol, 70% Methanol/30% Dichloromethane, 40% Methanol/10% Dichloromethane) were prepared and added to the herb powder (5 tubes for each solvent). Eppendorf tubes were closed, placed in an ultrasound bath for 10 minutes, and then centrifuged for 10 minutes (4400 RPM). Afterwards 500µl supernatants were carefully transferred to previously tarred Eppendorf tubes and allowed to evaporate overnight (about 16 hours) using the GeneVac (very low boiling point mixture). Finally dried extracts were weighed next day. Yields were calculated in an Excel sheet and compared with data of DWP.

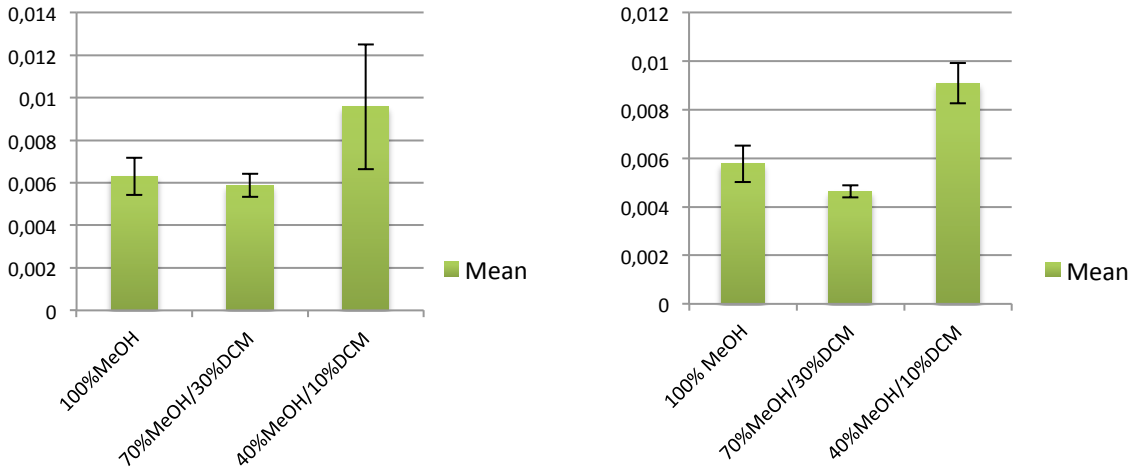


Figure 3.1.3.1.: Extraction optimization; comparison between extract yield of DWP and that of Eppendorf`s tubes. Left: DWP, Right: Eppendorf Tubes. Y-axis: yield in mg; X-axis: solvent composition

Results:

The two diagrams above show no great differences between yield of samples extracted in a 96 Deep well plate and those extracted in Eppendorf tubes which encouraged us to use them in the main experiment for extraction optimization.

After reaching this conclusion another question emerges whether Exhaustive Extraction can enhance reproducibility.

3.1.4 Exhaustive Extraction

Goal of the Experiment: to evaluate the benefit and necessity of exhaustive extraction in attempt to maximize the extraction efficiency and to improve the reproducibility of extraction procedures of herbal plants.

Material and Method:

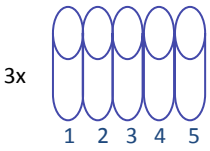
15 of 2ml Eppendorf tubes (1) were labeled as following:

Tubes A1 - A5: 100% MeOH; tubes B1 - B5: 70% MeOH/30% DCM; tubes C1 - C5: 40% MeOH/10% DCM; then tubes were weighed (use gloves when handling with tarred tubes!) and the weights were noted for later use; 20g of *Herba millefolii* were homogenized to a particle size of 0.25µm; 150mg herb powder were transferred to another 15 1.5ml Eppendorf tubes (2) which also were labeled the same way like the tarred ones: tubes A1 - A5: 100% MeOH; tubes B1-B5: 70% MeOH/30% DCM; tubes C1 - C5: 40% MeOH/10% DCM; 500µl extraction solvent was added to the Yarrow powder

Samples were sonicated for 10 minutes and then centrifuged for 10 minutes with 4400 RPM. Afterwards 200µl of supernatant were pipetted off in previously tarred 2ml Eppendorf tubes (1). Pellets of A2 - A5, B2 - B5 and C2 - C5 were then resuspended with 500µl fresh extraction solvents. A1, B1 and C1 represent the first extraction cycle. Afterwards samples were sonicated, centrifuged again. Additional 200µl supernatant were transferred to the tarred 2ml Eppendorf tubes (1). Pellets of A3-A5, B3-B5 and C3-C5 were resuspended and steps were repeated until a total of 5 cycles of extraction were accomplished. In the third, fourth and fifth extraction cycle volumes of transferred supernatants were modified to 300µl instead of 200µl to allow enough space for fresh solvents so that the final supernatant volume in each of 2ml Eppendorf tubes (1) were 1300µl.

The extracts were then dried overnight using GeneVac (very low boiling point mixture). The next day tubes were weighed and the extracts weights were calculated in an Excel sheet.

Extract weight = weight of tubes with dried extract´s - weight of empty Eppendorf tubes



1.150mg powder (0.25µ) + 500µl solvent

100% MeOH, 70%MeOH/70%DCM. 40% MeOH/10% DCM

2. Extraction 10min

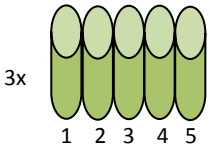
3. Centrifugation 10min

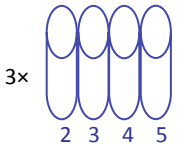
Supernatant

Pellet

4. Transfer 200 µl into tarred Eppendorf tubes

5.Resuspenion with 500µl solvent





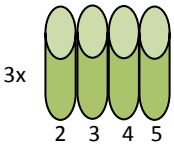
6. Repetition of steps 2 and 3

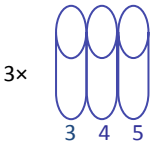
Supernatant

Pellet

7. Transfer 200 µl into tarred Eppendorf tubes

8. Resuspension with 500µl solvent





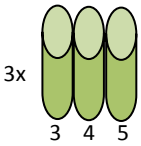
9. Repetition of steps 2 and 3

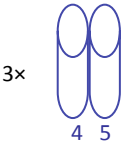
Supernatant

Pellet

10. Transfer 300 µl into tarred Eppendorf tubes

11. Resuspenion with 500µl solvent





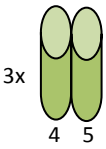
12. Repetition of steps 2 and 3

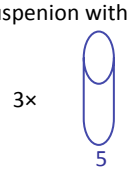
Supernatant

Pellet

13. Transfer 300 µl into tarred Eppendorf tubes

14. Resuspenion with 500µl solvent





15. Repetition of steps 2 and 3 and transfer 300 µl supernatant to the last 3 tarred Eppendorf tubes.

16. Evaporate solvents from 5 Eppendorf tubes (2) using Gene-vacuum (during night)

17. Weighing dried tubes and calculate the final extract weight

Figure 3.1.4.1: Procedure of exhaustive extraction experiment

Results:

As shown in figure 3.1.3.1 exhaustive extraction was not reached after 5 cycles, which might be contributed by unfitting of herbal drug ratios and their extraction solvent or the improper surface contact between sample and solvent since we used mini-size tools for the extraction process (DWP and Eppendorf tube). Decanting the whole supernatant at each step might be a solution for this problem. Eventually an additional intermediate step could be beneficial by transferring the original supernatant to a fresh Eppendorf tube which is centrifuged again to minimize the continuously volume increase in the tarred vial.

Finally we have to admit that the benefit, gained from exhaustive extraction, stands in inadequate relation to the extra effort required for such experiments.

