New analytical approaches for faster or greener phytochemical analyses

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Chapter 1

Introduction

1. General introduction

Natural products have been and continue to be an invaluable major source for drug development.[1-5] Newman and Cragg showed that ~40% of all new drugs introduced in the period 1981-2010 were either natural products or were derived from natural products.[3] As the large majority of natural products originates from plants, phytochemical analysis is of great interest not only for phytochemists involved in drug discovery programs but also for analytical chemists who work with natural products, e.g., for production or quality control (QC) purposes. The strategy for phytochemical analyses as part of the drug discovery process is screening, extraction, purification, separation and structure elucidation. When performing QC of herbal drugs, the purification is called sample preparation or sample pretreatment. The purification / sample pretreatment part is the most tedious and time-consuming step, often requiring more than 60% of the total analysis time. A still frequently used traditional technique for the pretreatment of raw plant materials or herbal drugs is liquid-liquid extraction (LLE). The most used instrumental technique for separating purified extracts is high performance liquid chromatography (HPLC) coupled with a UV detector or a mass spectrometer. HPLC can be used for quantitation, for structure elucidation (LC-MS) or for preparative purposes. Structure elucidation can also occur off-line by means of NMR or X-ray crystallography.

Phytochemical analyses are costly due to a combination of sample complexity, high manpower input, large use of solvents and stationary phases and expensive equipment. Besides the inherent demands for accuracy, sensitivity and selectivity that hold for any chemical analyses, there is a more general desire to speed up phytochemical analyses and make them less toxic and cheaper. This translates itself in a trend towards "miniaturized", "automatic", "in/on-site analysis / direct analysis", and "green". The concrete strategies used recently to achieve these aims are: 1) reducing consumption of chemical substances by a) miniaturization (minimizing size of set-up); b) shortening analysis time (simplifying or even omitting traditional steps or techniques, direct analysis); 2) increasing efficiency by automation and integration; 3) substituting toxic chemicals or solvents by less harmful ones or by reducing the amount of energy required. The last strategy is most important for large scale processes, like for production purposes. The microfluidic chip is most representative of miniaturization, automation and integration, ambient mass spectrometry (MS) allows the direct probing of plants

without any extraction or sample pretreatment steps, magnetic nanoparticles (MNPs) can streamline the purification process, and less toxic solvents help to make our chemistry greener. In the next few paragraphs, each of these techniques will be looked at in greater detail.

Microfluidic techniques have emerged at the end of the 20th century and so far they contributed a lot to chemical synthesis and analysis ("lab on a chip"), and bioanalysis.[6,7] The channels used have dimensions of tens to hundreds of micrometers and the amount of contained fluid is only 10^{-3} to 10^{-12} liters. This results in low solvent consumption, short analysis times, fast extractions due to small diffusion distances, easy integration and automation. Thus microfluidic chips are potentially environmentally friendly, time-saving and labor-saving. Within the group of LLE chips, which can be used for sample pretreatment, a design using parallel multiphase microflows[6] is one of the most popular formats. A LLE chip system with two microfabricated glass plates and a microporous membrane sandwiched in between was proposed for separating an aqueous feed phase using isobutanol as extractant.[8] A similar 3-phase microextraction in a microfluidic chip employing a 25 μm supported liquid membrane as middle phase could obtain high enrichments of analytes.[9] Another 3-phase chip was applied for the extraction of tanshinones from *Salvia miltiorrhiza*.[10] Ionic liquid-based aqueous two-phase systems are also interesting for microfluidic extractions.[11]

Ambient mass spectrometry was firstly reported by Graham Cooks' group in 2004.[12] Ambient ionization techniques named "Desorption electrospray ionization" (DESI) and "Direct Analysis in Real Time" (DART) allow analysis of a wide range of substrates under ambient conditions in seconds without any prior sample pretreatment.[13-16] Other ambient ionization techniques[17] like "Probe electrospray ionization" (PESI), "Laser ablation electrospray ionization" (LAESI), and "Desorption atmospheric pressure chemical ionization" (DAPCI) followed. However, no matter how different they are in name and how simple they are to operate, the ionization mechanisms are either ESI or APCI-related. So far, the most successfully commercialized ambient ionization sources are the initially developed DART and DESI. Compared to DESI, DART is easier to handle and more robust. For DART, after ionization of the analytes with excited helium species in the open air, they are selectively measured by either MS^n or high-resolution MS (HRMS).[18,19] DART has been widely applied in the analysis of a wide range of materials and mixtures, including pharmaceutical formulations, explosives, pesticides, beer, printing paper, textiles, chemical warfare agents, counterfeit

drugs, medicinal herbs, and propolis, and also for bioanalysis and food-quality and safety analysis.[20-31]

Besides various kinds of commercial or tailor-made ambient ionization sources, a new and simple technique to sample fresh or dry plants called "leaf spray mass spectrometry" was introduced.[32] The plant part with a natural or man-made spike is held in front of the MS inlet and a high voltage is applied. Fresh or dry plants after wetting with a suitable solvent, generate a Taylor cone and the formed spray ionizes the plant metabolites and carries them into the MS. The principle is derived from paper spray.[33] The method proved very simple and robust giving in real-time under mild conditions information about a wide variety of genuine phytochemicals. Since then, papers applying "leaf spray MS" have appeared focusing on urushiol allergens in poison ivy,[34] steviol glycosides from Stevia,[35] steroidal acids in tulsi[36] and extraneous pesticides on fruits,[37] respectively. Thus plant spray MS appears to be a simple, rapid, cheap and highly specific tool for the identification of botanical species. Recent publications on ambient mass spectrometry show that it can save a lot of time at the sample pretreatment stage.

Even though the development of novel instruments creates a lot of possibilities for quicker and simpler chemical analysis today, they cannot lift all the restrictions imposed by detection limits and matrix complexity. Especially for HPLC, sample pretreatment to remove impurities and enrich analytes as much as possible will remain necessary. Sample pretreatment by magnetic nanoparticles (MNPs) and an external magnetic field, has the advantage of being cheap and gentle, and MNPs are also suitable for large scale operations.[38,39] Functionalized magnetic nanoparticles, because of their large surface-area-to-volume ratio and specific adsorbent groups, have been used in food analyses, e.g., for lipase inhibitors,[40] and linuron.[41] Changing the purification process for specific compounds from traditional repeated LLE or tedious silica column chromatography to easily handled MNPs, cannot only save a lot of time and chemicals but also reduce the energy consumption.

Making their chemistry greener should be a goal for all chemists, no matter which research field they are working in. If a greener solvent is available and suitable for their research in terms of chemical properties, cost and logistics, and allowed by internal and external regulations, they will most likely prefer it, especially at an industrial scale, like for instance, for preparative separations. However, less research has been carried out towards greener preparative separations. Some of the popular greener techniques discussed above, e.g., miniaturization do not work for preparative separations at all because they do not decrease the amount of solvent needed per

gram of purified product. Replacement is the only option for greener preparative separations. Supercritical carbon dioxide (CO_2) and water are the greenest solvents that can be used. Organic solvents on the Pfizer green list,[42] include methanol, ethanol, 2-propanol, 1-propanol, 1-butanol, *t*-butanol, ethyl acetate, isopropyl acetate, acetone and methyl ethyl ketone. They offer the best compromise in terms of cost, energy aspects, toxicity, safety and environmental issues.[43-47]

2. Aim of this research

As a Chinese Sandwich PhD who works partially in a Chinese laboratory focusing on Traditional Chinese Medicines (TCMs) research, at the onset of my PhD trajectory I chose a project whose main aim was to improve the chemical analysis of TCMs in terms of simplicity, efficiency, cost and environmental friendliness. Thus all real sample applications should fall within the realm of TCMs. The following distinct aims were formulated:

1) Improve an existing 3-phase LLE chip and convert macroscale induced phase separation extraction (IPSE, an existing 2-phase LLE sample pretreatment technique) to a microfluidic chip-based format by optimizing parameters, evaluating efficiency, and hyphenating with commercial LC. Both chips in their optimized format were tested with TCMs samples.
2) Evaluate if ambient mass spectrometry like DART or direct plant spray MS can be used for quick authentication of Chinese star anise and Japanese star anise or for distinguishing between Scutellaria (important TCM ingredient) samples from different origins in China without any extraction or sample pretreatment.
3) Develop a process in which magnetic nanoparticles can substitute for a heptane LLE or preparative gravity column chromatography for the purification of ginkgolic acids from *Ginkgo biloba* leaves.
4) Determine if the three most commonly used organic modifiers (methanol, acetonitrile and tetrahydrofuran) for (preparative) reversed phase (RP) HPLC can be replaced by less toxic and greener modifier solvents (ethanol, acetone and ethyl acetate) without loss of resolution. Terpene trilactones from *G. biloba* will serve as test case.
5) Prepare an overview on the use, recent developments and prospects of HPLC in TCMs QC and drug discovery.

3. Outline of this thesis

The research carried out in this thesis is geared towards fulfilling the above aims. The content of the different Chapters is briefly summarized below.

Chapter 2 introduces an existing glass LLE microchip with three parallel 3.5 cm long and 100 μm wide interconnecting channels, which was optimized in terms of more environmentally friendly (greener) solvents and extraction efficiency. In addition, the optimized chip was hyphenated with nano-liquid chromatography with ultraviolet and mass spectrometric detection (nanoLC-UV-MS) for on-line analysis.

In **Chapter 3**, a highly efficient induced phase separation extraction (IPSE) chip is described, which can rapidly extract analytes into an aqueous or organic phase at a microscale. The sample, dissolved in acetonitrile-water (1:1), was separated in two immiscible phases by adding inducers (hydrophobic solvents). The two formed phases can be collected and analyzed separately. Extraction and fractionation were easily and efficiently achieved in this chip. Two groups of model compounds were investigated to check the efficiency.

In **Chapter 4** it is shown that DART coupled with orbitrap high resolution mass spectrometry (DART-HRMS) allowed the recording of mass spectra of the neurotoxin anisatin in solid Japanese star anise fruits in seconds without any prior sample pretreatment. A carpel of star anise and herbal tea containing anisatin-free Chinese star anise spiked with toxic Japanese star anise were tested in both positive and negative modes. Quick authentication proved possible.

Chapter 5 provides the application of direct plant spray ionization to differentiate Chinese star anise from Japanese star anise by determination of the concentration of anisatin. Direct plant spray and DART for quick authentication were compared.

In **Chapter 6**, it is detailed how inexpensive and easily prepared Fe_3O_4 magnetic nanoparticles in methanol were used to selectively adsorb ginkgolic acids (GAs) from crude extracts of Ginkgo leaves in the presence of various lipids including other alkylphenols (cardanols and cardols). In total, eight different GAs were captured by MNPs. The MNP adsorption method can be used to replace traditional column chromatography and liquid-liquid extraction steps and is superior in terms of time and solvent consumption, selectivity, labor and energy consumption.

The solvents ethanol, acetone and ethyl acetate are less toxic than the commonly used acetonitrile, methanol and tetrahydrofuran. In **Chapter 7** it is investigated if they can serve as alternative organic modifiers in preparative RP-HPLC. A group of terpene trilactones, i.e., ginkgolides A, B, C, and J, and bilobalide were used as model compounds. Systematic investigations were carried out regarding selectivity.

Chapter 8 presents an overview about applications and developments of HPLC-based methods such as multi-component quantitation, fingerprinting, bioaffinity chromatography and on-flow assays for screening of TCMs and other herbal drugs as well as new developments in sample pretreatment prior to HPLC analysis.

Chapter 9 gives a general conclusion and presents perspectives.

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Chapter 2

Hyphenation of Optimized Microfluidic Sample Preparation with NanoLC for Faster and Greener Alkaloid Analysis

Abstract

A glass liquid-liquid extraction (LLE) microchip with three parallel 3.5 cm long and 100 μm wide interconnecting channels was optimized in terms of more environmentally friendly (greener) solvents and extraction efficiency. In addition, the optimized chip was successfully hyphenated with nano-liquid chromatography with ultraviolet and mass spectrometric detection (nanoLC-UV-MS) for on-line analysis. In this system, sample pretreatment, separation and detection are integrated, which significantly shortens the analysis time, saves labour and drastically reduces solvent consumption. Strychnine was used as model analyte to determine the extraction efficiency of the optimized 3-phase chip. Influence of organic solvent, pH of feed phase, type of alkaloid, and flow rates were investigated. The results demonstrated that the 3-phase chip nanoLC-UV/MS hyphenation combines rapid (~25 sec) and efficient (extraction efficiency >90%) sample prep, with automated alkaloid analyses. The method was applied to real samples including *Strychnos nux-vomica* seeds, *Cephaelis ipecacuanha* roots, *Atropa belladonna* leaves, and *Vinca minor* leaves.

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1. Introduction

Liquid-liquid extraction (LLE) is a conventional and effective method to separate compounds based on their relative solubilities in two different immiscible liquids, e.g., water and a hydrophobic organic solvent. It is a standard technique in chemical laboratories and widely applied in analytical chemistry for sample pretreatment, partition coefficient determinations or preparative isolations. Advantages are its simplicity and high capacity relative to techniques involving solid stationary phases. Disadvantages are lack of automation, emulsion formation and still high solvent consumption. To circumvent these disadvantages, attempts have been made to carry out the LLE process in a microfluidic chip. Microfluidic techniques have emerged at the end of the 20th century and so far they contributed a lot to chemical synthesis and analysis ("lab on a chip") and biological analysis.[1] This is attributed to several advantages. At the microscale, the channels used have dimensions of tens to hundreds of micrometers and the amount of contained fluid is only 10^{-3} to10^{-12} liters. This results in low solvent consumption, short analysis times, fast extractions due to small diffusion distances and, easy integration and automation. Thus microfluidic chips are potentially environmentally friendly, time-saving and labour-saving.

LLE microfluidic chips have been used for separation/purification of analytes such as small drug molecules, proteins and even cells.[2-15] Within the group of LLE chips, parallel multiphase microflows[16] is the most popular format. Cai et al. proposed an LLE chip system with two microfabricated glass plates and a microporous membrane sandwiched in between, separating the aqueous feed phase and isobutanol as extractant. The analyte butyl rhodamine B was monitored by laser induced fluorescence.[17] Ramos Payánet et al. followed a similar approach and reported on a 3-phase microextraction in a microfluidic chip employing a 25 μm supported liquid membrane as middle phase. In stop-flow mode high enrichments could be obtained.[18] Xiao et al. developed a 2-phase laminar parallel flow chip for extracting ephedrine from aqueous solutions of different concentration to ethyl acetate with good repeatability and linearity.[19] Novak et al. showed that ionic liquid-based aqueous two-phase systems are also interesting in microfluidic extractions.[20] Surmeian et al. demonstrated that 3 parallel phases (aq/org/aq) are also feasible and constitute a kind of liquid membrane.[2] More recently Mu et al. used a 3-phase chip for the extraction of tanshinones from *Salvia miltiorrhiza*.[21] However as the two outer channels were both used for sample

infusion, it is essentially a 2-phase chip with increased contact area. Our group extended the above 2 and 3-phase concepts to a chip with 3 parallel channels containing alkaline water (feed phase), chloroform (transport phase) and formic acid in water (acceptor phase) respectively.[7] Three channels allow for two separate LLE steps in one device and the alkaloid strychnine was successfully purified from a *Strychnos nux-vomica* extract. However as mentioned in the discussion part of said paper,[7] there were several shortcomings like the use of chloroform, the unequal flows, and the limited extraction of 79.5%. Also the suitability for other alkaloids and dirtier matrixes (e.g., leaves) was not reported. Finally, there was no direct hyphenation with HPLC making the analysis of each sample tedious. In this follow-up paper, optimization of organic solvent and extraction efficiency, hyphenation with nanoLC and wider application in the field of alkaloid analysis of the 3-phase microfluidic chip are described.

2. Experimental

2.1. Chemicals and apparatus

Strychnine, vincamine, emetine dihydrochloride, berberine hydrochloride, and atropine sulfate were purchased from Sigma, The Netherlands. Acetonitrile and methanol (HPLC grade) were purchased from Biosolve, The Netherlands. Hexyl acetate, butyl acetate, ethyl acetate, chloroform, formic acid, and phosphoric acid were purchased from Merck, The Netherlands. *Strychnos nux-vomica* seeds and *Cephaelis ipecacuanha* roots were purchased from Jacob Hooy, Limmen, The Netherlands. *Atropa belladonna* leaves, and *Vinca minor* leaves were purchased from Hortus Botanicus Lovaniensis, Kruidtuin Leuven, Belgium. Gas tight syringes (500 μL), ferrules, nuts, Teflon tubing, Luer lock adapters, and filters were purchased from Alltech, The Netherlands. Syringe pumps (Harvard 11 PicoPlus, dual syringe) were purchased from VWR international, The Netherlands. Chips were obtained from Micronit, The Netherlands. The chip design (Fig. 1) and coating procedure are the same as those used in.[7]

Fig. 1. Schematic representation of the 3-phase chip. Channel dimensions: 100 μm width, 40 μm depth, 3.56 cm length (for each channel).

2.2. Photography

Phase separation in the microchip was monitored by using a binocular microscope (model SZ40) from Olympus, The Netherlands. A Canon Powershot A710 IS was used to obtain photographs of the three-phase microchips through the microscope.

2.3. Nano liquid chromatography

The nanoLC-DAD system was an UltiMate 3000 RSLC from Dionex, USA, including two pumps, a nano injector (10 nL) and a nano UV detector. The C_{18} column used was an Acclaim PepMap RSLC 15 cm × 75 μm, 2 μm particle size, 100 Å pore size, Thermo, USA. NanoViper connectors were used.

The nanoLC-ESI-MS system was home built and consisted of two pumps and a nano-injector (10 nL) from Shimadzu (LC 20AD nano, Shimadzu, Japan), while the ESI-MS was from Thermo (CE-MS housing and LCQ, Thermo, USA).

Mobile phases are A: 0.1% formic acid in water; B: 80% ACN-20% H_2O with 0.1% formic acid. Flow rate: 500 nL/min. Isocratic for strychnine, vincamine, berberine and atropine: 70% A for 10 min. Gradient for *Strychnos nux-vomica* seeds: 0 min 97% A; 18 min 75% A; 20 min 97% A. Gradient for *Cephaelis ipecacuanha:* 0 min 97% A; 18 min 80% A; 20 min 97% A. Gradient for *Atropa belladonna* leaves and *Vinca minor* leaves: 0 min 90% A; 10 min 40% A; 13 min 90% A.

Two nanoLC systems used here employed the same column. Flow rate and gradient were the same for analysis of the same analytes.

2.4. Optimization of conditions for 3-phase chip

For the evaluation of extraction efficiency, 20 μL samples were collected and analysed by an off-line HPLC system. Strychnine was initially used as

model compound, but later alkaloids with different pKa values such as atropine, vincamine and emetine were additionally investigated. Samples were collected in 0.5 mL Eppendorf tubes, and subsequently transferred to vials for being analysed by HPLC. Organic phase and flow rate optimisation. Based on their polarity, water solubility, toxicity and viscosity, ethyl acetate, n-butyl acetate and n-hexyl acetate were chosen as possible alternatives for chloroform. Each solvent was infused into the middle channel separately to act as the transport phase. The aqueous feed and acceptor phases were simultaneously infused into the first and third channel.

Flow settings in the 3-phase chip. The flow rate of organic phase was varied from 0.2-2.0 μL/min. The flow rate of the two aqueous phases tested was varied from 0.2-1.0 μL/min. Based on viscosity considerations, the flows of the basic aqueous phase/acidic aqueous phase were half of those of the ethyl acetate and chloroform flows but equal to that of n-hexyl acetate and n-butyl acetate.

pH optimization of basic aqueous phase. The influence of the pH of the basic feed phase on the extraction efficiency was investigated for alkaloids having different pKa values: vincamine 8.02, strychnine 8.37, emetine 8.92, atropine 9.98. The pH of the feed phase was varied from 7.31 to 11.78 by adding of 1% ammonia.

2.5. Hyphenation to nanoLC

3-phase chip hyphenated to nanoLC-DAD. The outlet of the feed or acceptor phase of the 3-phase chip was connected to the inlet of the nano-injector (VICI, USA) by means of a fused silica capillary (i.d. 50 μm). Between them there was a micro-filter (0.45 μm, Upchurch, USA) used for on-line removal of any particles > 0.45 μm. In the "load" position, the solvent was directed to the internal 10 nL nano-loop. After switching to the "inject" position, the liquid was injected into the nanoLC column. Flow rate: 0.50 μL/min; wavelength 254 nm for strychnine, 285 nm for vincamine, 272 nm for emetine, 265 nm for berberine.

3-phase chip hyphenated to nanoLC-ESI-MS. The connections between the 3-phase chip and the nano-injector valve were the same as described above. The outlet of the nanoLC column was connected to a 20 μm i.d. fused silica capillary, which was directly inserted into a CE-MS housing. Sheath liquid was ACN at a flow rate of 5.0 μL/min. This was used for supplying liquid for forming a liquid spray. MS conditions: ESI positive mode, sheath gas and auxiliary gas were set at 0; spray voltage: 6 kV; capillary temperature: 270 °C; capillary voltage: 5.12 V.

A micro-filter was installed after the outlet of the 3-phase chip to prevent the entry of any particles into the nano-injector. Longer capillaries (1.5 times as

long as the one used for connecting the outlet of the chip via the micro-filter to the nano-injector) were attached to the other two outlets of the 3-phase chip to balance the pressure difference.

2.6. Applications

Besides strychnine, other alkaloids with different pKa values including atropine, vincamine and berberine were investigated. As samples *Strychnos nux-vomica* seeds, *Cephaelis ipecacuanha* roots, *Atropa belladonna* leaves, and *Vinca minor* leaves were used. Of these plant materials, 0.5 g was initially extracted with 50 mL of 0.5 % formic acid as the crude extract, after which 1% ammonia solution was used for adjusting the pH to the pH value selected for each alkaloid in 2.4.

3. Results and discussion

3.1. Extraction efficiency

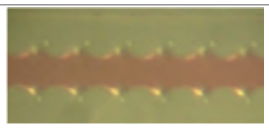
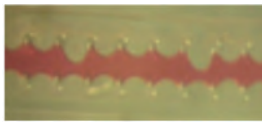
In the initial set-up of the 3-phase extraction chip, chloroform was used as the transport phase because it is an exceptionally good solvent for alkaloids due to being a strong proton donor. However, modeling showed that chloroform is actually a too good solvent, i.e., relatively poor extraction into the acceptor phase took place. Additionally its viscosity is rather low and it is toxic and environmentally unfriendly. Therefore three esters were selected as possible alternatives. For each, the quality of the three laminar flow profiles and the extraction efficiency of the model compound strychnine were investigated and compared with chloroform as benchmark solvent. Table 1 shows the physical and experimental parameters as well as a photo of the 3 parallel flows for each of the 4 systems. To compensate for pressure problems due to the low viscosity of chloroform and ethyl acetate (around half of that of water), their flow rate was set at twice that of water. The distribution ratio of strychnine in each phase as a function of the organic solvent used as transport phase is shown in Fig. 2. The distribution ratio is calculated by setting the whole area of strychnine in the three phases as 100%, strychnine in each phase accounts for its corresponding proportion. In Fig. 2 the extraction efficiencies after 25 sec for each solvent are presented. n-Butyl acetate gives with 92% the best result, followed by n-hexyl acetate. This is considerably better than for chloroform, which confirms our earlier modeling experiments.[7] The higher efficiency is primarily caused by a higher partition coefficient ($P_{\text{acidic aqueous/organic solvent}}$)

for butyl acetate relative to chloroform for the back extraction but also the fact that all three flows are equal in the case of butyl acetate is advantageous. The residence time was calculated by dividing the channel volume by the flow rate. Obviously, a longer residence time in the 3-phase channel increases the time for transporting analytes from the feed phase through the transport phase to the acceptor phase. This means lower flow rates lead to higher extraction efficiencies (Fig. 2). In practice, the lowest usable flow rate in the 3-phase chip was 0.20 μL/min when using butyl acetate or hexyl acetate as the transport phase. Stable laminar flows in each channel could be formed. In addition, the higher the difference in viscosity between the aqueous feed/acceptor phase and organic solvent at equal flows, the bigger the interfacial pressure difference is. This is not good for maintaining stable multiple flows in parallel channels especially at low flow rates (see images in Table 1). The interface between the aqueous solution and the organic solvent is more curved when the difference in viscosity between these two phases is larger[22] in spite of the flow rate adjustment. Also the interfacial tension (aq/org) plays a role. For butyl acetate this is much lower than for chloroform which also facilitates a more stable laminar flow.[19] Taking all these factors into consideration, 0.50 μL/min was chosen as the optimal flow rate for butyl and hexyl acetate. For chloroform and ethyl acetate, 2.0 μL/min was used.

3.2. pH and polarity effect

The extraction efficiency is not only determined by the choice of organic solvent and residence time but also by the pH of the feed phase and the polarity and pKa values of the alkaloids. To evaluate the applicability of the 3-phase chip, four tertiary alkaloids differing by 2 orders of magnitude in pKa value (from 8 to 10) and a factor 500 in Distribution coefficient (D, from 12 to 5900) were tested. In addition the polar quaternary alkaloid berberine was included. Table 2 shows the chemical information on the five alkaloids. Fig. 3 shows the distribution of alkaloids in each phase as a function of the pH of the feed phase. Firstly, as expected, the 3-phase chip does not work when the alkaloid is a quaternary one like berberine. Secondly, when the pH of the feed phase was 1 pH unit higher than the pKa value of the alkaloid, the extraction efficiency increased sharply. After a further increase of the pH of the feed phase, as expected, the extraction efficiency only gradually increased (see strychnine) according to the degree of dissociation of weak bases as a function of pH. Thus a high pH is necessary for reaching a high extraction efficiency within the fixed residence time of 25 sec. However a high pH also creates some problems in the microfluidic system, e.g., a high pH tolerance of

Table 1. Physical parameters and flow image for four organic solvents used as transport phase.

Solvent	Density (g/mL)	Viscosity (cps) 25 °C	Water Solubility (g/100mL) 20 °C	Flow rates[a] (µL/min)	Image of laminar flows[b]
Chloroform	1.483	0.58	0.8	1:2:1	
Ethyl acetate	0.897	0.426	8.3	1:2:1	
n-Butyl acetate	0.882	0.737	0.7	0.5:0.5:0.5	
n-Hexyl acetate	0.867	0.782	0.04	0.5:0.5:0.5	

[a] Values relate to feed, transport and acceptor phase respectively
[b] For these photos, a red dye was added to the organic phase

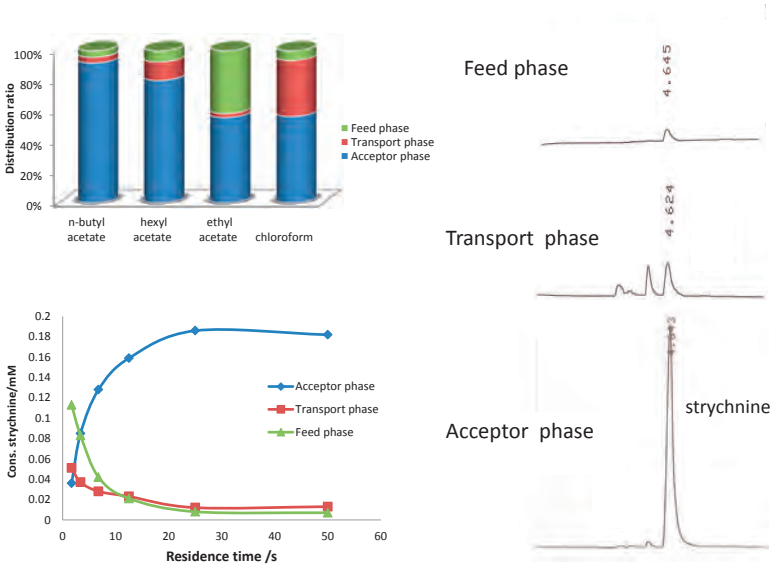
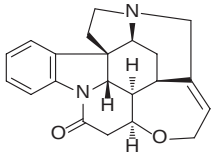
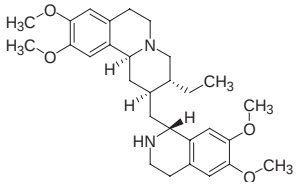
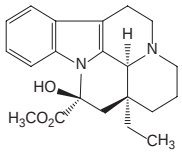
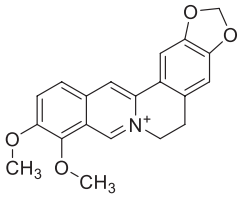
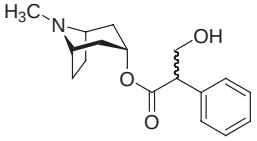


Fig. 2. Distribution ratio of strychnine in each phase as a function of the organic solvent used as transport phase (upper left); concentration of strychnine at different residence times (bottom left) and HPLC profiles of each phase (right) when butyl acetate is used as transport phase (flow rate: 0.5 : 0.5 : 0.5 µL/min, pH feed phase = 10.53).

the fused capillary and the connectors is needed, and upon direct injection of feed phase into the HPLC the retention time slightly shifts. The partition coefficient also plays a role and effects the extraction efficiency for very polar alkaloids like atropine (D=12) and very non-polar alkaloids like emetine (D=5900). Atropine is difficult to extract from the basic aqueous phase to the organic phase. In contrast, the non-polar emetine does not want to migrate from the organic solvent to the acidic aqueous phase. Thus the extraction efficiency of the 3-phase chip is not as good for atropine (72%) and emetine (86%) as for vincamine (94%) and strychnine (92%).

Table 2. Parameters and structures of five alkaloids.

alkaloids	conc. (mM)	pKa	log D (pH=10, 25 °C)	Structure
strychnine	0.20	8.4	1.58	
emetine	0.32	8.9	3.77	
vincamine	0.32	8.0	2.93	
berberine	0.33	-[a]	< 1	
atropine	0.33	9.98	1.09	

[a] quaternary alkaloid

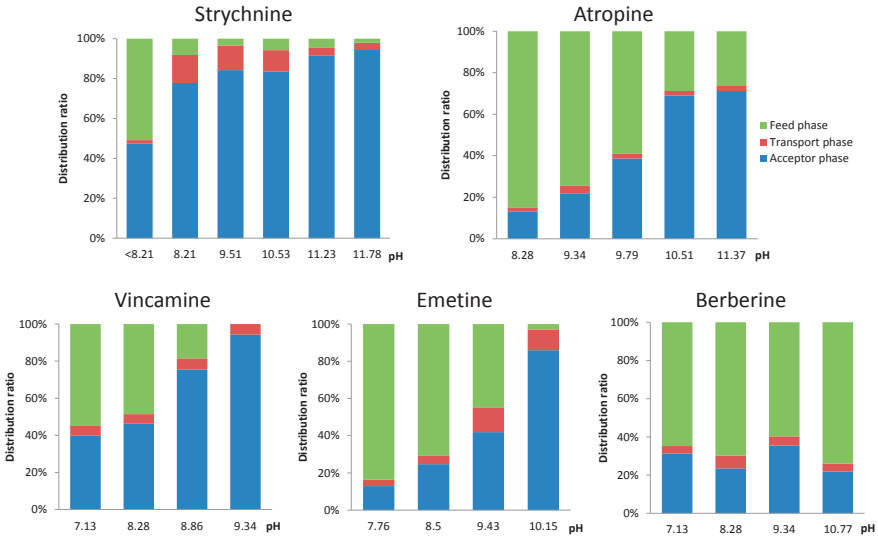


Fig. 3. Distribution of alkaloids in each phase as a function of the pH of the feed phase. Transport phase: butyl acetate, flow rate: 0.5 : 0.5 : 0.5 µL/min

3.3. 3-Phase chip hyphenated with nanoLC for on-line analysis

As a next step in our aim to arrive at an integrated extraction-purification-separation device, nanoLC was combined with the optimized 3-phase chip. As the flow rate is only 0.50 µL/min, the loop volume (10 nL) used in nanoLC fits the microfluidic system very well. The hyphenated chip with nanoLC system was initially used for on-line monitoring of alkaloids in different phases and later, more importantly, for automated on-line analysis of alkaloids. When the results obtained with the hyphenated system are compared with the off-line results obtained after manually collecting samples, transferring them into sample vials and off-line HPLC analysis, they show that the on-line hyphenation system is fast and fully automatic, has a low solvent consumption and is highly reproducible, as shown in Fig. 4.

Both an ultra-violet (UV) and a mass spectrometric (MS) detector were used to detect the alkaloids after the nanoLC separation. They are nowadays the most common and representative detectors in LC [23]. For MS hyphenation, a sheath liquid (5 µL/min) was used for forming a spray. A Taylor cone could be clearly observed. As sheath liquids, acetonitrile, methanol, methanol-water (8:2, v/v) and acetonitrile-water (8:2) were tested, with pure acetonitrile giving the best ionization.

The 3-phase chip in combination with nanoLC-UV/MS hyphenation gave good results for the on-line analysis of all four alkaloids. The LOD for strychnine and emetine by UV was 0.5 µg/mL. The LOD for atropine and vincamine by MS was 5 ng/mL. Peak areas were well reproducible with the intraday RSD being <1.5%, and the interday RSD ≤4%. This shows the hyphenation set-up is robust and suitable for quantification.

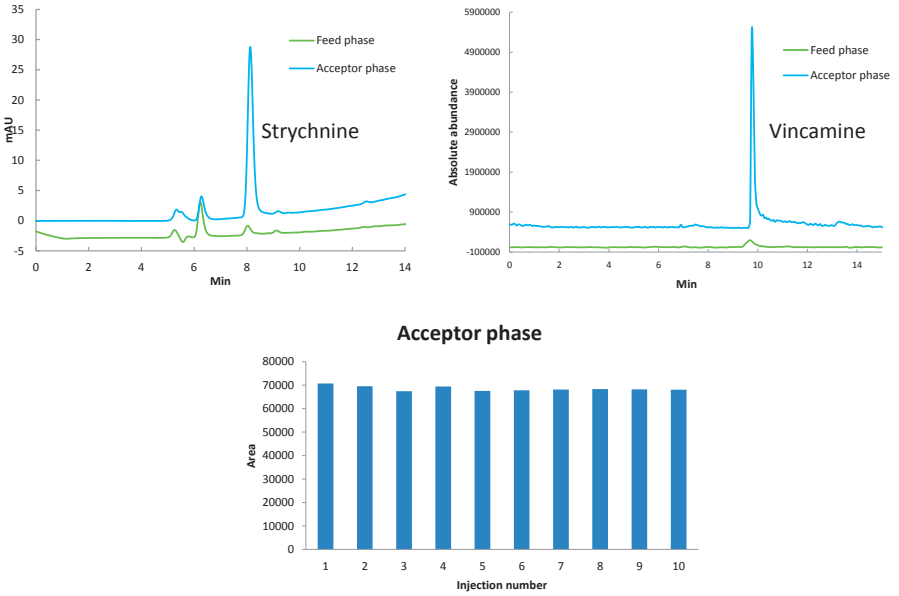


Fig. 4. On-line nanoLC profiles of strychnine standard (UV detection, upper left) and vincamine (MS detection, (+)-mode, SRM, upper right) in feed and acceptor phase of the 3-phase chip; area of strychnine standard peak in the acceptor phase for 10 individual injections (bottom).

3.4. Applications with real samples

The alkaloids present in crude extracts were successfully extracted from the feed phase to the acceptor phase, with impurities like sugars, salts and amino acids remaining in the alkaline feed phase, while sterols, waxes and other non-polars were eluted in the transport phase. In Fig. 5a the impurities at the retention time of ~7 min are not transferred to the acceptor phase. Alkaloids in *Cephaelis ipecacuanha* roots and *Strychnos nux-vomica* seeds e.g., emetine and strychnine could be successfully separated and analyzed by the 3-phase chip hyphenated with nanoLC-UV. The 3-phase chip hyphenated to nanoLC-MS shows better sensitivity

and selectivity for complex samples like *Atropa belladonna* leaves or *Vinca minor* leaves and for alkaloids having a low extinction coefficient e.g., atropine. The major alkaloids in *Atropa belladonna* and *Vinca minor* leaves, atropine and vincamine respectively, were efficiently purified from the crude extracts and analyzed by the 3-phase chip hyphenated with nanoLC-MS (Fig. 5b). The other two peaks in the acceptor phase at around 4.5 min and 7 min are also alkaloids and have the same MW as vincamine (354 Da). One of them is most likely epivincamine. Next a *Strychnos* seed extract was purified by either two classical LLE steps with a standard separatory funnel or by the 3-phase chip to compare extraction efficiency of strychnine, time demand and solvent use. The results have been summarized in Table 3. The optimized 3-phase chip hyphenated with nanoLC-UV/MS lowered the solvent consumption from milliliters to microliters, and saved a lot time due to the integrated on-line separation and analysis. The extraction efficiency and reproducibility were comparable.

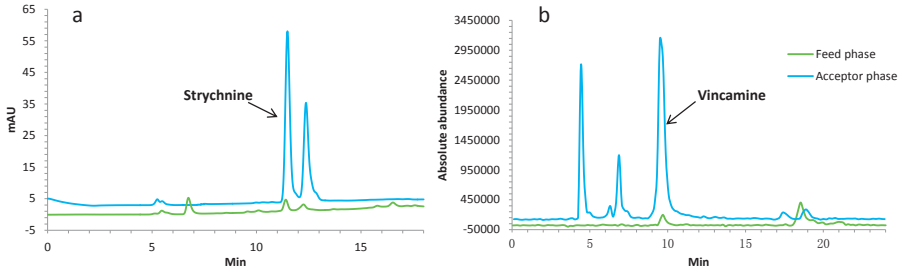


Fig. 5. The 3-phase chip-nanoLC UV/MS hyphenation system for on-line separation and analysis of alkaloids in *Strychnos nux-vomica* seeds. (a) chromatogram of *Strychnos nux-vomica* seed extracts in feed and acceptor phase; (b) the SIM (*m/z* 355) chromatogram of *Vinca minor* leaves in feed and acceptor phase.

Table 3. Comparison between traditional separatory funnel extraction of strychnine followed by off-line analysis and the 3-phase chip extraction with on-line nanoLC analysis.

Items	Normal separatory funnel extraction – off-line HPLC analysis	3-phase chip extraction – on-line nanoLC analysis
Time for extraction	Slow 30 min for extraction and back extraction, 30 min for HPLC analysis	Fast 25 s for extraction and back extraction, 18 min for nanoLC analysis
Organic solvent used	Chloroform	n-Butyl acetate
Organic solvent consumption (one sample)	50 mL for extraction, 12 mL for HPLC analysis	0.208 µL for 3-phase chip extraction, 3.6 µL for nanoLC analysis
Extraction efficiency for strychnine from *Strychnos nux-vomica* seed extract (average, n=3)	94% (RSD ≤5%)	92% (RSD ≤4%)
Labor intensity	Manual Labor-intensive	Automated Labor-saving
Environmental effect	A lot of harmful organic solvent waste	Environmentally friendly
Instrumental demand for sample preparation	Cheap (separatory funnel)	Expensive (3-phase chip plus syringe pumps)

4. Conclusion

The most alkaloid-favorable solvent, chloroform, was used in the initial development of the 3-phase sample pretreatment chip. We have shown that the use of the biodegradable and non-toxic butyl acetate leads to higher extraction efficiencies of ~90% within half a minute. The 3-phase chip was hyphenated with on-line nanoLC-UV/MS analysis. This set-up combines automated sample preparation with efficient and reproducible separations, thus saving time, organic solvents and labor and prolongs column lifetimes. After optimization of the pH of the feed phase, the 3-phase chip is capable of analyzing different classes of alkaloids with the exception of quaternary alkaloids. With high resolution mass spectrometry or tandem mass spectrometry unknown alkaloids could be elucidated. If the device could be further expanded with an on-line extraction cell and on-chip basification, total on-line alkaloid analyses become within reach. As many drugs, e.g., amitriptyline, contain a tertiary amine and behave similarly to alkaloids, such a set-up might find good use in the integrated analysis of

biofluids. By means of an internal standard true quantitation should be feasible. By exchanging the feed and acceptor phases, analyses of organic acids are also possible.

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Chapter 3

Conversion of Macroscale Induced Phase Separation Extraction to a Chip-based Version

Abstract

Induced Phase Separation Extraction (IPSE) is an efficient sample preparation technique that in some applications can replace liquid-liquid extraction (LLE). However, like LLE, it is difficult to automate or hyphenate. Therefore an IPSE chip was designed and evaluated for its ability to separate and purify samples on a microscale. The 5×2 cm chip was fed with a homogeneous sample solution consisting of acetonitrile-water (1:1) at 0.8 μL/min. In the 100 μm wide and 40 μm deep microchannels of the chip, the sample solution was efficiently separated into two immiscible phases by adding an inducer (hydrophobic solvent; butyl acetate-dichloromethane (4:1)) at 0.2 μL/min. The formed aqueous and organic phases were collected separately. Analytes present in the sample solution each migrated to their own favorable phase upon phase separation. After optimization, extraction and fractionation were easily and efficiently achieved. The behavior of analytes whose partitioning is pH-dependent, could be influenced by adjusting the pH of the sample solution. In this microscale system, the sample and solvent consumption is reduced to microliters, while the time needed for the sample pretreatment is less than one minute. Additionally, the extraction efficiency can reach nearly 100%, and emulsion formation is avoided. The chip is potentially multipurpose as it could be hyphenated with on-line extraction, chromatography or detection.