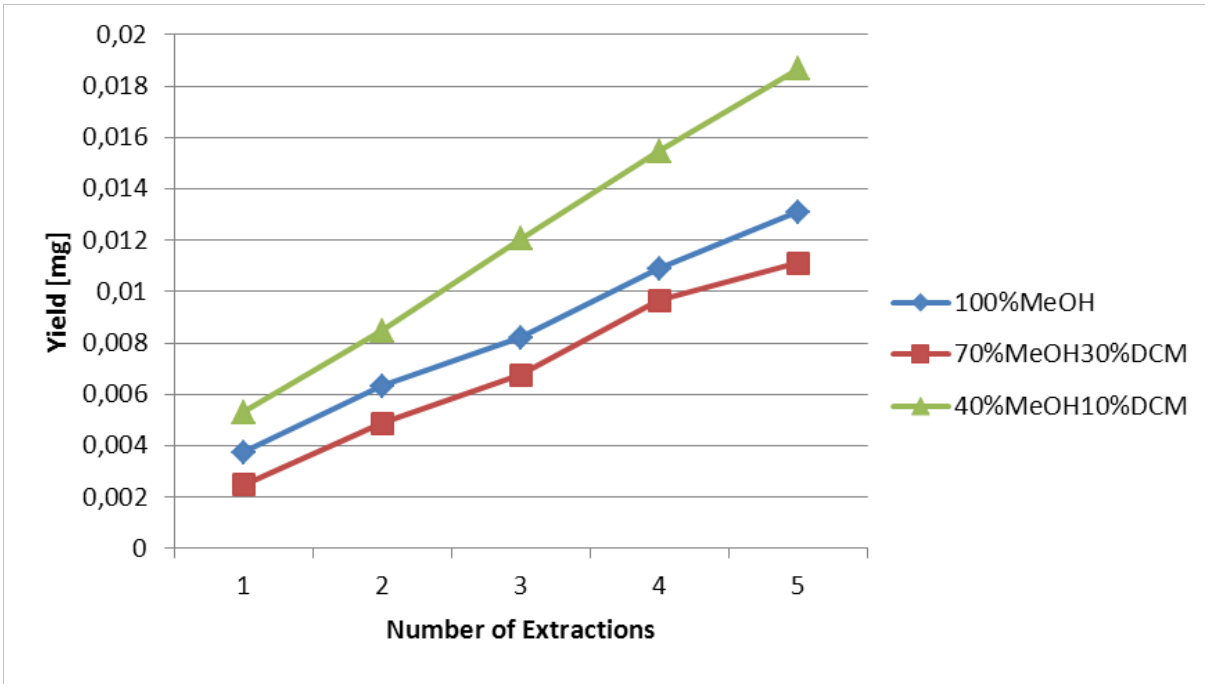


Figure 3.1.4.2: Extraction optimization; exhaustive extraction; Excel generated graph; X-axis: yield of H. Millefolii extracted by 3 different solvents; Y-axis: number of extractions

Name	Manufacturer	Quality
Water	In-house demineralization	-
Methanol	J.T. Baker	HPLC Gradient Grade
Dichloromethane	AnalaR NormaPuR	p.a.
Ethyl acetate	J.T. Baker	p.a.
Formic acid	Gatt-Koller	p.a.
Acetic acid	Gatt-Koller	p.a.
2-aminoethyl diphenylborinate, Natural Product (NP)	Roth	p.a.
Anisaldehyde reagent	Fluka Chemicals	p.a.
1-butanol	AnalaR NormaPuR	p.a.
Acetone	J.T. Baker	p.a.

Table 3.1.4.1: Solvents and chemicals (p.a: pro analysi)

Name	Manufacturer
Automatic TLC Sampler 4 (ATS 4)	CAMAG, Switzerland
Twin Trough Chamber 20cm×10cm	CAMAG, Switzerland
Automatic Developing Chamber (ADC 2)	CAMAG, Switzerland
Chromatogram Immersion Device	CAMAG, Switzerland
TLC Visualizer	CAMAG, Switzerland
Filter paper for saturation	CAMAG, Switzerland
Mill ZM 100	Retsch, Germany
Balance MA BA 200	Sartorius, Germany
Analytical Balance KB BA 100	Sartorius, Germany
Ultrasound bath Transsonic T 460	Elma, Germany
Centrifuge labofuge 400	Heraeus, Germany
Centrifuge Multifuge 1S-R	Heraeus, Germany
Centrifuge Pico 21	Heraeus, Germany
Evaporator Genevac EZ-2 Plus	Genevac, England

Table 3.1.4.2: Instruments

3.2 Visual evaluation of the extraction solvents efficiency

In this chapter the visual evaluation of the resulting fingerprints and the effect of extraction optimization through different extraction solvents are presented. Fingerprints are viewed in figures 3.2.1 - 3.2.18. Each figure consists of 2 sections (A&B):

A: fingerprints before derivatization

B: fingerprints after derivatization

The first 7 tracks of the fingerprint represent the samples of dilution series: 70% Methanol extracts in the following concentrations: 200%, 100%, 85%, 70%, 55%, 40% and 20%.

Each of the selected plant was extracted by 24 different solvents, which were then divided into groups of 8 samples. Therefore an analysis of samples from one plant resulted in 3 plates.

1. Yarrow:

Figure 3.2.1 shows the first group of samples. Fingerprints were almost similar in the upper part of the chromatogram (non-polar zone) characterized by clear zones. A very weak separation of substances was observed in the lower part (polar 2ry metabolite), except in track 9 and 11. Here, the associated extraction solvents contained 10% water and therefore extraction efficiency of polar 2ry metabolites was obviously increased.

The second group of samples is shown in figure 3.2.2. Here, fingerprints revealed slight variations in the intensity of the zones' colors, which were more noticeable in the lower part of the chromatogram. These variations indicate the differences in extraction power of the second group of solvents, especially of polar substances. According to tracks 9 and 15 samples seemed to be extracted with less efficient solvents than the others due to little water content of only 10-20%.

Fingerprints extracted with solvents of the third group are illustrated in figure 3.2.3. They looked almost similar in the lower part of the chromatogram. In track 8 the sample was extracted with 100% water and showed absence of zones in both upper and lower fields indicating poor extraction efficiency.

At the top of the chromatogram, the highly non-polar region, a number of zones were totally absent, namely in tracks 8, 9 and 10 because of the higher content of water and little or no content of DCM.

With regard to the visual aspect of all 3 plates we assume that 40-50% methanol/30-50% Dichloromethane solvent combinations have better extraction efficiency (for *Herba Millefolii*) than

the other solvents. Important to mention that extraction of polar 2ry metabolites were enhanced when solvent had a little amount of water (not more than 20 %).

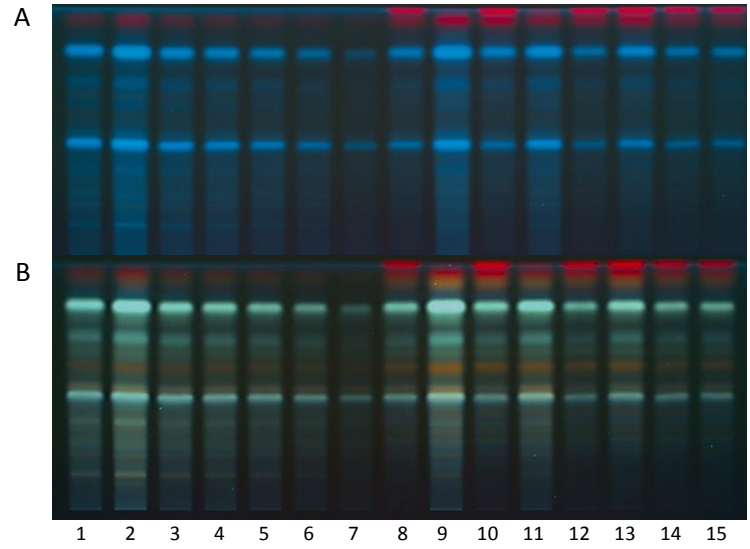


Figure (3.2.1): HPTLC fingerprints of Yarrow samples (group 1) extracted with different solvents under UV366nm A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-15): Yarrow samples extracted with different solvents → track8: 100% MeOH; track9: 40% MeOH/50% DCM; track10: 50% MeOH/50% DCM; track11: 50% MeOH/40% DCM; track12: 60% MeOH/40% DCM; track13: 70% MeOH/30% DCM; track14: 80% MeOH/20% DCM; track15: 90% MeOH/10% DCM