1. Introduction

A proper application of microfluidics can generate a lot of advantages in chemical analysis, such as saving solvents, time and labor, and potentially provide a high degree of integration and automation. Some of the traditional sample pretreatment methods have been successfully integrated into microfluidic chips, for example: mixing,[1-3] diluting,[4,5] liquid-liquid extraction (LLE),[6-12] solid phase extraction (SPE),[13-17] and liquid membrane transport extraction.[18-22] Thus it is possible to carry out a variety of chemical processes that are usually done with beakers, flasks and separation funnels by microfluidic chips. Overall lab-on-a-chip techniques ensure that experiments are simpler, more efficient, more environmentally friendly and less harmful for the researcher/analyst. Earlier we reported on two parallel LLE steps in a continuous-flow 3-phase chip.[6,12] On a microscale the high interfacial surface area between two immiscible liquids is one of the features that make multiphase microfluidics very interesting for analytical chemists. As Reynolds numbers are low, the multiphase flow is laminar, causing molecules to move rapidly from one fluid to the other by diffusion and affinity. This fast mass transfer makes LLE efficient in microchannels. In the 3-phase chip, due to polarity differences and a pH gradient, basic analytes migrated from a crude aqueous sample solution via an intermediate organic solvent to a much cleaner aqueous acidic acceptor fluid.[6,12]

Induced phase separation extraction (IPSE, also known as phase transition extraction (PTE) or solvent-induced phase transition extraction (SIPTE)) is a quick and efficient alternative for LLE. In essence, a water-miscible organic solvent, e.g., acetonitrile, is separated in seconds from water by adding salt[23-25] or a hydrophobic solvent,[26] or by cooling the solution to subzero temperatures.[27] Ideally analytes and matrix components migrate to their preferred and different phases. There are nice applications of IPSE such as in the analysis of drugs, for example, andrographolide, sildenafil, finasteride and nitrendipine from plasma,[26] the separation of glycosides and aglycones occurring in the medicinal plant *Scutellaria baicalensis*,[28] and the determination of angiotensin-converting enzyme activity by a induced phase separation extraction-spectrophotometric method.[29]

However, a disadvantage of IPSE up till now is that is difficult to automate and hyphenate. If the IPSE process could be miniaturized to chip-size, automation and hyphenation with extraction, chromatography or detectors would become possible, and the solvent usage would be even smaller. Altogether this would create

an alternative efficient sample pretreatment device at the μL scale. In this paper, we report on the integration of the IPSE technique using a hydrophobic solvent into a newly designed microfluidic chip and the optimization and evaluation thereof.

2. Experimental

2.1. Materials and Instruments

Chlorogenic acid, epigallocatechin gallate, rutin, quercetin, santonin, alizarin, 4-hydroxybenzoic acid, syringic acid, vincamine and emetine were purchased from Sigma. Acetonitrile, dichloromethane and chloroform (HPLC grade) were purchased from Biosolve. Hexyl acetate, butyl acetate, ethyl acetate, benzene, chloroform, dichloromethane and formic acid were purchased from Merck. Sudan red and Indigo blue were bought from Aladdin, Shanghai. Plant material (*Scutellaria baicalensis* Georgi) was purchased from a pharmacy in Changsha.

The IPSE chips were manufactured from borosilicate glass by means of photolithographic fabrication methods (Micronit, Enschede, The Netherlands) based on our own design. The IPSE chip was mounted in a PEEK holder from Micronit and connected to the syringe pumps via Teflon tubing (100 μm, i.d.). Gas tight syringes (500 μL, 3.26 mm i.d.), ferrules, nuts, Luer lock adapters, and filters were purchased from Alltech. Syringe pumps (Harvard 11 PicoPlus, dual syringe) were purchased from VWR international. Phase separation in the microchip was monitored by means of a digital microscope (DNT, the Netherlands).

The UHPLC was an Agilent 1290 Infinity, equipped with binary pumps, autosampler, thermostatted column compartment (25 °C), DAD and column (Agilent Zorbax Eclipse Plus C_{18}, 2.1 × 100 mm 1.8 μm). Mobile phase A: 0.1% formic acid in water; mobile phase B: acetonitrile. Flow rate: 0.20 mL/min. Gradient: 0-2 min 10% B; 2-15 min B increased from 10% to 60%; 15-16 min 60% B; 16.01-18 min 10% B. Wavelength: 254 nm. Injection volume: 1.0 μL. For acidic and basic compounds (polarity is dependent on pH): mobile phase A: 0.1% formic acid in water; mobile phase B: acetonitrile. Flow rate: 0.20 mL/min. Gradient: 0-10 min 10% B; 10.01-15 min 25% B; 15.01-18 min 10% B. Wavelength: 270 nm. Injection volume: 1.0 μL. For *Scutellaria baicalensis* analyses: Shimadzu SPD-M20A, DAD, and column (Shim-pack VP-ODS 4.6 × 250mm, 5 μm). Mobile phase A: 0.1% acetic acid in water; mobile phase B: acetonitrile. Flow rate: 1.0 mL/

min. Gradient: 0-5 min 20% B, 5-30 min 20-30% B, 30-60 min 30-60% B, 60-80 min 20% B. Wavelength: 254 nm. Injection volume: 20 μL.

Concentrations of the first set of model compounds in acetonitrile-water (1:1) solution were 49.8, 84.6, 62.9, 32.9, 38.0, and 24.1 μg/mL for chlorogenic acid, epigallocatechin gallate, rutin, quercetin, santonin, and alizarin, respectively. Concentrations of the second set of model compounds in acetonitrile-water (1:1) solution were 55.0, 62.0, 59.0, and 55.0 μg/mL for syringic acid, 4-hydroxybenzoic acid, emetine and vincamine, respectively.

Solvent distribution analysis was carried out by gas chromatography (GC) on a GC 2010 (Shimadzu, Japan). Column: DB-1701P, 30 m × 0.25 mm × 0.25 μm, Agilent; temperature program: 0-5 min, 40-100 ˚C, 5-10 min 100-200 ˚C; FID: 280 ˚C. Split ratio: 1:40. Injection volume: 0.1 μL, injection temperature: 180 ˚C. Relative solvent percentage (v/v) was calculated by separate calibration curves for each solvent.

2.2. IPSE Chip and Chip Modification

An all-glass two-phase chip was used for microscale IPSE. The design is depicted in Fig. 1a and further discussed in the Results and Discussion section. To facilitate full phase separation, the two parallel channels of 100 μm width and 40 μm depth are in open connection with one another. In between the parallel channels are pillar structures (semi-toroidal shape, represented by dots in Fig. 1a located at equal distances of 120 μm). Their width at half height is 20 μm. Prior to use, the IPSE chip was first washed with a basic 0.1 M NaOH solution, followed by water, 0.1 M sulfuric acid and again water. After washing, one of the parallel channels (the future organic phase channel) was selectively modified by octadecyltrichlorosilane to make it hydrophobic.

2.3. Parameter studies

Regarding the inducer-solvent composition, both single and mixed solvents were investigated by collecting the organic and aqueous phases at exits 4 and 5, followed by off-line GC analysis to study the relative solvent composition (% v/v). Physical parameters for each used solvent, such as viscosity and polarity were comprehensively considered with the aim of achieving a stable laminar flow in chip.

Acetonitrile-water (1:1) solution[26] was used as sample solution during all experiments, and contained during the initial experiments a non-polar and a

polar dye (Sudan red and Indigo blue, respectively) to make it easier to observe the phase separation and extraction during the optimization experiments.

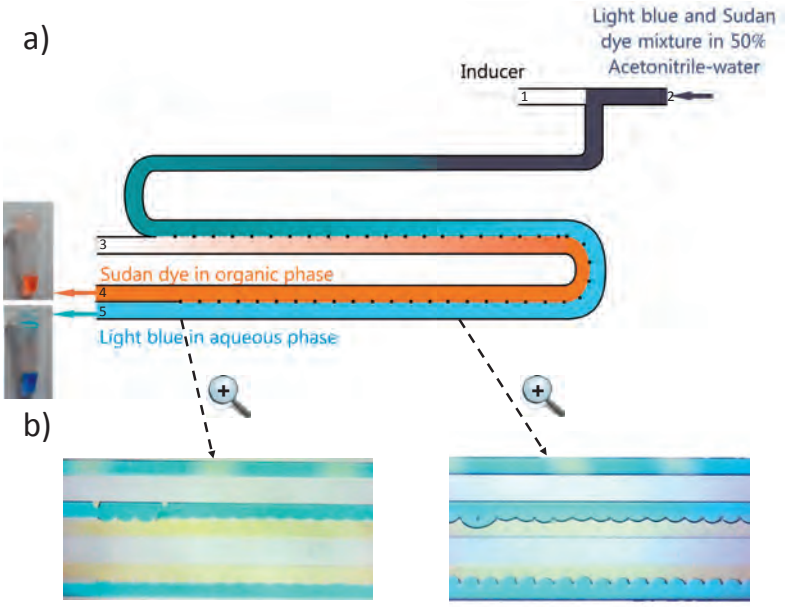

Fig. 1. Schematic representation of the induced phase separation extraction chip: a) lay-out of IPSE chip when filled with a dye mixture (indigo blue and Sudan red); b) photos of parts of IPSE chip while separating a blue-orange dye mixture by using the mixed inducer (dichloromethane – butyl acetate (2:8)).

2.4. Induced phase separation extraction conditions and extraction efficiency

The extraction efficiency was determined by off-line UHPLC analysis. The organic and aqueous phases eluting from the two outlets of the IPSE chip, were collected separately. The collected liquids were analyzed every hour (around 30 µL for each). pH buffer: $NaHCO_3$-Na_2CO_3 buffer 10 mM pH 9.95; acetate buffer 10 mM, pH 3.01. Preparation of real-life sample: 0.50 g powder of *S. baicalensis* was mixed with 50 mL of acetonitrile-water (1:1) solution and sonicated during 40 min followed by filtration through a 0.45 µm membrane.

3. Results and discussion

To carry out miniaturized IPSE using a hydrophobic solvent in a chip, we considered that a design should contain two inlet channels, namely one for the sample solution (in acetonitrile-water; Inlet 2) and one for the inducer (hydrophobic solvent; Inlet 1). These two channels should meet and mix for some time to trigger phase separation. Based on our earlier experiences with IPSE,[26,28] we expected that 12 sec should be sufficient to realize initial phase separation, which amounts at a flow of 1 μL/min to a microchannel of 4 cm length, 100 μm width and 40 μm depth. Thus in the chip design, Inlet 1 and Inlet 2 were placed opposite to each other followed by the 4 cm long channel. Based on our earlier experiences with obtaining stable laminar flows of immiscible liquids in 3-phase chips,[6,12] a 6.4 cm long 2-phase channel separated by small pillars was added. One of these channels should be made hydrophobic prior to use. The purpose of these two channels is to arrive at a complete phase separation so that at the two outlets of the chips, pure aqueous and organic phases can be collected for further off-line or on-line analysis. Finally for greater flexibility during method development, we decided to add an extra inlet channel (Inlet 3) where the single channel that contains the mixed aqueous and organic components splits in the two parallel, pillar-divided channels. This allows for the infusion of an auxiliary organic flow but, if not needed, it can also be blocked. Altogether this led to chips made according to the design depicted in Fig. 1. Fig. 1a shows schematically how a dye mixture behaves in the IPSE chip. The color of the dye mixture prior to infusion is dark blue, while after separation in the IPSE chip, the organic phase from Outlet 4 is orange (non-polar Sudan dye) and the aqueous phase from Outlet 5 is clear blue (polar Indigo blue dye). Fig. 1b shows photos of parts of the channels when the chip is in use. The left photo zooms in on the outlet part, showing well-separated phases each eluting from its own outlet.

For realizing a good phase separation in the chip as well as high extraction efficiency, several parameters influencing the IPSE process, including flow rates and inducer-solvent composition, were systematically investigated.

3.1. Inducer optimization

3.1.1. Single solvent inducer

To bring about phase separation between the less polar, aprotic acetonitrile and the highly polar water, initially we chose to add a small amount of a single

hydrophobic inducer, i.e., the non-oxygenated solvents dichloromethane or chloroform, or the oxygenated solvents ethyl acetate or butyl acetate, as these have been successfully applied before.[26]

Initially auxiliary Inlet 3 was kept blocked, and 200 nL/min of inducer and 800 nL/min of sample were infused. In all cases segmented flow when infusing a sample of Sudan red (in organic phase) and Indigo blue dye (in aqueous phase) was observed in the mixing channel. This proves phase separation occurred in the channel. Non-oxygenated solvents gave a more pronounced segmented flow, i.e., better initial phase separation, than oxygenated solvents, which is consistent with earlier macroscale data.

However, stable dual laminar flows in the two-phase parallel channels could not be achieved when using chloroform (η = 0.57 cP), dichloromethane (η = 0.44 cP) or ethyl acetate (η = 0.45 cP). A possible reason for this could be the significant viscosity difference between the aqueous and organic phases. Due to this, the aqueous phase (mostly water with some acetonitrile) will flow slowly, and the organic phase (acetonitrile with chloroform or dichloromethane or ethyl acetate) fast, which finally will cause a turbulent flow in the 2-phase channel part.[30] Thus using an inducing solvent with a viscosity closer to that of water (η = 1 cP), like butyl acetate (η = 0.74 cP) or hexyl acetate (η = 1.07 cP), might offer a solution to this problem. However, butyl and hexyl acetate were observed to possess a low inducing efficiency: less acetonitrile separated from the uniform acetonitrile-water sample solution. Thus the volume of the organic phase was considerably lower than that of the aqueous phase creating problems when the two phases were collected at the outlets of the chip. Therefore we decided to revert to the use of non-oxygenated solvents as inducer, and at the same time to open Inlet 3 to introduce a more viscous solvent (butyl acetate or hexyl acetate) as an auxiliary fluid.

With the help of the auxiliary fluid infused in Inlet 3, stable dual laminar flows in the two-phase parallel channels could be realized using non-oxygenated solvents as inducer. Various flow rates for sample, single inducer and auxiliary fluid were investigated: 700-1000 nL/min for sample, 50-400 nL/min for inducer and 100-500 nL/min for auxiliary flow. Stable laminar flows in the two parallel channels were obtained when flow rates of 800 nL/min, 200 nL/min and 400 nL/min were used for the sample, inducer and auxiliary fluid, respectively. A drawback of this approach was, however, that relatively a lot of halogenated solvent (200 nL/min) was needed and that the organic phase contained relatively little acetonitrile and much auxiliary solvent. This can complicate the injection of the organic phase into a reversed phase LC column in the case of hyphenation with HPLC.

3.1.2. Mixed solvent inducer

Thus mixed inducers consisting of a non-oxygenated solvent such as chloroform or dichloromethane, and an oxygenated solvent such as butyl acetate or hexyl acetate at various flow rates were investigated when Inlet 3 was blocked, that is, no auxiliary flow was used. As expected, stable laminar flows in the two-phase parallel channels could be observed because of the simultaneous contribution of a highly efficient phase separation-inducing solvent and a more viscous solvent. The observed phase separation and the solvent distribution in each collected phase were used as the criteria to select the best composition and flow rates. Two different ratios (2:8 and 4:6) of non-oxygenated solvent to oxygenated solvent, two different non-oxygenated solvents (dichloromethane and chloroform) and three different ratios of inducer to sample flow rates (100:800 nL/min, 150:800 nL/min, 200:800 nL/min) were tested (see Fig. 1 of SI). The ratio of the volumes of the organic and aqueous phases collected is shown in Fig. 2a. When the inducer flow rate was increased from 100 to 200 nL/min while keeping the sample flow constant at 800 nL/min, the volume ratio of organic phase to aqueous phase increased from 0.4 to >0.8 for both chloroform and dichloromethane. In addition, when setting the flow rate at 200 nL/min for the inducer and 800 nL/min for the sample, the most stable laminar flow was obtained. We did not increase the total flow rate further, as higher flow rates reduce the contact time below the duration necessary for a proper mixing of inducer and sample solution and achieving complete phase separation. The organic and aqueous effluents were collected from each outlet (n>3) and the relative solvent composition (% v/v) of each effluent was determined by gas chromatography (GC) (Fig. 2b). Based on the flow rates, solvent ratios and collected volumes of aqueous and organic phases, and under the assumption that almost all of the water will end up in the aqueous phase and almost all of the inducer and butyl acetate end up in the organic phase, one can calculate that the ~450 µL of organic phase will consist of ~250 µL of acetonitrile, ~160 µL of butyl acetate and ~40 µL of dichloromethane, and the ~550 µL of aqueous phase of ~150 µL acetonitrile and 400 µL of water. The data presented in Fig. 2b correspond well with this prediction. Acetonitrile is the major solvent in the organic phase, which proves that phase separation occurred in the chip. When the proportion of non-oxygenated solvent in the inducer was increased from 20% to 40% (Fig. 2a, right), there was neither a big increase in the separation of the acetonitrile from the water (see Fig. 1 in SI) nor a big change in phase ratio, but twice as much halogenated solvent was used. As a lower consumption of halogenated solvents is preferred, and also considering a more stable laminar flow

in the two parallel channels, 20% chloroform or dichloromethane was chosen as the optimal composition for the mixed inducer. The best condition for using the mixed inducer in the IPSE chip, therefore, is 20% of dichloromethane in butyl acetate as inducer at a flow rate of 200 nL/min in combination with a sample flow rate of 800 nL/min of acetonitrile – water (1:1).

Comparing the IPSE chip data to our earlier macroscale IPSE data without butyl acetate[26], the phase separation efficiency in the IPSE chip appears to be similar (Fig. 2b). There was still some water (around 5%) remaining in the organic phase and around 20% of acetonitrile was still present in the aqueous phase (Fig. 2b). For an even better comparison with the chip-based experiments, IPSE of 50% acetonitrile-water with the same mixed inducer was also carried out at a macroscale (Eppendorf tube) and the relative solvent distribution in each phase was analyzed by the same GC method. Results (Fig. 2b) show that both micro and macroscale gave similar relative solvent distributions, although the percentage acetonitrile was a bit lower and the percentage of butyl acetate a bit higher in the microscale IPSE. This could be due to non-equilibrium effects (kinetics of phase separation) at the microscale or occasional turbulence at the exit of the chip.

3.2. Extraction efficiency

3.2.1. Model compounds

The separation efficiency of the IPSE chip was characterized under the optimized conditions. Theoretically 100% separation efficiency is never attainable with any LLE-based system, nor will it be possible to get a good separation, like > 90% in one phase, for all analytes with a single solvent system. However, based on our earlier experience,[26] usable and fast results can be obtained reproducibly by IPSE. For the characterization we used a set of six model compounds of different polarities: chlorogenic acid, rutin, epigallocatechin gallate, quercetin, santonin and alizarin (see Fig. 2 of SI for structures). Their respective calculated log D values at pH = 7 are –3.04, –1.92, 0.57, 1.08, 1.15 and 2.05.[31] Fig. 3a shows the chromatograms of a mixture of the six model compounds and the collected organic and aqueous phase exiting the IPSE chip. The two most polar compounds, chlorogenic acid and rutin, were mostly found in the aqueous phase, while the three non-polar compounds, quercetin, santonin and alizarin, were almost exclusively present in the organic phase. The log D value of epigallocatechin gallate falls in between those of quercetin and rutin, and it is found in both phases. The extraction efficiency for

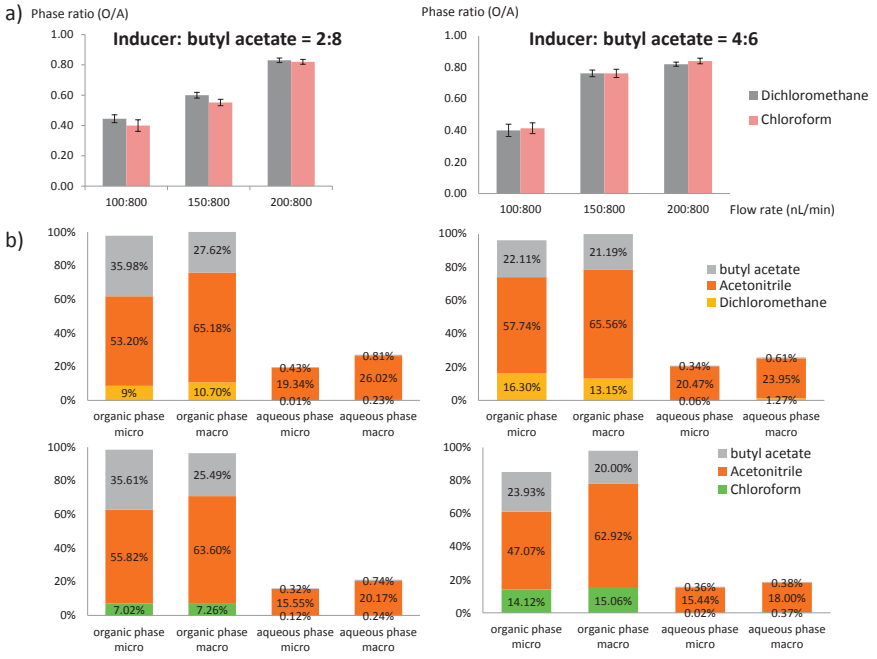

Fig. 2. (a) Phase ratio as a function of inducer flow rates (100, 150 and 200 nL/min), nature of inducer (dichloromethane or chloroform) and volume ratio of inducer and butyl acetate (2:8 or 4:6); (b) relative volume percentages of collected organic and aqueous phases at microscale (IPSE chip) and macroscale (Eppendorf tube) for both dichloromethane (upper) and chloroform (bottom) used at the optimal flow conditions. Volume ratio of inducer and butyl acetate is 2:8 (left) and 4:6 (right).

each compound both in the IPSE chip and by macroscale IPSE in an Eppendorf tube is shown in Table 1. With the exception of chlorogenic acid and santonin, the extraction efficiency on micro and macroscale is comparable. The reproducibility of IPSE, expressed as relative standard deviation (RSD) is ~3.5% and ~2% for chip and macroscale, respectively. These differences in extraction efficiency and RSD are most likely related to the fact that the IPSE process in the chip is much faster (< 30 sec). Once the two phases exit the chip, there is no more opportunity to reach equilibrium. Additionally for compounds like chlorogenic acid – whose log D varies a lot between pH 3 to 8[31] – small local changes in pH will influence its partitioning. What is furthermore interesting to observe, is that the partitioning behavior of analytes in the IPSE process does not correspond with the partioning behavior of those same analytes in RP-HPLC. Epigallocatechin gallate elutes before rutin (Fig. 3a) on an RP-HPLC column, yet rutin is for 94% present in the aqueous phase and epigallocatechin gallate only for 57%. This different selectivity offers interesting possibilities for IPSE to separate analytes during sample pretreatment, which co-elute on an RP-column.

Table 1. Extraction efficiency of six model compounds by IPSE on a micro (chip) and macroscale (Eppendorf tube)

Compounds	Extraction efficiency of IPSE chip in % (SD)		Extraction efficiency of IPSE in Eppendorf tube in % (SD)	
	Organic phase	Aqueous phase	Organic phase	Aqueous phase
Chlorogenic acid	10.4 (0.89)	89.6 (1.3)	19.1 (0.62)	80.9 (0.64)
Epigallocate-chin gallate	43.2 (0.69)	56.8 (1.0)	45.5 (2.5)	54.5 (2.7)
Rutin	6.10 (0.21)	93.9 (1.5)	6.60 (0.039)	93.4 (0.46)
Quercetin	94.0 (2.2)	6.00 (0.37)	94.4 (0.094)	5.60 (0.16)
Santonin	90.7 (1.9)	9.30 (0.37)	93.9 (0.46)	6.10 (0.11)
Alizarin	96.2 (1.9)	3.80 (0.29)	96.8 (0.38)	3.20 (0.10)

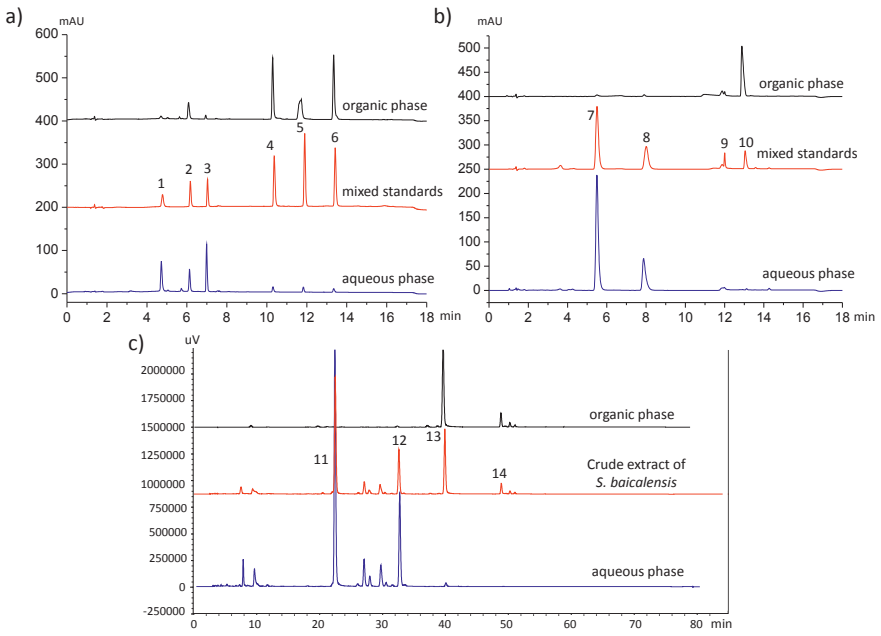

Fig. 3. (a) HPLC profiles of a mixture of six model compounds (chlorogenic acid (peak 1), epigallocatechin gallate (peak 2), rutin (peak 3), quercetin (peak 4), santonin (peak 5) and alizarin (peak 6)), and the corresponding organic and aqueous phases collected at the outlets of the IPSE chip; (b) HPLC profiles of the mixture of four acidic or basic model compounds (4-hydroxybenzoic acid (peak 7), syringic acid (peak 8), emetine (peak 9), and vincamine (peak 10)) and the corresponding organic and aqueous phases collected at the outlets of the IPSE chip; (c) HPLC profiles of a *S. baicalensis* extract and the corresponding organic and aqueous phases collected at the outlets of the IPSE chip; baicalin (peak 11), wogonoside (peak 12), baicalein (peak 13), wogonin (peak 14).

3.2.2. pH effect on IPSE characteristics of acidic and basic compounds

For compounds whose partitioning behavior depends on the pH, such as amines, acids and phenols, a change in the sample pH is expected to greatly affect their extraction behavior. To test this, we studied the IPSE behavior of two acids and two amines: syringic acid, 4-hydroxybenzoic acid, vincamine and emetine (see Fig. 2 of SI for structures). At pH 10, the two acids were mostly expected in the aqueous phase and the two alkaloids mostly in the organic phase. All four compounds were injected at pH 3 and 10, and both phases were collected and off-line investigated by HPLC (Fig. 3b). Both acids and vincamine behaved as expected (Table 2), but emetine showed erratic behavior at both pHs and at both micro and macroscale. Overall the extraction efficiencies at micro and macroscale were comparable. High extraction efficiencies of 94 - 99% into one phase were observed for syringic acid, 4-hydroxybenzoic acid and vincamine at pH 9.95. During the extraction of alkaloids by either traditional LLE and by macroscale IPSE, persistent emulsions are frequently formed. During the extraction of alkaloids by the IPSE chip, clean and stable laminar flows were observed at both pHs without emulsion formation. This is a significant advantage of IPSE in a chip versus traditional IPSE in a tube. This advantage would contribute to a reliable application of an IPSE chip in automated on-line sample clean-up.

Table 2. Extraction efficiency of four pH-dependent model compounds by IPSE on micro and macroscale

	Extraction efficiency at pH 3.07 in % (SD)				Extraction efficiency at pH 9.95 in % (SD)			
Compounds	IPSE chip		Eppendorf tube		IPSE chip		Eppendorf tube	
	Organic phase	Aqueous phase	Organic phase	Aqueous phase	Organic phase	Aqueous phase	Organic phase	Aqueous phase
4-Hydroxy-benzoic acid	78.7 (9.5)	21.3 (0.76)	72.7 (0.014)	27.3 (0.46)	1.20 (0.085)	98.8 (0.82)	2.20 (0.53)	97.8 (0.91)
Syringic acid	73.5 (9.2)	26.5 (0.64)	67.4 (2.6)	32.6 (0.35)	1.40 (0.056)	98.6 (3.1)	3.00 (0.72)	97.0 (2.5)
Emetine	47.2 (1.8)	52.8 (4.4)	33.6 (0.93)	66.4 (23)	48.5 (10)	51.6 (3.9)	43.1 (4.5)	56.9 (2.4)
Vincamine	19.4 (0.66)	80.6 (2.0)	16.7 (0.18)	83.4 (0.41)	94.0 (7.8)	5.96 (1.8)	97.2 (1.2)	2.81 (0.66)

3.3. Real-Life Sample Application of Microscale IPSE

Finally, the optimized chip was tested with a complex plant sample, *Scutellaria baicalensis*. This plant is an important constituent of Traditional Chinese Medicines (TCMs) and has earlier been separated by macroscale IPSE, allowing a comparison. Fig. 3c shows the chromatograms of the separation of aglycones and glycosides present in *S. baicalensis* by the IPSE chip. Aglycones, like baicalein **13** and wogonin **14**, were almost exclusively found in the organic phase, while glycosides such as baicalin **11** and wogonoside **12** were almost exclusively present in the aqueous phase. This application proves that the IPSE chip can also work well for more complex matrixes and confirms that the elution behavior in IPSE can deviate from elution behavior in RP-HPLC and thus can improve the analysis of complex natural mixtures as relevant for TCMs.

4. Conclusions

An Induced Phase Separation Extraction (IPSE) chip was designed and tested. It can be used for efficient miniaturized sample pretreatment. The newly developed IPSE chip could successfully separate acetonitrile – water (1:1) sample solutions into individual organic and aqueous phases by adding 20% hydrophobic inducer. The behavior of analytes in the sample solution is correlated to their log D values, but less so to the elution order in RP-HPLC. This different selectivity makes the combination IPSE-HPLC interesting for compounds, which co-elute in RP-HPLC. Adapting the pH for analytes whose pK_a value falls between 4 and 10 can further expand this difference in selectivity. The whole process can be finished in 30 sec at low flow rates: 0.2 µL/min for inducer (20% of dichloromethane in butyl acetate), and 0.8 µL/min for sample solutions. The extraction efficiency of the IPSE chip is equal to or even higher than that of macroscale IPSE carried out in Eppendorf tubes, as shown by the analysis of model compounds. The model compounds of variable polarity showed that similar to LLE, IPSE can never achieve a quantitative separation for all analytes. Although not investigated in this study, we expect that – again similar to LLE – this can be changed by the flow rates and relative percentages of water, acetonitrile and inducer. The reproducibility (RSD ~3.5%) of chip-based IPSE separations is similar to that of manual separatory funnel-based separations, and allows applications in quantitative analyses. As a real-life sample, an extract of *S. baicalensis* was well separated by the IPSE chip into its respective aglycones

and glycosides, and the results were virtually identical with those obtained with macroscale IPSE. When comparing our earlier 3-phase sample pretreatment chip[6,12] with the IPSE chip, the IPSE chip appears more robust. Future applications we envisage for the IPSE chip are hyphenation to a miniaturized extraction cell (upstream), HPLC or MS (both downstream) for automated on-line analysis to save time and increasing reproducibility while at the same time avoiding the introduction of impurities and possible analyte degradation. The optimal flow of 1.0 μL/min is ideal for combining chip-based IPSE with on-line UHPLC.

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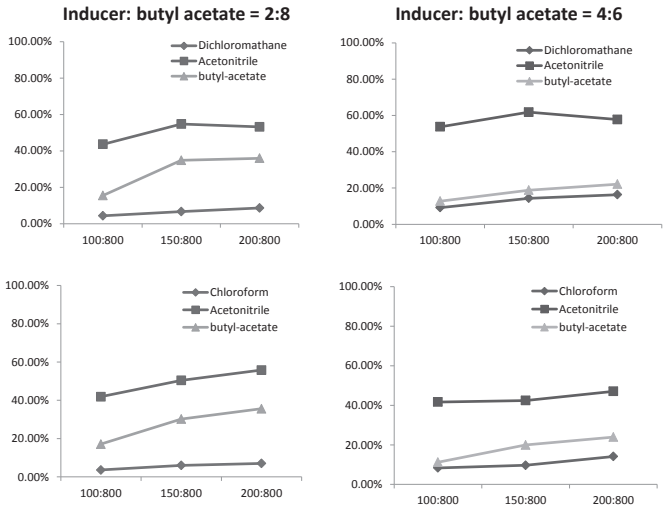
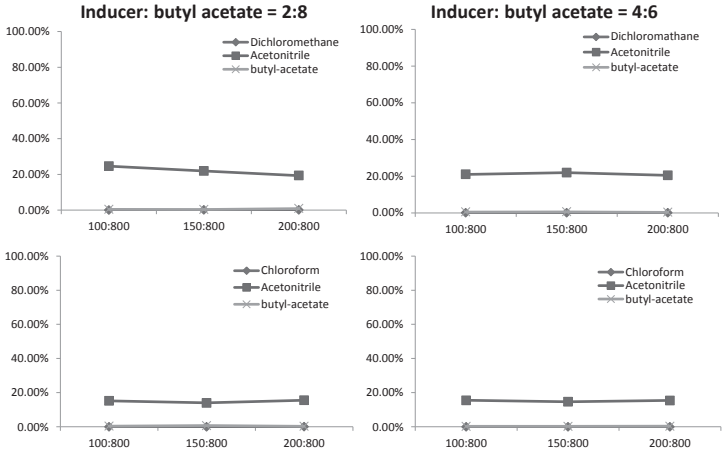
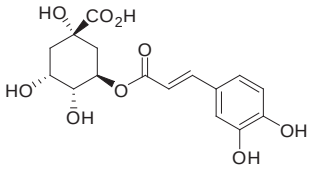
S1a. Relative volume percentages of collected organic phases at microscale (IPSE chip) for both dichloromethane (upper) and chloroform (bottom) used at the 3 flow rates.



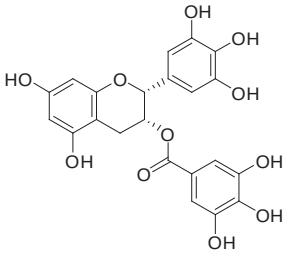
S1b: Relative volume percentages of collected aqueous phases at microscale (IPSE chip) for both dichloromethane (upper) and chloroform (bottom) used at the 3 flow rates.



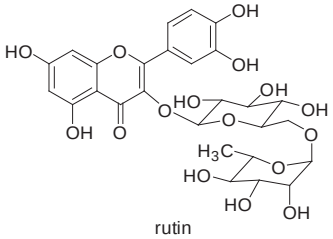
S2: Structures of 10 model compounds used for IPSE efficiency test



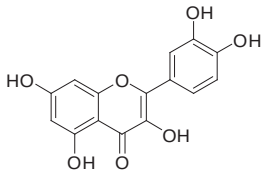
chlorogenic acid



epigallocatechin gallate



rutin



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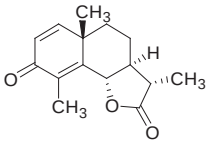

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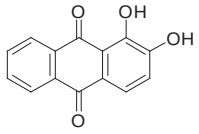
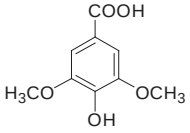


alizarin



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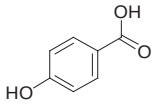

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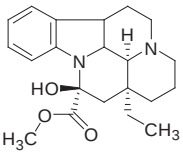

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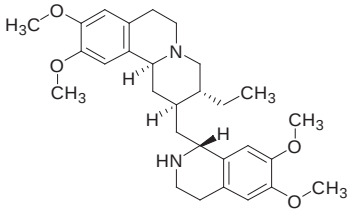

emetine

Chapter 4

Rapid control of Chinese Star Anise Fruits and Teas for Neurotoxic Anisatin by Direct Analysis in Real Time (DART) High Resolution Mass Spectrometry

Abstract

Ingestion of products containing Chinese star anise (*Illicium verum*) fruits contaminated or adulterated with Japanese star anise (*Illicium anisatum*) fruits can cause poisoning due to the neurotoxin anisatin that is present in Japanese star anise. Direct Analysis in Real Time (DART) ambient ionisation coupled with orbitrap high resolution mass spectrometry allowed the recording of mass spectra of anisatin in solid star anise fruits in seconds without any prior sample pretreatment. Spectra could be obtained in both positive and negative mode and gave the same outcome. The anisatin signal was typically > 1000 times larger in Japanese star anise than in Chinese star anise thus allowing an unequivocal qualitative determination. Herbal teas containing star anise fragments could be analysed by preparing a tea and subsequently sampling on a glass rod. Spiking a complex herbal tea containing Chinese star anise with an equally concentrated tea prepared from Japanese star anise provided a linear calibration curve ($R^2 \geq 0.995$) by standard addition method. This showed that adulteration down to 1% (w/w) is still measurable. Compared with existing PCR, TLC, GC-MS and HPLC-ESI-MS/MS procedures, the proposed DART-HRMS procedure is faster and simpler and moreover measures the actual biotoxin.

Chapter based on: Y. Shen, T.A. van Beek, F.W. Claassen, H. Zuilhof, B. Chen and M.W.F. Nielen, Rapid control of Chinese star anise fruits and teas for neurotoxic anisatin by direct analysis in real time (DART) high resolution mass spectrometry, *Journal of Chromatography A*, **2012**, 1259, 179-186.

1. Introduction

Chinese star anise fruits (*Illicium verum*) are a frequently used spice in Asian cuisine. In Latin America teas are used as a treatment for infant colic while in Europe teas are used for stress relief.[1-4] Further it is used as a substitute for anise (*Pimpinella anisum*) because of its high content of anethol.[2] This species, together with the morphologically similar Japanese star anise (*Illicium anisatum*), is also used for decoration purposes. Both species are a source of shikimic acid which can be synthetically converted to Tamiflu.[5] Japanese star anise fruits contain the rare sesquiterpene dilactone anisatin (Fig. 1),[6,7] which is a strong non-competitive antagonist of the $GABA_A$-receptor.[8,9] Due to the presence of anisatin, *I. anisatum* causes severe toxicity when ingested, i.e., nausea, hallucinations, and epileptic seizures. *I. verum* contains two less active neurotoxic sesquiterpene dilactones[10] in low concentration, which nonetheless might cause problems when Chinese star anise teas are administered in high doses to small babies.[11-13] A review on star anise is available.[14]

Recently, concern has been raised regarding the adulteration of Chinese star anise with Japanese star anise. Intoxications of babies, which are especially susceptible, in the form of adverse neurologic reactions have been reported from the USA, France, Spain and Switzerland.[11-13,15,16] In 2001, there was a large outbreak of toxicity among adults in The Netherlands which was caused by the accidental introduction of Japanese star anise in a tea blend containing six different plants. Sixty three persons were affected and 22 were hospitalised, 16 of them suffering from epileptic seizures.[9,17-19]

For the distinction between Chinese star anise and toxic Japanese star anise, different methods have been proposed. According to the literature it is possible to distinguish the two species morphologically but it requires specific expertise in botanical microscopy and does not work when the fruits have partially disintegrated as in teas.[1] Fluorescent microscopy has also been proposed.[20] However both Fritz et al. and Ize-Ludlow et al. concluded that star anise anatomy is widely variable and differentiation thus difficult.[4,21] According to the former group only hexagonal crystals may serve as indicator for the presence of *I. anisatum*. For finely powdered samples chemical analytical methods are the first choice.[21]

Several chemical methods focus on the essential oil which differs among Illicium species. Anethol is the main constituent of *I. verum* while asaricin, methoxyeugenol and O-prenyleugenol are characteristic of *I. anisatum*.[3,4,20,22-25]

SPME, steam distillation, and thermal desorption have been used for the isolation of volatile analytes prior to GC-MS. However, this approach does not analyse anisatin itself and might fail in the case of herbal teas containing multiple plant species incl. anise seeds or limited adulteration. Techen et al. have proposed a molecular method.[26] After DNA extraction, PCR, and digestion with an endonuclease, electrophoresis on agarose or polyacrylamide made a distinction possible. Kawazu et al. also used PCR in combination with microscopy and LC-MS.[27] The best assay so far has been published by Lederer et al. who actually quantitated anisatin itself. After a 12-step extraction and sample pretreatment procedure, they used LC-MS/MS in combination with external standardization. The LOQ was 3.9 μg/kg which allowed for the first time the detection of anisatin in Chinese star anise albeit at a more than 10,000 times lower concentration than in Japanese star anise.[24] After being commissioned by the FDA, the American Herbal Pharmacopoeia (AHP) prepared a monograph on star anise and its adulteration.[28]

All of these methods are rather slow and several of them are labour-intensive and/or do not measure the analyte of interest. Recently a new ambient ionisation technique named "Direct Analysis in Real Time" (DART), has been proposed that allows analysis of a wide range of substrates in seconds without any prior sample pretreatment.[29-32] After ionisation of analytes with excited helium species in the open air, analytes are selectively measured by either MS^n[33] or high-resolution MS (HRMS)[34]. DART has been widely applied to different analytical fields, e.g. pharmaceutical formulations,[35] explosives,[36] pesticides,[37] beer characterisation,[38] printing paper,[39] textiles,[40] chemical warfare agents,[41] counterfeit drugs,[42] medicinal herbs,[43,44] propolis,[34] bioanalysis[33] and food-quality and safety analysis.[45,46] The results show DART-MS to be a very promising tool for the rapid analysis of important markers in crude products.