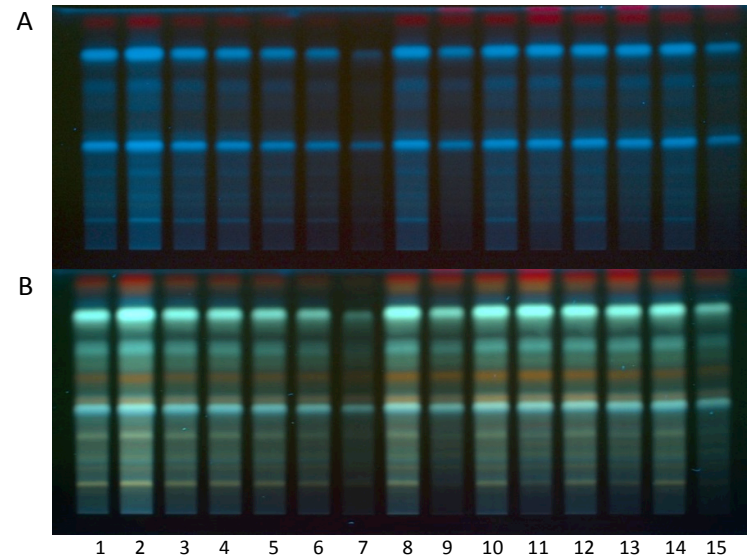


Figure (3.2.2): HPTLC fingerprints of Yarrow samples (group 2) extracted with different solvents under UV366nm A: developed and B: after derivatization; tracks(1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-15): Yarrow samples extracted with different solvents → track8: 50% MeOH/30% DCM; track9: 60% MeOH/30% DCM; track10: 60% MeOH/20% DCM; track11: 70% MeOH/20% DCM; track12: 70% MeOH/10% DCM; track13: 80% MeOH/10% DCM; track14: 80% MeOH; track15: 90% MeOH

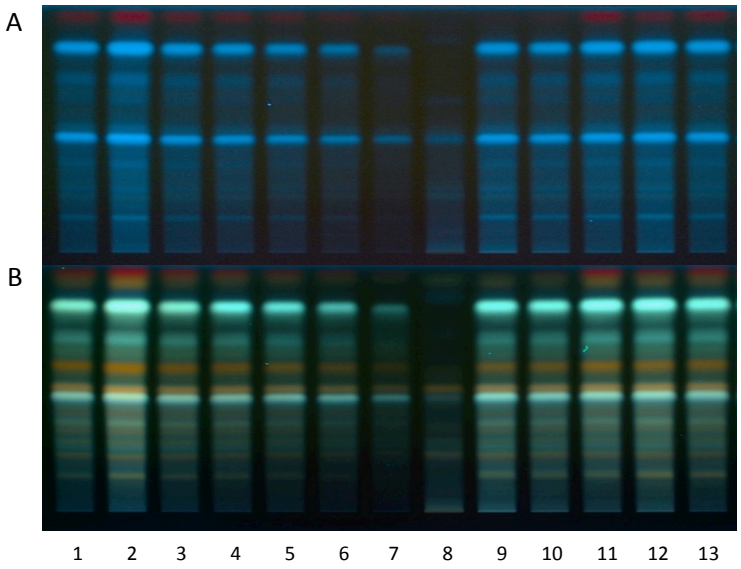


Figure (3.2.3): HPTLC fingerprints of Yarrow samples (group 3) extracted with different solvents under UV366nm A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-13): Yarrow samples extracted with different solvents → track8: 100% water; track 9: 40% MeOH; track10: 50% MeOH; track11: 60% MeOH/10% DCM; track12: 60% MeOH6; track13: 70% MeOH

2. Thyme:

The chromatograms of thyme samples, extracted by the first group of solvents, can be seen in figure 3.2.4. We observed no visually detectable differences between the fingerprints, neither in number of zones nor in their color intensity. Among this group of solvents it is hard to point out which solvent has best extraction efficiency.

In figure 3.2.5 a minor variability in the upper part of the chromatogram can be revealed, especially in track 10. There were additional weak bands in the non-polar zone. Furthermore, absence of the upper red zone of chlorophyll could be noticed in track 12. Other fingerprints looked similar without obvious differences in the quality of sample contents. However tracks 8 and 9 seem to display more intense zones than others.

The third group of samples is represented in figure 3.2.6. There, track 12 shows a better color intensity than all other fingerprints. In all fingerprints the second red zone of chlorophyll was missed and both zones were totally absent in tracks 8 and 9. This was the result of higher water content of the extraction solvents represented by this group. According to the visual evaluation of thyme fingerprints we consider that a combination of 40-50% Methanol, 30-40% Dichloromethane and 20-30% water has better effects on the overall fingerprint.

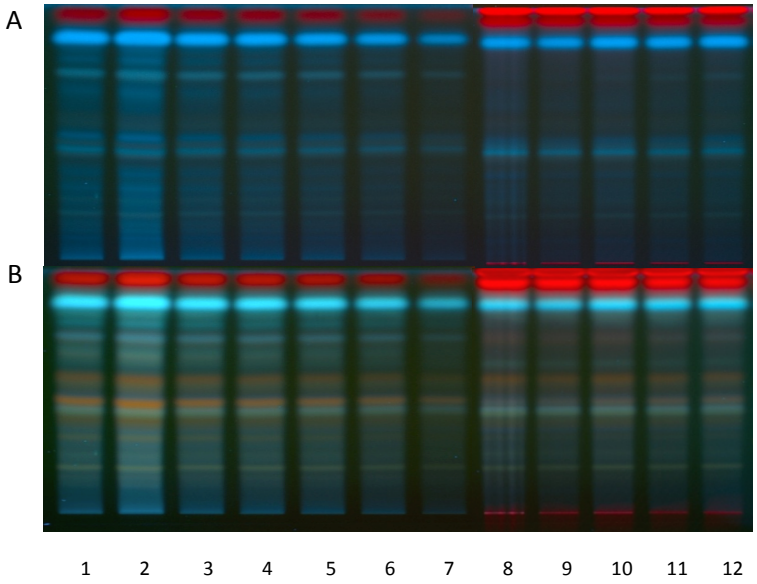


Figure 3.2.4: Extraction optimization: HPTLC fingerprints of thyme samples (group1) extracted with different solvents under UV 366nm; A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12): Thyme samples extracted with different solvents → track8: 40% MeOH/60% DCM; track9: 50% MeOH/50% DCM; track10: 60% MeOH/40% DCM; track11: 70% MeOH/30% DCM; track12: 80% MeOH/20% DCM

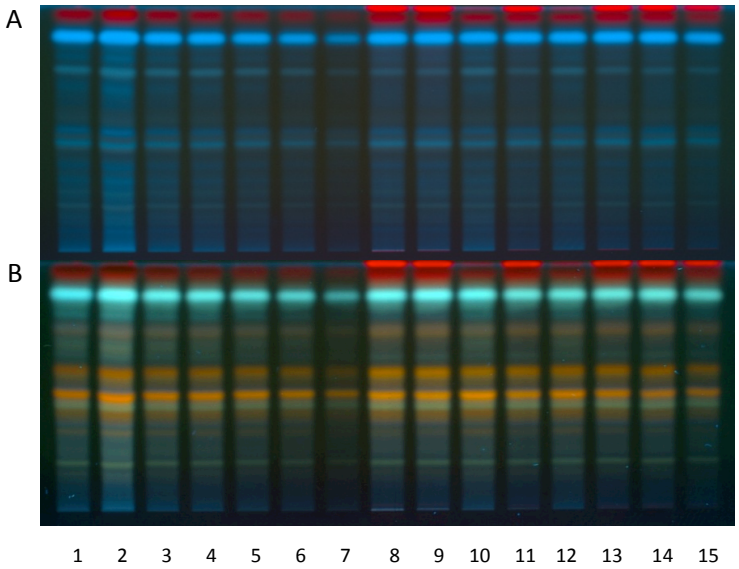


Figure 3.2.5: Extraction optimization: HPTLC fingerprints of thyme samples (group2) extracted with different solvents under UV 366nm, A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12): Thyme samples extracted with different solvents → track8: 40% MeOH/50% DCM; track9: 50% MeOH/40% DCM; track10: 50% MeOH/30% DCM; track11: 60% MeOH/30% DCM; track12: 60% MeOH/20% DCM; track13: 70% MeOH/20% DCM; track14: 80% MeOH/10% DCM; track15: 90% MeOH