As anisatin is an oxygenated sesquiterpene dilactone of intermediate polarity, it should be suitable for rapid sampling by DART. In this contribution we report on the development of an ultrafast qualitative assay and a rapid semi-quantitative assay for anisatin in fruits of Japanese and Chinese star anise, and teas derived thereof by means of DART-HRMS.

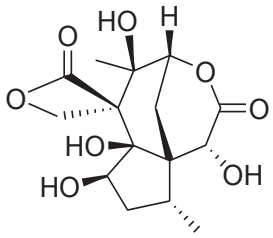

Fig. 1. Chemical structure of anisatin ($C_{15}H_{20}O_8$, exact mass = 328.1153 Da)

2. Experimental

2.1. Materials and reagents

Chinese star anise (*Illicium verum* Hook. f.) samples were obtained from RIKILT (Wageningen, The Netherlands), from a local market in Sumatra, Indonesia and from retail teas. Japanese star anise (*Illicium anisatum* L.) samples were obtained from RIKILT, from PhytoLab (Vestenbergsgreuth, Germany) and from Quarryhill Botanical Garden (Glen Allen, CA, USA). The following teas containing Chinese star anise tea fruits were purchased locally around Wageningen, The Netherlands in 2011: (1) Taste and Tools sterrenmix tea (batches 2013 & 2014); (2) sterrenmix tea De Tuinen; (3) Piramide sterrenmix tea (tea bags); (4) Jacob Hooy sterrenmix tea (tea bags); (5) C1000 Sterrenmunt (tea bags); (6) Zonnatura sterrenmix (tea bags); (7) Albert Heijn sterrenmix tea (tea bags). According to the labels the investigated teas contained beside Chinese star anise (*Illicium verum*), three or more of the following herbs: liquorice (*Glycyrrhiza glabra*), anise seeds (*Pimpinella anisum*), juniper berries (*Juniperus communis*), meadowsweet (*Filipendula ulmaria*),cornflower (*Centaurea cyanus*), spearmint (*Mentha spicata*), fennel seeds (*Foeniculum vulgare*), peppermint (*Mentha* × *piperita*), common mallow (*Malva sylvestris*), large-leaved linden blossoms (*Tilia platyphyllos*), stinging nettle leaves (*Urtica dioica*), rosemary (*Rosmarinus officinalis*) and fenugreek (*Trigonella foenum-graecum*). High-purity helium gas (grade 6.0) was provided by Linde Gas Benelux B.V. Ultrapure water was freshly prepared in-house by means of a Seradest SD 2000 water filtration unit (18 MΩ cm quality). Dip-it capillaries for DART-MS measurements of teas were purchased from IonSense Inc. (Saugus, USA).

2.2. Preparation of star anise tea

Commercial teas. Loose teas: a representative sample of 5 g of a "sterrenmix" tea containing Chinese star anise fruits was added as such to 50 mL of boiling water in an open 100 mL beaker and gently boiled for another 6 min. After forced cooling to room temperature, any evaporated water was replaced and next the leaves were removed by filtration through a filter paper. The resulting clear light brown solution was frozen and kept at −18 °C. Prior to DART-MS analyses, it was thawed and brought to room temperature. Tea bags: a number of tea bags equivalent to 4-6 g of dry plant material was extracted in ten times the amount of water as described above for loose teas. Also the remaining procedure was the same except that if the obtained tea was clear upon cooling, no filtration was carried out.

The procedure was the same as for the commercial tea blends except that 1 g of sample and 10 mL of water were used. Fruits were extracted intact, i.e., without grinding.

2.3. DART-orbitrap MS

2.3.1 Analysis conditions

The DART-Orbitrap MS system consisted of a DART ion source (model DART-SVP, IonSense, Saugus, USA) coupled to an Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The mass spectrometer was calibrated at the beginning of each day. XCALIBUR software (v. 2.1) was used for instrument control, data acquisition and data processing.

DART settings positive mode: He as ionising gas, fixed flow of ~3.5 L/min; gas beam temperature 350 °C; grid electrode voltage +350 V. MS: capillary voltage +60 V; tube lens voltage +100 V; skimmer voltage +20 V; capillary temperature: 250 °C. The resolution was set at "ultra high" and a scan rate of 1 Hz was used. The mass range was *m/z* 300-400.

DART settings negative mode: He as ionising gas, fixed flow of ~3.5 L/min; gas beam temperature 400 °C; grid electrode voltage −350 V. MS: capillary voltage −60 V; tube lens voltage −125 V; skimmer voltage −28 V; capillary temperature: 250 °C. The resolution was set at "ultra high" and a scan rate of 1 Hz was used. The mass range was *m/z* 300-400.

2.3.2. Qualitative DART-HRMS measurements of dry Japanese star anise and Chinese star anise fruits

One carpel (typically 1/8th of an intact fruit) was taken by means of tweezers. Then the DART-MS measurement was started by recording background. Next the star anise was held in the DART sampling area and slightly moved until a clear signal related to star anise was observed. During 15-25 sec the star anise piece was held in this position. This was repeated twice more, i.e., an intermittent measurement of background and star anise. The ion at *m/z* 327.107 (negative mode) or *m/z* 346.150 (positive mode) was observed. For each of the 3 measurements, the average signal height over the middle 80% of one star anise measurement was taken as a measure of the amount of anisatin. The average value of the 3 measurements was considered representative for the anisatin content of this particular star anise carpel. No background subtraction was performed as never any signal (signal <5, n=6) for *m/z* 327.107 (negative mode) or *m/z* 346.150 (positive mode) was observed in blank measurements.

2.3.3. Qualitative DART-HRMS measurements of anisatin in teas

A DART-MS measurement was started by recording background. Next a glass Dip-it was dipped in a tea as prepared under 2.2. On average 1.8 mg ± 0.5 mg (n=10) of tea adhered to the Dip-it stick. Immediately afterwards the Dip-it was placed by means of a special holder in the DART sampling area and left there for 15-25 sec. This was repeated twice more, i.e. an intermittent measurement of background and star anise tea. The ion at *m/z* 327.107 (negative mode) or *m/z* 346.150 (positive mode) was observed. For each of these 3 replicate measurements, the average signal height over the middle 80% was taken as a measure of the amount of anisatin. The average value (n=3) was considered representative for the anisatin content of this star anise tea. One analysis of 3 replicates including determining the average response of each replicate and entering the data in a spreadsheet took less than 5 min.

2.3.4. Semi-quantitative DART-HRMS analysis of anisatin in tea

To Taste & Tools 2013 sterrenmix tea, 5 different volumes of Japanese star anise "tea" (see 2.2) were added. The final concentrations of Japanese star anise tea were 0%, 1%, 2%, 5%, 20%, and 50% (v/v). These solutions were measured by DART-HRMS as described under 2.3.3 and the anisatin signal recorded. In addition the average height of *m/z* 242.052 (negative mode) or *m/z* 216.120

(positive mode) was measured. As these ions occurred only in Chinese star anise tea, they could be used for internal normalisation, i.e., the anisatin signal was adjusted according to the strength of the signal of intrinsic Chinese star anise constituent. After correction for the relative volumes present, calibration curves were constructed (Fig. 6).

2.4. MS/MS studies

An aqueous extract of Japanese star anise fruits was infused at 5 µL/min into a Thermo LXQ linear ion trap mass spectrometer in negative mode and ESI-MS/MS studies were carried out on the *m/z* 327 ion. MS settings: sheath gas 30, spray voltage 6 kV, capillary temperature 300 °C, capillary voltage −3 V and tube lens voltage −80 V. This was repeated with the DART connected to the ion trap mass spectrometer. For DART parameters, see 2.3.1; MS capillary temperature and voltage, and tube lens voltage same as for infusion experiment.

2.5. Precision

The intra-day precision was determined from a set of three replicate analyses of the same sample of an aqueous extract of Japanese star anise fruits (see 2.2) on a single day carried out by the same operator. Each measurement was itself an average of a triplicate measurement (see 2.3.3). The inter-day precision was evaluated from a set of three replicate analyses of the same sample on three different days by the same operator.

3. Results and discussion

3.1. DART-HRMS conditions

Due to the worldwide unavailability of anisatin at the time of this research, Japanese and Chinese star anise fruits were used as a positive and negative control respectively for the presence of anisatin. According to Lederer et al. the concentration difference is ~10,000.[24] In negative mode, the mass spectrum of a carpel of Japanese star anise recorded at 400 °C is shown in Fig. 2a. A similar spectrum of a carpel of Chinese star anise can be seen in Fig. 2b. This shows that anisatin is well detectable in dry fruits of Japanese star anise by DART-MS. In Chinese star anise fruits either no signal or very weak responses (signal < 130 in negative mode and < 90 in positive mode) for anisatin were observed. Both mass

spectra contained an additional peak at *m/z* 327.072 with as most likely elemental composition $C_{14}H_{16}O_9$. This could be bergenin, a C-glycoside of 4-O-methyl gallic acid and a relatively common plant metabolite. This first very clear difference between Japanese and Chinese star anise, which was obtained in seconds without any sample preparation, looked very promising and prompted us to see if also positive mode DART-MS could be used and confirm the result.

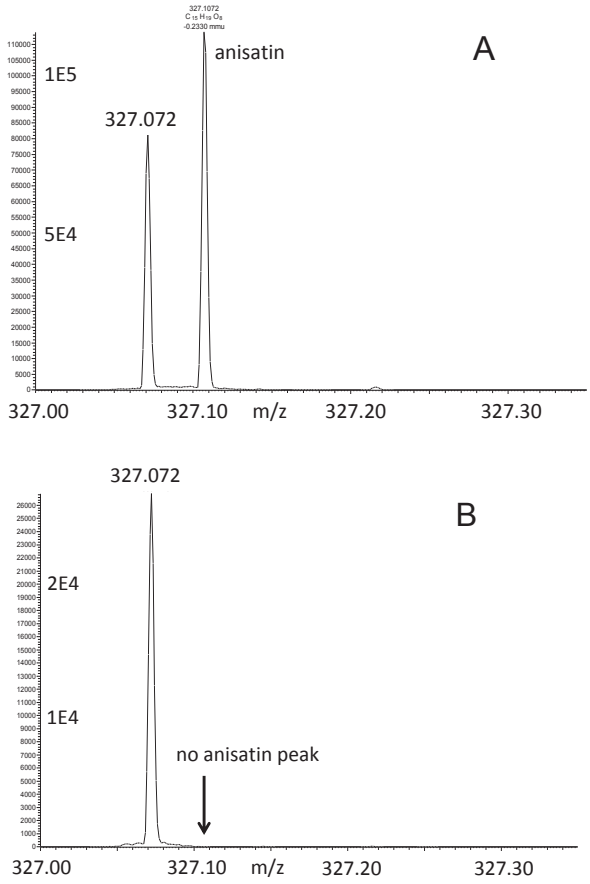

Fig. 2. Negative mode DART-orbitrap mass spectra from *m/z* 327.00-327.35 of one carpel of Japanese star anise (A) and Chinese star anise (B). Vertical scale in (B) is expanded 4 times. Peak at *m/z* 327.107 is anisatin $[M–H]^-$ ($C_{15}H_{19}O_8$), peak at *m/z* 327.072 is of an unknown constituent ($C_{14}H_{15}O_9$).

The positive mode spectra of Japanese and Chinese star anise fruits are presented in Fig. 3. In this case not $[M+H]^+$ but rather $[M+NH_4]^+$ at *m/z* 346.150 ($C_{15}H_{24}NO_8$) was obtained for anisatin, which is fairly common in (+)-DART-MS. Again a similar "day and night" difference was observed substantiating the results obtained in negative mode. To prove that anisatin is present in the investigated Japanese star anise fruits and indeed responsible for the ion at *m/z* 327.109, the DART was mounted on a linear ion trap mass spectrometer and MS/MS studies on an aqueous extract were performed. Selection of the ion at *m/z* 327 followed by collision induced dissociation, gave a mass spectrum very similar to the negative mode ESI-MS/MS spectrum published by Lederer et al. with fragments at *m/z* 309, 297, 283, 265 and 127.[24] Direct infusion of the extract followed by ESI-MS/MS provided the same spectrum. Thus it was ascertained that anisatin was indeed present and can be ionised and analysed by DART-HRMS.

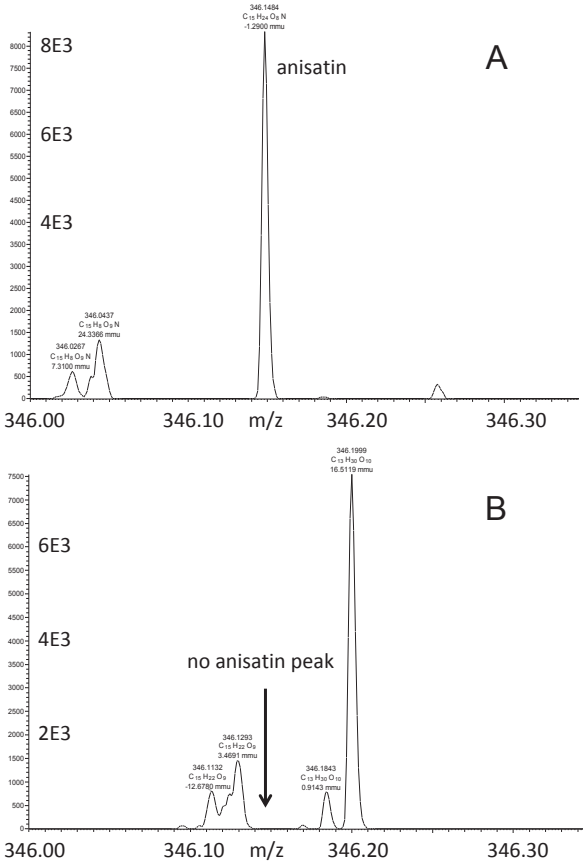

Fig. 3. Positive mode DART-orbitrap mass spectra from *m/z* 346.00-346.35 of one carpel of Japanese star anise (A) and Chinese star anise (B). Peak at *m/z* 346.148 is anisatin, $[M+NH_4]^+$ ($C_{15}H_{24}NO_8$).

The temperature of the helium gas in the DART was optimised in steps of 50 °C. Below 300 °C no signal could be observed. Above 450 °C the signal intensity decreased, most likely due to thermal decomposition, e.g., loss of water. The strongest signal in negative and positive mode was obtained at 400 and 350 °C respectively. In all further experiments these temperatures were used.

As many different compounds with masses almost identical to that of anisatin occur in both Japanese and Chinese star anise samples, a very high mass resolution is required for reliable results. For instance the vertically expanded positive mode mass spectrum from *m/z* 346.08-346.35 of a Chinese star anise fruit in Fig. 4 shows no signal for anisatin but peaks of at least 12 other constituents having a different elemental composition. The one closest to anisatin differs only by 0.012 Da. To resolve this ion from that of anisatin a mass resolution of at least 27,000 is required. The used orbitrap mass spectrometer has a mass resolution of ~90,000 in this mass spectrum. Thus only FT, orbitrap or high-end TOF mass spectrometers should be used for the analysis of star anise in combination with DART. A comparison of Fig. 3b and 4 also shows that the chemical composition of Japanese star anise varies with its origin.

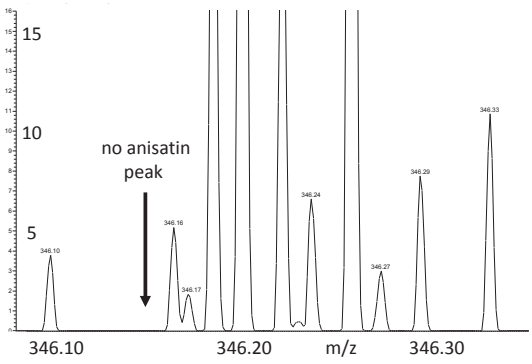

Fig. 4. Positive mode DART-orbitrap mass spectrum from *m/z* 346.08-346.35 of one carpel of Chinese star anise showing compounds with 12 different elemental compositions.

3.2. Distinction of Japanese and Chinese star anise fruits and teas by DART-orbitrap MS

To evaluate whether DART-HRMS is really conclusive for distinguishing Japanese and Chinese star anise, Japanese star anise fruits originating from three sources and Chinese star anise fruits from five different sources were investigated.

From all fruits three different carpels were measured and from two of the three different Japanese sources two fruits were analysed. Each sample was analysed in triplicate. The results are presented in Table 1. Although standard deviations are large due to the large influence of the exact position of the fruit in the sampling area, the natural variation in anisatin concentration between fruits and carpels, the probably uneven distribution of anisatin over the fruit and the DART ionisation process itself, the results nevertheless allow a clear distinction between the two types of star anise. In negative mode the average signal height of 12 carpels originating from four different fruits was 39,000 (range: 14,000 - 65,000). For 18 carpels from six different Chinese fruits this value was 33 (range: 0 - 130), i.e., a difference of more than three orders of magnitude. In positive mode these values were 13,250 (range: 950 - 35,000) and 6 (range: 0 - 50), again a difference of three orders of magnitude. Negative ionisation gave on average a four times higher response. As expected, none of the three investigated Dutch retail teas with intact star anise fruits contained Japanese star anise. A sample of dried unripe (green) Japanese star anise gave an anisatin response in between mature Japanese and Chinese fruits, suggesting that anisatin is biosynthesised or accumulated late during the ripening process. Overall negative spectra are preferred as they give a higher signal, and are simpler and more reproducible.

Table 1. Average signal height and standard deviations of anisatin in different dry star anise fruits. Each sample was measured in triplicate.

Sample	Carpel 1		Carpel 2		Carpel 3		Average	
Ionisation mode	Negative mode	Positive mode	Negative mode	Positive mode	Negative mode	Positive mode	Negative mode	Positive mode
Japanese star anise RIKILT fruit 1	5.7×10^4 ± 2.2×10^4	2.0×10^4 ± 1.0×10^4	3.3×10^4 ± 2.6×10^4	2.9×10^4 ± 1.0×10^4	3.4×10^4 ± 1.7×10^4	3.5×10^4 ± 1.6×10^4	4.1×10^4 ± 1.4×10^4	2.8×10^4 ± 0.3×10^4
Japanese star anise RIKILT fruit 2	4.0×10^4 ± 0.1×10^4	1.1×10^4 ± 0.3×10^4	5.1×10^4 ± 1.4×10^4	1.5×10^4 ± 0.3×10^4	2.6×10^4 ± 1.9×10^4	1.0×10^4 ± 0.5×10^4	3.9×10^4 ± 1.3×10^4	1.2×10^4 ± 0.1×10^4
Japanese star anise PhytoLab fruit 1	1.4×10^4 ± 0.6×10^4	2.2×10^3 ± 0.7×10^3	3.0×10^4 ± 0.8×10^4	1.5×10^4 ± 1.9×10^4	2.6×10^4 ± 1.8×10^4	9.5×10^2 ± 1.6×10^2	2.3×10^4 ± 0.6×10^4	6.1×10^3 ± 7.8×10^3
Japanese star anise PhytoLab fruit 2	1.6×10^4 ± 0.5×10^4	4.5×10^3 ± 3.2×10^3	2.7×10^4 ± 2.3×10^4	1.2×10^4 ± 0.8×10^4	6.5×10^4 ± 2.3×10^4	4.9×10^3 ± 0.5×10^3	3.6×10^4 ± 1.1×10^4	7.0×10^3 ± 3.9×10^3
Unripe Japanese star anise	2.2×10^2 ± 0.8×10^2	nd	2.3×10^2 ± 1.6×10^2	8.7×10 ± 2.3×10	2.9×10^2 ± 0.9×10^2	1.5×10^2 ± 0.4×10^2	2.5×10^2 ± 0.5×10^2	7.9×10 ± 7.5×10
Chinese star anise RIKILT fruit 1	1.3×10^2 ± 0.4×10^2	1.4×10 ± 1.3×10	1.0×10^2 ± 0.13×10^2	nd	4.5×10 ± 2.8×10	nd	9.2×10 ± 4.3×10	5 ± 7
Chinese star anise RIKILT fruit 2	1.3×10 ± 1.9×10	nd	5.9×10 ± 1.6×10	nd	9.7×10 ± 7.1×10	nd	5.6×10 ± 3.1×10	nd
Chinese star anise from Sumatra	1.2×10 ± 0.5×10	nd	1.9×10 ± 1.0×10	nd	3.7×10 ± 1.0×10	nd	2.3×10 ± 0.3×10	nd
Chinese star anise "Taste & Tools" batch 2013	1.4×10 ± 1.3×10	5.0×10 ± 5.6×10	6 ± 10	nd	7 ± 10	1.7×10 ± 2.3×10	9 ± 4	2.2×10 ± 2.8×10
Chinese star anise "Taste & Tools" batch 2014	1.2×10 ± 1.1×10	nd	3 ± 5	3 ± 5	4 ± 7	3 ± 6	7 ± 3	2 ± 3
Chinese star anise from "De Tuinen"	1.7×10 ± 2.7×10	6 ± 9	1.5×10 ± 1.9×10	nd	nd	1.4×10 ± 1.7×10	1.1×10 ± 1.4×10	6 ± 9

nd = not detectable, i.e. signal < 5.

The method was not applicable to star anise material in tea bags as the small particle size (~1 mm) of the 3-6 different plant species precluded the unambiguous selection of star anise pieces. To investigate tea bags, a tea was prepared by boiling a tea bag during 6 min in water. It was argued that if no anisatin was detectable in the aqueous extract ("tea"), the tea should also be safe to drink. The tea was sampled by immersing a glass rod designed for DART-MS ("Dip-it") in the solution and placing it directly via a prefixed holder in the DART sampling area. Approximately 1.5-2 µL of tea adhered to the Dip-it. After evaporation of the water in a couple of seconds, a more or less stable signal of anisatin was obtained for more than 1 min. Like for the dry fruits, no sample preparation was necessary. For comparison also aqueous extracts with the same ratio plant material/water, i.e., 1:10, of Japanese and Chinese star anise fruits were prepared. The results are presented in Table 2. To be able to evaluate the results in Table 2 better, intra-day and inter-day variation was determined with an aqueous extract of Japanese star anise (Table 3).

Teas prepared from eight retail Dutch teas containing Chinese star anise gave on average a signal of 28 (range: 0-73) and 118 (range: 0-400) in negative and positive mode respectively. Compared with "teas" prepared from Japanese star anise fruits at the same concentration, the anisatin signal in the retail teas was 1000 and 200 times lower for negative and positive modes respectively allowing once again a kind of "digital" distinction. We hypothesise that the relatively smaller difference in positive mode is caused by the occurrence of another constituent in one of the many additional herbs in the tea bags giving also an ion at *m/z* 346.150. Extracts made of pure Chinese star anise gave weaker anisatin signals in positive mode than some of the retail teas even though the star anise concentration was higher. Thus for investigating teas, negative mode is favoured over positive mode measurements. Taking into account the relative standard deviations of ~25% for Japanese star anise teas and ~75% for Chinese star anise/retail herbal teas, none of the investigated eight Dutch teas contained Japanese star anise fruits. The outcome of the inter and intra-day analysis (Table 3) showed that the inter-day variation of ~40% is twice as big as the intra-day variation of ~20%. These figures show that the DART-HRMS data should only be used for qualitative analyses.

Table 2. Average anisatin response of different teas plus standard deviations. Each sample was measured in triplicate.

Sample	Average signal/standard dev.		Sample	Average signal/standard dev.	
	Negative mode	Positive mode		Negative mode	Positive mode
Japanese star anise extract RIKILT	$3.2\times10^4 \pm 0.6\times10^4$	$3.6\times10^4 \pm 2.2\times10^4$	Tea "De Tuinen"	9 ± 8	$4.0\times10^2 \pm 2.8\times10^2$
Japanese star anise extract PhytoLab	$1.0\times10^5 \pm 0.1\times10^5$	$2.4\times10^4 \pm 1.9\times10^4$	Tea "C1000"	nd	nd
Chinese star anise extract RIKILT	$3.4\times10^2 \pm 1.6\times10^2$	$1\times10 \pm 0.9\times10$	Tea "Jacob Hooy"	4 ± 3	$1.6\times10^2 \pm 1.1\times10^2$
Chinese star anise extract Sumatra	$3.0\times10^2 \pm 0.5\times10^2$	6 ± 10	Tea "Zonnatura"	$3.6\times10 \pm 0.3\times10$	$8.2\times10 \pm 6.3\times10$
Tea "Taste & Tools" batch 2013	$7.3\times10 \pm 6.3\times10$	$1.0\times10^2 \pm 0.2\times10^2$	Tea "Albert Heijn"	$4.3\times10 \pm 2.0\times10$	$2.1\times10 \pm 1.9\times10$
Tea "Taste & Tools" batch 2014	5 ± 9	$8.2\times10 \pm 4.9\times10$	Tea "Piramide"	$5.2\times10 \pm 2.0\times10$	$1\times10^2 \pm 0.8\times10^2$

Table 3. Inter-day and intra-day variation of anisatin signal in an aqueous Japanese star anise extract. Each day three analyses were performed. Each analysis is the average of 3 replicates.

Sample	Analysis 1		Analysis 2		Analysis 3		Average	
Ionisation mode	Negative mode	Positive mode	Negative mode	Positive mode	Negative mode	Positive mode	Negative mode	Positive mode
Day 1	$2.0\times10^4 \pm 0.2\times10^4$	$1.9\times10^4 \pm 1.0\times10^4$	$0.9\times10^4 \pm 0.4\times10^4$	$1.9\times10^4 \pm 0.5\times10^4$	$1.6\times10^4 \pm 0.2\times10^4$	$1.3\times10^4 \pm 0.3\times10^4$	$1.5\times10^4 \pm 0.6\times10^4$	$1.7\times10^4 \pm 0.4\times10^4$
Day 2	$3.2\times10^4 \pm 0.6\times10^4$	$3.6\times10^4 \pm 2.2\times10^4$	$4.7\times10^4 \pm 1.3\times10^4$	$2.6\times10^4 \pm 1.1\times10^4$	$3.9\times10^4 \pm 1.6\times10^4$	$3.3\times10^4 \pm 1.3\times10^4$	$3.9\times10^4 \pm 0.5\times10^4$	$3.2\times10^4 \pm 0.6\times10^4$
Day 3	$2.6\times10^4 \pm 0.7\times10^4$	$1.8\times10^4 \pm 0.5\times10^4$	$2.0\times10^4 \pm 0.5\times10^4$	$1.6\times10^4 \pm 0.8\times10^4$	$2.3\times10^4 \pm 0.13\times10^4$	$1.6\times10^4 \pm 0.3\times10^4$	$2.6\times10^4 \pm 0.25\times10^4$	$1.7\times10^4 \pm 0.3\times10^4$

Due to the lack of pure anisatin the limit of detection (LOD) of this DART-HRMS assay could not be experimentally determined. However if the anisatin concentration as measured by Lederer et al. is assumed, i.e., 1.2 mg/g Japanese star anise,[24] and an extraction efficiency of 90% after 6 min, the anisatin concentration in the tea is ~100 µg/mL. This concentration gave a signal of ~30,000. The smallest detectable peak had a height of 5 arbitrary units. Assuming a linear response, this leads to a detectable concentration of ~0.2 µg/g, which is in the same range as the anisatin concentration reported by Lederer et al. for Chinese star anise fruits.[24] The 90% efficiency is based on a DART-MS analysis of the extracted carpels. According to both negative and positive mode measurements ~10% of the original signal remained. Most likely however, this 90% is an overestimation as the DART samples only the surface. The extraction efficiency for anisatin deeper inside the

fruits is probably lower. If this is the case, the detection limit would be lower than 0.2 μg/g.

We also explored if veranisatins A ($C_{16}H_{22}O_8$, mass = 342) and B ($C_{16}H_{20}O_9$, mass = 356) could be used as additional markers. These compounds have been reported as trace convulsants (1-2 μg/g) in Chinese star anise.[10] Indeed signals at *m/z* 341.123 ($[M-H]^-$) / 343.139 ($[M+H]^+$), and *m/z* 355.102 ($[M-H]^-$) / 357.118 ($[M+H]^+$) could be observed in Chinese star anise at low intensities (~300). However the identification remained tentative and additionally these same ions were also present in Japanese star anise (intensity ~100). Thus they cannot be used to distinguish Chinese from Japanese star anise.

3.3. Semi-quantitative assay for the detection of anisatin

Most of the variation in signal intensity appeared to be due to the DART ionisation process itself as the response of many different ions fluctuated over time in the same manner as that of anisatin. It was therefore investigated if more quantitative measurements were possible by normalising on a component that was already present in star anise. In a standard addition experiment, Japanese star anise tea was added to a herbal tea containing Chinese star anise at different ratios. In negative mode normalisation took place against the ion at *m/z* 242.052 which was only present in the herbal tea. In positive mode, the ion at *m/z* 216.120 was used for normalisation. Also the latter ion occurred only in the herbal tea. After correction for the volume ratios, straight 5-point calibration curves were obtained in both negative and positive modes (Fig. 5). They show there is a linear relation between response and concentration of anisatin and additionally the linearity indicates that ion suppression is not a problem with DART ionisation. Fig. 6a and b show the corresponding negative mode mass spectra of the herbal tea and the same herbal tea spiked with 1% (v/v) of an equally concentrated aqueous extract of Japanese star anise. An almost 5-fold increase in anisatin signal intensity was observed, showing that 1% adulteration of a tea with Japanese star anise is clearly detectable. If anisatin would become commercially available, the DART-HRMS method would allow the determination of its true concentration in a tea. In that case an added internal standard would be preferable. Bilobalide, a commercially available sesquiterpene trilactone might be a suitable candidate. It gives strong signals with DART-MS with ions at *m/z* 325.092 ($[M-H]^-$, $C_{15}H_{17}O_8$) and 344.134 ($[M+NH_4]^+$, $C_{15}H_{22}NO_8$).

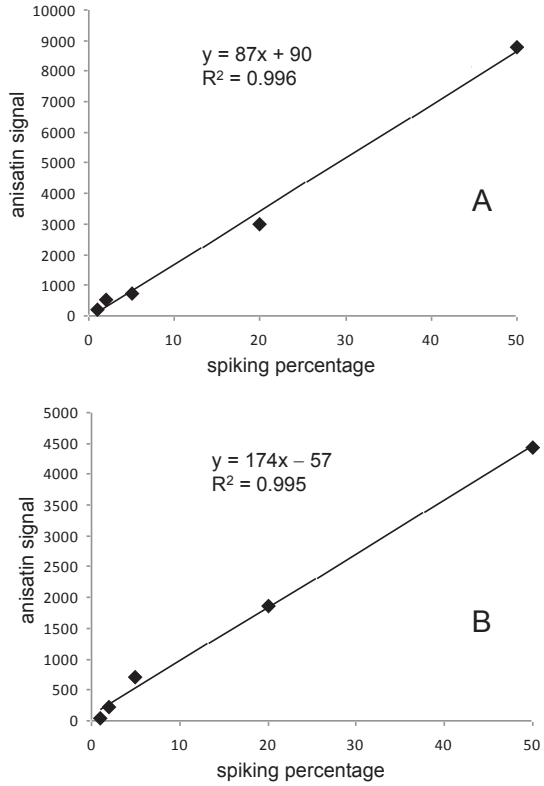

Fig. 5. Calibration curves of a herbal tea containing Chinese star anise spiked with an aqueous extract of Japanese star anise (standard addition). Plotted is the normalised height of the anisatin signal against the % of the spike. A: negative mode, normalised against *m/z* 242.052; B: positive mode, normalised against *m/z* 216.120.

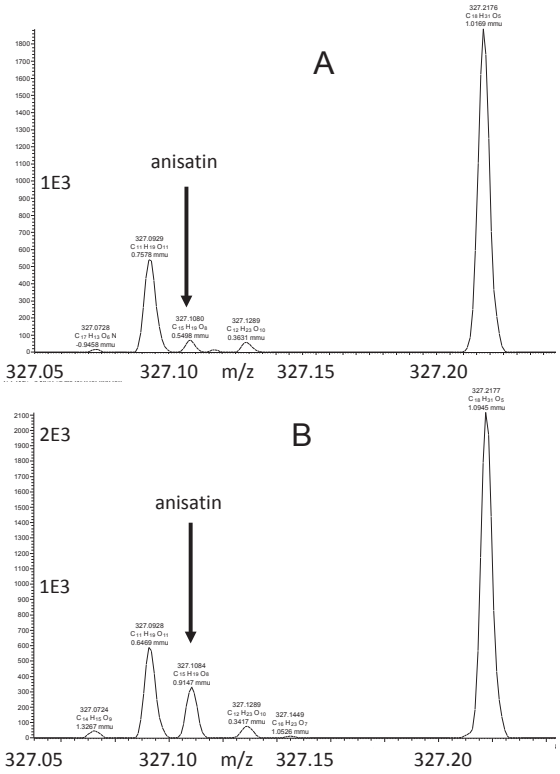

Fig. 6. Negative mode DART-orbitrap mass spectra from *m/z* 327.05-327.25 of a herbal tea containing Chinese star anise fruits (A) and the same tea spiked with 1% of an aqueous extract of Japanese star anise fruits at the same concentration (B). Peak at *m/z* 327.108 corresponds with anisatin $[M-H]^-$ ($C_{15}H_{19}O_8$) and increases significantly in height (A: 70; B: 330) upon spiking relative to the other peaks.

4. Conclusion

DART-orbitrap MS technology allows for an unambiguous distinction between toxic Japanese star anise and non-toxic Chinese star anise fruits within seconds without any sample pretreatment. This is possible in both positive and negative mode although the latter mode is preferred because of higher sensitivity and cleaner spectra. As star anise is chemically highly complex, a mass resolution of ≥ 27,000 is needed, which can only be provided by FT, orbitrap or high-end TOF instruments. Samples like herbal teas that no longer contain recognisable plant parts, can be analysed after making a tea. Probing the aqueous solution with a glass rod and introducing it into the DART provides a clear signal of anisatin if Japanese star anise is present. If the anisatin signal is normalised on

another peak present ("standard addition"), semi-quantitative measurements (R^2 0.995) are possible in spite of the relatively poor repeatability of DART-HRMS. If anisatin becomes available, the method could be used to determine the actual concentration in teas. The proposed method is much faster than any existing methods for detection of adulteration of Chinese star anise, less labour-intensive, measures the actual biotoxin and the required equipment is of a comparable price level as LC-triple quad MS or thermal desorption GC-MS. In Table 4 we compared the DART-HRMS method with the best LC-MS/MS method for anisatin.[24] DART-HRMS scores especially good in terms of required time, robustness and simplicity. Thus it may be beneficial for improved food safety control.

Table 4. Comparison of HPLC-MS/MS [24] and DART-HRMS method (this paper) for anisatin analysis.

Parameter	HPLC-MS/MS	DART-HRMS
Equipment cost	high	high
Sample pretreatment	12 steps	0 or 2 steps
Cost for one analysis (manpower)	high	low
Cost for one analysis (chemicals)	high	none
Technical training level required	high	moderate
Sensitivity (LOD)	1.2 µg/kg	< 200 µg/kg
Speed of analysis	several hours (quantitative)	seconds (qualitative) < 1 hour (quantitative)
Selectivity	very high	moderate - high
Linearity calibration curve (R^2)	0.999	≥ 0.995 (with internal standard)
Reproducibility (intra-day/ inter-day precision)	3.0 / 6.6%	20% / 40% (without internal standard)
Robustness	fair	good
Final outcome	accurate anisatin concentration in fruit	approximate anisatin concentration in tea

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Chapter 5

Rapid and Simple Neurotoxin-based Distinction of Chinese and Japanese Star Anise by Direct Plant Spray Mass Spectrometry

Abstract

After adding ~200 µL of methanol to one star anise carpel placed at 7-10 mm from the inlet of a mass spectrometer and applying a potential of ~5 kV to the carpel, an immediate, robust and stable electrospray is created. The presence or absence of anisatin could be monitored by orbitrap high resolution mass spectrometry (HRMS) in negative mode by observing the $[M-H]^-$ ion at *m/z* 327.1074 ($C_{15}H_{19}O_8$) or in positive mode the $[M+K]^+$ ion at *m/z* 367.079 ($C_{15}H_{20}KO_8$). Several parameters like extraction solvent, voltage, distance and set-up were optimised. The anisatin signal was ~250 times higher in Japanese than in Chinese star anise. An existing Direct Analysis in Real Time (DART) HRMS for anisatin was used for benchmarking. Direct plant spray and DART ionisation are both robust and provided the same yes/no answer in seconds without any prior sample preparation. Compared with the DART-HRMS procedure, the direct plant spray method is simpler in terms of equipment, yields a more stable signal, does not require heating of the sample but is slightly less selective and requires working with high voltages.

Chapter based on: M. Schrage*, Y. Shen*, H. Zuilhof, M. Nielen, T.A. van Beek and B. Chen, Rapid and simple neurotoxin-based distinction of Chinese and Japanese star anise by direct plant spray mass spectrometry, *Journal of Chromatography A*, **2013**, 1317, 246-253.

* These two authors contributed equally to this paper.

1. Introduction

Botanical species identification of ingredients is an essential part of the quality control of plant-derived medicines as well as certain foods like herbal teas. Normally, identification proceeds according to methods published in pharmacopoeias or other protocols using e.g. morphological analysis, i.e., macroscopic and microscopic identification, simple chemical tests, TLC analysis and olfactory analyses. However, the specificity of such methods is not in all cases adequate.

Recently a new and simple technique to sample fresh and dry plants called "leaf spray mass spectrometry" was published.[1] The plant part with a natural or manmade spike is held in front of the MS inlet and a high voltage is applied. Fresh plants or dry plants after wetting with a suitable solvent, generate a Taylor cone with ionised spray carrying ionised plant metabolites. The principle is derived from paper spray.[2] The method proved very simple and robust giving in real-time under mild conditions information about a wide variety of genuine phytochemicals. Since then, four papers applying "leaf spray MS" have appeared focussing on urushiol allergens in poison ivy,[3] steviol glycosides from Stevia,[4] steroidal acids in tulsi[5] and extraneous pesticides on fruits,[6] respectively. As the method is simple and robust and does not require any add-on, it appears to be a promising new tool for simple, rapid and highly specific identification of botanical species.

Chinese star anise (*Illicium verum*) is used as a spice in Asian cuisine while in America and Europe it is used in teas. In addition it is a substitute for anise and together with Japanese star anise (*Illicium anisatum*) it is used for decoration purposes. Finally both species are a source of shikimic acid, which can be synthetically converted to Tamiflu.[7-8] Only Japanese star anise fruits contain the neurotoxic anisatin (Fig. 1), which is a strong non-competitive antagonist of the $GABA_A$-receptor.[9] Due to the presence of this rare sesquiterpene, *I. anisatum* causes severe toxic effects when ingested, and several reports of poisoning of babies[10] but also adults[11] have been reported. More references on star anise uses and toxicity are available in references.[7,12]

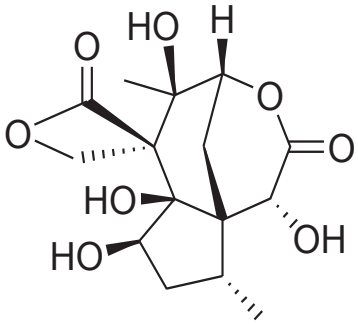

Fig. 1. Chemical structure of anisatin ($C_{15}H_{20}O_8$, exact mass = 328.1153 Da)

Fig. 2. Photographs of Japanese (left, Phytolab) and Chinese star anise (right, YadanO).

Thus unambiguous methods for distinguishing phytomedicinal Chinese star anise and neurotoxic Japanese star anise (Fig. 2) are of importance for food safety. Morphological distinction requires expertise in botanical microscopy and even then differentiation is challenging due to the widely variable anatomy of star anise.[10,13] Chemical assays based on other constituents than anisatin are less reliable than chemical analysis based on the neurotoxin content.[7] The best direct HPLC assay quantitates anisatin by LC-MS/MS after a 12-step extraction and clean-up procedure.[14] More recently a qualitative method was published using "Direct Analysis in Real Time" (DART) coupled to high-resolution (orbitrap) mass spectrometry (HRMS), which is capable of unambiguously distinguishing between Japanese and Chinese star anise without any extraction or sample preparation and, in mere seconds.[7] Typically the anisatin signal was ~1000 times larger in Japanese versus Chinese star anise fruits allowing for a "digital" differentiation. The only disadvantages were strong variations in signal strength as a function of the exact location of the carpel in the DART sampling zone and the necessity to buy a DART add-on.

In view of the above, we investigated if direct plant spray MS could function as a tool for identification at the species level. Below we report on the development of a method for analysing anisatin in star anise fruits to enable a rapid and unambiguous distinction of Japanese and Chinese star anise fruits.

2. Experimental

2.1. Materials and reagents

Anisatin (HPLC purity: 98%; absolute purity: 73%) was purchased from PhytoLab GmbH, Vestenbergsgreuth, Germany. Chinese star anise (*Illicium verum* Hook. f.) samples were obtained from Dutch retail teas (Taste and Tools sterrenmix tea, batches 2013 & 2014), from a local market in Sumatra, Indonesia and from a Belgian supermarket (YadanO steranijs). Japanese star anise (*Illicium anisatum* L.) samples were obtained from RIKILT (Wageningen, Netherlands) and from PhytoLab (Vestenbergsgreuth, Germany). High-purity helium gas (grade 5.0 for DART and 6.0 for LXQ) was provided by Linde Gas Benelux B.V. Methanol was from J.T. Baker (Deventer, Netherlands), acetone from Sigma-Aldrich (Germany), ethyl acetate from Biosolve (Valkenswaard, Netherlands) and methyl *tert*-butyl ether from Acros Organics (Belgium).

2.2. Analysis conditions

2.2.1. Direct plant spray measurements

One carpel of a dry star anise fruit was placed in a steel alligator clip. This clamp was placed at an angle of 90° in a second fixed alligator clip, which was connected to the high voltage supply of the MS. Multiple clamps of the former type prepared in advance with samples allowed a sample change within 10 s. The latter clamp was in turn placed via a non-conductive plastic arm to a laboratory clamp on a ring stand. This allowed an easy and safe positioning of each star anise sample relative to the MS inlet even with the high voltage switched on. Ideally the natural spike of a star anise carpel was positioned both horizontally and vertically directly in front of the MS inlet at a distance of 7-10 mm. This distance is quite relevant, as distances < 4 mm caused electrical discharges, while at distances > 12 mm usually no electrospray was observed. Depending on the size and shape of the star anise sample, 100-300 µL of methanol was added by means of a 500 µL HPLC syringe taking care that no droplets fell off the sample. This quantity was sufficient

for at least 1 min of stable signal. If needed, more measurements could be run on the same carpel by adding 50-150 µL of fresh methanol after each measurement. Measurements were started by activating the mass spectrometer and next applying 5 kV. This caused the immediate formation of a stable electrospray. The average signal height over a stable portion of one measurement was taken as a measure of the amount of anisatin. For each carpel at least 3 measurements were taken and averaged. The set-up was used in combination with either a HRMS (see 2.3.1) or a linear ion trap MS in SRM mode at nominal mass resolution (see 2.3.2). Caution: high voltages are involved! The sample and the two alligator clips should never be touched during a measurement. Prior to adding more methanol or removing the clamp with the sample, the voltage should be set to 0 V, followed by a waiting time of 5 sec and observing the actual voltage to be indeed zero.

2.2.2. DART measurements

A DART ion source (model DART-SVP, IonSense, Saugus, USA) was used in positive or negative mode and was connected to either a high-resolution MS (see 2.3.1) or to a linear ion trap MS (see 2.3.2). Settings positive mode: He as ionising gas, fixed flow of ~3.5 L/min; gas beam temperature 350 °C; grid electrode voltage +350 V. Settings negative mode: He as ionising gas, fixed flow of ~3.5 L/min; gas beam temperature 400 °C; grid electrode voltage −350 V.

Measurement: one carpel (typically $1/8^{th}$ of an intact fruit) was taken by means of tweezers. Then the DART-MS measurement was started by recording background. Next the star anise was held in the DART sampling area and slightly moved until a clear signal related to star anise was observed. During 15-25 sec the star anise piece was held in this position. This was repeated twice more, i.e., an intermittent measurement of background and star anise. In HRMS mode the ion at *m/z* 327.108 (negative mode) or *m/z* 346.150 ($[M+NH_4]^+$, positive mode) was observed. For each of the 3 measurements, the average signal height over a stable portion of one star anise measurement was taken as a measure of the amount of anisatin. The average value of the 3 measurements was considered representative for the anisatin content of this particular star anise carpel.

2.3. Mass spectrometers

2.3.1. Orbitrap High Resolution Mass Spectrometer

An Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used. Settings (+)-mode: capillary voltage +60 V; tube lens

voltage +100 V; skimmer voltage +20 V; capillary temperature: 250 °C. Settings (−)-mode: capillary voltage −60 V; tube lens voltage −125 V; skimmer voltage −28 V; capillary temperature: 250 °C. The resolution was set at "ultra high" and a scan rate of 1 Hz was used. The mass range was either from *m/z* 150-750 or from *m/z* 300-400. The mass spectrometer was calibrated at the beginning of each day and was so stable that no internal lock mass was needed. XCALIBUR software (v. 2.1) was used for instrument control, data acquisition and data processing.

2.3.2. Linear Ion Trap mass spectrometer

A Thermo LXQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used in negative selective reaction monitoring (SRM) mode. The transition from *m/z* 327 (selection width 326.1-328.1 Da) to *m/z* 265 (selection width 264.6-265.6 Da) was monitored. Settings: capillary temperature 200 °C, capillary voltage −25 V, tube lens voltage −100 V and CID (Collision induced dissociation) 25%.

3. Results and discussion

3.1. Direct plant spray conditions

In a first experiment to test the applicability of direct plant spray HRMS, the conditions as described in the original leaf spray MS paper were reproduced.[1] One carpel of Japanese star anise was wetted with methanol, −5 kV was applied and in negative mode the ion corresponding to anisatin ($[M-H]^-$ at *m/z* 327.108, $C_{15}H_{19}O_8$) was observed. As discussed in our earlier paper on star anise, this is a very rare elemental composition and characteristic of the neurotoxic sesquiterpene anisatin.[7] Immediately a strong and stable electrospray was observed for several minutes. The spray stopped by itself when most of the methanol had evaporated but after the addition of fresh methanol spraying continued as before. Usually we terminated the spray after one minute by switching off the voltage (Fig. 3A) but if there was sufficient methanol left, the spray would reform after switching on the voltage (Fig. 3B). Changing of samples was easy and fast by using two alligator clips. One was fixed and connected to the high voltage supply and held the second clamp with the sample (Fig. 4). Between *m/z* 327.00 - 327.35 the spectrum looked very clean (Fig. 5A) and the signal strength was high (~1E6). Applying the same conditions to a Chinese star anise carpel gave a much weaker

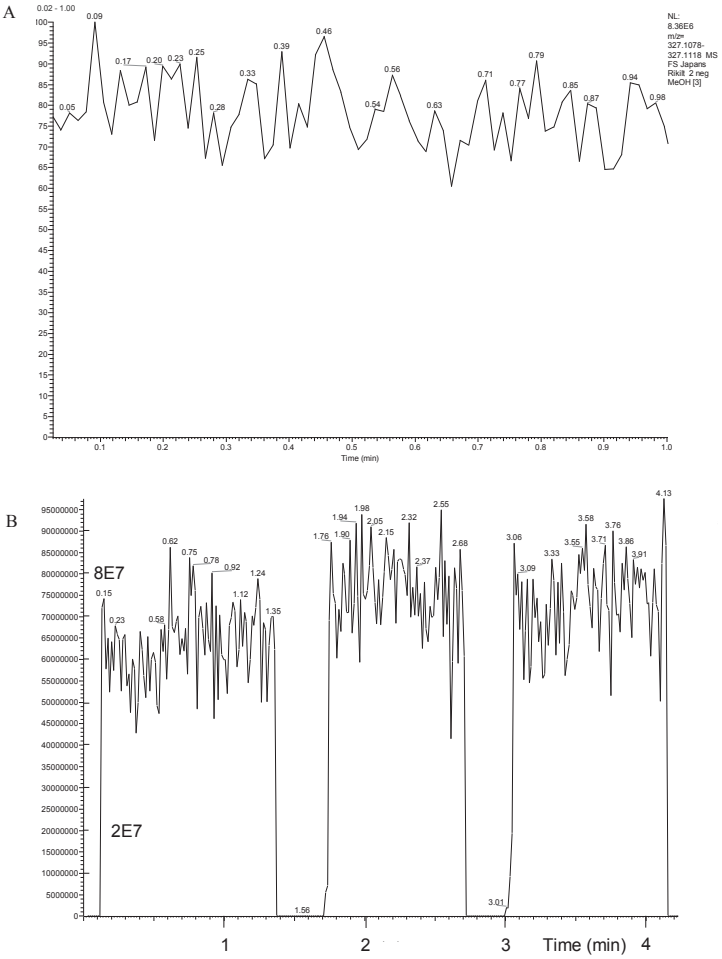

Fig. 3. (A) Selected ion trace (*m/z* 327.110) during 1 min of one star anise carpel in negative direct spray mode on an orbitrap high resolution MS. Voltage (−5 kV) was applied continuously. (B) Total ion current (TIC) signal of one star anise carpel in positive direct spray mode on an orbitrap high resolution MS. Voltage (+5 kV) was applied at ~0.12, 1.70, and 3.00 min and turned off at ~1.37, 2.72 and 4.16 min respectively.

signal for anisatin (~1E4) and several other masses were now prominent (Fig. 5B). Next the experiment was repeated in (+)-mode. In this case neither an $[M+H]^+$ at *m/z* 329.123 ($C_{15}H_{21}O_8$) as in LC-ESI-MS nor an $[M+NH_4]^+$ at *m/z* 346.150 ($C_{15}H_{24}NO_8$) as in DART-MS could be observed but instead a strong signal at *m/z* 367.079 ($C_{15}H_{20}KO_8$) corresponding with $[M+K]^+$ was present (Fig. 6A). In the first communication on leafspray mass spectrometry by Liu et al. also several potassium adducts of plant metabolites were described,[1] as well as in the paper on stevioside[4] and thus this seems rather typical of direct plant spray MS. In general

potassium levels in plants are 25 times higher than sodium levels.[15-16] Subsequent analysis of a carpel of Chinese star anise showed the anisatin content to be much lower similar to the measurements in (–)-mode (Fig. 6B). Thus a clear proof of principle for distinguishing Japanese and Chinese star anise was obtained.

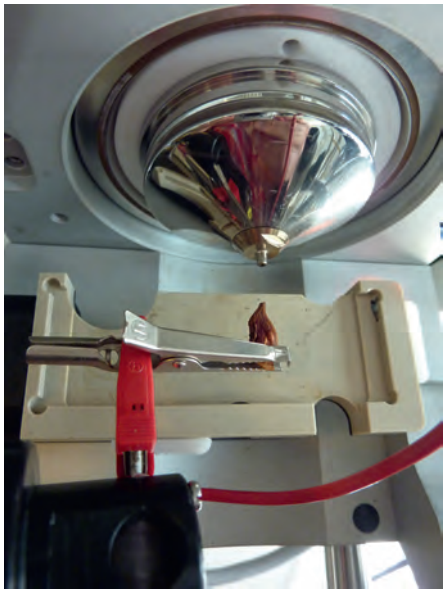

Fig. 4. Direct plant spray set-up with 2 alligator clips one of which is easily replaceable and holds the star anise carpel. The other (red) one is fixed and connected to the high voltage supply via the red cable. The natural star anise spike from which the electrospray forms is pointing towards the inlet of the mass spectrometer.

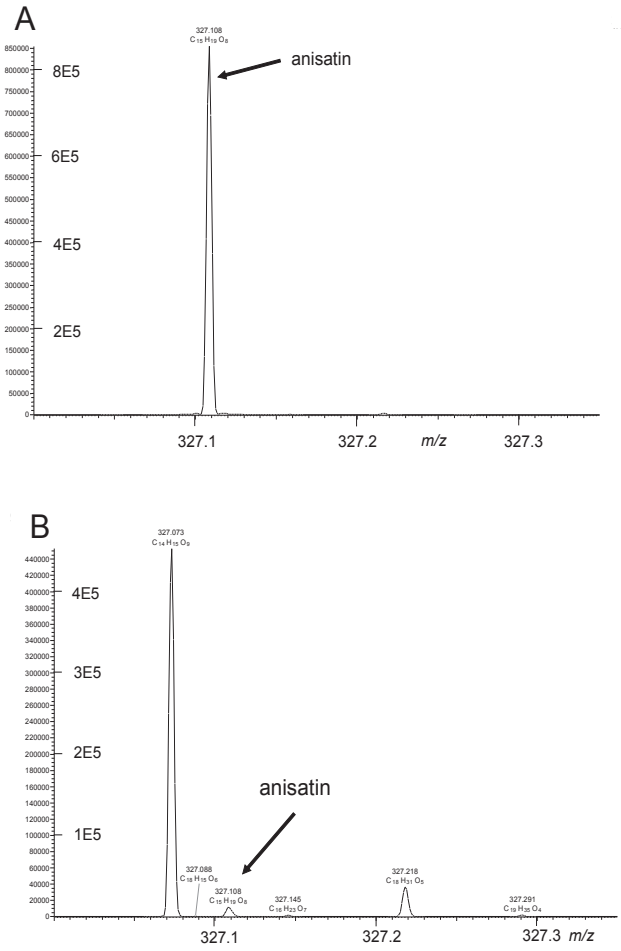

Fig. 5. Negative mode direct plant spray - orbitrap mass spectra from *m/z* 327.00-327.35 of one carpel of Japanese star anise (A) and Chinese star anise (B). Peak at *m/z* 327.108 is of anisatin, $[M-H]^-$ ($C_{15}H_{19}O_8$).

Next some optimisation was carried out. Apart from methanol, also acetone, ethyl acetate and methyl *tert*-butyl ether (MTBE) were tested as wetting solvents. Anisatin is well soluble in all four solvents. With acetone and ethyl acetate no electrospray could be formed, while with MTBE the electrospray was not as stable and long-lasting as with methanol. Therefore methanol was used in all further experiments. Also distances were varied both at constant voltages and with a fixed voltage/cm. However in this case no real optimum was observed, rather there either

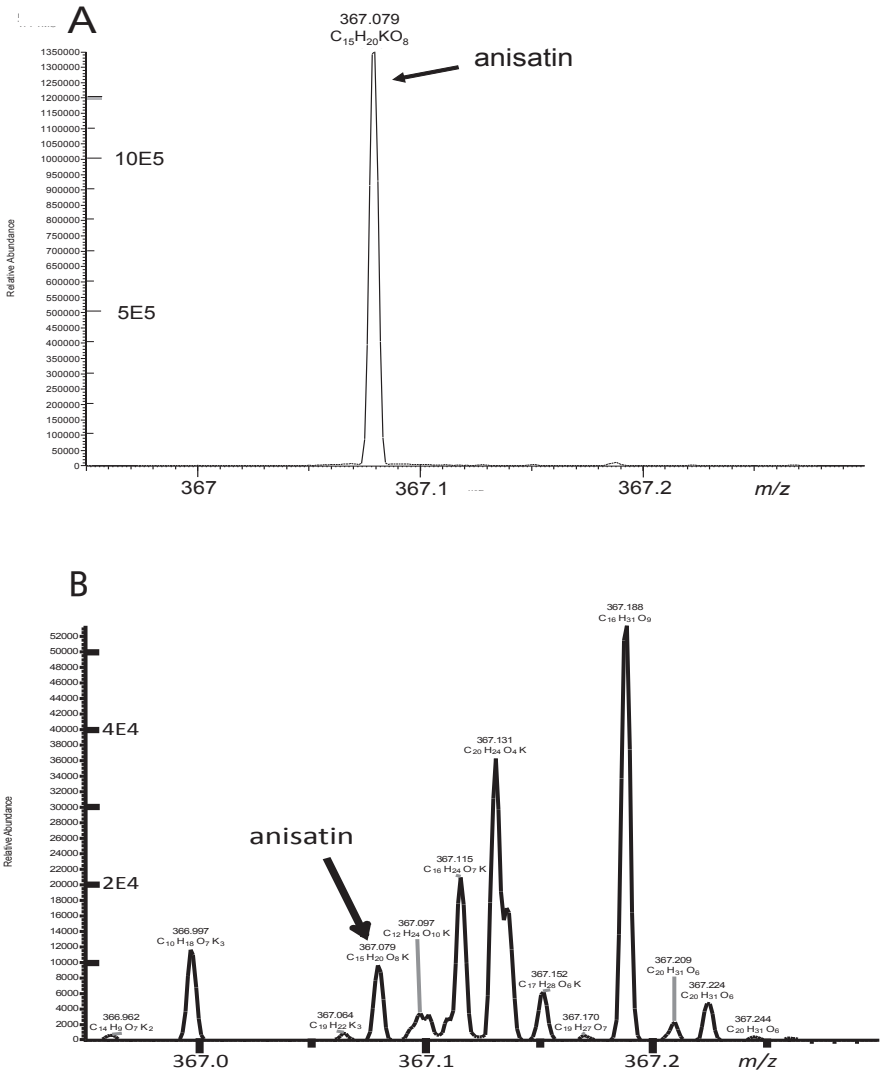

Fig. 6. Positive mode direct plant spray - orbitrap mass spectra from *m/z* 366.95-367.30 of one carpel of Japanese star anise (A) and Chinese star anise (B). Peak at *m/z* 367.079 is anisatin, $[M+K]^+$ ($C_{15}H_{20}KO_8$). Signal is averaged over 2.13 min (A) and 1.07 min (B).

was an electrospray or none at all. The most robust conditions were a combination of 7-10 mm and 5 kV. Small distances, i.e., < 4 mm led to electrical discharges and should be avoided. One additional observation was that the sharpness of the spike on the star anise carpel significantly influenced the formation and strength of the electrospray. This coincides with the observation by Liu et al. [1]

3.2. Analysis of star anise fruits by direct plant spray and DART in combination with HRMS

To evaluate whether direct plant spray HRMS is really conclusive for distinguishing Japanese and Chinese star anise, Japanese star anise fruits originating from two sources and Chinese star anise fruits from three different sources were investigated. Each sample was analysed at least in triplicate and results are presented in Fig. 7. Direct plant spray analysis of a carpel always took place prior to the DART analysis of the same carpel to avoid any influence of the charring, which results from DART analyses. The signal obtained with direct plant spray in both (+) and (−)-mode was considerably stronger than the signals obtained with DART. Thus absolute signals cannot be compared between the two approaches. On average Japanese star anise fruits gave a 285 and 215 times stronger signal for anisatin in (−) and (+)-mode respectively than Chinese star anise fruits. Although this difference is smaller than that obtained with DART (~1000), it nonetheless allows a clear distinction between the two types of star anise. Probably the higher selectivity of DART is due the higher selectivity of APCI relative to ESI ionisation. Relative standard deviations (RSDs) for direct spray are smaller than those of DART due to the large influence of the exact position of the manually held fruit in the DART sampling area and the DART ionisation process itself. RSDs for Japanese star anise analyses by direct plant spray were 28% and 26% in (−) and (+)-mode respectively while these same values were 55% and 86% for DART. The signal during direct plant spray analyses is more stable (Fig. 3A). Even after adding fresh methanol and resuming the electrospray process the signal has approximately the same strength (Fig. 3B). The DART-HRMS results (Fig. 7) are very similar to those reported earlier by us with the exception of the RIKILT 2 carpel in (+)-mode whose value now appears lower.[7]

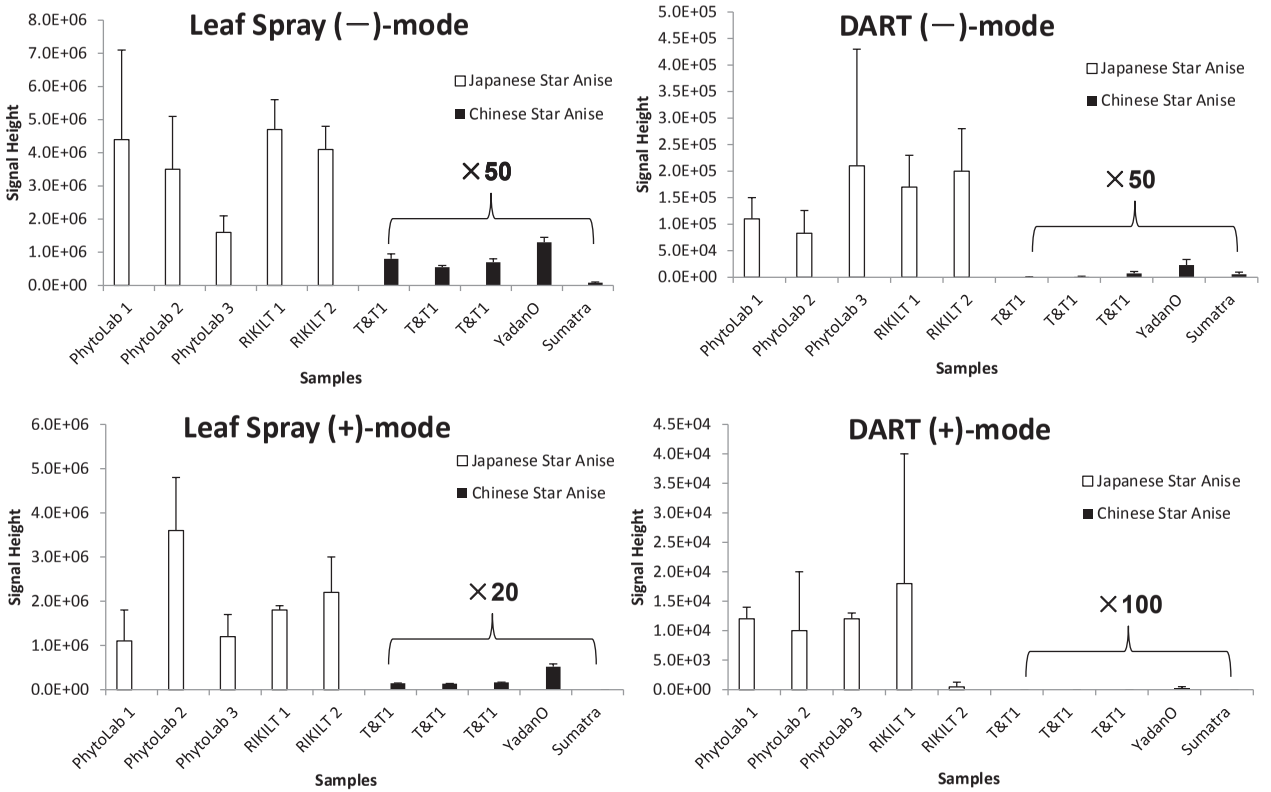

Fig. 7. Average signal height and standard deviations of anisatin in different dry star anise fruits as measured by direct plant spray (left) and DART ionisation (right) in combination with high resolution orbitrap MS. Each sample was measured at least in triplicate in negative mode (top) and positive mode (bottom).

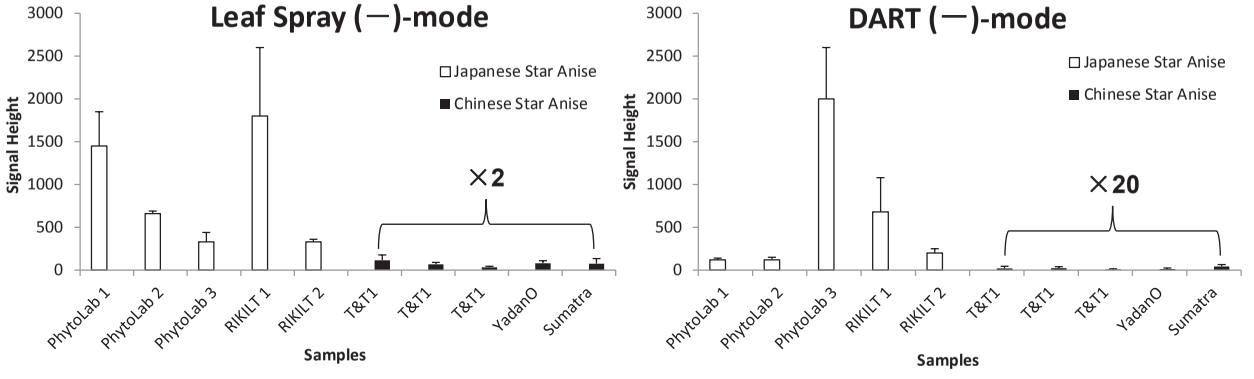

Fig. 8. Average signal height and standard deviations of anisatin in different dry star anise fruits as measured by direct plant spray (left) and DART negative ionisation (right) in combination with a linear ion trap MS in negative SRM mode (transition 327 → 265). Each sample was measured at least in triplicate.

As high-resolution mass spectrometers are less common than ion trap mass spectrometers, next we investigated if an ion trap mass spectrometer in selective reaction monitoring (SRM) mode could also distinguish between Japanese and Chinese star anise, both in combination with direct plant spray and DART ionisation. First, we infused pure anisatin to a linear ion trap MS and carried out collision induced dissociation (CID) experiments. In (−)-mode, CID of the $[M-H]^-$ peak at *m/z* 327 ion yielded fragments at *m/z* 309, 297, 283, 265, 247 and 127. We chose to monitor the relatively strong fragment at *m/z* 265 $[M-H-H_2O-CO_2]^-$ in subsequent SRM experiments. As expected, CID of the $[M+K]^+$ peak at *m/z* 367 did not yield any usable fragments thus making SRM experiment in (+)-mode impossible.

The SRM results are depicted in Fig. 8. With direct plant spray ionisation the ratio of the anisatin content in Japanese and Chinese star anise is reduced to a factor of 30, i.e., considerably worse than the ratio obtained with HRMS. This lower selectivity must be caused by the fact that other isobaric ions from Chinese star anise (see Fig. 5B) are also selected in the low-resolution ion trap and exhibit the same neutral losses as anisatin. This was substantiated by comparing the MS/MS spectra of pure anisatin, Japanese star anise and Chinese star anise. In all cases the fragments mentioned above were present but their ratios differed significantly. As expected, the spectra obtained with DART and leafspray for the same sample were identical, showing that this difference is purely sample related and not due to the ionisation method. The too high value for anisatin in Chinese star anise in turn gives rise to a lower ratio. Similar to HRMS, the APCI-type of ionisation in DART appears more selective and the ratio Japanese/Chinese obtained with SRM varies for DART between 100 to more than 1000.

4. Conclusion

Direct plant spray ionisation in combination with orbitrap HRMS, like DART-HRMS, allows for an unambiguous distinction between toxic Japanese star anise and non-toxic Chinese star anise fruits within seconds without any sample pretreatment. Positive and negative mode gave similar results. When direct plant spray is compared with DART ionisation, the latter has the advantage of a slightly higher selectivity, the absence of high voltages, no need of adding solvents and no requirements regarding shape of the sample. Direct plant spray ionisation

has the advantage of low cost, simplicity, room temperature and lower standard deviations. Neither method is very suitable for quantitative measurements of solid samples like star anise fruits. A mass spectrometer with nominal mass resolution in SRM mode showed lower selectivity in combination with direct plant spray MS relative to an orbitrap MS but could be used in combination with DART ionisation. All in all direct plant spray ionisation appears to be powerful yet simple tool for characterising plants at the species level, i.e., for rapid analyses of the "present/absent" type.

Acknowledgement

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Chapter 6

Efficient Purification of Ginkgolic Acids from *Ginkgo biloba* Leaves by Selective Adsorption on Fe_3O_4 Magnetic Nanoparticles

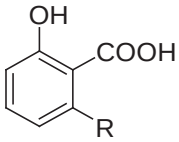
Abstract

Ginkgolic acids (GAs; anacardic acids; 6-alkylsalicylic acids) are both unwanted constituents in standardized *Ginkgo biloba* (Ginkgo) extracts and desirable constituents for pharmacological assays. Thus, for the quality control of Ginkgo extracts, the availability of pure GAs is important. In this investigation, inexpensive and easily prepared Fe_3O_4 magnetic nanoparticles (MNPs) in methanol were used to selectively adsorb GAs from crude petroleum ether extracts of Ginkgo leaves in the presence of various lipids including other alkylphenols (cardanols and cardols). The adsorption capacity of the MNPs is with 4-5% (w/w) high. The moiety responsible for the adsorption is the salicylic acid group, which binds strongly to Fe(III). Desorption with acidified methanol gave an extract with a GA content of 73%. This could be further separated by preparative HPLC on a C_8 column. In total, eight different GAs were captured by MNPs. The MNP adsorption step can replace more traditional column chromatography and liquid-liquid extraction steps and is superior in terms of solvent consumption, selectivity, labor and energy consumption. MNPs might become an efficient separation technique for selected high-value phytochemicals that contain a salicylic acid moiety.

Chapter based on: R. Li*, Y. Shen*, X. Zhang, M. Ma, B. Chen and T.A. van Beek, Efficient purification of ginkgolic acids from *Ginkgo biloba* leaves by selective adsorption on Fe_3O_4 nanoparticles, *Journal of Natural Products*, **2014**, 77, 571-575.
* These two authors contributed equally to this paper.

1. Introduction

Ginkgolic acids (GAs), which are also known as anacardic acids, are natural 6-alkylsalicylic acids. The alkyl group usually has 13, 15 or 17 carbons and 0-2 double bonds. They occur in various plant materials, including cashew and pistachio nuts, nutmeg, liverworts, brown algae and, the leaves and sarcotestas of *Ginkgo biloba* L. (Ginkgoaceae).[1] Both negative and positive properties have been related to the GAs. Positive attributes include potential antitumor, antidepressant, anti-HIV, antistress, possible antiamnestic, antifungal, antifeedant, acaricidal, molluscicidal, insecticidal and larvicidal activities.[1-3] Recently, the putative anticancer activity of GAs is of renewed interest.[4-6] Negative properties include contact allergenic, cytotoxic, embryotoxic, immunotoxic, mutagenic and slight neurotoxic properties.[1, 7, 8] Due to their supposed allergenic properties, a limiting concentration of 5 μg/g for all ginkgolic acids combined has been specified in the monographs for standardized Ginkgo extracts in the European and U.S. pharmacopoeias, and a limit of 10 μg/g in the Chinese Pharmacopoeia.[9] Thus, for the purpose of both quality control of standardized Ginkgo extracts[10] and pharmacological assays, an efficient production and purification of GAs is of considerable economic importance. For example, the two most abundant GAs (C15:1 and C17:1) are currently sold commercially at over $ 400 for 10 mg, which makes them belong to the more precious phytochemicals.



ginkgolic acids

R = $C_{13}H_{27}$ (C13:0) R = $C_{15}H_{31}$ (C15:0) R = $C_{15}H_{29}$ (C15:1)

R = $C_{17}H_{33}$ (C17:1) R = $C_{17}H_{31}$ (C17:2) R = $C_{17}H_{29}$ (C17:3)

Due to the high price of purified GAs, many synthetic studies have been carried out to develop efficient routes to obtain GAs for biological investigations. Tyman et al. applied a sequential alkylation and carboxylation of 2-fluoroanisole with alkyllithium and CO_2, followed by demethylation with BCl_3, which afforded GA (15:0) in low yield.[11,12] Fürstner et al. prepared GA (15:0) using the Suzuki cross-coupling reaction and directly constructed the side chain.[13] In turns, Satoh et al. successfully synthesized

GA (15:0)[14,15] and GA (15:2)[16] using an annelation reaction of isoxazoles with ethyl acetoacetate and a Wittig reaction. Very recently, Fu et al. successfully developed a novel synthesis of GA (13:0) from 2,6-dihydroxybenzoic acid through a state-of-the-art palladium-catalyzed cross-coupling reaction and catalytic hydrogenation with an overall yield of 34% in five steps.[17] However, so far no simple generic way to prepare all major GAs (C13:0, C15:0, C15:1 and C17:1) is available.

Thus, another strategy for the production of pure GAs is separation and purification from plant sources, like Ginkgo or Anacardium extracts. Gellermann and Schlenk saponified their non-polar extract and separated the resulting phenolic acid and fatty acid methyl ester fraction by column chromatography (CC) on silica.[18] Itokawa et al. separated an intermediate chloroform extract by silica CC in a GA fraction and a cardol-cardanol fraction.[19] Nagabhushana and Ravindranath achieved a similar class separation by using silica CC in combination with an amine-containing eluent.[20] Irie et al. and Lepoittevin et al. both processed non-polar Ginkgo extracts by means of CC on silica and Sephadex LH-20 using chloroform-containing solvents.[8,21] Similarly Koch et al. purified a heptane extract by a series of CC procedures (twice with Sephadex LH-20, Lichroprep RP-18, and silica using chloroform) giving a semi-pure mixture of GAs, which still contained some cardanols.[22] Van Beek and Wintermans isolated, through a four-step preparative chromatographic procedure GAs C13:0, C15:0, C15:1, C17:1, C17:2 and tentatively C17:3 from *G. biloba* leaves.[23] Fuzzati et al. developed a combined liquid-liquid extraction (LLE)-silica CC procedure to prepare a mixture of GAs to be used as reference standard and C17:1 GA from Ginkgo leaves.[24] Gandhi et al. avoided CC by selectively precipitating GAs with calcium hydroxide, followed by treatment with HCl and LLE.[25] The final separation of the GA fraction into individual GAs is best carried out via preparative RP-HPLC on a C_8 phase.[24] The conclusion is that the bottleneck in the production of pure GAs resides in the pre-purification steps. This is because LLE, and CC separation on silica are tedious, always consume large amounts of (halogenated) solvents and require long separation times. Therefore, a simple pre-separation method for GAs requiring neither LLE nor CC is of significant economic significance.

In this paper, we report on a simple and selective preparative purification procedure for GAs involving adsorption on magnetic nanoparticles (MNPs). The MNPs adsorption step exhibits high structural selectivity. The whole purification process using MNPs is solvent-efficient and also energy-efficient when using a permanent magnet.

2. Experimental

2.1. General Experimental Procedures

Dry Ginkgo (*G. biloba*) leaves (collected in June - August 2013 in a Changsha suburb, People`s Republic of China; dried in sunlight) were purchased from Hunan Province Medicine Company (Changsha, People`s Republic of China). A voucher specimen (No. 2013-8-23-Chen) is deposited at the Key Laboratory of Chemical Biology & Traditional Chinese Medicine Research, Ministry of Education, Hunan Normal University. Standard of GA (13:0) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, People`s Republic of China). Analytical grade 1,4-dimethoxybenzene (99%) as an internal standard for quantitative NMR[35] was purchased from Sigma (Sigma Chemical Co., St. Louis, Mo, USA). Other chemicals and reagents (Analytical grade) were purchased from Hunan Chemical Reagent Company (Changsha, People`s Republic of China). IR spectra were recorded on a Bruker Vector 22 IR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ on a DRX-500 Bruker spectrometer. ESIMS/MS data were obtained on a Finnigan LCQ Advantage Max mass spectrometer with an ESI probe (Thermo-Finnigan, USA).

2.2. HPLC Analysis

HPLC analysis was carried out on a Shimadzu LC-20A HPLC system (Shimadzu, Japan) consisting of two LC-20AT HPLC pumps, an SPD-M20A detector (190-800 nm), and a 20AC column temperature controller. Data processing was performed on an LC-solution chromatographic workstation (Shimadzu, Japan). LC separations were accomplished on an Ultimate XB-C_8 column (5 μm, 250 mm × 4.6 mm; Welch Materials, Inc., Ellicott City, MD, USA). The mobile phase for isocratic elution was CH_3CN-H_2O (90:10, v/v) including 0.01% (v/v) trifluoroacetic acid (TFA) (Fig. 1) or CH_3CN-H_2O (80:20, v/v) including 0.01% TFA (Fig. 2). The flow rate was set at 1.0 mL/min, the injection volume was 10 or 20 μL, and DAD detection was set at the range of 190-400 nm. Preparative HPLC was carried out on a Shimadzu LC-8A HPLC system (Shimadzu, Japan) consisting of two LC-8AT HPLC pumps and an SPD-20A detector with a preparative cell. Data processing was performed with an LC-solution chromatographic workstation. LC separations were accomplished on an Ultimate XB-C_8 column (10 μm, 250 mm ×

10 mm; Welch Materials, Inc.). The mobile phase for isocratic elution was CH_3CN-H_2O (75:25, v/v) including 0.01% (v/v) TFA. The flow rate was set at 3.0 mL/min, with the injection volume being 1.0 mL. UV detection was set at 310 nm.

2.3. Determination of GA Content

The analysis of the GA content in Ginkgo leaves and extracts was based on a method in the Chinese Pharmacopoeia.[9] Chromatographic conditions: Hypersil C_{18} column (200 × 4.6 mm, 5 μm, Dalian, People`s Republic of China); mobile phase: MeOH-acetic acid (99:1, v/v), 1.0 mL/min; detection at 310 nm; 10 μL injection. Standard solution for quantitation: 50 μg/mL C13:0 in methanol. Eight peaks with a similar UV spectrum to GA (13:0) were specified as GAs. The total peak area of the eight GA peaks was used to calculate the total GA content vs. C13:0. Sample treatment: 200 mg of Ginkgo leaves was extracted by petroleum ether (3 × 10 mL) in an ultrasonic bath. The extracts were combined in a 50 mL volumetric flask and petroleum ether was added to the mark. Ten mL of this solution was evaporated to dryness, and re-dissolved in 10 mL of methanol. After filtration, this sample solution was injected into the HPLC.

2.4. Preparation of MNPs

The Fe_3O_4 MNPs were prepared via a previously reported co-precipitation method.[36] Five mL of a 1 M ferric chloride ($FeCl_3$) aqueous solution in 2 M HCl and 1 mL of 2 M ferrous chloride ($FeCl_2$) aqueous solution in 2 M HCl were mixed and deoxygenated by purging with nitrogen gas for 20 min. Then, 50 mL of deionized water and 10 mL of 25% ammonia solution were mixed and deoxygenated with nitrogen. Finally, the solution with iron ions was added to the ammonia solution under nitrogen and stirred vigorously for 30 min at room temperature. After the reaction, the precipitate obtained was separated from the reaction medium by a magnet, washed with 80 mL of deionized water five times and 80 mL of methanol three times. The Fe_3O_4 MNPs were kept in methanol and stored at 4 °C in a refrigerator until use.

2.5. Extraction of Ginkgo Leaves and Purification by MNPs

A 150 g aliquot of dried Ginkgo leaf powder was extracted three times with petroleum ether (30-60 °C) (1.5 L each) in an ultrasonic bath and filtered. The combined extracts were evaporated at 60 °C under a vacuum in a rotary evaporator. Recovered petroleum ether could be reused later on. The residue (about 13.4 g)

was re-dissolved in 500 mL of methanol in an ultrasonic bath. After filtration over filter paper, the GAs in the sample solution were treated with 50 g of MNPs. The mixture was swirled for 15 min to adsorb GAs, after which the MNPs were separated from the solution by a magnet. After removal of the supernatant, the MNPs were washed three times with methanol (each time 250 mL). Afterwards, GAs on the MNPs were desorbed in 500 mL of methanol-formic acid (90:10 v/v) in an ultrasonic bath for 15 min. The desorption solution was evaporated to dryness in a rotary evaporator under a vacuum to remove the solvent. Methanol was recovered for reuse. The residue (about 3.48 g) was re-dissolved in 100 mL of petroleum ether to remove some colored polar impurities formed during the desorption procedure. After filtration, the colorless petroleum ether extract solution was evaporated to dryness under vacuum. The residue (about 2.13 g, total GA content 73.4%) was re-dissolved in 250 mL of methanol. After filtration through a 0.45 μm membrane, the solution was separated by preparative HPLC (vide supra) for the preparation of pure GAs.

3. Results and discussion

Catechol (1,2-dihydroxyphenol) derivatives are often used as dispersant anchors for Fe_3O_4 magnetic nanoparticles (MNPs), because of the high affinity of catechols for the nanoparticles.[26,27] This "grafting to" approach suggested that Fe_3O_4 nanoparticles could be applied to selectively adsorb phytochemicals containing a carboxylic acid group or ortho-phenolic hydroxyl groups. We demonstrated that Fe_3O_4 MNPs could indeed selectively adsorb ascorbic acid, cichoric acid, and citrinin as well as some flavones containing a catechol moiety from *Scutellaria baicalensis* Georgi roots.[28] Assuming that the Fe(III) ion is responsible for the complex formation, it was reasoned that phytochemicals containing a salicylic acid moiety might be even more strongly bound. The ferric ion is known to give an intense violet-red-colored complex with salicylate ions under slightly acidic or neutral conditions and this phenomenon is even used in spot tests.[29] In further research it was proven that salicylic acid binds even stronger than catechols and carboxylic acids to bare Fe_3O_4 nanoparticles (B. Chen, unpublished data). This opened the way to apply these easily prepared, stable and inexpensive ($ 0.05/g) nanoparticles for the selective analytical or preparative enrichment of phytochemicals possessing a salicylic acid (2-hydroxybenzoic acid) moiety. An

important representative of this class is ginkgolic acid (GA, syn. anacardic acid), which occurs in Ginkgo leaves and sarcotestas as well as in cashew nut oil.

To obtain proof of principle for this hypothesis, first the adsorption and desorption characteristics of pure GAs on MNPs were studied. A nice property of MNPs is that the adsorption can be carried out in pure organic solvents. We compared the adsorption capacity and selectivity of MNPs for GAs in methanol, ethanol, acetonitrile, acetone, hexane and petroleum ether. Using a reconstituted mixture of pure GAs in different solvents (in total 0.9 mg GAs/mL) as test solution, 20 mg of MNPs were added to 2.0 mL of test solution. The adsorption was monitored by comparing the peak area ratio of each GA in the test solution before and after adsorption. The results are listed in Table 1. The adsorption is similar in methanol, ethanol, acetonitrile and acetone. The capacity is very high. Assuming saturation of the MNPs, the capacity is 4-5% (w/w). When 200 mg instead of 20 mg of MNPs were used, indeed all GAs were adsorbed (results not shown). The adsorption capacity in hexane and petroleum ether was lower than that in polar solvents. This phenomenon might be caused by the fact that in non-polar solvents there is no proton acceptor, which hampers the formation of the salicylate ion. This ion is the actual species, which complexes with Fe(III). Alternatively an oxygenated solvent might facilitate the stabilization of the Fe(III)-salicylate complex. In further experiments methanol was used in the adsorption step and to avoid saturation effects the MNP weight was increased 10-fold (200 mg).