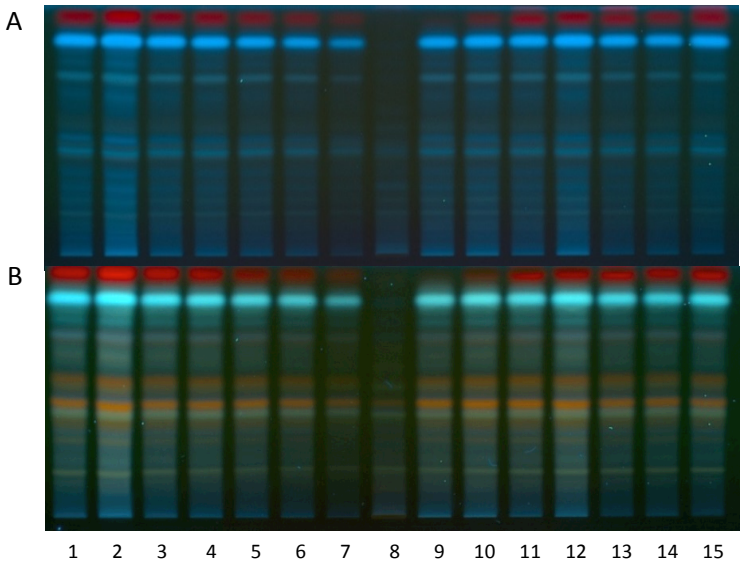


Figure 3.2.6: Extraction optimization: HPTLC fingerprints of thyme samples (group3) extracted with different solvents under UV 366nm, A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12): Thyme samples extracted with different solvents → track8: 100% water; track9: 40% MeOH; track10: 50% MeOH; track11: 60% MeOH/10% DCM; track12: 60% MeOH; track13: 70% MeOH/10% DCM; track14: 70% MeOH, track15: 80% MeOH

3. Senna:

Fingerprints of Senna, extracted by the first group of solvents looked almost the same with slight variations in the intensity of the colors in particular zones in the lower part of the chromatogram. In track 9 nearly all zones were missed except of the chlorophyll zone. This effect can be attributed to the extraction with pure dichloromethane, which is highly non-polar.

Similar effects were observed in the second group (figure 3.2.8). There were no great visible differences between the fingerprints aside in track 9 again, which attracted attention due its brightness of zones.

Tracks 8 and 9, as depicted in figure 3.2.9, showed brighter and better separated zones in comparison to the other tracks. Remarkable were fingerprints of the samples 14 and 15, which showed total absence of a number of zones in the upper part of chromatogram indicating poor extraction efficiency of highly polar solvents. Solvent mixtures containing 40-50% Methanol, 40-50% Dichloromethane and 10% water are considered to be an effective choice for the extraction of Senna.

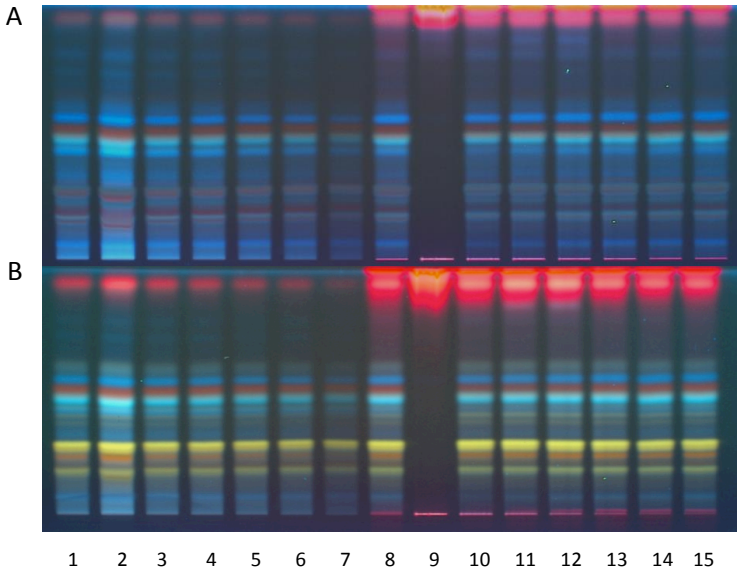


Figure 3.2.7: Extraction optimization: HPTLC fingerprints of Senna samples (group1) extracted with different solvents under UV 366nm, A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12): Senna samples extracted with different solvents → track8: 100% MeOH; track9: 100% DCM; track10: 40% MeOH/60% DCM; track11: 50% MeOH/50% DCM; track12: 60% MeOH/40% DCM; track13: 70% MeOH/30% DCM; track14: 80% MeOH/20% DCM; track15: 90% MeOH/10% DCM

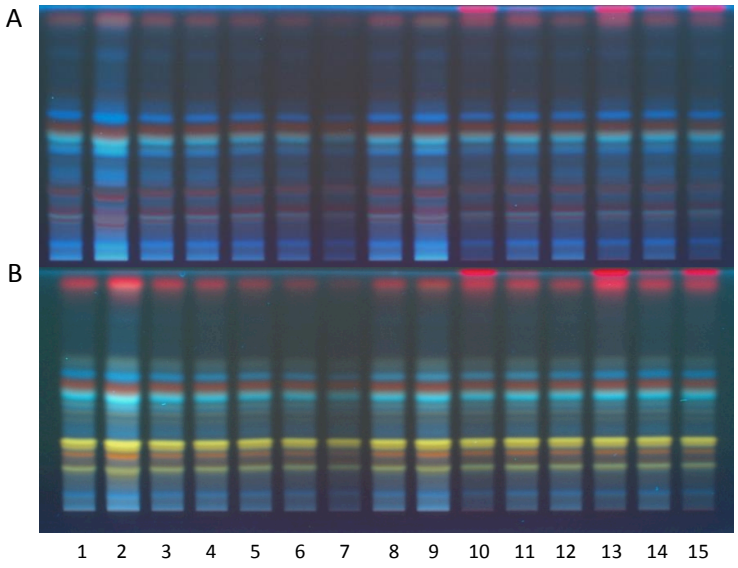


Figure 3.2.8: Extraction optimization: HPTLC fingerprints of Senna samples (group2) extracted with different solvents under UV 366nm, A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) Senna samples extracted with different solvents → track8: 60% MeOH/10% DCM; track9: 60% MeOH; track10: 70% MeOH/20% DCM; track11: 70% MeOH/10% DCM; track12: 70% MeOH; track13: 80% MeOH/10% DCM; track14: 80% MeOH; track15: 90% MeOH

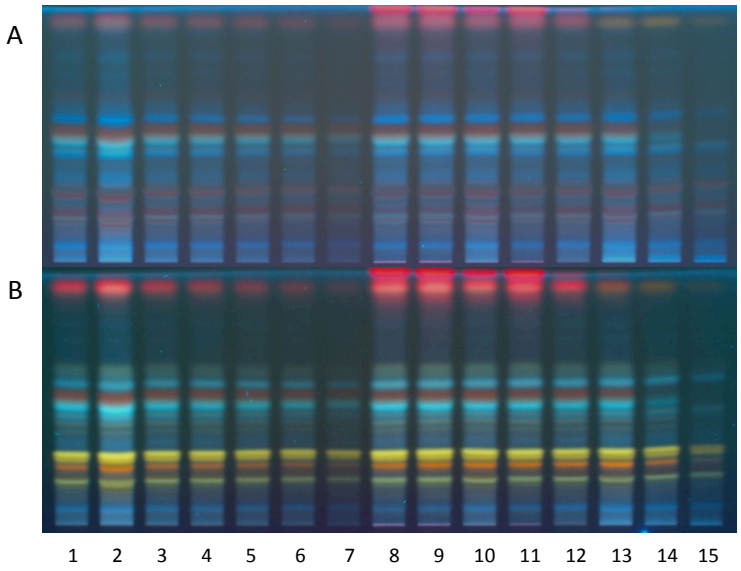


Figure 3.2.9: Extraction optimization: HPTLC fingerprints of Senna samples (group3) extracted with different solvents under UV 366nm, A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) Senna samples extracted with different solvents → track8: 40% MeOH/50% DCM; track9: 50% MeOH%/40% DCM; track10: 50% MeOH/30% DCM; track11: 60% MeOH/30% DCM; track12: 60% MeOH/20% DCM; track13: 50% MeOH; track14: 40% MeOH; track15: 100% water

4. Atropa Belladonna:

All fingerprints of Belladonna, as depicted in figure 3.2.10, 3.2.11 and 3.2.12, looked quite similar with respect to the number of zones and their brightness. The only remarkable difference was the absence of chlorophyll zones in the upper part of the chromatograms in many fingerprints of the third plate.

Track 9 of the first plate and track 8 of the third plate showed many missing zones which indicates weak extraction efficiency of both pure Dichloromethane (non-polar) and pure water (polar).

Track 8, as shown in figure 3.2.11, seemed to reveal a visually preferable separation of substances so that we can suggest a solvent`s combination of 50% Methanol/40% Dichloromethane/ 10% water as optimal extraction solvent for Belladonna.

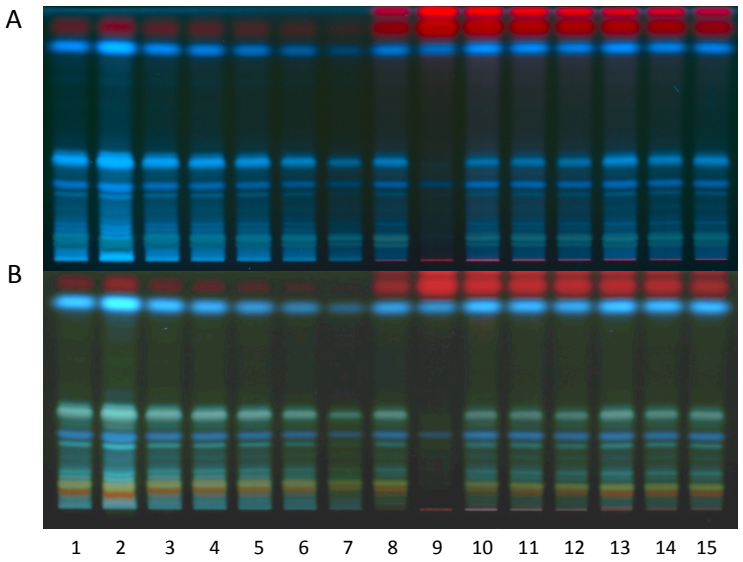


Figure 3.2.10: Extraction optimization: HPTLC fingerprints of Belladonna samples (group1) extracted with different solvents under UV 366nm, A:developed and B: after derivatization; tracks(1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) Belladonna samples extracted with different solvents → track8: 100% MeOH; track9: 100% DCM; track10: 40% MeOH/60% DCM; track11: 50% MeOH/50% DCM; track12: 60% MeOH/40% DCM; track13: 70% MeOH/30% DCM; track14: 80% MeOH/20% DCM; track15: 90% MeOH/10% DCM

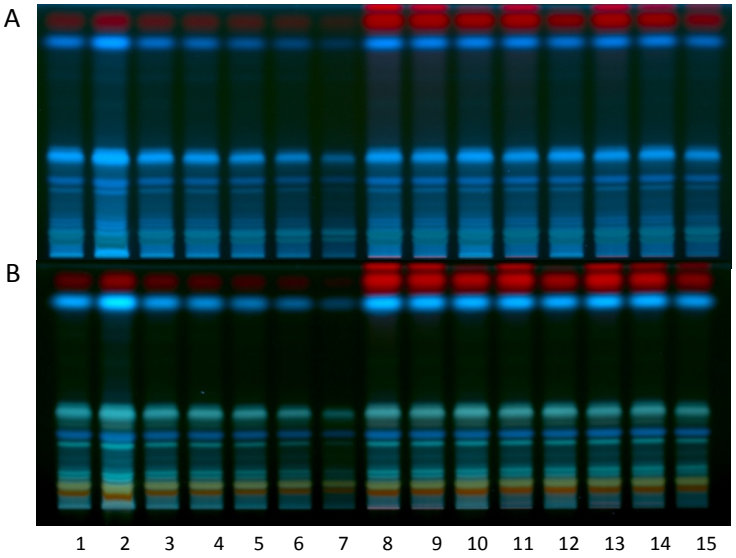


Figure 3.2.11: Extraction optimization: HPTLC fingerprints of Belladonna samples (group2) extracted with different solvents under UV 366nm, A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) Belladonna samples extracted with different solvents → track8: 40% MeOH/50% DCM; track9: 50% MeOH/40% DCM; track10: 50% MeOH/30% DCM; track11: 60% MeOH/30% DCM; track12: 60% MeOH/20% DCM; track13: 70% MeOH/20% DCM; track14: 80% MeOH/10% DCM; track15: 90% MeOH

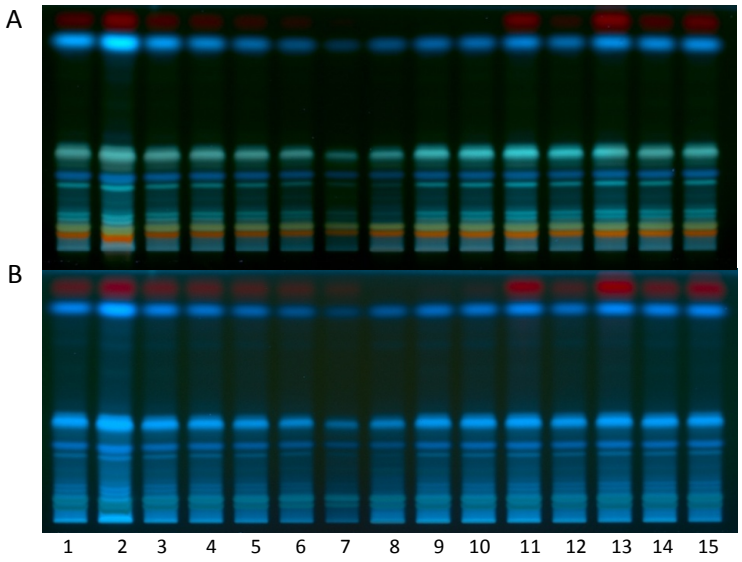


Figure 3.2.12: Extraction optimization: HPTLC fingerprints of Belladonna samples (group3) extracted with different solvents under UV 366nm, A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) Belladonna samples extracted with different solvents → track8: 100% water; track9: 40% MeOH; track10: 50% MeOH; track11: 60% MeOH/10% DCM; track12: 60% MeOH; track13: 70% MeOH/10% DCM; track14: 70% MeOH; track15: 80% MeOH

5. Liquorice:

Fingerprints extracted by the first group are illustrated in figure 3.2.13, which showed no noticeable variations except in track 10. There, differences in width of all equivalent zones could be observed caused by a high extraction yield of this sample.

Fingerprints extracted by the second group (figure 3.2.14) were all similar without visually detectable distinction aside a slight variation in brightness of white zones in the upper part of the chromatogram.

Figure 3.2.15 shows fingerprints of the third group with slight variations in brightness and width of zones in the upper part of the chromatograms.

A remarkable observation is that fingerprints of the second and third groups (figures 3.2.14 and 3.2.15) included additional greenish zones in the upper part of the chromatogram, which are totally absent in fingerprints of the first group (figure 3.2.13). A 50% Methanol, 30% Dichloromethane and 20% water solvent`s combination (track 9, second plate) can be considered as productive extraction solvent for liquorice roots.