Table 1. Adsorption of MNPs to GAs[a]

Solvents	C13:0	C15:0	C15:1	C17:1	C17:2	C17:3
methanol	48	46	43	48	46	44
ethanol	47	48	46	46	47	49
acetonitrile	50	49	46	48	49	49
acetone	45	42	39	40	45	43
hexane	19	17	19	21	19	18
petroleum ether	20	18	20	20	19	18

[a]In %; defined as 100% − (100% × HPLC peak area ratio after adsorption/before adsorption).

To optimize the desorption conditions, different desorption agents, i.e., acetic acid, formic acid and ammonia were investigated. Using the same mixture of pure GAs in methanol as before, 200 mg of MNPs was added to 2.0 mL of test solution. After the adsorption step, the MNPs with the attached GAs were washed three times by 2 mL of methanol, and then desorbed in 2.0 mL of methanol containing

the desorption agent. The combined adsorption-desorption ability was investigated by comparing the HPLC peak area ratio of each GA in the test solution after desorption and before adsorption, respectively (Table 2). Both acids and ammonia effectively desorb GAs from the MNP surface. When the concentration of acid is 10% (v/v) or higher, GAs can be desorbed for ~90% in one step, which is in line with the statement of Vogel that the presence of a large excess of organic acids (or dilute mineral acids) destroys the complex.[29] In all further experiments, 10% formic acid in methanol was used for desorption of GAs.

Table 2. Combined Adsorption and Desorption of GAs Using MNPs[a]

	C13:0	C15:0	C15:1	C17:1	C17:2	C17:3
acetic acid 1% (v/v)	23	26	21	22	24	26
5%	45	51	53	42	44	43
10%	89	92	91	93	86	90
formic acid 1% (v/v)	31	29	34	27	25	26
5%	51	53	53	46	48	51
10%	94	92	93	89	89	91
ammonia 1% (v/v)	21	25	27	22	22	21
5%	46	44	43	49	45	45
10%	78	82	74	69	76	71

[a]In %; defined as 100% × (HPLC peak area ratio after desorption/before adsorption).

In the next step, it was investigated as to whether MNPs exhibit sufficient selectivity with real-life samples, for instance Ginkgo leaf extracts. Ginkgo leaves contain a wide range of phytochemicals including ginkgolides, bilobalide, flavonols and flavonol glycosides,[30] biflavones and many non-polar constituents like GAs,[31] cardanols, cardols, fatty acids, sesquiterpenes, polyprenols, sterols, carotenoids, chlorophylls, and waxes.[1,32] To avoid possible interference of flavonol glycosides during the MNP enrichment step, petroleum ether was used as the initial extraction solvent. Flavonol glycosides as well as other polar phytochemicals will not be extracted by this non-polar solvent. Although GAs can be directly adsorbed by MNPs from non-polar solvents, the desorption of GAs from MNPs in petroleum ether will be difficult because of the immiscibility of petroleum ether with formic acid. Therefore, the petroleum ether extract solution of Ginkgo leaves was evaporated and re-dissolved in methanol. All recovered petroleum ether could be reused for subsequent extractions. At this stage, HPLC

investigations with diode-array detection showed the presence of two different classes of alkyphenols (GAs and cardanols). Many other even more non-polar species present (vide supra) without UV absorbance at 310 nm were not detected or, more likely, did not elute. Of all these constituents, only GAs possess a salicylic acid moiety, and were expected to be adsorbed by MNPs. Fig. 1 is showing, in turn, three chromatograms, of a crude petroleum ether extract of 15 g of Ginkgo leaves re-dissolved in 300 mL of methanol, 100 mL of the same extract after treatment with 5 g of MNPs, and finally the acidic desorption solution. After being adsorbed by MNPs, GAs almost completely disappeared from the solution (Fig. 1, middle). In this fraction some compounds, like the peak at 30 min, are likely to be cardanols on the basis of their UV spectrum (λ_{max} ~230 and ~270 nm). In the desorption solution (Fig. 1, lower), all eight peaks exhibited the typical UV spectrum of a GA (λ_{max} ~242 and ~310 nm). Only five of these could be matched on the basis of retention times (vide infra).

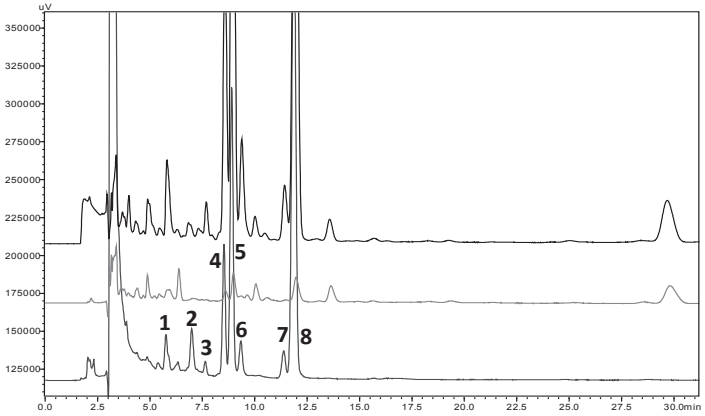


Fig. 1. Overlay HPLC traces on a C_8 column of a petroleum ether extract of Ginkgo leaves (upper), supernatant after adsorption step with MNPs (middle), solution after desorption of MNPs by methanol - formic acid (lower). **1**: unknown GA, **2**: unknown GA, **3**: GA (17:3), **4**: GA (13:0) **5**: GA (15:1), **6**: GA (C17:2), **7**: GA (C15:0), **8**: GA (C17:1). Note: the large peak at 3.2 min in desorption solution is caused by 10% formic acid.

Next, this procedure was repeated on a ten-fold larger scale, i.e., with 150 g of Ginkgo leaves. According to a quantitative assay performed on the leaves, the GA content of the leaves, expressed as GA (13:0), was 1.34%. The leaves were extracted with petroleum ether to give 13.4 g of extract. This extract was treated in

methanol with 50 g of MNPs for 15 min. After desorption and evaporation, 3.48 g of extract resulted. Re-dissolving in petroleum ether to remove some colored polar side products, gave 2.13 g of enriched GA fraction with a GA content of 73.4%. Calculated back to the original leaves, this amounts to an overall GA yield of 78%. The less than 100% recovery is caused by a combination of a five-fold lower solvent/plant material ratio in case of the preparative extraction, a 4.5-fold lower MNP/GA ratio, the ~90% recovery of the desorption step (vide supra) and other scale-up problems. Still, in two simple steps the GA concentration, without involving chromatography or LLE, was increased from 1.34 to 73.4%, i.e., more than 50-fold. Most importantly, cardanols were removed. Thus, the method appears suitable for a simpler and cheaper production of GAs.

As the last step, a small part of the enriched GA extract was separated into its individual, pure GAs by means of semi-preparative HPLC on a 10 mm i.d. C_8 column (Fig. S5, Supporting Information). Sufficient amounts of the three major GAs ((13:0), (15:1) and (17:1), original concentration in leaves 0.14, 0.44 and 0.59% respectively) were isolated for recording carbon and proton NMR spectra (Fig. S2, Supporting Information). These data compared well with published NMR data.[23] Their chromatographic purity according to HPLC-UV was >98%, while according to quantitative NMR the absolute purity for GA (13:0), (15:1) and (17:1) was 95.7%, 96.2% and 97.4%. Three minor GAs ((17:3), (17:2) and (15:0), original concentration in leaves 0.01, 0.05 and 0.03% respectively) were identified by means of their retention time, UV spectrum and ESIMS/MS spectra (Fig. S3 and S4, Supporting Information). Two minor, unknown and more polar GAs (original concentration in leaves 0.015 and 0.05% respectively), with lower retention times were also isolated. On the basis of their MS/MS data, they are presumably oxidized GA (15:1) and GA (17:1), possibly an epoxy or hydroxy-GA. Such compounds have been described as metabolic products of GA (15:1) in rat liver microsomes.[33] Once sufficient quantities have been produced for NMR, their structures will be elucidated. To exclude that they were oxidation products of GA (15:1) and GA (17:1) formed during the interaction with the MNPs, pure GA (15:1) and GA (17:1) were treated with MNPs and then desorbed. The two early eluting peaks could not be detected by HPLC in the desorption solution. Thus they are probably genuine compounds, or at least not formed during the MNP purification step. This also nicely illustrates the untargeted characteristics of the MNPs, due to the shared salicylic acid group they adsorb any GA irrespective of polarity. An HPLC profile of a reconstituted mixture of the eight isolated GAs is shown in Fig. 2.

This confirms that the current approach involving only one chromatographic step yields GAs of sufficient purity for use in quality control by means of HPLC. For the efficient production of larger quantities, a larger-sized HPLC column should be used.

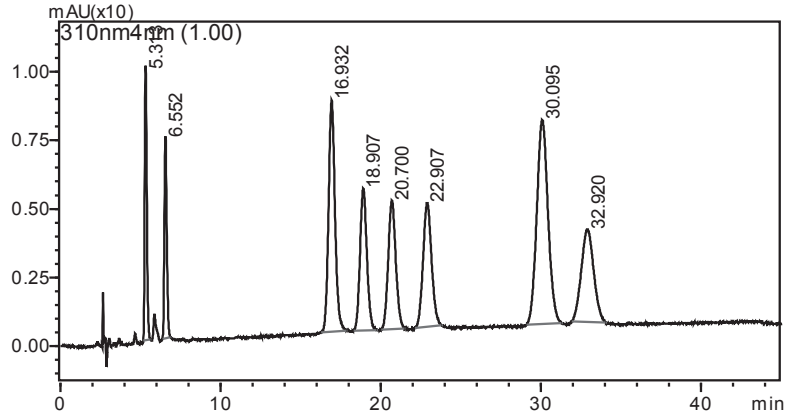


Fig. 2. HPLC-UV (310 nm) trace on a C_8 column of a reconstituted mixture of pure GAs (concentration 50-130 µg/mL). 5.3 min: unknown GA, 6.5 min: unknown GA, 16.9 min: GA (17:3), 18.9 min: GA (13:0), 20.7 min: GA (15:1), 22.9 min: GA (C17:2), 30.1 min: GA (C15:0), 32.9 min: GA (C17:1). Eluent: CH_3CN-H_2O (80:20) including 0.1% TFA (v/v), 1.0 mL/min, 20 µL injection.

4. Conclusion

In conclusion, ginkgolic acids (GAs) were for the first time efficiently purified from raw plant material (*G. biloba*) in only three steps, namely by (1) extraction; (2) selective purification by MNPs; (3) preparative HPLC on a C_8 column. The three main constituents occurring at concentrations of 0.15% - 0.60% were enriched to >95% absolute purity without using tedious (gravity) column chromatography or halogenated solvents, thus saving time and chemicals. The high selectivity, stability and loadability of the MNPs open up more possibilities for phytochemical applications in the field of GAs. For instance, MNPs might be used on an analytical scale for the sample preparation prior to quantitative HPLC analyses of standardized Ginkgo extracts. Due to their high capacity, they might be industrially used as an alternative for the expensive heptane, which is now used to deplete crude acetone-water Ginkgo extracts of GAs. Apart from GAs, these MNPs could also be applied for other phytochemicals having a salicylic acid group, e.g., to separate tetrahydrocannabinolic acid (THCA, 2-COOH-THC) from other

cannabinoids in *Cannabis sativa* L. THCA is the main cannabinoid in Cannabis and upon smoking is converted into the psychoactive tetrahydrocannabinol (THC). A very recent paper published by material scientists described independently from this paper a completely different application of Fe_3O_4 MNPs: coated with anacardic acid they were proposed as hyperthermia agents.[34]

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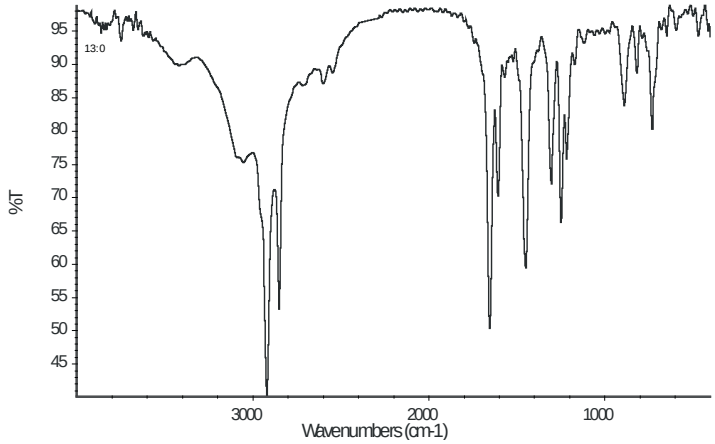
S1. IR spectra of purified GA (13:0), (15:1) and (17:1)



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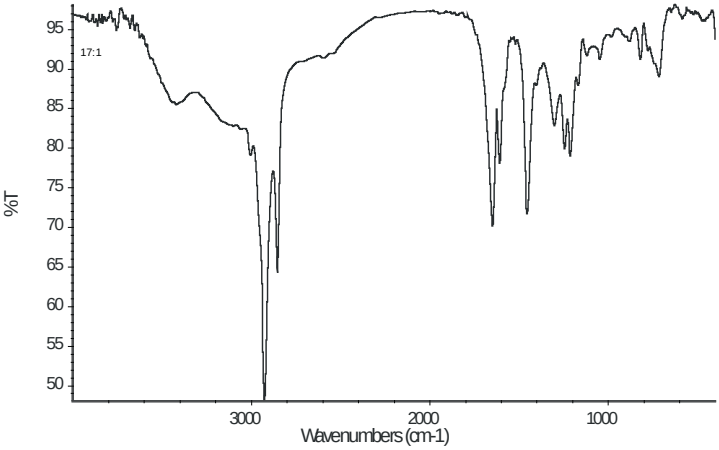

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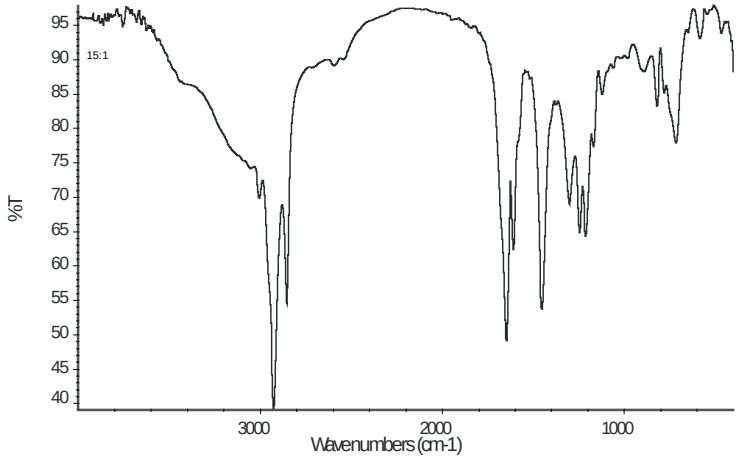
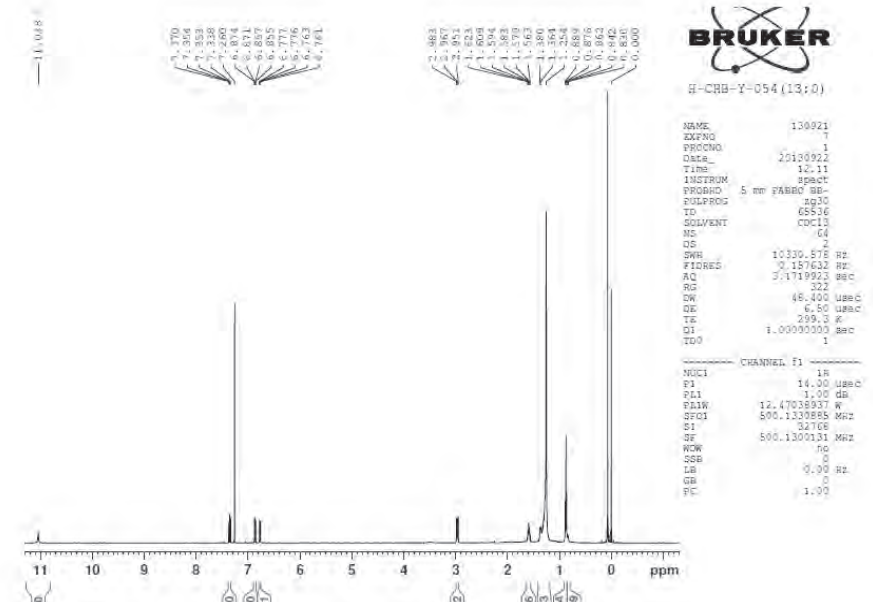
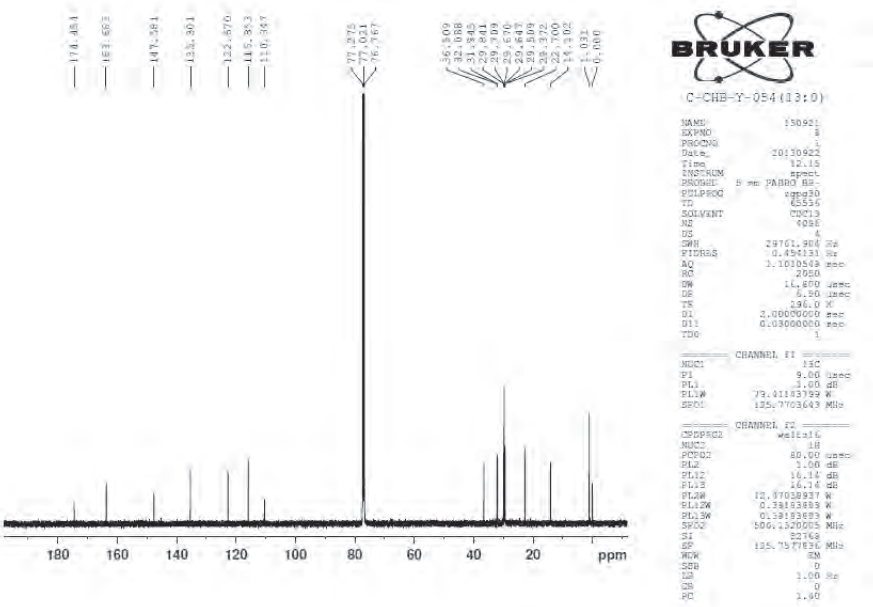


GA (17:1)

S2. NMR spectra of purified GAs





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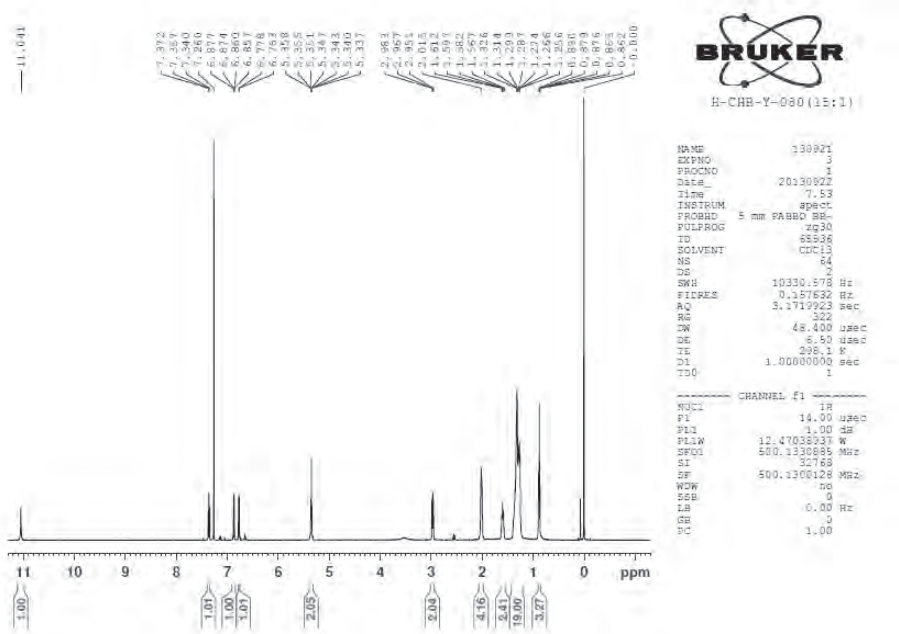
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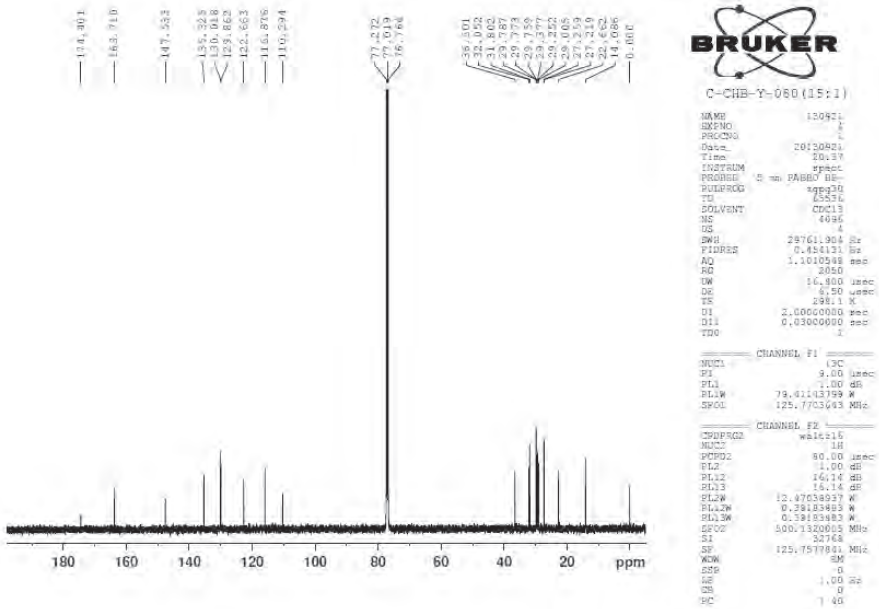
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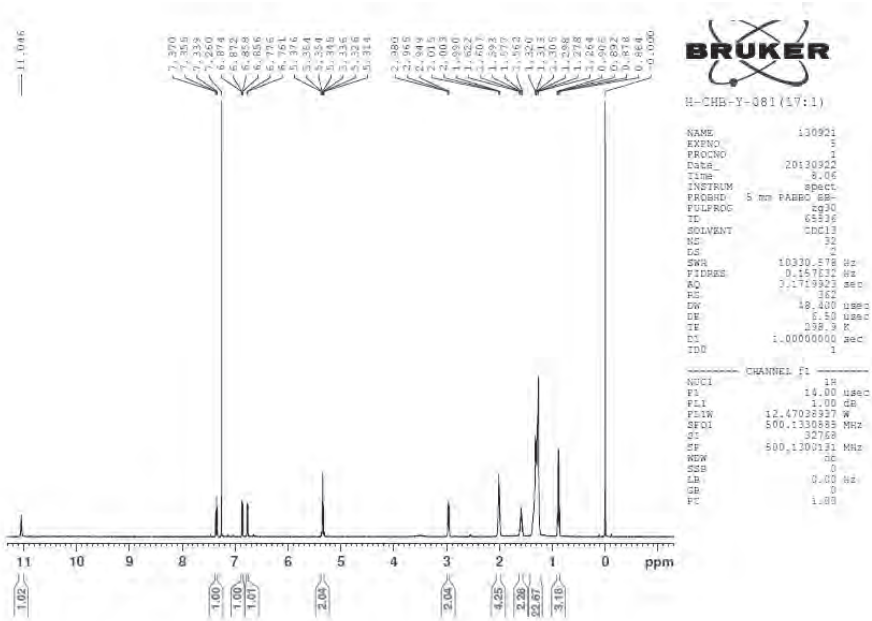
GA (15:1)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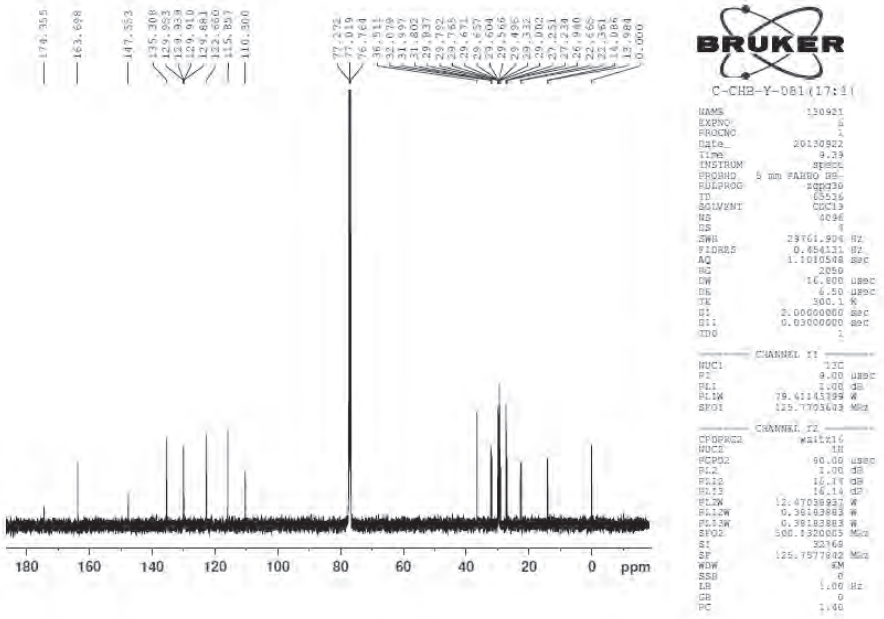
DE 6.50 usec
TE 298.9 K
D1 1.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 14.00 usec
PL1 1.00 dB
PL1W 12.47038937 W
SFO1 600.1330885 MHz
SI 32768
SF 600.1300131 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.00
11.046
7.390
7.355
7.339
7.280
6.872
6.858
6.856
6.776
6.761
5.376
5.364
5.349
5.336
5.326
5.314
2.380
2.365
2.344
2.005
1.990
1.622
1.607
1.593
1.577
1.562
1.330
1.313
1.305
1.288
1.278
1.264
0.905
0.887
0.878
0.864
0.000
11 10 9 8 7 6 5 4 3 2 1 0 ppm
1.02
1.00
1.00
1.01
2.04
2.04
4.25
2.26
22.67
3.16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FIDRES 0.454131 Hz
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D11 0.03000000 sec
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NUC1 13C
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SFO1 125.7703603 MHz
CHANNEL f2
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NUC2 1H
PCPD2 90.00 usec
PL2 1.00 dB
PL12 16.14 dB
PL13 20.14 dB
PL1W 12.47038937 W
PL12W 0.36103803 W
PL13W 0.09183883 W
SFO2 500.1320005 MHz
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SF 125.7577902 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
174.355
163.698
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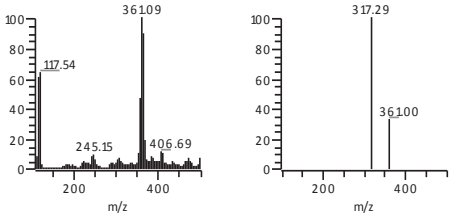
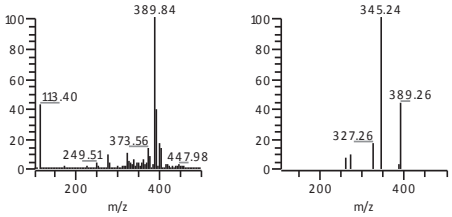
135.308
129.993
129.939
129.910
124.681
122.460
115.837
110.300
77.272
77.019
76.764
36.911
32.079
31.997
31.802
29.837
29.782
29.735
29.671
29.657
29.604
29.566
29.496
29.332
29.082
27.251
27.230
26.940
22.566
22.561
14.086
13.984
0.000
180 160 140 120 100 80 60 40 20 0 ppm

GA (17:1)

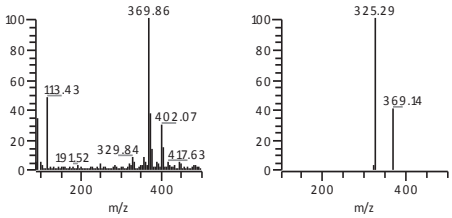
S3. ESI-MS and ESI-MS/MS spectra of purified GAs



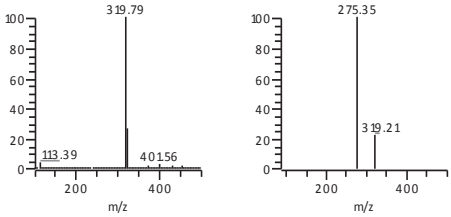
(unknown 1) $C_{16}H_{33}$ (*m/z* 361, 317)



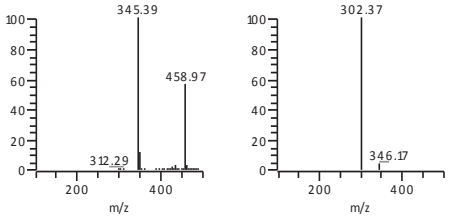
(unknown 2) $C_{18}H_{37}$ (*m/z* 389, 345)



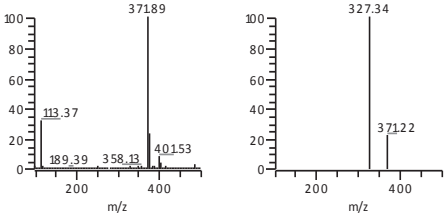
(C17:3) $C_{17}H_{29}$ (*m/z* 369, 325)



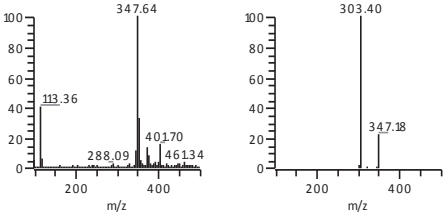
(C13:0) R= $C_{13}H_{27}$ (*m/z* 319, 275)



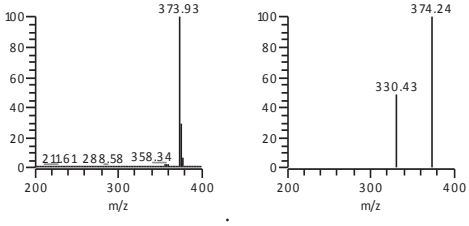
(C15:1) R= $C_{15}H_{29}$ (*m/z* 345, 301)



(C17:2) R= $C_{17}H_{31}$ (*m/z* 371, 327)

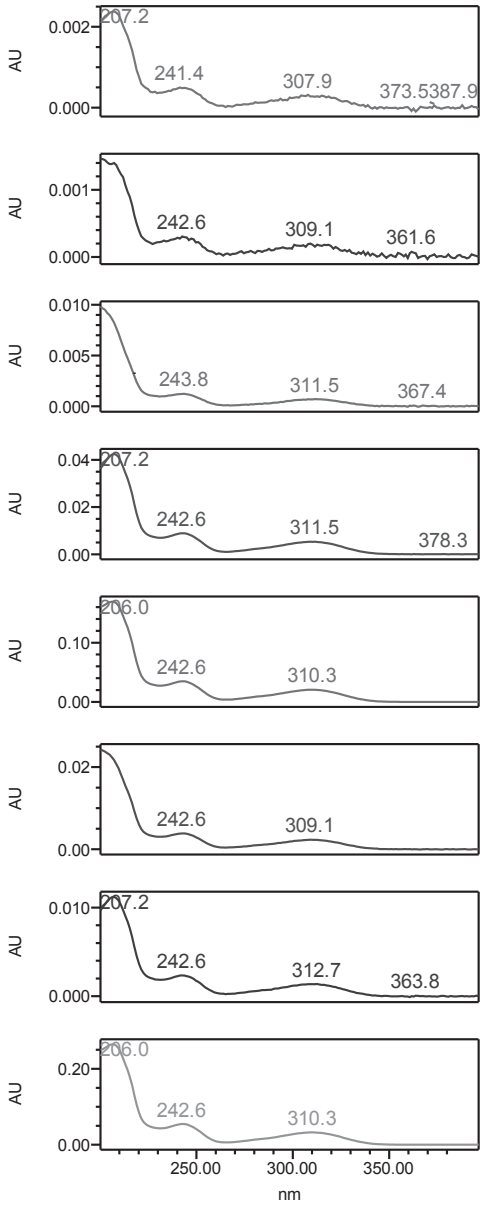


(C15:0) R=$C_{15}H_{31}$ (*m/z* 347, 303)

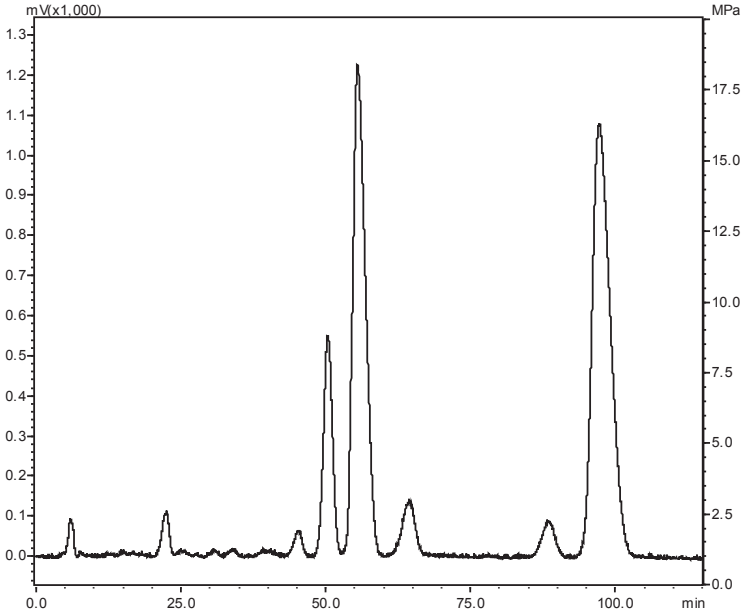


(C17:1) . R=$C_{17}H_{33}$ (*m/z* 373, 329)

S4. HPLC-UV spectra of purified GAs. From top to bottom in order of HPLC elution: unknown 1 → unknown 2 → C17:3 → C13:0 → C15:1 → C17:2 → C15:0 → C17:1



S5. Chromatogram of semi-preparative HPLC separation on a 10 mm i.d. C8 column. Peaks correspond with the 8 compounds mentioned in **S4** on page 115 of this supplementary info



Chapter 7

Alternative Solvents can Make Preparative Liquid Chromatography Greener

Abstract

To make preparative Reversed-Phase High Performance Liquid Chromatography (RP-pHPLC) greener, alternative solvents were considered among others in terms of toxicity, cost, safety, workability, chromatographic selectivity and elution strength. The less toxic solvents ethanol, acetone and ethyl acetate were proposed as possible greener replacements for methanol, acetonitrile and tetrahydrofuran (THF). For testing their feasibility, five Ginkgo terpene trilactones were used as model analytes. The best "traditional" eluent, i.e., methanol-THF-water (2:1:7) was used as benchmark. A generic two-step chromatographic optimization procedure by UHPLC consisting of (1) a simplex design using the Snyder solvent triangle and (2) HPLC modelling software was used. In the first step, two ternary mixtures were found (acetone-ethyl acetate-water (20.25:3.75:76) and ethanol-ethyl acetate-water (9.5:7.5:83)), which already gave better results than the benchmark. The second step in which the influence of the gradient time, temperature and ratio of the two best ternary isocratic solvents was studied, led to an optimal 10.5 min gradient and a minimum resolution of 5.76. In the final step, scale-up from 2.1 to 22 mm i.d. pHPLC columns proceeded successfully. When 0.5 g of sample was injected, baseline separation was maintained. Chromatographic and absolute purities for products exceeded 99.5% and 95% respectively. This example shows that using less-toxic and cheaper solvents for pHPLC can go hand in hand with higher productivity and less waste.

1. Introduction

As part of a global drive towards sustainability, during the last 15 years much has been done on making chemistry, including analytical chemistry greener. To assess whether analyses are more or less green, one needs to take a comprehensive view as there are so many aspects to reckon with: worker health, process safety, environmental impact, sustainability, energy requirements, life cycle analysis (LCA), efficiency, regulations, amounts of solvents and chemicals needed, waste disposal and last but not least, costs. The buzz words in several reviews to achieve this are: smaller sample sizes, solvent-free extraction, simpler and more selective sample preparation, miniaturization, more benign solvents and greener chromatography.[1-8] To make phytochemical analysis greener, we have developed a solvent-free assay for the neurotoxin in Japanese star anise,[9] on-chip sample preparation for alkaloid extracts using only µL of solvents[10] and highly selective and energy-efficient magnetic nanoparticles replacing a column chromatographic step with halogenated solvents during the large-scale purification of Ginkgo allergens.[11] In most cases however, an HPLC step is involved in the quantitation of non-volatile analytes and is not so easily replaced although Capillary Electrophoresis (CE) is sometimes a fine but somewhat less rugged alternative. As Gaber et al. estimated the total amount of waste generated by HPLC instruments worldwide at 34 million liters/year, even an incremental improvement would lead to a significant reduction of waste.[12] Recycling is difficult because most runs are gradient runs, and additionally there are safety, reproducibility, regulatory and manpower aspects to consider. In making HPLC greener, most can be gained in terms of solvents needed, by going to smaller internal diameters. In this respect nanoLC is the best (< 1 µL/min) but requires expertise and again is less rugged and therefore not popular. Nowadays 2.1 mm i.d. columns (80% reduction of solvent usage relative to traditional 4.6 mm i.d. columns) offer the best compromise between reproducibility and solvent usage. UHPLC allows the use of more efficient and thus shorter sub 2 µm columns and many protocols now make use of 5 cm columns leading to faster analyses and reduced solvent consumption. The two most benign solvents for chromatography are water and carbon dioxide. There are several possibilities to increase the percentage of water in the mobile phase at the expense of organic modifiers. An example is superheated (or subcritical) water chromatography at temperatures of 100 to 250 °C.[13] For some analytes, 60 or 80 °C sufficed to use pure water as eluent.[14] Problems are stationary phase

stability, analyte degradation and non-polar analytes. Other approaches include less retentive stationary phases[15, 16] or the addition of surfactants (Micellar Liquid Chromatography, MLC).[8, 17-21] In some cases, the latter technique works without the addition of any organic solvent. MLC is less suitable for highly non-polar analytes and unsuitable for preparative HPLC (pHPLC) because of the additives needed. The use of carbon dioxide requires the use of specialized Supercritical Fluid Chromatography (SFC) equipment and usually the addition of 10-30% of an organic modifier, like methanol or ethanol. It is efficient, especially for enantioselective separations[22] but less suitable for highly polar analytes. Due its normal phase-like mechanism, it cannot always replace reversed phase HPLC (RP-HPLC). Van der Vorst et al. questioned the sustainability of SFC when taking into account all aspects.[23] Finally to reduce the heavy demand of HPLC for toxic solvents like acetonitrile, methanol and tetrahydrofuran (THF), many studies have proposed to replace them by less toxic alternatives like ethanol,[24-27] acetone, isopropanol, propylene carbonate[24, 25] and ethyl lactate.[28]Almost all of the above studies concern analytical separations and relatively little attention has been paid to making preparative separations greener, probably because it is harder to make them green anyway.[12] All of the miniaturization approaches do not work as they do not decrease the amount of solvent needed per gram of purified product. Also pHPLC precludes the use of non-volatile additives as in MLC or the use of high-boiling (and viscous) green solvents such as propylene carbonate (b.p. 242 °C) and ethyl lactate (b.p. 151 °C) and preparative high-efficiency columns with sub 2 μm particles do not exist. Thus, only replacement is an option for RP-pHPLC. However it is unclear if any less toxic alternatives could really substitute for acetonitrile, methanol and THF with respect to chromatographic selectivity and efficiency, cost and workability. The aim of this paper is to evaluate various organic solvents for their suitability in RP-pHPLC, select the best three and test them in a real-life preparative separation.

Table 1 Comparison of properties of traditional RP-HPLC organic modifiers and alternative ones

Name solvent	methanol	ethanol	acetonitrile	acetone	THF	ethyl acetate
toxicity TLV* (ppm) [29]	200	1000	40	750	200[29], 50[30]	400
safety**[31]	1.65	1.6	2.2	1.7	1.7	1.65
cost (€/L)[32]	2.82	2.41	7.20	3.44	10.56	3.54
energy to pre- pare (MJ/kg)[31]	41	50	88	75	271	96
elution strength[33]	3.0	3.6	3.1	3.4	4.4	***
boiling point (°C)	65	78	82	56	66	77
viscosity (cP)****	0.6	1.2	0.37	0.32	0.46	0.45
chromatogr. selectivity[34]	II	II	VI	VI	III	VI
UV (nm) transparency	> 210	> 210	> 193	> 335	> 230	> 260
odor thres- hold (ppm)[29]	100	84	170	13	2	4
PEEK compatibility	+	+	+	+	±	+
stability*****	+	+	+	±	-	±
NMR signals	+	±	+	+	±	-
solubility in water	miscible	miscible	miscible	miscible	miscible	8.3 g/100 mL

* by inhalation, TLV = Threshold Limit Value (in ppm)
** score on release potential, fire/explosion hazard and reactivity/decomposition as part of EHS evaluation of solvents; a lower score, indicates a safer solvent
*** no value could be found, our estimation is 4.5
**** viscosity of methanol-water and ethanol-water mixtures is considerably higher than the viscosity of either pure methanol/ethanol or pure water; this is a disadvantage of methanol/ethanol
***** based on our own assessment/experience

2. Experimental

2.1. Materials and instruments

HPLC-grade methanol, tetrahydrofuran, acetone, ethanol, acetonitrile, and ethyl acetate were purchased from Merck, Germany. The Ginkgo terpene trilactones (TTLs) ginkgolide A (GA), B (GB), C (GC), J (GJ), and bilobalide (BB) were isolated in our lab.[35] Uracil used for determining of the dead time was purchased from Sigma, the Netherlands. The UHPLC was an Agilent 1290 Infinity, equipped with binary pumps, autosampler, thermostatted column compartment, and Sedex 90 LT-ELSD; column: Agilent Zorbax Eclipse Plus C18, 2.1×100 mm 1.8 μm; flow: 0.20 mL/min, injection volume 1.0 μL. For getting an aqueous solution saturated with ethyl acetate, ethyl acetate was gradually added to water

until there was an organic layer on top of the solution. The whole solution was stirred overnight to obtain full saturation. The bottom layer was used as HPLC eluent (9% v/v ethyl acetate in water).

The prepHPLC consisted of two LC-8A pumps, SIL-10AP auto injector, FRC-10A fraction collector, LT-ELSD and active splitter, all from Shimadzu, Japan; the column selector allowed the use of either an analytical column: Alltima C18, 250×4.6 mm 5 µm, Alltech, (the Netherlands); flow: 1.0 mL/min, injection volume 20 µL, no split; or a preparative column: Alltima C18, 250×22 mm 5 µm, Alltech; flow 20 mL/min, injection volume 4 mL, active split to ELSD, 120:1.

Drylab Software (version 4; Molnár-Institute, Berlin) was used for chromatographic solvent optimization.

2.2. Optimization of solvent strength of water – organic modifier mixtures for use in simplex design

According to Glajch et al., the first step in the solvent optimization according to the simplex design, is to determine the solvent composition at the 3 apices of the solvent triangle.[36] To this purpose various isocratic runs were carried out with mixtures of water with ethanol, acetone and ethyl acetate respectively to arrive at approximately the same solvent strength as the benchmark eluent (average k´ for the 5 TTLs 4).

2.3. Selection of the two optimal solvent compositions by means of simplex design

The five TTLs were analyzed under isocratic conditions with the 13 solvent compositions indicated in Fig. 1b as mobile phase. Their retention factors (k´) and critical resolution (Rs_{min}) were calculated based on the obtained chromatograms (see supplementary information (SI)). The two optimal compositions corresponded with point 6 (ethanol-ethyl acetate-water=9.5:7.5:83) and point 15 (acetone-ethyl acetate-water=20.25:3.75:76) and were used for further optimization by HPLC modelling software.

2.4. HPLC eluent optimization by HPLC simulation software

To use the HPLC simulation software (DryLab, Berlin) with a 100 mm column, a number of gradient runs with two different gradient times of 6 and 18 min are required and all analytes need to elute before the end time of the short gradient (t_r < 6 min). This meant that the isocratic conditions needed to be converted to

gradient conditions. This was accomplished by using water as the weak mobile phase (A) and the two best eluents from the simplex design (see 2.3) without water as the strong solvent (B1 and B2). The ratio of ethanol-ethyl acetate (B2) and acetone-ethyl acetate (B1) remained unchanged. In total 12 gradient separations of the 5 TTLs were carried out: 2 gradients (6 min and 18 min) × 2 temperatures (25 °C and 50 °C) × 3 different eluents (B1 (Point 15 in Fig. 1b), B2 (Point 6 in Fig. 1b) and B1-B2 (1:1)). The chromatograms were entered into the software for further optimization. One million simulated chromatograms were obtained from which the one giving the highest minimal resolution was selected.

2.5. Preparative HPLC of 5 TTLs with the optimal mobile phase

After minor adaption of the mobile phase composition and gradient, the simulated chromatogram corresponding with the optimal solvent composition was compared with the experimental chromatogram obtained by means of UHPLC. Then, the UHPLC conditions were scaled up by calculation, first to HPLC (250×4.6 mm column) and then to pHPLC (250×22 mm column).

An enriched Ginkgo biloba extract containing approximately 94% of TTLs (GA 27.85%; GB 11.92%; GC 5.50%; GJ 0.90%; BB 47.92%) was used as a real-life sample for evaluating the transferability of the optimal solvent. 480 mg of sample dissolved in 4 mL of acetone-water (1:1), were injected into the pHPLC. Each TTL was collected separately and the solvent was removed in vacuum by means of a rotary evaporator. The crude yield was 85%. After recrystallization,[34] the yield of GA, GC and BB was 75%. The purity of all 5 TTLs was assessed by means of UHPLC (area normalization) and quantitative NMR. qNMR: an accurately weighed amount of each TTL and the internal standard 1,4-dimethoxybenzene (99.8%) was dissolved in deuterated methanol-benzene (2:1) and NMR spectra were recorded.[37]

3. Results and discussion

3.1. Selection of alternatives for methanol, acetonitrile and THF

Liquid chromatography (LC), and especially RP-HPLC, is the most used universal high-resolution separation technique for small organic molecules. Acetonitrile, methanol, water, and to a lesser extent THF, are the four most common solvents used for preparing RP mobile phases. In combination with 22 mm i.d.

columns, a pHPLC consumes about 30 L of solvent per 24 h and produces an equal amount of waste. All three organic constituents as well as the waste are significantly toxic and potentially environmental pollutants.[38,39] "Reduce" and "replacement" are two of the most followed rules for green separations in the well-known "12 principles of green chemistry"[1] but for pHPLC only replacement is a viable option. Unfortunately there are only few less toxic organic solvents and there are also practical considerations in pHPLC such as chromatographic selectivity (preferably different for each replacement), boiling point (< 100 °C), viscosity (< 1.5 cP), smell, cost (also reflects in part the energy requirements in its production), stability, miscibility with water, UV transparency, corrosiveness, and elution strength. Methanol is the easiest to replace as ethanol has proven itself in many analytical HPLC papers (see Introduction for references). Advantages over methanol include much lower toxicity, slightly lower costs and higher elution strength, which means that less ethanol than methanol is needed for comparable retention times. Its lower vapor pressure will lead to less evaporation and consequently to lower inhaled concentrations. Its chromatographic selectivity is the same as that of methanol, which is desired. Disadvantages include higher viscosity, harder to remove and, if not removed completely, more residual solvent signals in the NMR spectrum of the isolate. On the Pfizer list of 11 preferred solvents,[40] there are also the alcohols: n-propanol, isopropanol, n-butanol and t-butanol, but as these are not less toxic or cheaper than ethanol and possess much higher viscosities and boiling points, they were not considered as serious alternatives for pHPLC purposes.

The remaining solvents on the Pfizer green list are acetone, methyl ethyl ketone, ethyl acetate and isopropyl acetate. According to Snyder's classification of organic solvents (Fig. 1a), acetonitrile, acetone, methyl ethyl ketone and ethyl acetate are all in group VI, which means they have similar proton acceptor, proton donor and dipole moments.[32] However in our experience, the experimental selectivity in RP-HPLC of solvents from the same group can still vary significantly. Of the two ketones, we have a clear preference for acetone over methyl ethyl ketone on the basis of availability as HPLC grade, cost, smell and miscibility with water. Thus we selected acetone as a replacement for acetonitrile in this study. A constraint of acetone is its UV absorbance below 335 nm. Although the working range may extend to 320 nm with short preparative UV cells, it still significantly hampers its applicability. For all non-volatile analytes an evaporative light scattering detector (ELSD) as used in this study, is an interesting alternative for pHPLC work and

then acetone can be used without problems. Also a mass spectrometer, which is becoming more and more popular detector for pHPLC, can cope with acetone.

Of the three solvents to be replaced, THF is the least desirable one to work with as it is toxic, attacks PEEK tubing, forms peroxides and is expensive. Of the two remaining solvents, we prefer ethyl acetate over isopropyl acetate for reasons of availability, cost, boiling point and water miscibility. Thus in this study ethyl acetate was used as a replacement for THF. Although THF is in group III (Fig. 1a), actually ethyl acetate differs mainly in its dipole moment from THF.[33] With respect to the energy required to make 1 kg of solvent and CO_2 production during manufacture and incineration, ethyl acetate scores worse than ethanol and acetone but much better than THF.[41] A significant disadvantage is the poor miscibility of ethyl acetate and water, 100 mL of a saturated ethyl acetate solution contains only 9.2 mL of ethyl acetate. However in mixtures with ethanol or acetone, the solubility was not an issue in this study. The only other possible alternative for ethyl acetate is methyl acetate. The latter solvent is not on the Pfizer list[40] but does occur in other studies on green solvents, where it scores better than ethyl acetate on the basis of EHS and LCA analyses.[31] An additional advantage is its higher water solubility. Disadvantages are availability/costs, higher vapor pressure/flammability and hydrolysis to methanol instead of ethanol upon inhalation.[42] This is also reflected by the twice lower Threshold Limit Value.[29] Thus we decided to continue this study with ethanol, acetone and ethyl acetate as possible greener replacements for pHPLC. Their properties and of the three solvents they replace are summarized in Table 1.

3.2. Set-up of optimization process

Nowadays reliable HPLC optimization software exists, which in the case of the package we use, allows one to simulate 1 million chromatograms after recording 12 scouting chromatograms. In these 12 runs, two organic modifiers mixed with water can be tested (pure or in 1:1 ratio) as well as two temperatures and two gradient times. However the software does not allow the comparison of three different organic modifiers. We therefore resorted first to the simplex design as published by Glajch et al.[36] to find two solvent compositions, which give a fair separation of the analytes of choice. These would then form the starting point in a further optimization step with the help of modern HPLC modelling software. This should finally lead to an optimal solvent and elution conditions, which will be tested in preparative runs.

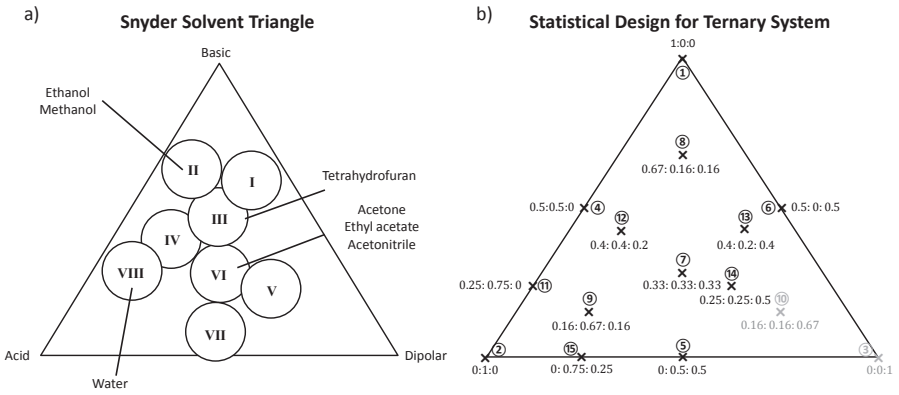


Fig. 1 a) Snyder solvent triangle; b) Simplex design for ternary system triangle. Point 1: ethanol-water (19:81); point 2: acetone-water (27:73); point 3: ethyl acetate:water (15:85). Points 3 & 10 are in grey as they cannot be prepared due to immiscibility problems.

3.3. Model analytes

A group of terpene trilactones (TTLs), i.e., ginkgolide A (GA), B (GB), C (GC), J (GJ) and bilobalide (BB) were used as the model analytes to evaluate the scope of the proposed solvents as greener mobile phases in pHPLC. There is an old but still excellent study comparing the selectivity of methanol, THF and acetonitrile for the separation of TTLs.[43] This study showed that acetonitrile-water mixtures cause co-elution of GA and GB and are best avoided. Methanol-water (30:70) and methanol-THF-water (15:5:75) gave fair separations. Later methanol-THF-water (20:10:70) was shown to give an even better separation of the five TTLs.[44] The latter eluent was used as the benchmark solvent in this study. In combination with a 100 mm UHPLC column, the Rs_{min} was 3.13 for GC and BB while the total run time was less than 10 min. As TTLs do not significantly absorb UV light, evaporative light scattering detection (ELSD) was used, which is also perfectly compatible with the organic solvents used in this study for optimization.

3.4. Optimization by means of simplex design

In the simplex design study, 15 points on a triangle were chosen: 3 apices, 5 points on the outside and 7 points inside the triangle (Fig. 1b). Each point represents a different composition of ethanol, acetone, ethyl acetate and water. The top apex (1) is a mixture of ethanol and water, the bottom left apex (2) represents an acetone-water mixture while the most right apex is a mixture of ethyl acetate

of water (Fig. 1b). For obtaining the right composition for the 3 apices (points 1-3 in Fig.1b) several ratios of water-organic modifier were tested isocratically with the analytes and the average retention factor (k´) of the five TTLs was compared to those of the benchmark eluent methanol-THF-water (20:10:70). The results are shown in Fig. 2. The average k´ values (~4) obtained with ethanol-water (19:81; in blue) and acetone-water (27:73; in green) were close to the average benchmark k´ value (in purple). The red line presents the data for ethyl acetate. Clearly for ethyl acetate-water (9:91) the average k´ (~8.5) is too low however it is impossible to dissolve more than 9% ethyl acetate in water. To arrive at the virtual but correct composition for the ethyl acetate/water mobile phase at apex 3, a calculation according to[45, 46] was carried out. The following Equation (1) shows the relationship between retention factor (k´) and mobile phase composition (φ).

$$\ln k' = A\varphi^2 + B\varphi + C \qquad (1)$$

Based on the experimentally determined relationship between k´ and , it is possible to calculate A, B, C. If k´ ~3.8 is desired, should be around 0.85. Thus the virtual apex for ethyl acetate-water was fixed at 15:85. This virtual composition allowed the preparation of all other eluents corresponding with points on the simplex triangle (Fig. 1b) with the exception of point 10. For all other points, the presence of ethanol and/or acetone sufficiently increased the solubility of ethyl acetate in the total mixture.

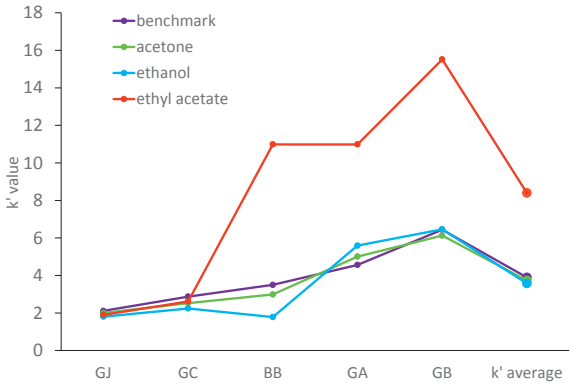


Fig. 2 Individual k´ values and average k´ value of five TTLs with different mobile phases; benchmark (in purple): methanol-THF-water (20:10:70); ethanol (in blue): ethanol-water (19:81); acetone (in green): acetone-water (27:73); ethyl acetate (in red): ethyl acetate-water (9:91).

Average retention factors (k´) and minimum resolution (Rs_{min}; resolution of worst separated pair of analytes) were experimentally determined in triplicate for the 13 mobile phases compositions indicated in Fig. 1b. The results are shown in Fig. 3. All compositions gave a good average k´ value (from 3.5 to 5.3), however, the minimum resolution varied from 0 to 4.4. For example, BB & GJ were not separated at Point 1 and BB & GC were not separated at Point 4. Ethanol strongly reduced the retention of BB, similar to methanol. Ethyl acetate showed the opposite effect: relative to the other TTLs, it strongly increased the retention of BB, similar to THF. Acetone did not show such effects, confirming that solvents in the same Snyder class, can exhibit a very different selectivity in RP-HPLC. Points 6, 7, 9, 13 and 15 all showed a good minimum resolution (~4) for the separation of the five TTLs. The average k´ value for each of them was also similar to the benchmark. As all of them, but point 9, are on a straight line, we assumed that the optimum solvent should be on or close to this line. Thus the solvent compositions corresponding with points 6 and 15 were selected as the starting solvents for further optimization by means of the HPLC simulation software. If an Rs_{min} of 4.4 would be considered sufficient for pHPLC, which would normally be the case, at this point it would be a valid option to skip further optimization and proceed with eluent composition 6: water-ethanol-ethyl acetate (83:9.5:7.5). This would already constitute a significant advantage over the THF and methanol-containing benchmark eluent both in toxicity and Rs_{min}. As more often than not an Rs_{min} of 4.4 will not be achieved after one round of optimization, for proof of principle we continued with the second optimization step. Apart from this, an even higher Rs_{min} is always desirable as it simply means more sample can be injected before baseline separation is lost.

3.5. Further optimizing mobile phase by means of HPLC simulation software

The HPLC optimization software requires slow and a fast gradient runs as input. For the 100 mm UHPLC column, 6 and 18 min are customary. This allows the software to deal with the effect of water on the chromatographic selectivity, which is something the simplex design used so far does cannot take into account. Thus, the isocratic conditions corresponding with points 6 & 15 had to be converted to gradient conditions. The ratio between acetone and ethyl acetate at point 15 is 20.25:3.75 and should remain constant during the gradient, only the percentage of water may vary. Similarly the ratio between ethanol and ethyl acetate at point 6 should remain fixed at 9.5:7.5 during all gradients. After a few scouting runs, it

was established that the 5 TTLs could all be eluted within 6 min by a 5% to 80% B gradient for the acetone-ethyl acetate-water system. For the ethanol-ethyl acetate-water system a 5% to 70% B gradient in 6 min sufficed (Table 2). To satisfy software requirements and to make them more uniform and reproducible, in their final form both gradients ran from 6% to 100%. This was possible by adding a small percentage of water to the mixed organic phase. Finally, 12 runs using 3 mobile phases for B1, B2 and 50% B2 in B1 (shown in Table 2) at 2 gradient times (6 min and 18 min) and 2 temperatures (25 °C and 50 °C) were carried out. Consequently 12 experimental chromatograms were obtained, which were imported as raw data into the modelling software. In the software, B1 is mobile phase B for point 15, and B2 is mobile phase B for point 6.

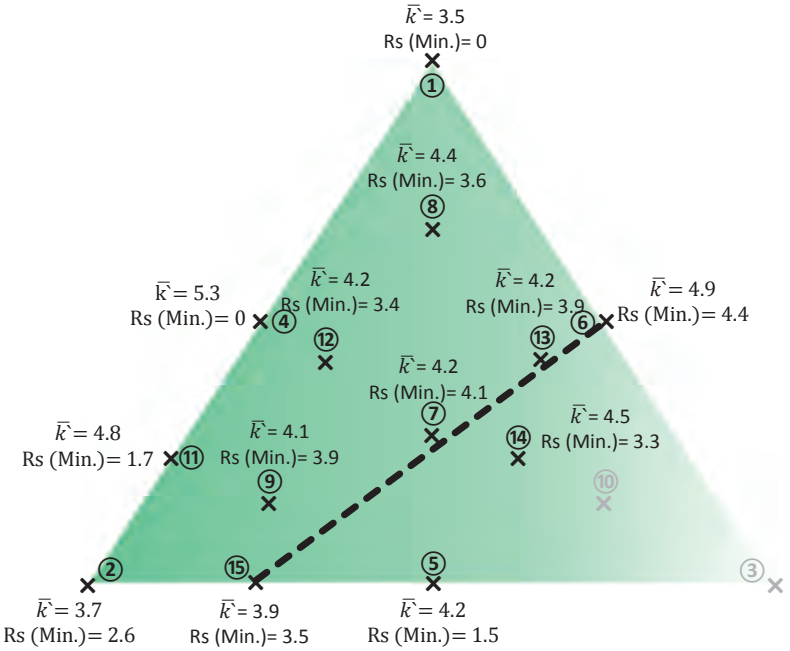


Fig. 3 Average retention factor (k´) and minimum resolution (Rs_{min}) for each mobile phase in the ternary triangle simplex system. Points 3 and 10 could not be measured because ethyl acetate and water are not miscible with each other at these ratios. Dashed line shows compositions potentially providing a high Rs_{min}.

Table 2 Conversion of isocratic conditions to gradient conditions and final three mobile phase compositions for use with HPLC modelling software

		B1	50% B2 in B1	B2
Isocratic condition	Mobile phase	acetone-ethyl acetate-water	--	ethanol-ethyl acetate-water
	ratio	20.25:3.75:76	--	9.5:7.5:83
Gradient condition	Mobile phase A composition		water 100%	
	Mobile phase B composition	acetone-ethyl acetate-water 67.5:12.5:20	acetone-ethanol-ethyl acetate-water 33.75:19.55: 21.7:25	ethanol-ethyl acetate-water 39.1:30.9:30

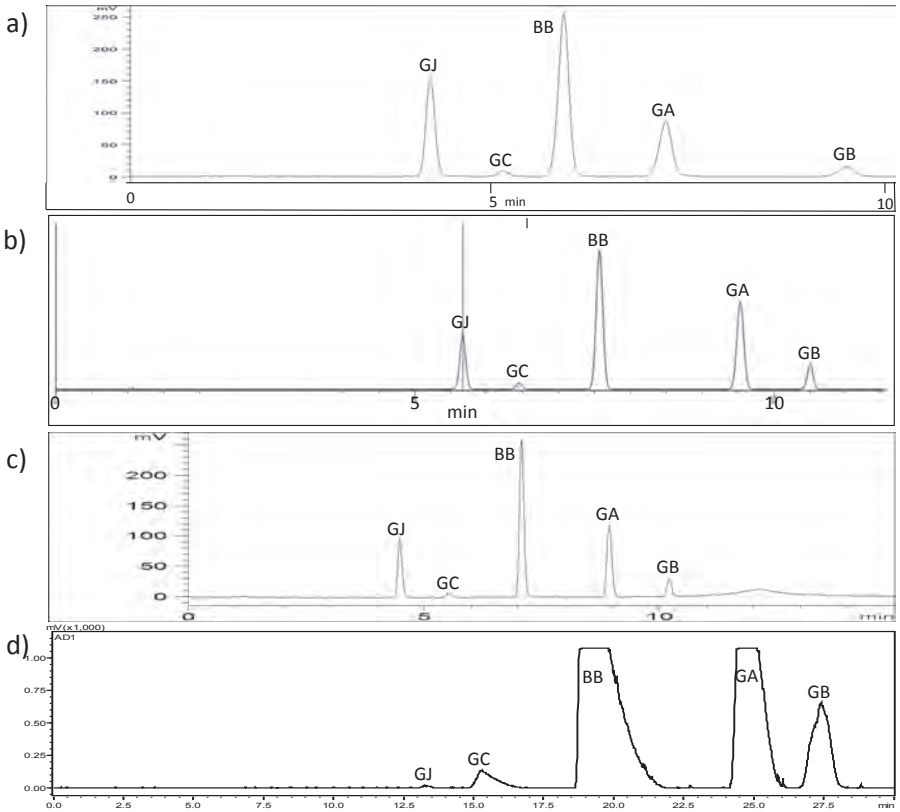


Fig. 4 a) Benchmark chromatogram of 5 TTLs with methanol-THF-water (2:1:7) as mobile phase; b) simulated chromatogram for the optimal UHPLC mobile phase; c) experimental chromatogram for the optimal UHPLC mobile phase; a & c on a 100×2.1 mm C18 column at 0.20 mL/min; d) pHPLC chromatogram for the optimal mobile phase condition (250×22 mm C18 column, 480 mg injected).

After assigning the chromatograms, the software automatically calculates 1 million chromatograms and presents the optimal gradient. The best gradient for separation of the 5 TTLs was 22% B2 in B1 used as mobile phase B (corresponding with water-acetone-ethanol-ethyl acetate (22.2:52.65:8.6:16.55), a column temperature of 22.5 °C, and a linear gradient from 1% to 38.56% in 12.28 min). Under these conditions, the predicted minimal resolution was 4.8 (for GC and BB), which was, as expected, a bit higher than the best Rs_{min} of 4.4 found in the simplex design study. As BB is normally the component present in the highest concentration in GBE, a higher resolution for BB with GC and GA is desirable as it would allow the injection of more sample/run. The optimum temperature of 22.5 °C means effectively room temperature, which is convenient for pHPLC as it requires some trouble and energy to pre-heat the high flow of eluent and to cool it afterwards. The software allowed for a further optimization of the gradient aiming at a higher resolution for BB, while keeping the mobile phase composition and column temperature fixed. The final stepwise UHPLC gradient (Table 3, Fig. 4b) should give a minimum resolution of 5.14 for GJ and GC with a gradient time of 10.46 min. It was selected as the best condition for the next steps of experimental verification and preparative application.

Table 3 Best gradient conditions and scale-up from UHPLC via HPLC to pHPLC

UHPLC		Analytical HPLC		Preparative HPLC	
Time (min)	B%	Time (min)	B%	Time (min)	B%
0	21	0	21	0	21
3.72	21	11.5	21	12.25	21
4.86	30	11.66	30	12.38	30
7.37	30	17.68	30	18.78	30
10.46	59	25.09	59	26.65	59
15	21	35.99	21	30	21

3.6. Experimental verification

First the best predicted condition was carried out on a real UHPLC instrument. The simulated and experimental chromatograms are compared in Fig. 4b and 4c. The predicted and experimental retention times of each TTL do not match perfectly but the deviation stays within ± 0.6 min. Resolution and peak profile, however, are quite coherent with each other. The experimentally determined minimal resolution (5.76) was even higher than the simulated one (5.14). The worst resolution (GC and BB) obtained with the THF-methanol benchmark

solvent (Fig. 4a) is only 3.13, so much lower than the minimal resolution (5.76) of the new less toxic mobile phase system. The analysis time for both conditions is around 10 min. This means the acetone-ethyl acetate-ethanol mobile phase should display a higher throughput when scaled up to pHPLC.

The UHPLC gradient was then adapted to correct for column lengths, first to analytical HPLC (4.6 mm i.d.) and then to preparative HPLC (22 mm i.d.) (Table 3). We knew from other applications that all three columns used are very comparable so no major shifts in selectivity were expected. The flow rate was also adapted to maintain the same linear flow. Preparative HPLC experiments using a Ginkgo leaf extract highly enriched in TTLs (mostly GA, GB and BB) were carried out for real-life testing of the greener eluent. The pressure during the run was approximately 150 bar. Per 30 min pHPLC run around 99 mL of acetone, 16 mL of ethanol and 31 mL of ethyl acetate were consumed besides water. After some trial runs, it proved possible to inject per run 480 mg of sample dissolved in acetone-water (1:1, v/v) while still preserving baseline separation of all five TTLs (Fig. 4d). White crystals were obtained after evaporation of the solvent *in vacuo* and recrystallization. The final yield for GA, GB, GC and BB was 98, 38, 18 and 176 mg respectively. The amount of GJ was very small. The purities of the 5 TTLs as determined by UHPLC were all above 99.5% (see SI). Quantitative NMR showed the absolute purity for GA, GB, GC and BB to be 95.3%, 96.2%, 95.7% and 95.3% respectively (see SI).

4. Conclusion

After taking into account both green and practical issues of which toxicity for the user counted most, ethanol, acetone and ethyl acetate were proposed as possible replacements for methanol, acetonitrile and THF in preparative RP-HPLC. During a two-step optimization procedure it became obvious that all three alternatives exhibited significantly different selectivity, at least for our model analytes, five closely related terpene trilactones. This is desirable. The results also showed that a one-step chromatographic optimization procedure would not have led to current optimal outcome, that is, the two optimization procedures are complementary. The best separation is achieved by a gradient in which the weak solvent is water and the strong ternary solvent is water-acetone-ethanol-ethyl acetate (22.2:52.65:8.6:16.55). This gave a minimal resolution of 5.76, which

is much higher than that of the best traditional (Rs_{min} = 3.13) solvent containing THF and methanol. This shows that greener can actually mean more efficient too. An additional advantage of the green alternative is that on average its elution strength is higher meaning that less organic solvent is needed. For this reason, and because the expensive THF is not needed, in this case greener also equals cheaper. The alternative mobile phase system was successfully scaled up from 100 × 2.1 mm UHPLC to 250 × 22 mm pHPLC with a real sample while preserving baseline separation. Selectivity was not affected. All the data prove convincingly for at least this sample that alternative less toxic, cheaper solvents can work equally well if not better than traditional solvents for RP-HPLC. As the combined optimization procedure is generic, it is expected that it will also work for other analyte mixtures. For other applications however the proposed solvents do have their limitations: acetone is problematic if UV detection is mandatory and the limited miscibility of ethyl acetate is a problem if it is the major organic modifier and the analytes are non-polar. Methyl acetate might serve as a substitute for ethyl acetate in some cases. The positive results might inspire others to move away from the acetonitrile-methanol mindset and experiment with more benign solvents for their pHPLC separations.

Acknowledgements

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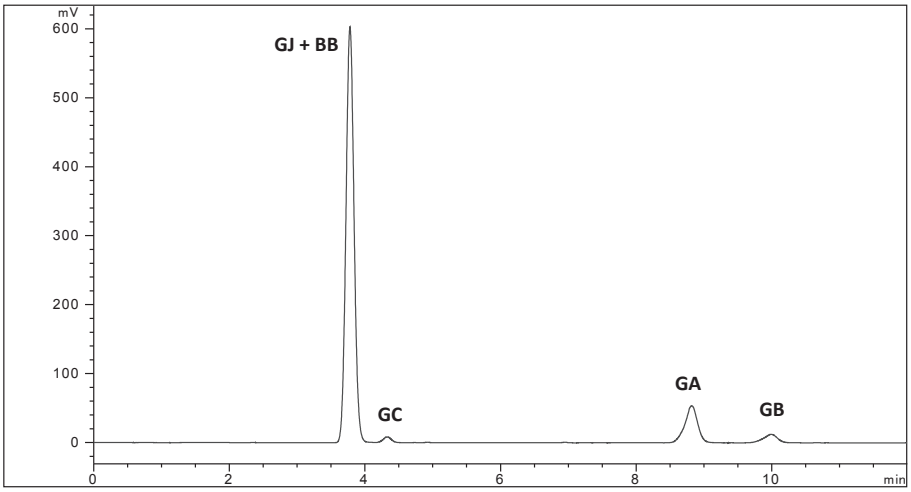
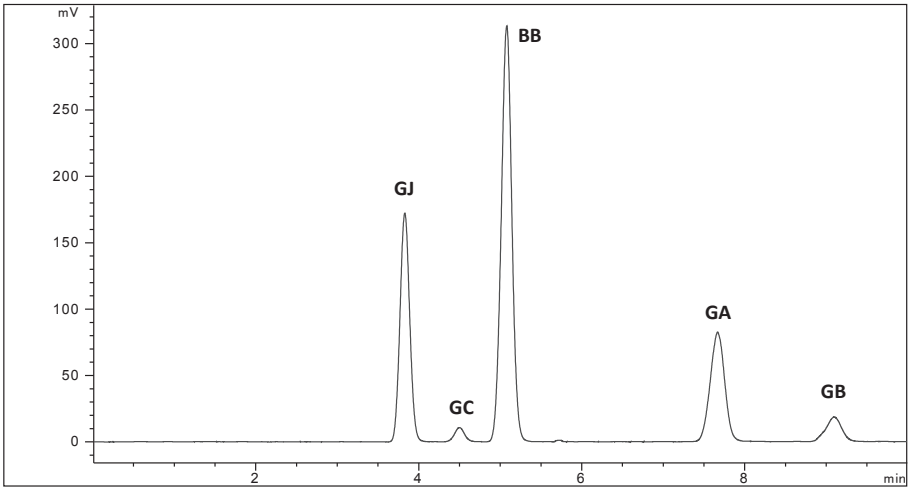
S1. Chromatogram of 5 TTLs under isocratic conditions with the 13 solvent compositions indicated in Fig. 1b as mobile phase.



Point 1



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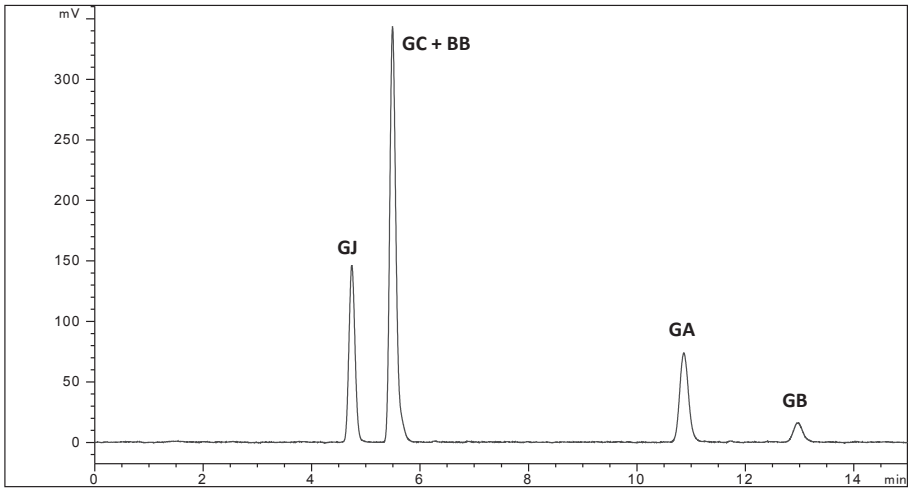

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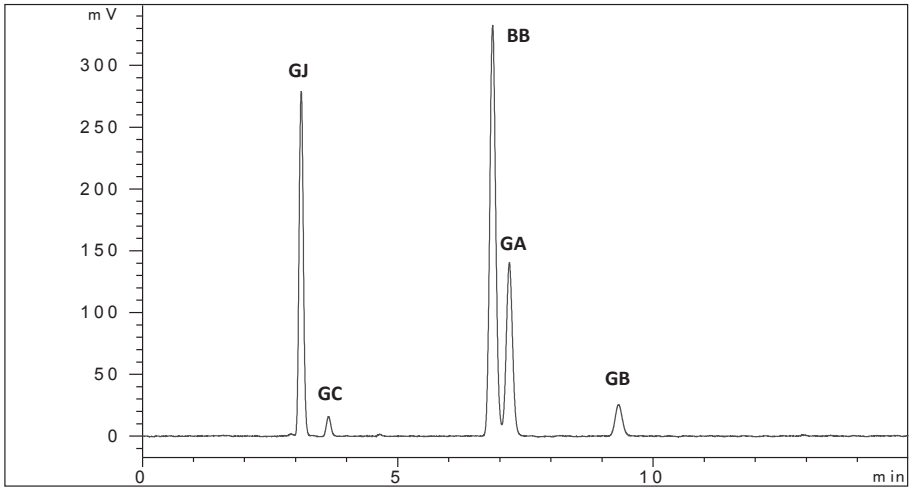

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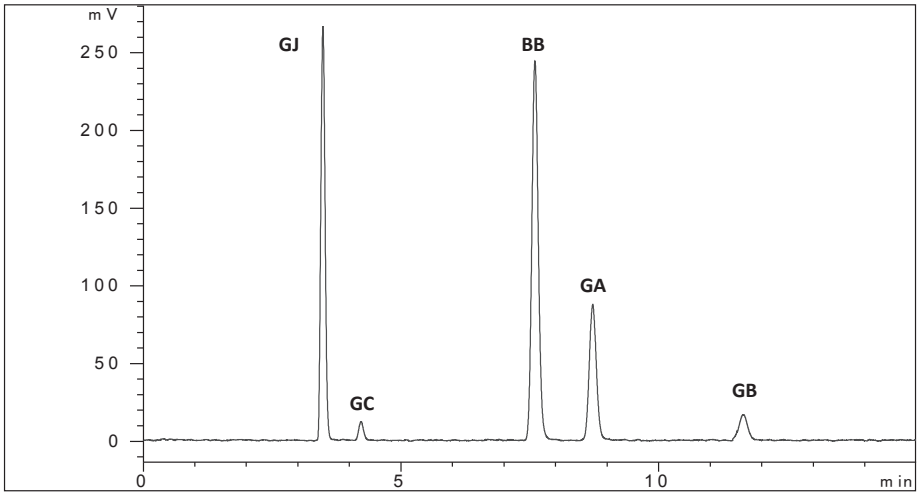

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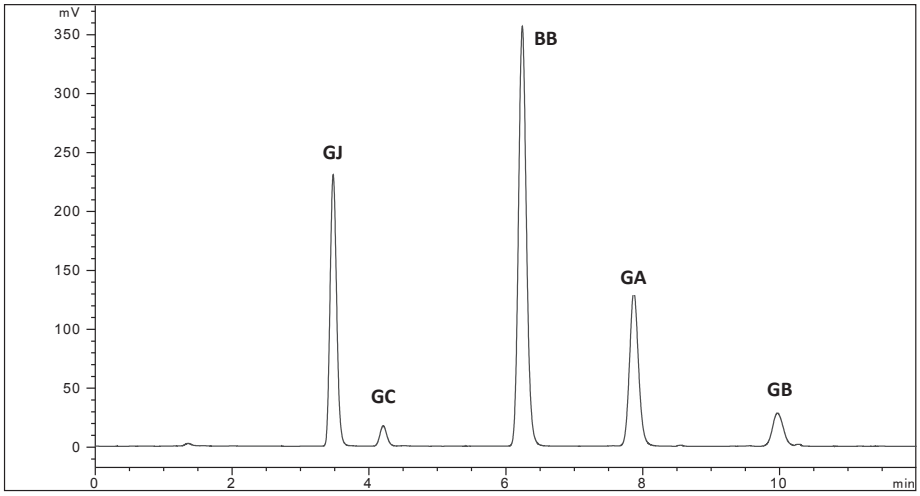

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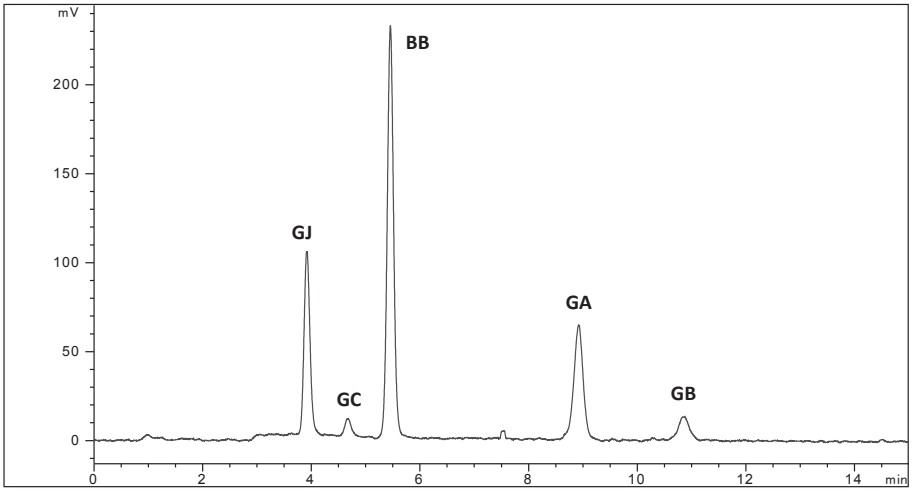

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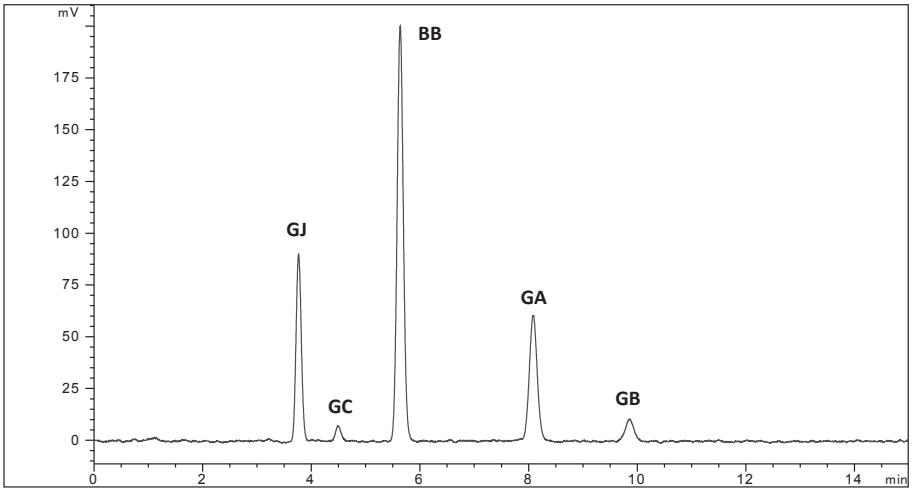

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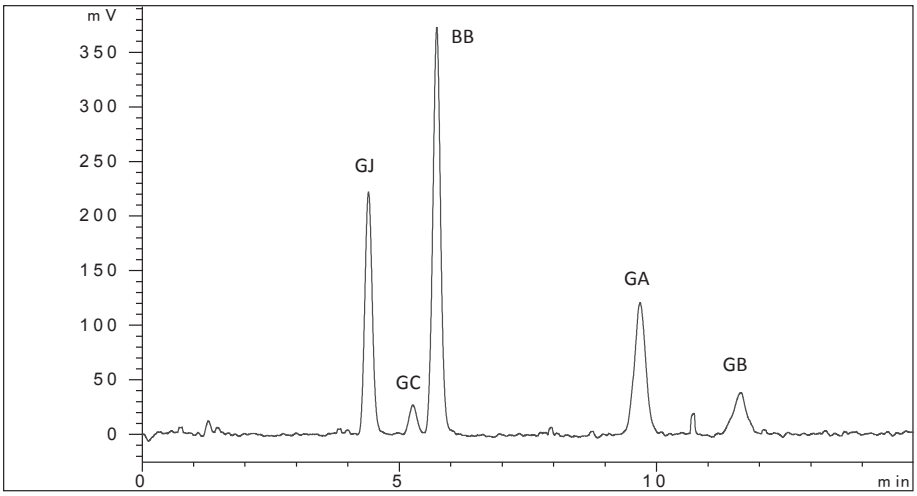