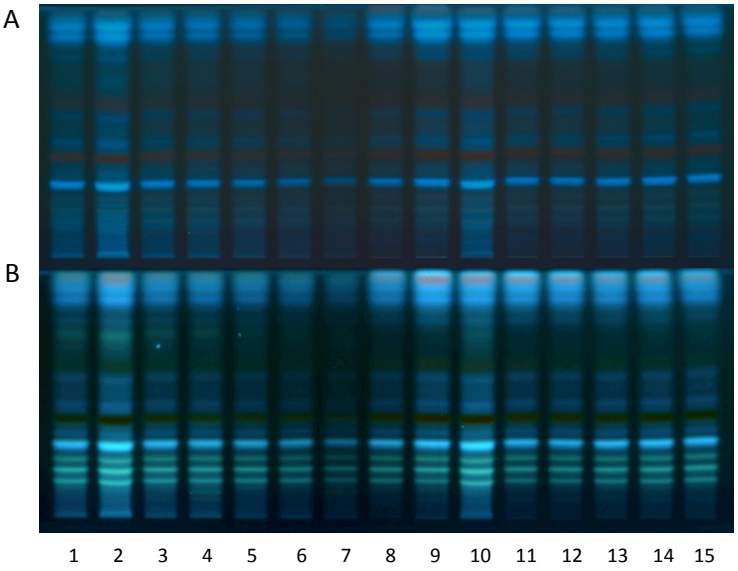


Figure 3.2.13: Extraction optimization: HPTLC fingerprints of liquorice samples (group1) extracted with different solvents under UV 366nm; A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) liquorice samples extracted with different solvents → track8: 100% MeOH; track9: 40% MeOH/60% DCM; track10: 40% MeOH/50% DCM; track11: 50% MeOH/50% DCM; track12: 60% MeOH/40% DCM; track13: 70% MeOH/30% DCM; track14: 80% MeOH/20% DCM; track15: 90% MeOH/10% DCM

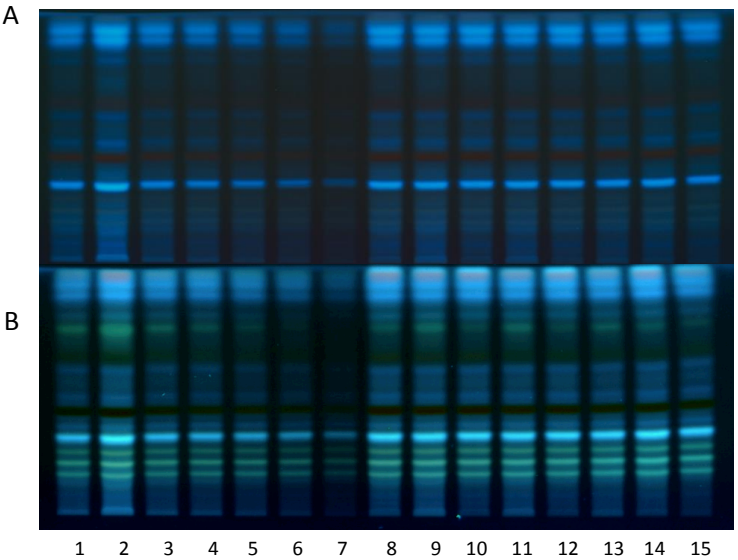


Figure 3.2.14: Extraction Optimization: HPTLC fingerprints of liquorice samples (group2) extracted with different solvents under UV 366nm; A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) liquorice samples extracted with different solvents → track8: 50% MeOH/40% DCM; track9: 50% MeOH/30% DCM; track10: 60% MeOH/30% DCM; track11: 60% MeOH/20% DCM; track12: 70% MeOH/20% DCM; track13: 70% MeOH/10% DCM; track14: 80% MeOH/10% DCM; track15: 90% MeOH

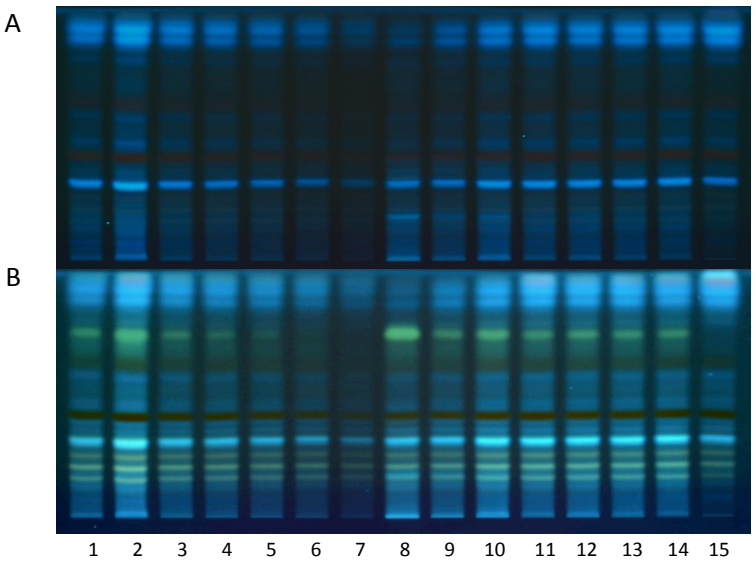


Figure 3.2.15: Extraction optimization: HPTLC fingerprints of liquorice samples (group3) extracted with different solvents under UV 366nm; A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) liquorice samples extracted with different solvents → track8: 100% water; track9: 40% MeOH; track10: 50% MeOH; track11: 60% MeOH/10% DCM; track12: 60% MeOH; track13: 70% MeOH; track14: 80% MeOH; track 15: 50% MeOH/50% DCM

6. Hops:

Fingerprints of Hops are depicted in figure 3.2.16 and showed some variations among the corresponding zones as 5 of 8 fingerprints presented additional orange zones in the second part of the chromatogram. In track 9 almost no visible zones are detected except of few ones in the top of the chromatograms.

The second group, shown in figure 3.2.17, revealed better distinction of zones in both upper and lower sections of the chromatogram concerning brightness and widths of the zones. Effects of solvents, attributed to their polarity (water and dichloromethane), could be clearly noticed in this group of fingerprints whereby higher percentage of dichloromethane resulted in additional zones in the non-polar part which is in contrast to absence of others in the polar part of the chromatogram and vice versa.

Figure 3.2.18 illustrates no great differences aside some zones in the upper part of the chromatograms, which seemed to be brighter than the others. In track 8 extraction was performed by water whereby many zones are missed due to its high polarity. For optimum extraction of hops we suggest a solvent composition of between 60-70% Methanol, 10-20% Dichloromethane and 20-30% water.

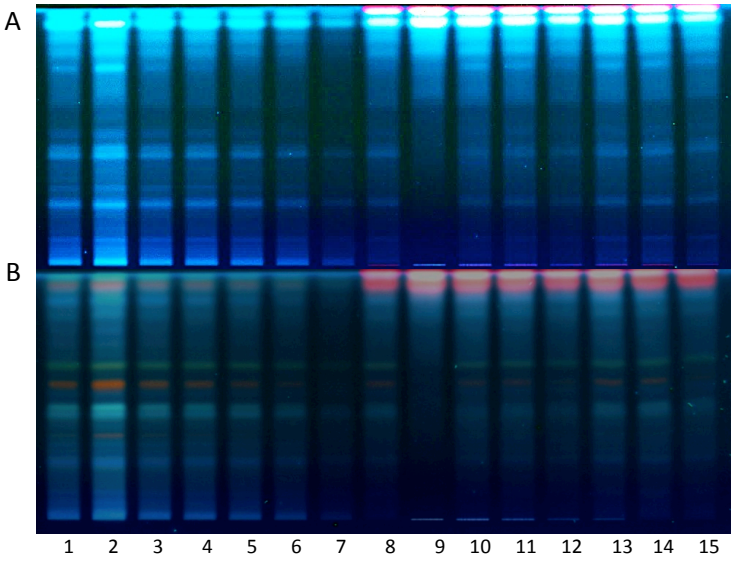


Figure 3.2.16: Extraction optimization: HPTLC fingerprints of Hops samples (group1) extracted with different solvents under UV 366nm; A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) Hops samples extracted with different solvents → track8: 100% MeOH; track9: 100% DCM; track10: 40% MeOH/60% DCM; track11: 50% MeOH/50% DCM; track12: 60% MeOH/40% DCM; track13: 70% MeOH/30% DCM; track14: 80% MeOH/20% DCM; track15: 90% MeOH/10% DCM