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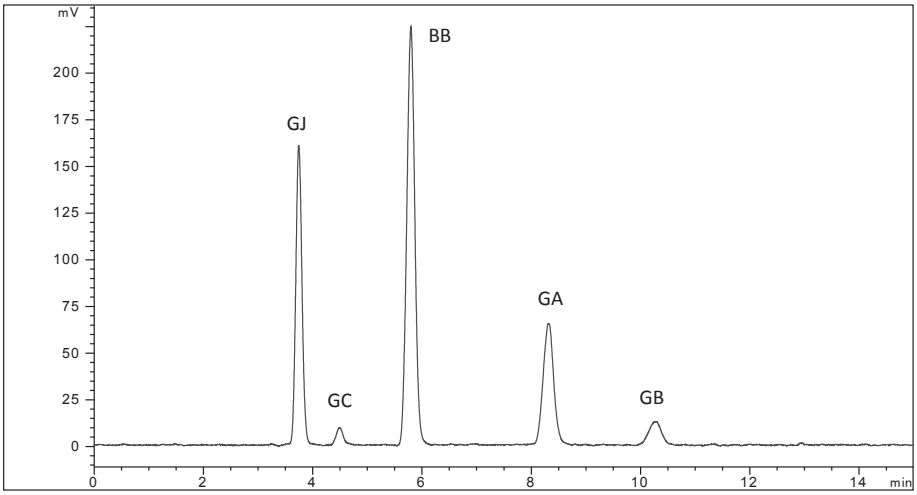

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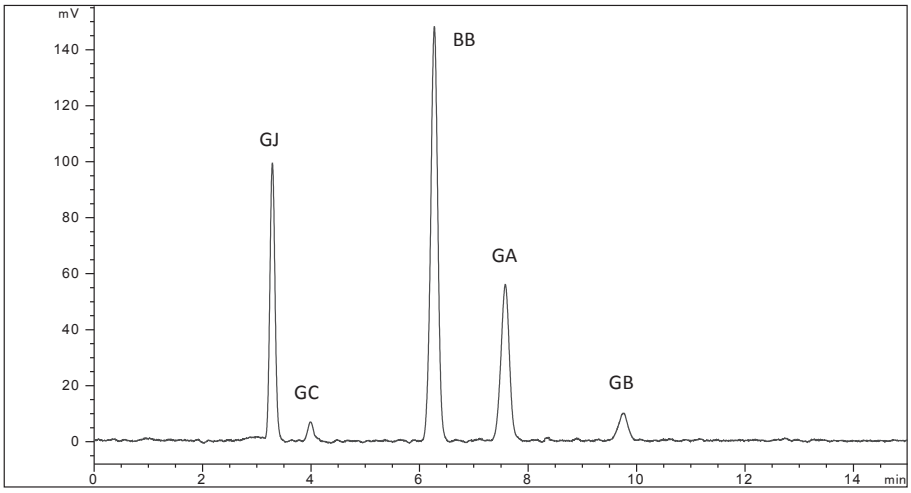

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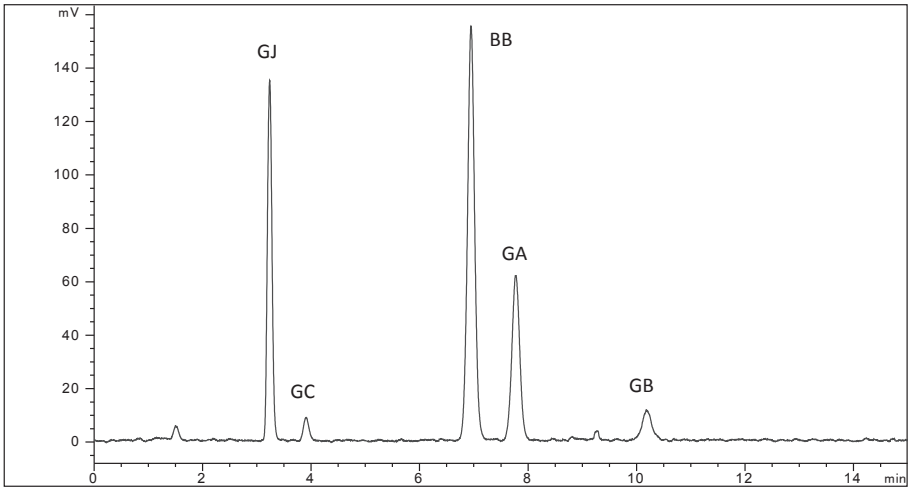

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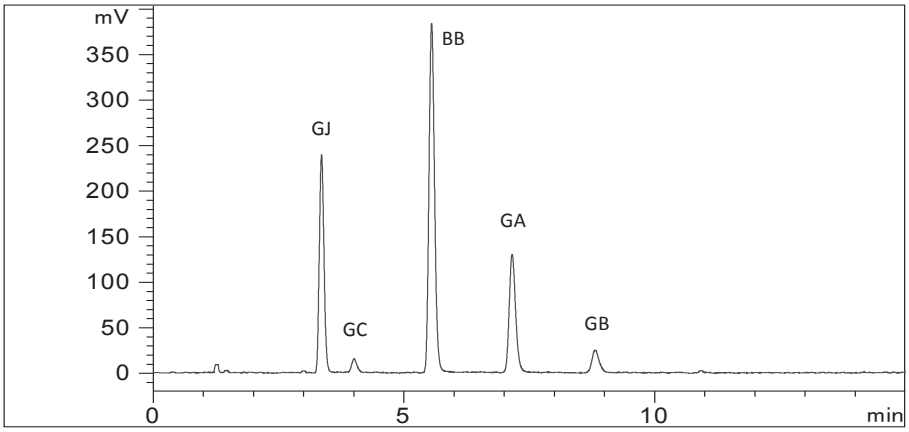
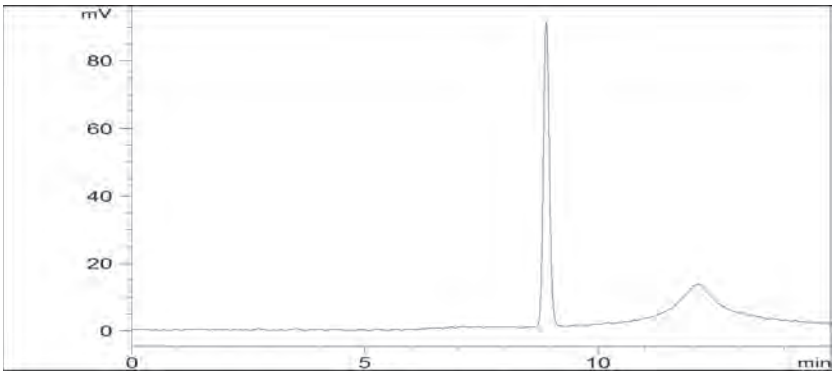


Point 15

S2. UPLC Chromatogram of purified GA, GB, GC, GJ and BB



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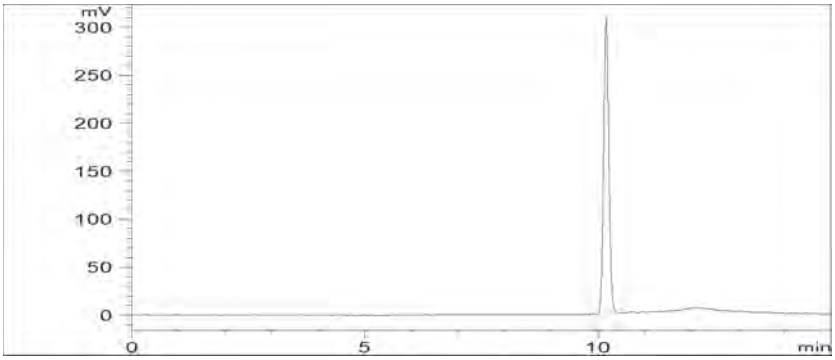

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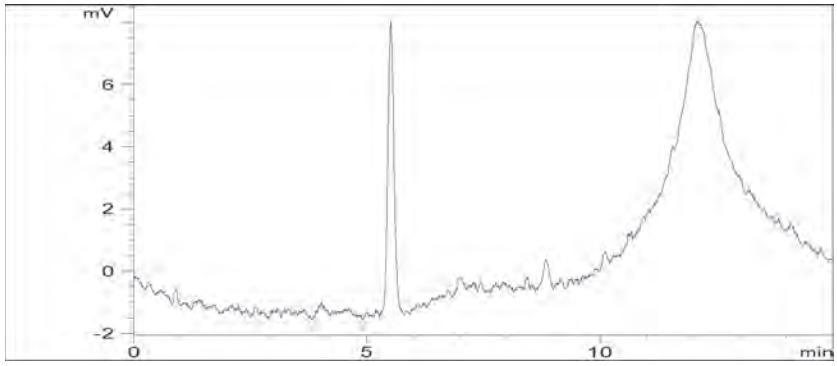
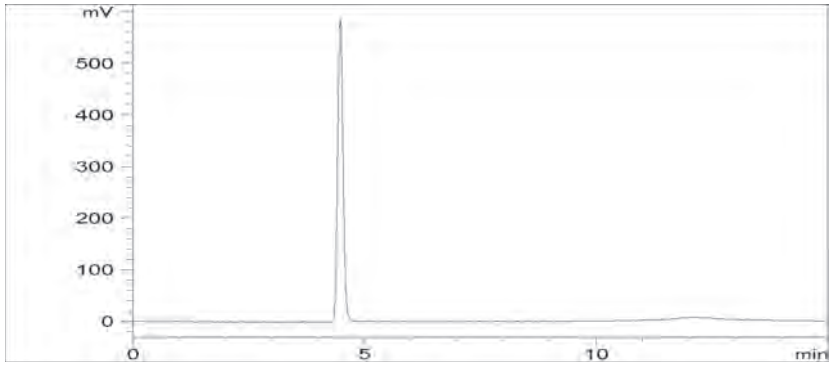

mV
6
4
2
0
-2
0
5
10
min

GC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5
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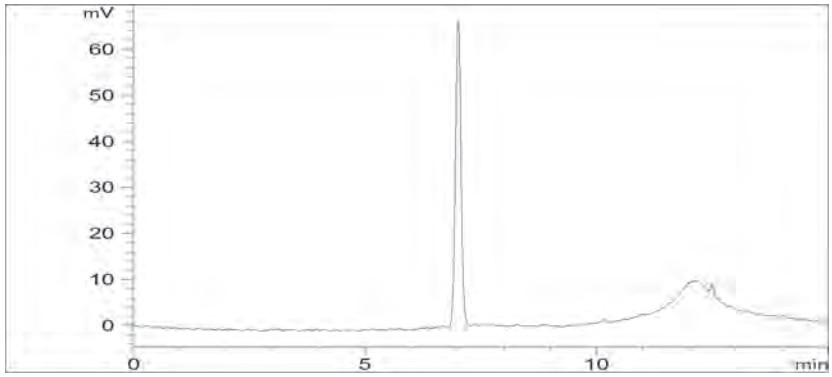
GJ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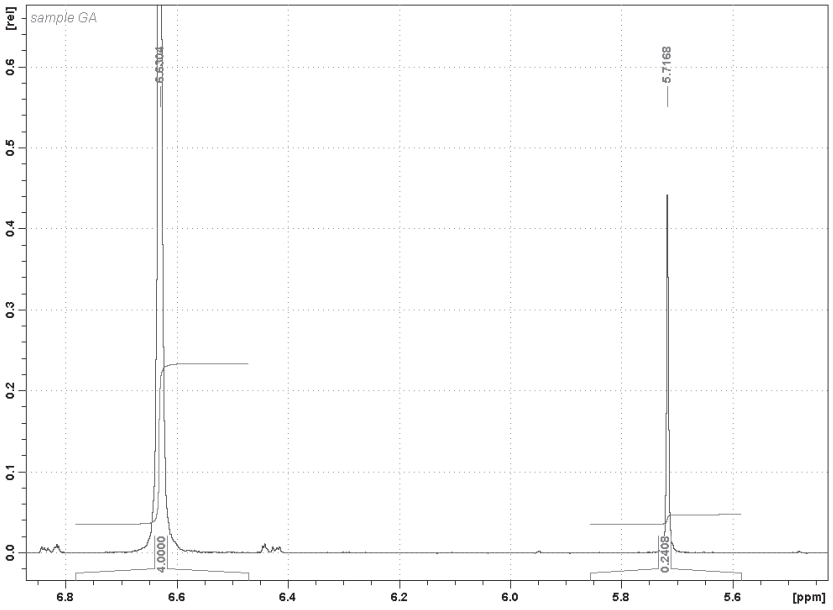
min

BB

S3. Relevant part of quantitative NMR spectra of purified GA, GB, GC and BB



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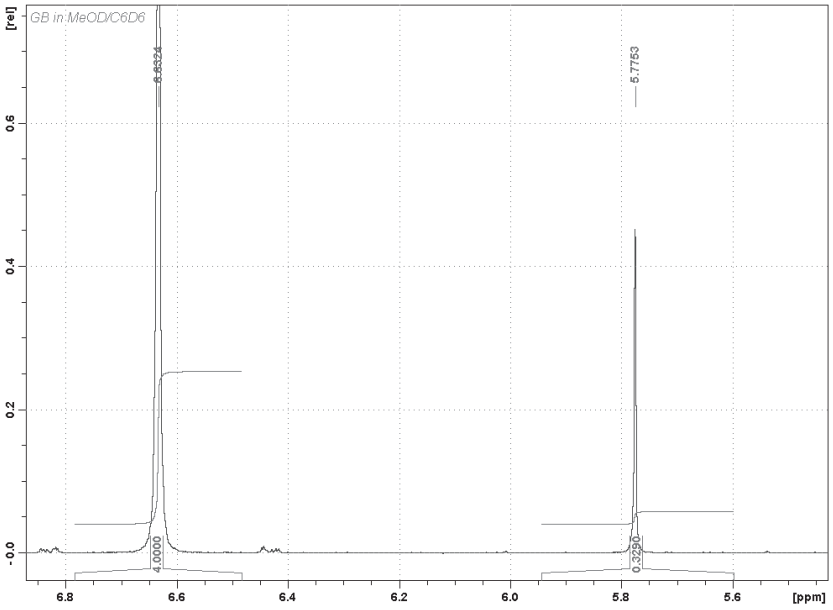

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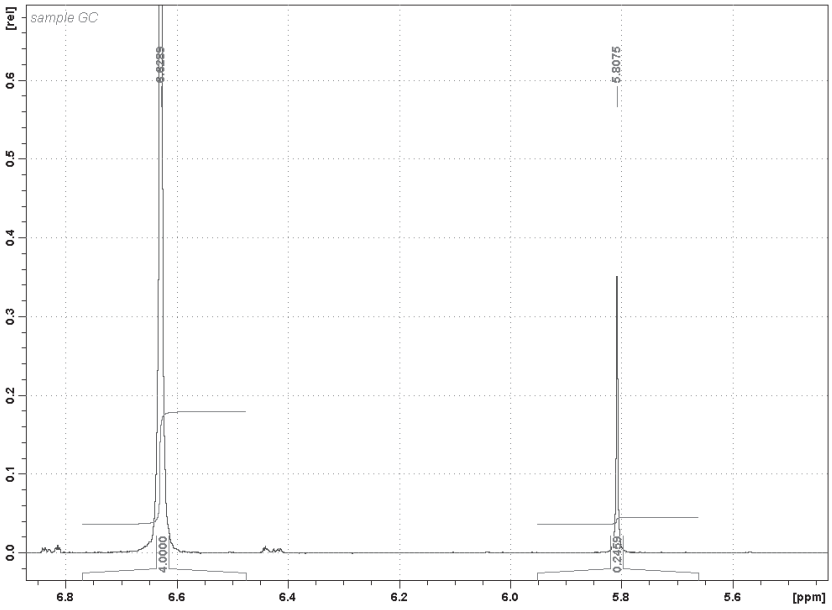
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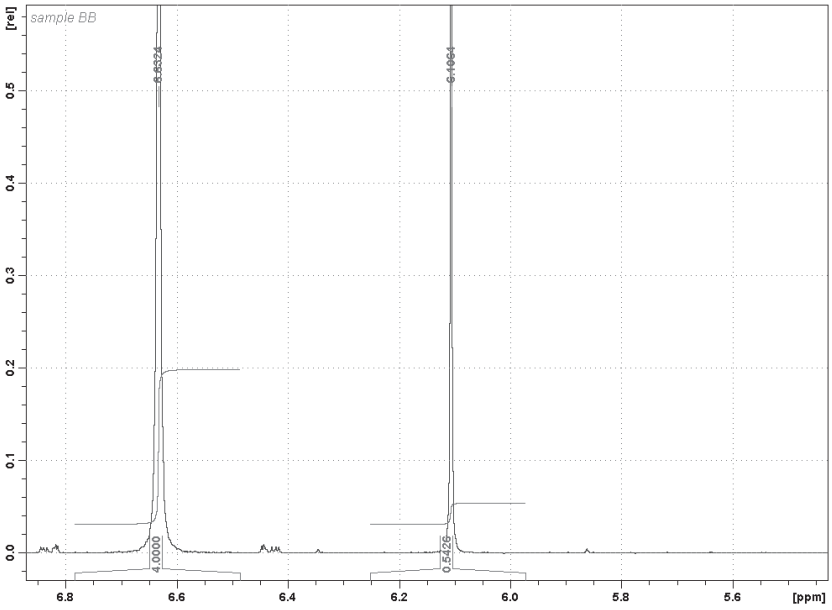
GC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BB

Chapter 8

Applications of High Performance Liquid Chromatography in the Quality Control of Traditional Chinese Medicines, an Overview

Abstract

A characteristic of traditional medicinal practices, e.g., Traditional Chinese Medicines (TCMs) and Indian Ayurveda is the abundant use of plants as drugs. However, the bottleneck in the use of herbal drugs is the control of their safety, effectiveness and quality. Good Quality Control (QC) is necessary to achieve the standards of modern medicines. HPLC, being a powerful universal separation technique, is widely available in laboratories and easily hyphenated with other spectroscopic and biochemical techniques. A number of HPLC-based strategies – such as multi-component quantitation, fingerprinting, bioaffinity chromatography and on-flow assays for screening – have been used for the comprehensive QC of TCMs, lead-finding and identification of active constituents. In this chapter, applications and developments of such HPLC-based methods for TCMs and other herbal drugs as well as new developments in sample pretreatment prior to HPLC analysis are presented and discussed.

Chapter based on: Y. Shen, T.A. van Beek, H. Zuilhof and B. Chen, Applications of Liquid Chromatography in the Quality Control of Traditional Chinese Medicines, an Overview. Chapter 19 in: S. Fanali, P.R. Haddad, C.F. Poole, P. Schoenmakers and D. Lloyd (eds.), Liquid Chromatography Applications, Elsevier, Amsterdam, **2013**, pp. 519-540.

1. Introduction

Phytomedicines are among the oldest forms of healing, and are globally common to all cultures. There are Western Herbal Medicines, Traditional Chinese Medicines, Indian Ayurveda, etc. Although they are based on different concepts and theories, e.g., TCM formulas are based on yinyangnism, and Ayurveda is derived from Indian philosophy, they employ a common material substance, i.e., herbs. It is noteworthy that Traditional Chinese Medicine (TCM) is the most ancient and best-documented one among them, and uses a wide variety of plant species. The healing efficacy of TCMs has gradually improved by trial and error over thousands of years.[1,2]

To date, 12,806 different natural resources have been used for medicinal purposes in China, including 11,145 plants, 1581 animals, and 80 minerals.[3] TCMs are multi-component systems with an abundant diversity in composing chemical structures, which contributes to the complexity of TCMs. The concept of combining different compounds and increasing therapeutic effectiveness through synergism, minimizes toxicity and side effects, and optimizes the therapeutic effect of individual components. For example, there may be fifteen plants each containing 100 compounds in one TCM formula.[4] This feature not only makes taxonomy, authentication, isolation, elucidation of chemical constituents, quality control, pharmacology, and toxicology of TCMs hard work for researchers, but also slows down the globalization and modernization of TCMs. Therefore, the application of modern analytical techniques is important for the elucidation of the composition of TCMs and for the quality control of TCMs. A large number of papers and reviews on this topic has recently been published.[5-11] Among these techniques, High Performance Liquid Chromatography (HPLC) takes a prominent position as it is in principle a universal high-resolution separation technique capable of analyzing widely different classes of compounds in complex matrixes.

In this chapter, recent approaches for analysis of TCMs by means of liquid chromatography (LC) are reviewed, including new techniques for sample pretreatment of TCMs prior to LC analysis, multi-component quantitation, fingerprinting, and activity screening. Although all applications relate to TCMs, the LC techniques and methodologies discussed can also be applied to the quality control of other herbal drugs.

2. Novel techniques for sample pretreatment of TCMs

Being complicated multi-component systems consisting of many plants, sample pretreatment of TCMs to diminish peak overlap, column degradation or ion suppression is often mandatory prior to LC analysis. In addition, for elucidating the mode of action of TCMs, some scientists focus on investigating the pharmacology of TCMs. To achieve this, the active compounds of TCMs need to be isolated and enriched not only from materia medica but also from biofluid samples (e.g., plasma, serum, urine, tissues, and cells) by bioassay-guided fractionation. The extraction of TCMs is routinely performed by conventional methods, such as simple refluxing, Soxhlet extraction, ultrasound-assisted extraction (UAE) or microwave-assisted extraction (MAE). The most used sample pretreatment approaches are liquid-liquid extraction (LLE) and solid-phase extraction (SPE).[12-15] However, they suffer from some disadvantages. For example, normally extraction methods have a low selectivity, and sample pretreatment steps are typically time-consuming and labour-intensive. New technologies are needed for faster and more selective approaches. In this section, several potentially promising new developments in sample pretreatment of analysis of TCMs (including materia medica and biofluids) are highlighted.

2.1. Molecularly-imprinted polymers (MIPs)

The molecular imprinting technique (MIT) has emerged as a useful tool for highly selective sample preparation or separations.[16] Due to their high affinity, selective analyte recognition, and physical and chemical robustness, MIPs have been successfully used in the analysis of flavonoids, alkaloids, polyphenols, steroids and coumarins from TCMs.[9, 17, 18] For instance molecularly imprinted surface layer-coated core-shell silica nanoparticles have been used for the highly selective separation of diosgenin (DG) from *Dioscorea nipponica*.[19] The DG-imprinted layer was prepared via a condensation/gelation reaction between silanol groups of the silica nanoparticles and the ethoxysilane groups of DG-3-isocyanatopropyltriethoxysilane and tetraethoxysilane. Removal of the templates by a simple thermal cleavage reaction of the urethane bond created the recognition sites of DG in the silica coating layer. The DG-imprinted silica nanoparticles were evaluated by binding experiments. The advantages of MIPs together with advantages like high specific-surface area, better site accessibility and lower mass-transfer resistance of nanoparticles, caused the adsorption efficiency of the

imprinted nanoparticle silica for DG to reach 99%, which was almost 5 times as much as commercial silica (21%) and non-imprinted core-shell silica (25%). Fig. 1[19] shows the comparison of the bound amount of DG and three other analogous structures (sarsasapogenin, digitoxigenin and oleanolic acid) on the DG-imprinted silica nanoparticles. This shows the imprinted silica had high molecular selectivity for DG.

If they possess enough capacity and selectivity, MIPs can uniquely speed up the sample preparation. However they are only useful for a targeted approach, i.e., either for a preparative isolation or the quantitative analysis of one or more very similar bioactives or marker compounds. For multi-component quantitation or fingerprinting of TCMs they have no merit. A clear downside is their time-consuming synthesis and validation. Thus we expect them to play a niche role in QC of TCMs but a useful one for that.

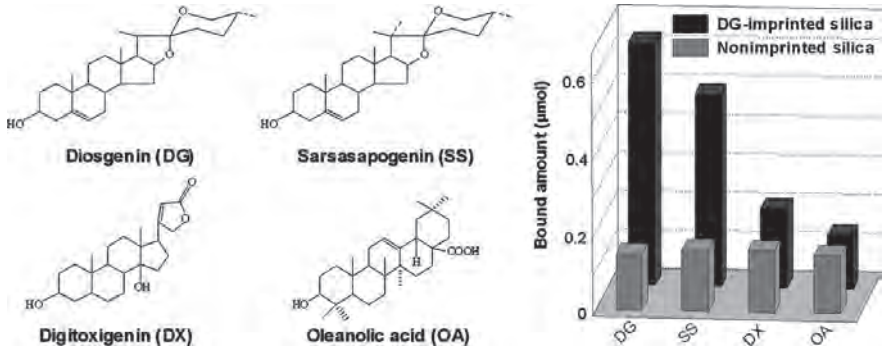


Fig. 1. Molecular selectivity of DG-imprinted silica nanoparticles and non-imprinted nanoparticles. Reprinted with permission from Elsevier.[19] Copyright (2009) Elsevier.

2.2. Microfluidic devices

With the development of microfluidic techniques, chip-based approaches have been introduced for the pretreatment of chemical and biochemical samples. Advantages of miniaturised devices are the high speed of analysis, the possibilities for automation, low sample and solvent consumption, possible integration of sample pretreatment, separation and detection, and as a result of all this significant savings in labor costs.

LLE, as one of the oldest and most robust sample preparation techniques, is especially valuable for the isolation of organic acids and bases. Natural bases like

alkaloids can be effectively purified by two consecutive LLE steps. Polar impurities like sugars, salts and amino acids are retained in the alkaline aqueous phase, while non-polar , triacylglycerols, sterols, waxes and other lipids remain in the organic phase after an extraction with acid. However, these steps are labor-intensive, solvent-intensive and slow. Tetala et al. have developed a microfluidic 3.5 cm three-phase chip, which realized both extraction and back extraction within 30 sec in a chip.[20] Strychnine which occurs naturally in *Strychnos nux vomica* was used as model compound. According to the Chinese pharmacopoeia,[21] seeds of *S. nux vomica* are often incorporated with other herbs to treat conditions such as pain and swelling, inflammation of the throat, arthralgia and rheumatism, while the leaves are applied as a poultice on sloughing wounds and ulcers. Because of its high toxicity, its use is more restricted than that of other TCMs, i.e., quality control of Strychnos and other toxic plants material is very important. The general design of the three-phase chip is shown in Fig. 2. The three solvents (basic aqueous feed phase with alkaloids, organic phase and acidic aqueous trapping phase) were infused into the chip from one side and collected at the other side of the chip for off-line HPLC detection. With chloroform as organic phase, 79.5% of strychnine was extracted to the acidic aqueous phase in 25 sec. This rapid transport is due to large surface area-to-volume ratio and high mass transfer efficiency at this small scale. Strychnos seed was investigated in this three-phase chip as a real sample. Fig. 3 shows that the purification of a Strychnos seed extract was successful, as pure strychnine and brucine were extracted into the acidic aqueous phase, leaving polar compounds in the feed phase, and non-polar (retention time > 50 min) impurities in the organic phase.

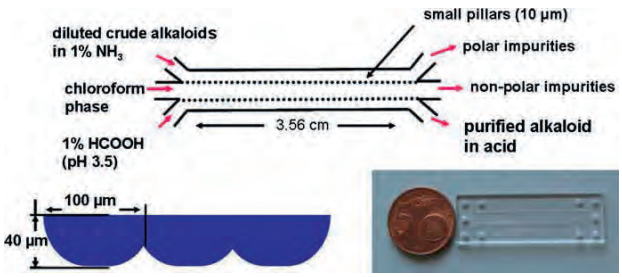


Fig. 2. Upper drawing: general lay-out of 3-phase microreactor capable of continuously separating a crude alkaloid fraction into three separate fractions; bottom left: lay-out (side view) with dimensions of the three parallel channels, the solvents in the channels make direct contact; bottom right: real image of 5 × 2 cm glass 3-phase chip, the holes at the side are for the fluid connections. Five Euro cents coin is for size comparison only. Reprinted with permission from Springer.[22] Copyright (2009) Springer.

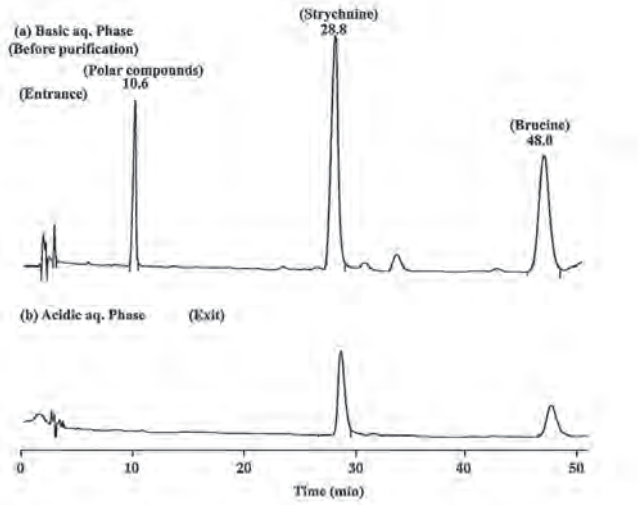


Fig. 3. HPLC profiles of a Strychnos seed extract before and after purification using a three-phase chip at a flow rate of 1 : 2 : 1 µL/min: (a) basic aqueous phase before purification, (b) acidic aqueous phase. Reprinted with permission from Royal Society of Chemistry.20 Copyright (2009) Royal Society of Chemistry.

If 3-phase chips could be robustly integrated on-line with extraction cells, nanoHPLC and/or MS in a such way that it would allow for quantitative analyses, it would really give a boost to the fast and low manpower analysis of alkaloid-containing plants and drugs. Also a replacement for chloroform needs to be found as this solvent is toxic, expensive and non-degradable.

2.3. Hydrophobic solvent induced phase transition extraction

Phase transition extraction is an extraction by means of a sudden phase separation of an initially homogeneous mixed solution followed by a selective partitioning of analytes in one of the two phases. The phase separation can be brought about by changing the temperature or adding modifiers. Advantages of this technique are the high extraction yield, the direct concentration effect and simple operation compared to liquid-liquid extraction. Examples of this technique include salting-out extraction, subzero-temperature extraction and cloud-point extraction.[23-27] However, there are also potential disadvantages, like the resulting high salt concentrations in the organic solvent, which are harmful to any MS detector, and the subzero-temperature conditions that make transferring the extractant difficult. A recent study by Liu et al. revealed that hydrophobic organic solvents such as dichloromethane can be used as the modifier to induce

the phase separation of a homogeneous acetonitrile-water mixture. This is called hydrophobic solvent-induced phase transition extraction (SIPTE).[28] Six non-oxygenated solvents and four oxygenated solvents were investigated as modifiers. At least 0.3 mL of an oxygenated modifier was needed to induce the phase separation of 2 mL of an acetonitrile-water mixture (1 : 1, v/v). However, when using a non-oxygenated solvent, only 0.05 mL was needed. Fig. 4 shows the effects of the amount of modifier (chloroform) and acetonitrile on the recovered volume of the organic phase. Smaller amounts of modifier resulted in smaller volumes of the recovered organic phase. In addition, the recovered volume of the organic phase has a linear relationship with the amount of acetonitrile used.

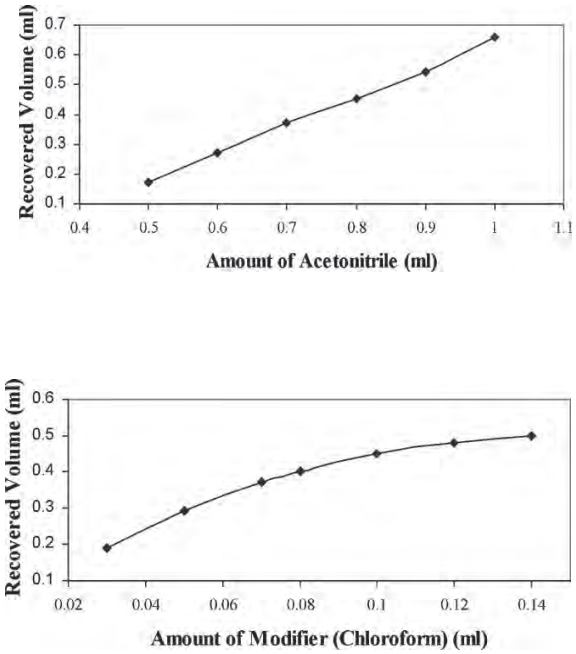
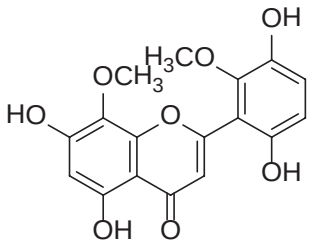


Fig. 4. Hydrophobic solvent-induced phase transition extraction (SIPTE). Left: Effect of amount of acetonitrile on recovered volume of the organic phase. The ratio between the amount of modifier (chloroform) added and the total volume is fixed at 0.1. Right: Effect of amount of modifier on recovered volume of the organic phase. The amount of acetonitrile is fixed at 0.7 mL. Reprinted with permission from Elsevier.[28] Copyright (2010) Elsevier.

SIPTE was successfully applied for analysis of three TCMs (*Scutellaria baicalensis*, *S. barbata* and *Citrus reticulata*).[29] Taking *S. baicalensis* as an example, Fig. 5 shows the HPLC profiles of the acetonitrile and aqueous phases. This approach not only shows the high recovery of SIPTE (up to 100%), but also the high selectivity for flavonoid aglycones *versus* flavonoid glysosides in *S. baicalensis*. In the HPLC profile (Fig. 5C), aglycone **3** (5,7,2',5'-tetrahydroxy-8,6'-dimethoxyflavone) coelutes with glycoside **4** (baicalin) in the initial extraction solution. However, after performing the SIPTE process, **3** and **4** appear in the acetonitrile and aqueous phases respectively. The results were confirmed by ESI-MS.



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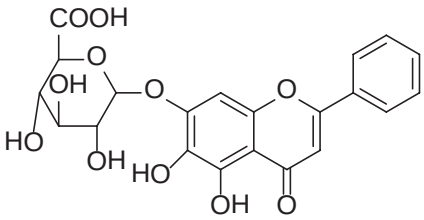

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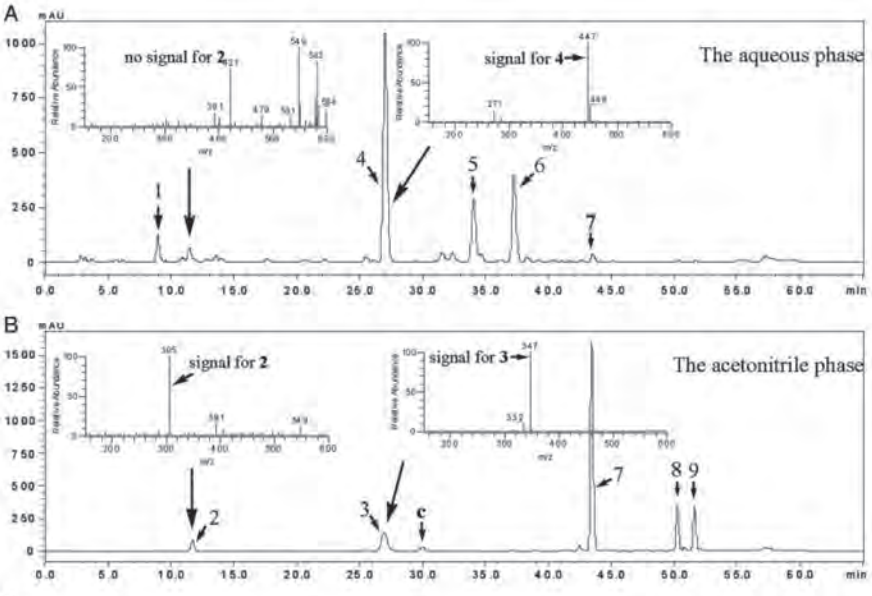


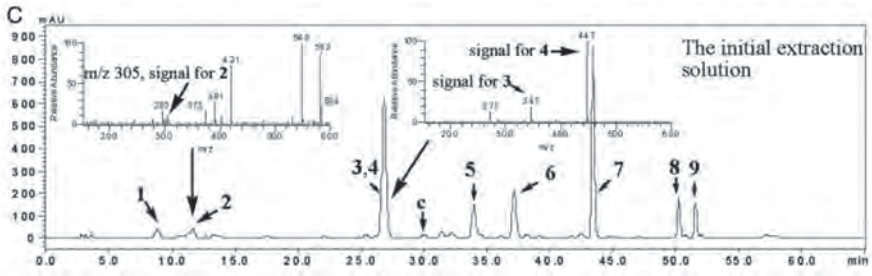


Fig. 5. Top: structures of **3** and **4**; Below: HPLC-UV profiles of aqueous SIPTE fraction (A), organic SIPTE fraction (B) and total extract of *S. baicalensis* prior to SIPTE (C). **1**: Chrysin-6-C-arabinosyl-8-C-glucoside, **2**: 5,7,3,2',6'-pentahydroxyflavanone, **3**: 5,7,2',5'-tetrahydroxy-8,6'-dimethoxyflavone, **4**: baicalein-7-O-glucuronide (baicalin), **5**: oroxylin A-7-O-glucuronide, **6**: wogonin-7-O-glucuronide (wogonoside), **7**: 5,6,7-trihydroxyflavone (baicalein), **8**: 5,7-dihydroxy-8-methoxyflavone (wogonin), **9**: 5,7-dihydroxy-6-methoxyflavone (oroxylin A), c: unkown. **1**, **4**, **5** and **6** are flavone glycosides, while **2**, **3**, **7**, **8** and **9** are flavone aglycones. Reprinted with permission from John Wiley & Sons Limited.[29] Copyright (2011) John Wiley & Sons Limited.

This approach was also employed for *in vivo* TCM pharmacokinetic research. Liu et al. extracted the diterpene lactone andrographolide by SIPTE from plasma, and analyzed it by HPLC-ESI-MS afterwards.[28] Andrographolide, the main bioactive component of the plant *Andrographis paniculata*, has antinflammatory and antiischemic properties[30] besides antiviral properties, and is widely used in many TCMs. The SIPTE recovery of andrographolide from spiked plasma was 90%, which is higher than when using LLE. Additionally there was almost no ion suppression due to matrix effects, i.e., samples were very clean apart from the analyte. When using LLE or protein precipitation (PPT), matrix effects were substantial and signals were 25-41% lower. The results indicate that SIPTE appears to be an attractive new addition to existing sample pretreatment methods. It combines sample clean-up and preconcentration and the acetonitrile phase after removal of the aqueous phase is still suitable for HPLC-MS analysis unlike acetonitrile phases after a salting-out sample pretreatment. Automated SIPTE, e.g., via selective sampling with a robotic autosampler or on-chip with on-line analysis of both phases by HPLC would make this new technique even faster and less susceptible to human errors. However it also has limitations. There are only two phases and not in all cases will it be possible to get a perfect distribution of each class of analytes. All depends on the distribution coefficient and method development could prove time-consuming.

3. HPLC analysis of TCMs

HPLC is such a powerful separation tool because multiple different separation mechanisms can be employed: partitioning (RP), adsorption (NP), HILIC, ion-exchange, or gel permeation. This makes it suitable for both volatile and non-volatile, polar and non-polar, and low and high MW analytes. Due to the developments in packing, chemistry, sub 2 μm particles, and hardware (pressure > 1000 bar) the separation efficiency has steadily increased with UHPLC using multiple core-shell columns (N_{th} ≈ 100,000) being the current state-of-the-art. Such developments allow for either 1 min analyses with medium resolution or > 1 hour separations with extremely high resolution. Not surprisingly then, (U) HPLC is the cornerstone of TCM QC. It first started with determining one or two marker compounds but has evolved into the simultaneous quantitation of preferably all known bioactive constituents. This is the topic of section 3.1. However sometimes the mode of action of a TCM has not been clarified and then an overall image of preferably all constituents relative to a standard is the best solution. This is highlighted in section 3.2. Combining two different separation mechanisms or hyphenation with multiple detectors can increase the information content. Finally HPLC can also be hyphenated with post-column (bio) chemical assays for faster lead-finding and examples are presented in section 3.3.

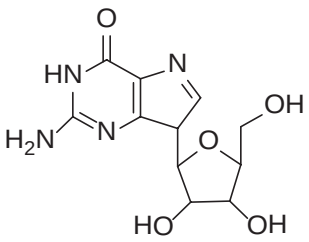
3.1. Multi-compound quantitation

Having many active synergistic constituents (multi-component character) interacting with multiple receptors in the human body is seen as a major advantage of TCMs and pivotal for the overall therapeutic effect.[2] However the multi-component character is also a major obstacle for the quantitation of components, identification of structures, explanation of the mode of action and elucidating metabolic pathways. To date, there are hundreds of published papers on the simultaneous determination of multiple components in crude materia medica or finished TCMs.[10,31-39]

Developing a quantitative multi-compound assay is a challenging task, especially so if several analytes from different compound classes need to be simultaneously analyzed. The validation may take many months. ESI-MS as detector is becoming more and more common as it combines high sensitivity with high selectivity. In our view a state-of-the-art assay should fulfill as many requirements as possible of those listed below.