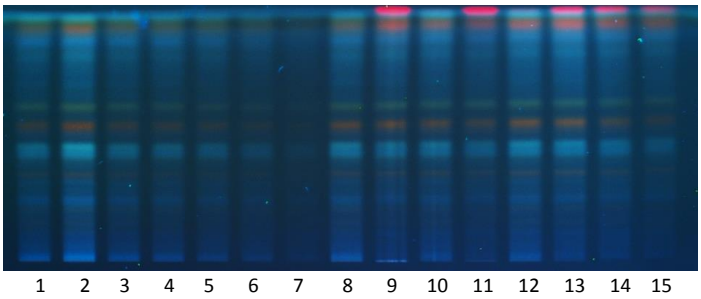


Figure 3.2.17: Extraction optimization: HPTLC fingerprints of hops samples (group2) extracted with different solvents under UV 366nm, A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) Hops samples extracted with different solvents → track8: 60% MeOH/10% DCM; track9: 50% MeOH/40% DCM; track10: 70% MeOH/10% DCM; track11: 60% MeOH/30% DCM; track12: 60% MeOH/20% DCM; track13: 70% MeOH/20% DCM; track14: 80% MeOH/10% DCM; track15: 90% MeOH

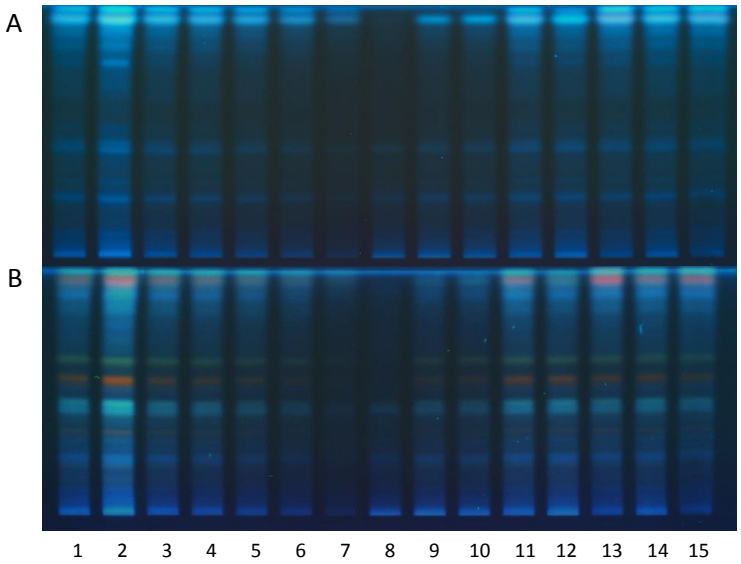


Figure 3.2.18: Extraction optimization: HPTLC fingerprints of Hops samples (group3) extracted with different solvents under UV 366nm; A: developed and B: after derivatization; tracks (1-7) represent the dilution series; reference materials: 200%, 100%, 85%, 70%, 55%, 40% and 20%; tracks (8-12) Hops samples extracted with different solvents → track8: 100% water; track9: 40% MeOH; track10: 50% MeOH; track11: 60% MeOH/10% DCM; track12: 60% MeOH; track13: 70% MeOH/10% DCM, track14: 70% MeOH; track15: 80% MeOH

HPTLC results can be also presented in a different perspective by creating an image that includes samples extracted with following solvents: 100% Methanol, 70% Methanol and 2 additional solvents which provide, according to my opinion, visually improved extraction efficiency for each of the 6 investigated plants when compared to the "standard extraction solvents" (i.e. 100% and 70% methanol). These results are depicted in figures 3.2.19 – 3.2.24.

1. Yarrow:

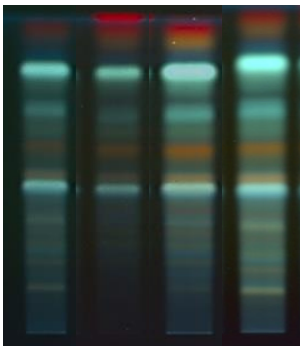


Figure 3.2.19: Extraction optimization; HPTLC fingerprints of Yarrow; track1: 100% MeOH; track2: 70% MeOH; track3: 40% MeOH/50% DCM; track4: 50% MeOH/30% DCM

2. Thyme:

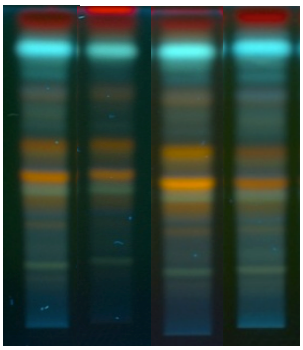


Figure 3.2.20: Extraction optimization; HPTLC fingerprints of Thyme; track1: 100% MeOH; track2: 70% MeOH; track3: 50% MeOH/30% DCM; track4: 60% MeOH

3. Senna:

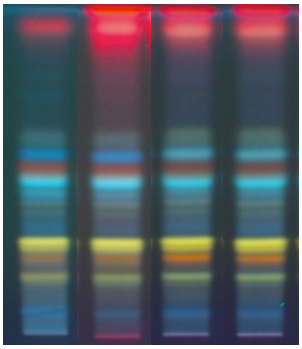


Figure 3.2.21: Extraction optimization; HPTLC fingerprints of Senna; track1: 100% MeOH; track2: 70% MeOH; track3: 40% MeOH/50% DCM; track4: 50% MeOH/40% DCM

4. Atropa Belladonna:

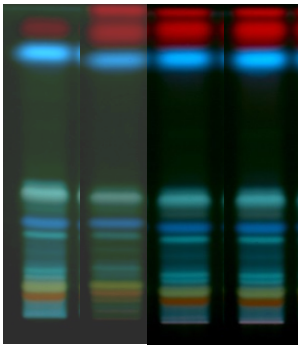


Figure 3.2.22: Extraction optimization; HPTLC fingerprints of Atropa Belladonna; track1: 100% MeOH; track2: 70% MeOH: track3: 40% MeOH/50% DCM; track4: 50% MeOH/40% DCM

5. Liquorice:

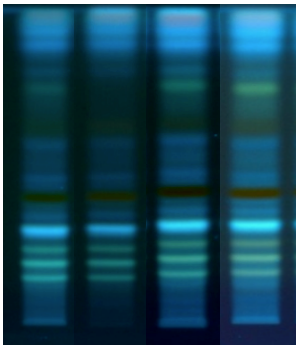


Figure 3.2.23: Extraction optimization; HPTLC fingerprints of Liquorice; track1: 100% MeOH; track2: 70% MeOH: track3: 60% MeOH/10% DCM; track4: 50% MeOH/30% DCM

6. Hops:

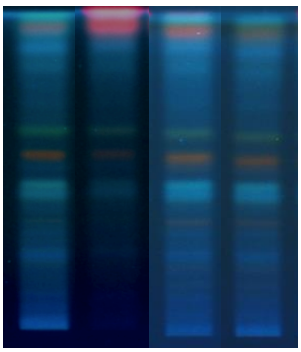


Figure 3.2.24: Extraction optimization; HPTLC fingerprints of Hops; track1: 100% MeOH; track2: 70% MeOH; track3: 60% MeOH/20% DCM; track4: 60% MeOH/10% DCM

4 Discussion

As extraction is considered to be a vital step in QC of HMs, big emphasis has been placed in optimization of this process to save time, raw materials and money and to gain more information rich fingerprints from the examined herbal drugs.

There are many published studies discussing the idea of extraction optimization to achieve the maximum extract yield with regard to quality and quantity. In this context possible influencing factors such as temperature, extraction solvents, extraction method, extraction time etc. have been investigated in detail using different analytical methods for evaluation [44-48].

Through this work we tried to optimize the extraction solvents using a 96 DWP as extraction bowel, which is unusual for such experiments. Then HPTLC was deployed for evaluation of results, as it is the only chromatographic system, which has the ability to show and document the results of multiple samples together in one colorful picture.

This study focused primarily on detecting a universal solvent or solvent´s combination, which is able to extract out of the majority of herbal plants main secondary metabolites (single factor study), which characterize them via relevant fingerprints. 24 solvents/solvent combinations consisting of methanol, dichloromethane and water in different ratios and polarity ranges were selected according to our own protocol, which does not refer to a standardized, validated system. Thus, in the future application of statistical approaches like design of experiment (DOE) and surface response techniques are highly recommended as potential tools to derive optimal parameters for HPTLC analysis. However, since current state-of-the art HPTLC did not allow direct comparison between tracks from different plates, further efforts should focus on the development of novel strategies that resolve this issue.

HPTLC technique was chosen to evaluate visually the extraction´s efficiency of different groups of solvents. A further argument favoring HPTLC is the possibility of saving results in terms of images so that a comparison of results from different plates can be performed at any time. However many factors can affect the instrument itself as well as the imaging process leading to inaccurate results and inability of convenient comparison of data generated from different plates. These variations in results can be referred to certain factors which are related to the plate itself such as manufacturing defects, unintentional failures by packaging and storage which may alter reproducibility not only from one plate to another but also across the same plate. Another factor that is related to environment is relative humidity. Humidity stability during the development operation is required. Inappropriate unsaturation of the developing chambers produce chromatograms with sharp zones,

secondary fronts and also affects the R_f values. Therefore it is important to use systems that stand changes in humidity[2, 49].

By Image acquisition we have to keep in mind that exposure is changing from plate to plate and from image to image and thus influence the correct evaluation of results from different plates. In order to diminish such a drawback we took some precautions in consideration:

Firstly we always recorded a 366nm clean plate image to see whether oversaturation of the image occurs. Secondly we prepared a pure compound dilution series to establish a calibration curve and to see if quantitative comparison of tracks between different plates is possible (results no shown).

We also considered using the same gain- and exposure time for all plates to be able to quantitatively compare tracks from different plates. Ultimately we decided to apply HDR imaging technique, which is supported by the TLC visualizer together with VisionCATS and results optimal visual appearance of chromatograms.

The visual evaluation of generated fingerprints is usually done by qualified persons, however the interpretation is susceptible to human errors because of the variations of individual´s comprehension, experience, and own sensation as well as the unintentional different perception of colors.

Yarrow was the first herb to be tested (it was also our first choice in all precondition experiments) which contains valuable varieties of 2ry metabolites such as flavonoids, alkaloids, tannins, terpenes, coumarins, saponins and phenol carbonic acids[50]. These variations had a positive impact on the HPTLC results and the images displayed distinct variations in zone´s brightness and widths as well as quantitative differences among the fingerprints.

A solvent combination of 40-50% Methanol, 30-50% Dichloromethane and 10-20% water presented a better differentiation of fingerprints. Consequently we argue that these are the solvent combinations of choice to extract efficiently Yarrow samples. Unfortunately the other 5 selected plants did not show the same clear results as Yarrow. We observed less variation considering the visual discrimination of fingerprints.

From my point of view I generally consider 40-50% Methanol/30-50% Dichloromethane as more efficient than other solvent`s combinations in extraction optimization experiments. Unlikely these results could not be statistically analyzed, as we did not find a proper method to normalize chromatograms obtained from different plates. Such circumstances do not allow reliable comparison of various images despite the use of dilution series.

By establishing a dilution series in each plate it was necessary to investigate the effect of concentration of samples on the R_f values of the equivalent zones, hoping that this procedure may enable the statistical analysis of data. As mentioned in the result´s section the retention index shifts, which were visually intensified by the blurring and tailing of zones, were affected by the mobile phase and its pH-values. Therefore we consequently used Ethyl Acetate: Acetic Acid: Formic Acid: water (100:11:11:27) as standard mobile phase in all experiments.

Before using the 96 DWP in the main experiment we needed to prove its reproducibility and efficacy concerning the extraction process. In this context an experiment of 2 steps was performed and the results suggested the possibility to try out these plates and benefit from extraction of more samples at the same time. During practical work and handling of 96 DWP many difficulties were observed. Deep wells (well center to well center distance: 9 mm, well depth: 41.2mm)[43] allow only limited contact between samples and solvents even by appropriate mixing. In contrast vigorous mixing led to leakage of samples despite closing plates with silicon lids. This problem was partially solved by using a multi-channel pipette to mix samples.

The next adverse observation was that pipetting off supernatant was exhausting, time consuming and sometimes confusing especially when two different herbal drugs occur in one plate. Another problem was the difficult cleaning of the DWP because of its pigmentation by extracts.

In summary we have to admit that these problems overweighed the advantage of the possibility to test large numbers of samples. Another idea of optimizing the extraction process was to investigate the effect of exhaustive extraction in improving the extract yield, but no beneficial results were reached.

In conclusion we suggest a certain range for extraction solvents, which give quantitative and qualitative better extract yield. However exact determination of a universal solvent is still a challenging task, which could not be solved by this method. Unlikely we failed to find a way to normalize such a great amount of data obtained from different plates and affected by many external factors. Future development of convenient statistical methods would be a valuable support to overcome the difficulties that were defined in this study.

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6 Appendix

Folium Menyanthidis Trifoliatae	*Herba Herniariae*	*Radix Althaeae mund.*
Folium Orthosiphonis	*Herba Hyperici*	*Radix Gentianae*
Folium Rosmarini	*Herba Millefolii*	*Radix Valerianae*
Folium Menthae Crispae	*Herba solidaginis giganteae*	*Radix Taraxaci*
Folium Althaeae	*Herba Anserinae*	*Radix Zingiberis rhizome*
Folium Betulae	*Herba Marrubii*	*Radix Rhei Chin*
Folium Malvae	*Herba Passiflorae inc.*	*Radix Ratanhiae*
Folium Melissae	*Flos Hibisci*	*Radix Levistici*
Folium Menthae pip.	*Flos Calendulae*	*Radix Imperatoriae*
Folium Plantaginis	*Flos Lavandulae*	*Radix Harpagophyti*
Folium Salviae	*Flos Lupuli*	*Radix Galangae*
Folium Sennae	*Flos Matricariae*	*Radix Caryophyllatae*
Folium Utricae	*Flos Sambuci*	*Radix Curcumae xanthorrhizae*
Folium Salviae-trilobae	*Flos Tiliae*	*Radix Angelicae*
Folium Stromanii	*Flos Verbasci*	*Radix Ononindis*
Folium Hamamaelidis	*Flos Arnicae*	*Radix Liquiritiae nat.*
Folium Crataegi c.f.	*Flos Chamomillae rom.*	*Amylum Tritici*
Folium Belladonnae	*Flos Crayophylli*	*Semen Sinapis nigrae*
Folium Digitalis lan.	*Flos Crataegi*	*Semen Lini*
Folium Digitalis Purp.	*Flos Malvae*	*Semen Trigonellae*
Folium Vitis-idaeae	*Flos Paeoniae*	*Fructus Anisi Vulg.*
Folium Uvae-ursi	*Flos Prunispinosae*	*Fructus Sennae acutif.*
Folium Hyoscyami	*Pseudo-fri Rosae*	*Fructus Petroselini*
Herba Marrubii	*Cortex Sassafras*	*Fructus Crataegi*
Herba Passiflorae inc.	*Cortex Sambuci*	*Fructus Coriandri*
Herba Thymi	*Cortex Quercus*	*Fructus Carvi*
Herba Carduibenedicti	*Cortex Cinnamomi*	*Fructus Capsici Cayenn.*
Herba Alchemillae	*Cortex Cinchonae*	*Fructus Anisi stel.*
Herba Centaurii	*Cortex Salicis*	*Fructus Myrtilli*
Herba Epilobii	*Cortex Rhamni Pursh.*	*Fructus Juniperi*
Herba Absinthii	*Cortex Hamamelidis*	*Fructus Piperis nigri*
Herba Agrimoniae	*Cortex Frangulae*	*Fructus Foeniculi*
Herba Equiseti	*Cortex condurango*	

Table 6.1: List of plants (in powder form) measured using FT-IR spectrometer

7 Curriculum vitae

Personal Summary

Name:	Abdelmajeed Moshira
Date of Birth:	11.04.1985
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Academic Education:

2003-2008:	College of Pharmacy, Cairo University/Egypt
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