– Simple, i.e. using standard equipment, fast sample pretreatment and low manpower input
– Green character, i.e., using small volumes of non-halogenated organic solvents
– Purity of reference substances determined by quantitative NMR[40]
– Extraction efficiency of all analytes ≥ 95%
– Accuracy (absolute recovery after spiking) between 95% - 105%
– Relative recovery of entire analytical process ≥ 90%
– Inter-day precision for all analytes (expressed as RSD) < 5%
– Intermediate precision for all analytes < 10%
– Preferably baseline resolution of all analytes, peak purity ≥ 98%
– Fast HPLC separation, preferably < 30 min
– Selective detection
– Linear calibration curves, $r^2 \geq 0.998$
– Sample stability during HPLC analysis ≥ 98%
– LOQ less than 10% of lowest analyte concentrations in plant or drug
– Easy quantitation (internal or external standardization)

To date no published method meets all the demands in Table 1. However a good procedure is that published by Fan et al. who simultaneously quantified hydroxysafflor yellow A **2**, anhydrosafflor yellow B **9**, the kaempferol glycosides **3**, **4**, **6**, **7** and **10**, and 6-hydroxyapigenin 6-O-glucoside-7-O-glucuronide **8**, together with two other active compounds named guanosine **1** and syringin **5** (Fig. 6) in *Carthamus tinctorius* by HPLC-DAD.[41] The optimized extraction (water, 60 °C, 45 min) was simple, green and fairly selective as no additional sample preparation other than filtration proved necessary. The 10 active compounds were separated on a 5 μm 250 × 4.6 mm C_{18} column in 70 min. Thus the chromatography was rather traditional, i.e., slow and solvent-intensive. All compounds, which were characterized by UV, NMR, MS and HPLC, showed good linearity ($r^2 \geq 0.9989$) and the recoveries at three spiking levels varied from 94.8% to 105.4%. Repeatability and intermediate precision varied between 0.95-3.4% and 0.9-4.65% respectively. Peak purity and sample stability were also checked. The quantitative analysis of those 10 components in 46 batches of *C. tinctorius* samples of different origin was carried out. The total amounts of 10 components varied from 4.88 to 37.55 mg/g, i.e., a 7-fold variation. The concentration of the main active constituent, hydroxysafflor yellow A **2**, varied almost 9-fold. These results indicate that comprehensive QC of *C. tinctorius* is absolutely necessary.



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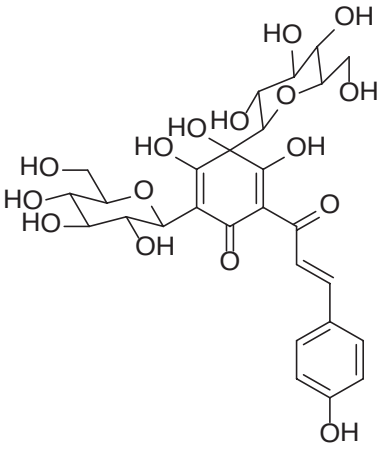

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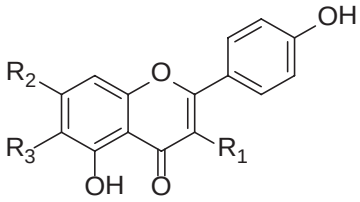
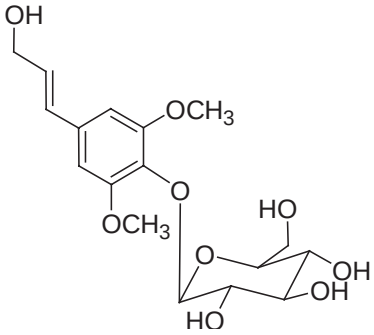


3: R1=R3=*O*-Glucose, R2=*O*-Glucosamine
4: R1=R2=R3=*O*-Glucose,
6: R1=*O*-Rutinose, R2=H, R3=*O*-Glucose
7: R1=R3=*O*-Glucose, R2=H
8: R1=H, R2=*O*-Glucosamine, R3=*O*-Glucose



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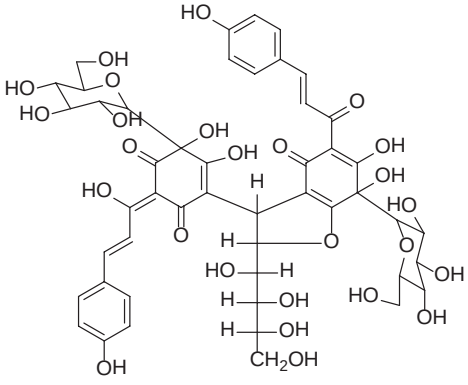

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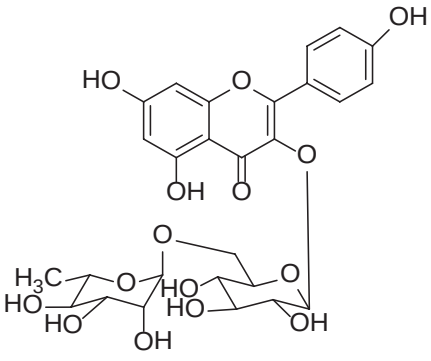


10

Fig. 6. Structures of 10 major active components in *Carthamus tinctorius*

Another example is the simultaneous determination by LC-MS^n of seven active compounds in a plasma sample collected after oral administration of a Zi-Shen pill (ZSP), i.e. xanthone glycosides (neomangiferin and mangiferin), timosaponins (timosaponin E1, timosaponin B-II and timosaponin B) and two alkaloids (palmatine and berberine).[42] Ginsenoside Re (for xanthones and timosaponins) and tetrahydroberberine (for alkaloids) were chosen as internal standards (IS) for MS quantitation. Sample preparation consisted only of protein precipitation with acetonitrile, centrifugation and subsequent dilution with water. For the two xanthones this was apparently not enough as for neomangiferin and mangiferin, ~40% ion suppression and ~70% ion enhancement respectively were observed. Other procedures such as LLE were tried but gave unsatisfactory recoveries for the timosaponins. This shows that it is not easy and perhaps not desirable to aim at a quantitative procedure for multiple chemical classes. Chromatographic separation was achieved on a 3.5 μm 150 mm x 2.1 mm Agilent Zorbax SB-C_{18} column in 9 min at a flow rate of 0.25 mL/min, which is both fast and economical. The MS detection was carried out either in negative (for xanthones and timosaponins) or positive ion (alkaloids) mode with multiple reaction monitoring (MRM). This method shows high selectivity and sensitivity and fair precision (3.8 - 9.2% inter-day) and accuracy (–6.9 - 7.0% bias). The linear range was 5 - 1000 ng/mL for mangiferin, 0.5 - 100 ng/mL for neomangiferin, tinosaponin E1, timosaponin B-II and timosaponin B, 0.05 - 10 ng/mL for palmatine and berberine. The limit of quantitation (LOQ) was 0.2 ng/mL for mangiferin, 0.5 ng/mL for neomangiferin, timosaponin E1, timosaponin B-II and timosaponin B, and 0.05 ng/mL for palmatine and berberine. Extraction recoveries were greater than 80% for all analytes, with the exception of palmatine (range 73.9 - 77.5%) and berberine (range 64.7 - 69. 1%). The latter two figures are a bit low, again bringing up the question whether it is always wise to aim at a multi-class quantitation. Fig. 7 shows the representative chromatograms of blank plasma, blank plasma spiked with analytes and IS, and plasma obtained 4 h after oral administration of ZSP. The concentration-time profiles and pharmacokinetic parameters of these seven constituents in rats could be obtained with this method.

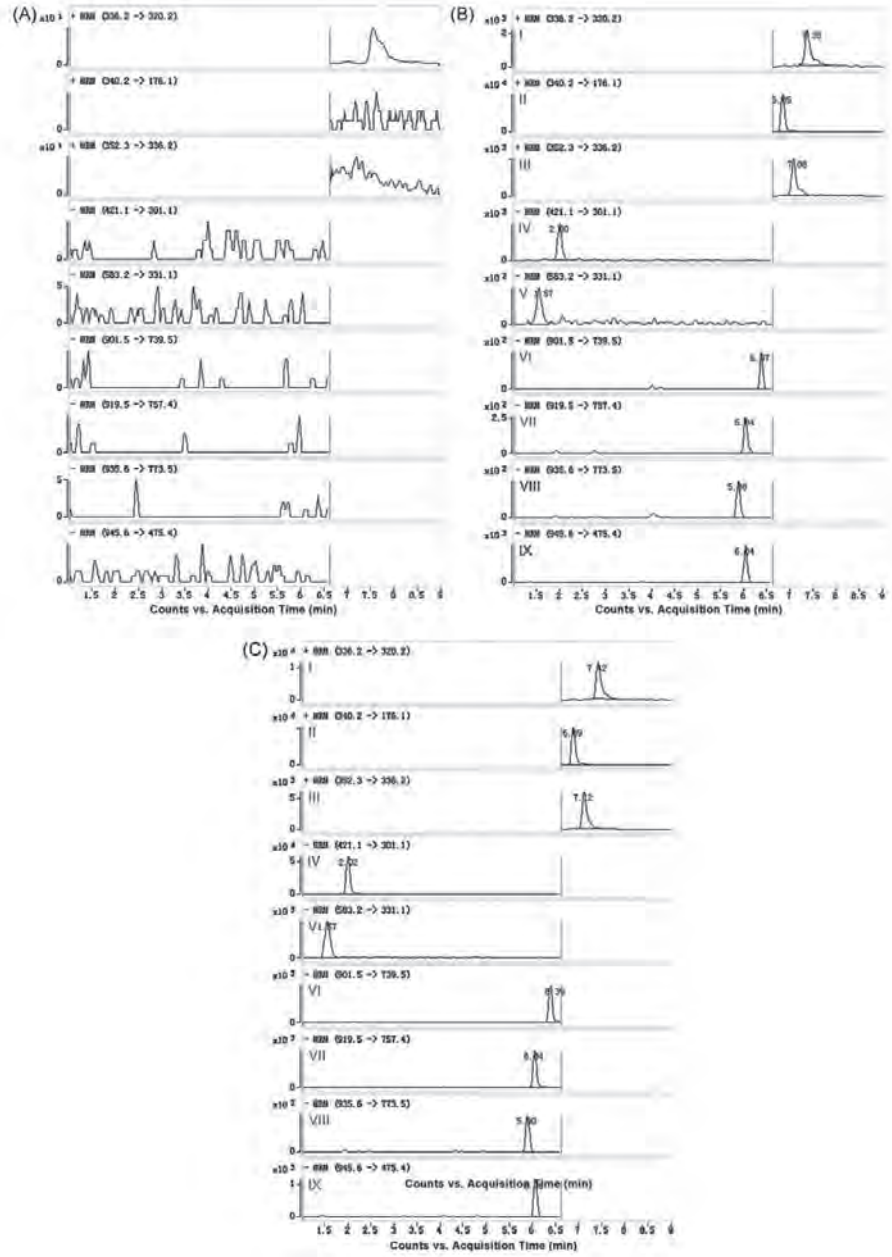


Fig. 7. Representative MRM chromatograms of I: berberine, II (IS): tetrahydroberberine, III: palmatine, IV: mangiferin, V: neomangiferin, VI: timosaponin B, VII: timosaponin B-II, VIII: timosaponin E1, and IX (IS): ginsenoside Re. (A) blank plasma sample, (B) blank plasma sample spiked with seven analytes at LOQ and IS, and (C) plasma sample collected from a rat 4 h after oral administration of ZSP extract at a dose of 1.94 g/kg. Reprinted with permission from Elsevier.[42] Copyright (2010) Elsevier.

3.2. High-resolution separation and fingerprinting.

Ideally HPLC separates mixtures into individual constituents. However, as the technique evolved and separation performance increased, in several cases it was discovered that isolated components previously thought to be pure were actually combinations of coeluting species. This means that 1D HPLC cannot always give a satisfactory separation of each analyte. This fact, together with the need to evaluate complex samples like TCMs, has triggered the development of multiple hyphenation. Two main routes are used to resolve more peaks and produce "fingerprints": either HPLC is combined with other chromatographic systems (two dimensional (2D) separation methods, section 3.2.1),[43-45] or HPLC is coupled with a detector capable of providing spectroscopic data, i.e., DAD, MS or NMR (section 3.2.2).[46-50]

Fingerprinting analysis is considered an effective method to control the quality of herbal drugs including TCMs because it provides a better overall picture of the content and the relative concentration of constituents. In the specific chromatographic patterns multiple compounds in TCMs can be recognized and allow for conclusions regarding the product's "phytoequivalence".[51] The WHO accepted fingerprint analysis as a methodology for the assessment of natural products.[52] HPLC is the most popular analytical technique for the fingerprinting analysis of TCMs. HPLC hyphenated techniques, with the help of data recognition methods, such as similarity analysis, exploratory data analysis, clustering, pattern recognition, and multivariate calibration, have been introduced to the fingerprinting research for obtaining more chemical information on TCMs.[7,8,11]

3.2.1. Multi-dimensional LC for TCMs analysis

The attractiveness of 2D-HPLC is its potential to significantly improve the separation power. The peak capacity for 2D separations is defined as a linear combination of peak capacities in both separation dimensions. For example, when combining orthogonal LC modes with peak capacities of 50 and 100, theoretically the resulting 2D capacity would be 5000.[53] In reality, the 2D-LC performance is less as columns are never 100% orthogonal and peaks are never evenly distributed over the entire run time.[54,55] Problems related to 2D-LC include instrumental complexity, long run times, incompatibility of 1st and 2nd dimension eluents, required data processing and sometimes poor reproducibility. Nonetheless a number of 2D-HPLC methods have been developed for the analysis of TCMs and some are listed in Table 1.

Table 1. Examples of 2D HPLC for analysis of TCMs.

TCMs	Separation conditions	Detection	Purpose	Ref.
Ligusticum chuanxiong and *Angelica sinensis*	comprehensive, Kromasil-CN (5 µm, 150 mm×4.6 mm) × Chromonolith Speed ROD (50 mm×4.6 mm)	DAD-APCI-MS	Separation and identification of compounds	56
Swertia franchetiana	off-line, Intersil CN-3 (5 µm, 250 mm×4.6 mm) × XTerra MS C18 (5 µm, 150 mm×2.1 mm)	UV-ESI-MS	Separation and identification of compounds	57
Dracaena cochinchinensis xylem	off-line, Ultimate XB-CN (5 µm, 250 mm×4.6 mm) × Ultimate XB-C18 (5 µm, 250 mm×4.6 mm)	UV	Separation and determination of compounds	58
Lonicera caprifolium	off-line, Kromasil-SCX (5 µm, 120 Å, 150 mm×4.6mm) × Kromasil-ODS (5 µm, 120 Å, 150 mm×4.6mm)	DAD-APCI-MS; MALDI-TOF-MS	Isolation and identification of compounds	59
Coptis chinensis	comprehensive, SCX Poly-SEA column (3 µm, 150×0.3 mm) × Magic C18 AQ, (5 µm, 50 mm×0.3 mm)	UV	Separation and analysis of compounds	60
Corydalis yanhusuo	off-line, XTerra Prep MS C18 OBDTM (5 µm, 100 mm×19 mm) × XTerra Prep MS C18 OBDTM column (5 µm, 100 mm×19 mm)	UV and MS-triggered collection	Isolation and purification of alkaloids	61
Dalbergia odorifera	off-line, Click oligo(ethylene glycol prep column (10-20 µm, 300 mm×70 mm) × XTerra Prep MS C18 OBDTM column (5 µm, 100 mm×19 mm)	UV-QTOF-MS	Purification of compounds	62
Isatis tinctoria	off-line, XTerra MS C18 (5 µm, 150 mm×50 mm) × homemade polar-copolymerized C18 (5 µm, 150 mm×10 mm)	UV-MS	Purification of polar compounds	63
Longdan Xiegan decoction	comprehensive, immobilized liposome chromatography column (5 µm, 300 Å, 150 mm×4.6 mm) × ODS (100 mm×4.6 mm)	APCI-MS	Screening and analysis of membrane-permeable compounds	44
Rheum palmatum	silica-bonded HSA column (5 µm, 300 Å, 150 mm×4.6 mm) × Chromonolith Speed ROD (50 mm×4.6 mm)	DAD-APCI-MS	Analysis of biological fingerprinting	64

From Table 1 it follows that mostly commercial columns with different separation mechanisms have been combined, e.g., strong cation exchange chromatography (SCX) × reversed phase liquid chromatography (RP)[59,60] or RP × normal phase liquid chromatography (NP, -CN column).[57] Also a number of tailor-made columns immobilized with biological macromolecules have been used in the 1st dimension for TCMs analysis.[44,62,64] Due to a specific interaction with these bio-macromolecules, such separations may provide significantly more information on components. In most of the cases, the two columns have been combined off-line, which is simpler. However a few are truly comprehensive LC×LC.

An example of the latter is the method developed by Wang et al. for the frequently used TCM Longdan Xiegan Decoction (LXD). In the 1st dimension an immobilized liposome chromatography column (ILC) was used and in the 2nd dimension a reversed-phase column coupled with DAD and APCI-MS.[44] The separation in the first dimension column was accomplished in 70 min and equal volumes were collected at 10 min intervals. The separation on the ODS column was completed in 10 min to match the interval. A modulation time of 10 min should be considered too long and some of the separation obtained in the 1st dimension might have been lost again. Nevertheless with this method more than 50 components in the ethyl acetate fraction of an LXD extract were separated. Fig. 8 shows the contour plot of the ethyl acetate fraction of the LXD extract. Among them, eight flavonoids (**3**-**10**) and two iridoids (**1** and **2**) were identified by their UV and mass spectra. The 1st dimension retention of this 2D HPLC system reflects interaction of analytes with membranes, a first prerequisite to penetrate membranes *in vivo*. As such it can be regarded as a kind of biological fingerprinting of a complex TCM. A disadvantage of this column is its moderate chromatographic efficiency.

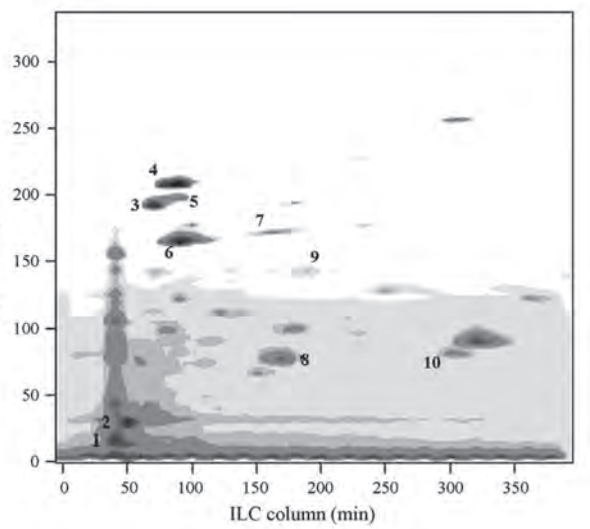


Fig. 8. 2D chromatogram of the LC×LC-UV separation of 5 μL of a Longdan Xiegan decoction. Peak assignment: **1**: geniposide; **2**: gentiopicroside; **3**: oroxylin A-7-O-glucuronide; **4**: wogonoside; **5**: 7-O-β-D-glucuronopyranosylchrysin; **6**: baicalin; **7**: ononin; **8**: liquiritin apioside; **9**: 3′,4′-dihydroxy-5,6-dimethoxyflavone 7-O-glucoside; **10**: liquiritin. Chromatographic conditions: ILC column (1st dim.): isocratic elution with 10 mM ammonium acetate solution (pH 6.8) at 0.05 mL/min; C18 column (2nd dim.): linear gradient elution from 10% ACN to 70% ACN in 7 min, 7-10 min 10% ACN (re-equilibration) at 2.0 mL/min; detection wavelength, 210 nm. Modulation time: 10 min. Reprinted with permission from Elsevier.[44] Copyright (2009) Elsevier.

3.2.2. Fingerprinting and chemometric analysis

Fingerprint chromatograms obtained by LC coupled to one or more detectors can be considered as characteristic profiles, which together reflect the complex chemical composition of the TCM sample. A typical example is the LC-DAD fingerprinting for QC of Qingkailing injections. This TCM shows good efficacy in case of blood circulation diseases, phlogistic disease, virosis and some inexplicable fevers.[45] Fingerprints (Fig. 9) of retention time versus UV response at multiple wavelengths (derived from a time versus wavelength contour plot) are used for a quality evaluation of injections produced by different manufacturers by means of principal component analysis (PCA). In comparison with a simple LC-UV profile at one wavelength, these combined fingerprints were more informative.

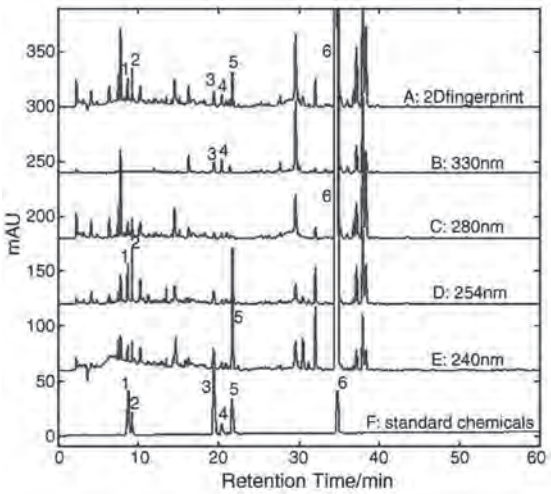


Fig. 9. Fingerprints of Qingkailing injection at various wavelengths as obtained from a time versus wavelength contour plot. (A) so called "max" plot; (B-E) fixed wavelength chromatographic fingerprints; (F) chromatogram of standard compounds at 254 nm. **1**: uridine; **2**: adenosine; **3**: chlorogenic acid; **4**: caffeic acid; **5**: geniposide; **6**: baicalin. Reprinted with permission from Elsevier.[45] Copyright (2005) Elsevier.

A total of 14 Qingkailing injection samples (marked as 1–14) were collected from two TCMs manufacturers and a mixed sample of 11–14 (from manufacturer B) in equal proportions (named authentic sample AUS) were evaluated by this method. After performing PCA on the data of the fingerprints, the differences between the samples are shown clearly in the scores plot of PC1 versus PC2 (Fig. 10). It can be seen that the samples belonging to groups I and II, which are all from manufacturer A, are well separated from those of B (group III). This suggests

that not only the products from manufacturer B are different from those from manufacturer A but also that A sells two distinctly different types of Qingkailing injections, the difference being caused by their different preparation procedures.

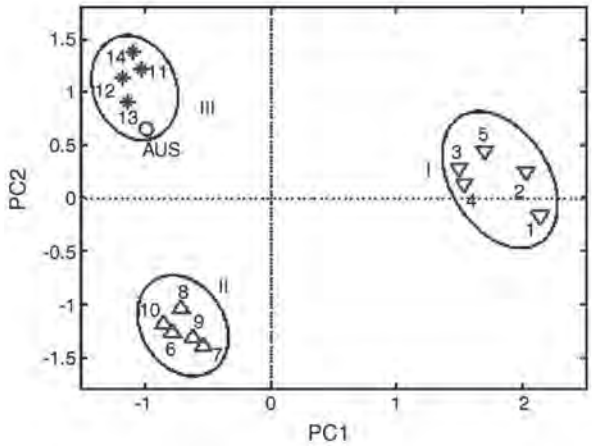


Fig. 10. Representation of LC-DAD contour plots of various Qingkailing injections and authentic sample (AUS) on PC1 and PC2 (83.4% variance explained): (▽) samples of manufacturer A with initial preparation procedures; (△) samples of manufacturer A with adjusted preparation procedures; (*) samples of manufacturer B; (○) AUS. Reprinted with permission from Elsevier.[45] Copyright (2005) Elsevier.

3.2.3. Fingerprinting analysis in Chinese Pharmacopoeia 2010

The Chinese Pharmacopoeia is the book most involved with the QC of TCMs. It contains monographs (analytical protocols) for many materia medica (crude products) and finished drugs. As a supplementary technique beside marker compound quantitation (section 3.1), official chromatographic fingerprinting protocols and the so-called specific chromatogram technique are included in the 2010 version. The HPLC fingerprintings of 1 injection, 5 solid oral dosage forms and 6 extracts and the so-called HPLC specific chromatograms of 2 solid oral dosage forms and 6 extracts were standardized (Table 2).[21]

The major difference between fingerprintings and specific chromatograms is the evaluation method used after obtaining the chromatogram. For fingerprinting chromatograms, the characteristic peaks are used to evaluate the similarity to a reference sample by chemometric methods and software. The relative retention time (RRT) and relative peak area (RPA) of characteristic peaks versus a reference peak are used as evaluation parameters. The similarity analysis is based on the

Table 2. Official HPLC fingerprintings and specific chromatogram monographs occurring in Volume 1 of the Chinese Pharmacopoeia 2010[21]

Form of TCMs	Name of TCM or materia medica	Reference compound*	Characteristic peaks
Fingerprintings			
1 injection	Shuanghuanglian injection	chlorogenic acid	7 peaks including chlorogenic acid
5 solid oral dosage forms	Guizhi fuling capsule	paeoniflorin	6 peaks including paeoniflorin
	Tianshu capsule	ferulic acid	7 peaks including ferulic acid
	Fufang Danshen Diwan	salvianic acid A	8 peaks including salvianic acid A
	Yaotongning capsule	strychnine	10 peaks including strychnine
	Nuodikang capsule	salidroside	13 peaks including salidroside
6 extracts	Notoginseng root extract (triol saponins)	ginsenoside Re	5 peaks including notoginsenoside R1, ginsenoside Re & Rg1
	Notoginseng root and stem extract (total saponins)	ginsenoside Re	5 peaks including notoginsenoside R1, ginsenoside Rb1, Rd, Re & Rg1
	Salvia root and stem extract (total phenolic acids)	salvianolic acid B	8 peaks including rosmarinic acid, salvianolic acid B, protocatechuic aldehyde, shikonin
	Salvia root and stem extract (total tanshinones)	tanshinone IIA	13 peaks including cryptotanshinone, tanshinone I & IIA, dihydrotanshinone I
	Zedoary turmeric oil	furanodiene	8 peaks including germacrone, furanodiene
	Centella extract	asiaticoside	3 peaks including asiaticoside, madecassoside
Specific chromatograms			
2 solid oral dosage forms	Wuling capsule	5-methylmellein	7 peaks including 5-methylmellein
	Bailing capsule	adenosine	6 peaks including uridine, adenosine
6 extracts	Ginseng stem and leaf extract	ginsenoside Rd	6 peaks including ginsenoside Rg1, Re, Rc, Rb2 & Rd
	Ginseng root extract	ginsenoside Rd	7 peaks including ginsenoside Rg1, Re, Rf, Rb1, Rc, Rb2 & Rd
	Hawthorn leaf extract	rhamnosylvitexin	4 peaks including rhamnosylvitexin, vitexin, glucosylvitexin, hyperin
	Weeping forsythia extract	phillyrin	4 peaks including phillyrin, pinoresinol-β-D-glucoside, forsythoside A, phillygenin
	Glabrous sarcandra extract	isofraxidin	6 peaks including chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, isofraxidin, rosmarinic acid, rosmarinic acid-4-glucoside
	Capillary wormwood extract	chlorogenic acid	7 peaks including chlorogenic acid

*: marked as S in the chromatograms.

correlation coefficients (r) of the evaluation parameters of the sample fingerprint and the fingerprint of a reference sample, as supplied by the State Food and Drug Administration (SFDA) of China. The similarity ranges from 0% to 100% with 0% meaning no similarity between the evaluated fingerprinting and the reference fingerprinting. For a regular product, the similarity (evaluation index) should be higher than 90%.

For example, a fingerprint of Fufang Danshen Diwan (see Table 2) is obtained by means of UHPLC with an Acquity HSS T3 1.8 µm 100 × 2.1 mm column in combination with a 10 min gradient at 0.4 mL/min at 40 °C. Mobile phase A is water containing 0.02% H_3PO_4, B is water-acetonitrile (20:80, v/v) containing 0.02% H_3PO_4. The detection wavelength is 280 nm. Eight characteristic (common) peaks in the sample according to the reference fingerprint (Fig. 11) recorded in the Pharmacopoeia are evaluated by chemometric software (reference peak: salvianic acid A (peak 1)).[21] Because standardized reference samples are not always available for reference fingerprinting, a so-called specific chromatogram method is used to discriminate the high and low quality TCMs.

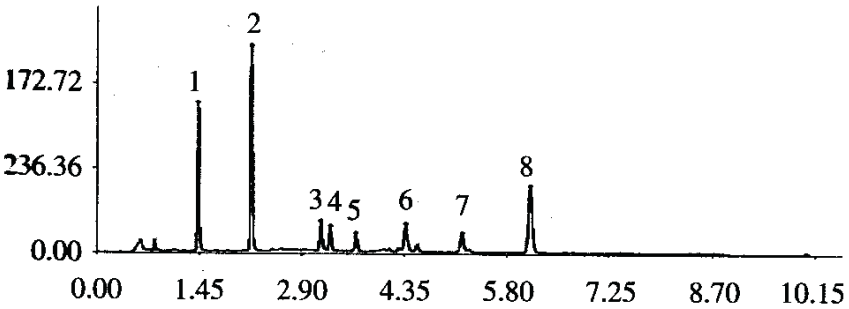


Fig. 11. Reference fingerprint HPLC-UV profile of Fufang Danshen Diwan as occurring in the Chinese Pharmacopoeia 2010.[21]

The specific chromatogram approach uses only the RRT of several characteristic peaks versus a reference peak to evaluate the quality similarity of TCMs. In this case no software is used. The relative deviation (RD) of the retention times should be less than ±5% of the specified values listed in the Pharmacopoeia. For example, the specific chromatogram of ginseng root extract (see Table 2) is obtained by using a linear gradient of water with 0.1% H_3PO_4 (A) to acetonitrile (B) on a C18 column with UV detection at 203 nm. There are 7 characteristic peaks, i.e., ginsenoside R_{g1} **1**, R_e **2**, R_f **3**, R_{b1} **4**, R_c **5**, R_{b2} **6** and R_d **7** in the chromatogram (Fig. 12). According

to the Pharmacopeia the specified values of the characteristic peaks **3** - **7** relative to the reference peak **7** are 0.84, 0.91, 0.93, 0.95 and 1.00, respectively. This means that if the retention time of **7** is 60 min, the peak with retention time from 47.88 min to 52.92 min can be considered as ginsenoside R_f **3**.[21]

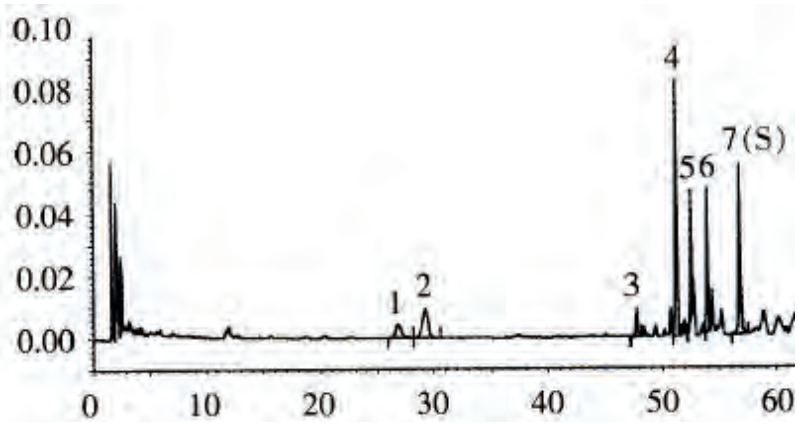


Fig. 12. Reference chromatogram of Ginseng root extract for the specific chromatogram method as occurring in the Chinese Pharmacopoeia 2010.[21]

Although it is a positive QC development that fingerprinting methods have been introduced in the Chinese Pharmacopoeia 2010, at the same time it should be remarked that the techniques and evaluation are still somewhat primitive. Neither of the two proposed methods takes the absolute concentration of constituents into consideration. The specific chromatogram method also does not take the relative concentration into account. From a quality control point of view, it is obvious that differences in absolute as well as relative concentrations will influence the quality of a TCM. A good HPLC fingerprint can provide this information and enables discrimination between samples. Thus improved chemometric evaluation protocols are necessary.

3.3. On-line HPLC mining of active compounds

Finding new leads in TCMs for new drugs will remain high on the agenda. To date the pharmaceutical industry is facing an unprecedented challenge: more funds are invested, but fewer new drugs are generated. This predicament is partially attributed to the limitations of the current one-drug-one-target paradigm

in drug discovery.[65] Thus, more and more attention is given to multi-component therapeutics, which incorporate two or more active ingredients in one drug to hit multiple targets, e.g., TCMs. It is furthermore a fact that in the past 30 years, around half of the new drugs that came to the market were natural products, natural products derivatives or synthetic analogues of natural products.[1] For drug screening, the hit rate in natural products libraries (0.3%) is significantly higher than in combinatorial and synthetic compound libraries (0.001%). When TCM libraries are used, the hit rate can be as high as 0.5% and the time to market is shorter.[66,67] As a result, the overall costs of development can be significantly reduced. Examples of leads from TCMs include: tetramethylpyrazine and 3-*n*-butylphthalide (from Rhizoma Chuanxiong) as neuronal injury inhibitors; miltirone (from *Salvia miltiorrhiza*) as an anxiolytic drug; baicalin (from Radix Salviae miltiorrhizae) as antiinflammatory drug, and 1-hydroxy-3,6,7-trimethoxyxanthone (from Radix Polygalae tenuifoliae) as an antidiabetic and aldose reductase inhibitor.[68]

Thus knowing which compounds are responsible for the beneficial effects of TCMs is indispensable for finding new leads, elucidating the mode of action, and indirectly for achieving better QC. For the screening of TCMs for active compounds, pharmacological models involving experimental animals, organ and tissue models, cellular models, receptors and enzymes have been employed. However, off-line screening of extracts does not provide information on individual compounds and sometimes suffers from false positive or negative results. To solve this problem various approaches have been proposed. In biological fingerprinting analysis (BFA), a TCM is incubated with the target macrobiomolecule and LC fingerprints before and after the interaction are compared. A decrease is indicative of an interaction.[69,70] In bioaffinity chromatography, drug targets such as enzymes, antibodies, liposomes, DNA or a cell membrane are immobilized on a stationary phase and any bioactive components are chromatographically retarded due to their specific interactions (section 3.3.1). The third and most advanced option is HPLC-based on-line screening (syn. high-resolution screening)[71] in which separated analytes react post-column in an assay. This allows for the direct pinpointing of active constituents in complex mixtures (section 3.3.2). Examples are screening for the presence of enzyme inhibitors[72] or antioxidants.[73]

3.3.1. Bioaffinity Chromatography

To screen for bioactive components in a complex matrix by means of affinity chromatography, usually frontal affinity chromatography (FAC) with mass spectrometric detection is used.[74-77] An example of FAC in the TCMs field, is the screening of the extract of *Phyllanthus urinaria* for interaction with immobilized polyclonal antibodies against compound A, structure see Fig. 13.[74] Compound A is a strong inhibitor of hepatitis C virus (HCV) NS3 protease and thus each clone of the antibody contains some similarity with the active site of the protease. The *Phyllanthus* extract containing potential ligands was continuously infused through the column. The order of elution of compounds depends on their affinities for the receptor with the compound showing the strongest interaction eluting last. Brevifolin **3**, brevifolin carboxylic acid **4**, corilagin **11**, ellagic acid **5**, and phyllanthusiin U **13** in the TCM showed activity (Fig. 14) and their high inhibitory activities could be confirmed in off-line analyses.

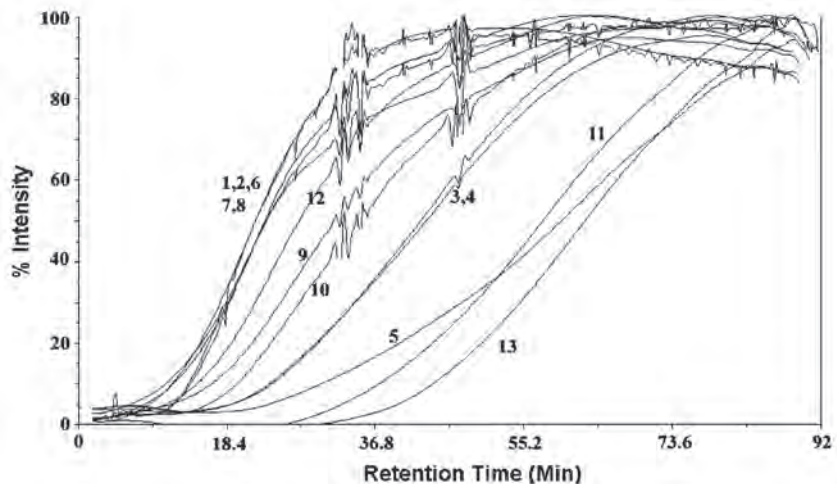
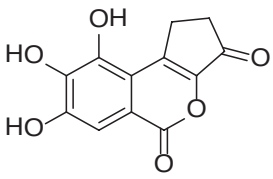
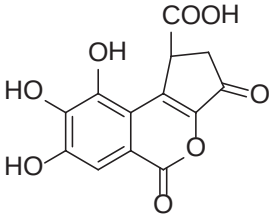


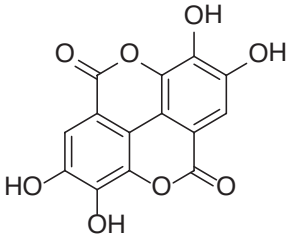
Fig. 13. Frontal affinity selected ion chromatograms of each compound in a *Phyllanthus urinaria* extract. The infusion flow rate was 5 µL/min. ESI-TOF-MS was used to measure the concentration of each compound. The chemical structures of compounds **3**, **4**, **5**, **11**, **13** are shown in Fig. 14. Reprinted with permission from American Chemical Society.[74] Copyright (2003) American Chemical Society.



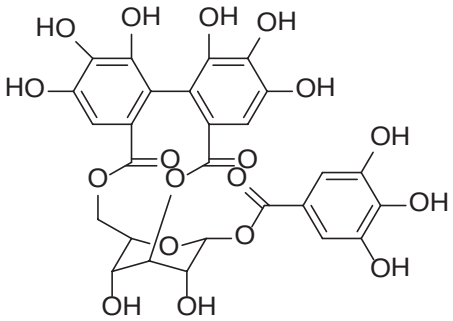
3 Brevifolin



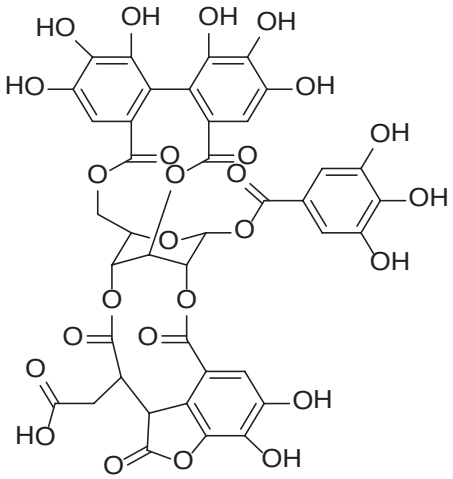
4 Brevifolin carboxylic acid



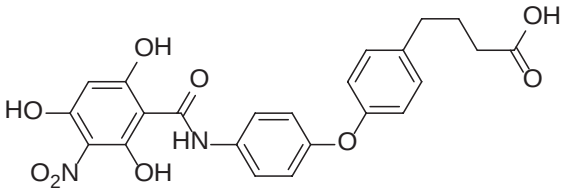
5 Ellagic acid



11 Corilagin



13 Phyllanthusiin U



Compound A

Fig. 14. Structures of **3**, **4**, **5**, **11**, **13** and compound A

3.3.2. HPLC high-resolution screening

Coupling of HPLC with a post-column reactor is a powerful method for the fast individual screening of active components in complex mixtures.[78-83] Due to the superior separation power of HPLC, no tedious isolation steps are necessary. High-resolution screening consists of HPLC separation, reaction of analytes

with target solution (e.g., an enzyme) in a first reaction coil, and interaction of a reporter ligand or substrate with the target in a second coil, and finally dual detection (Fig. 15). Sometimes the two coils are combined. Full integration of the bioassay into the HPLC flow can be viewed as the ultimate achievement in interfacing bioactivity and chromatography. Parallel spectrometric detectors (MS, DAD, or NMR) provide chemical characterization.

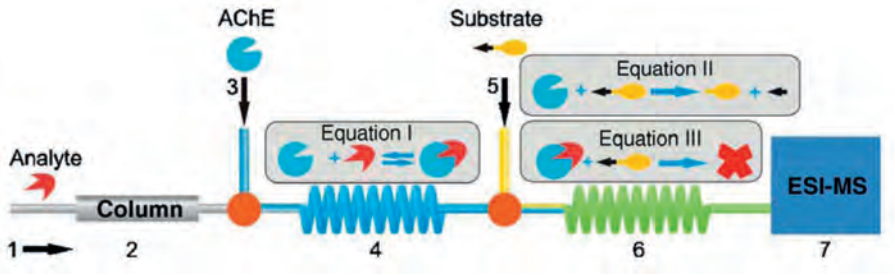


Fig. 15. Principle of high resolution screening as used in the detection of acetylcholineesterase inhibitors in a *Narcissus* extract. Reprinted with permission from Elsevier.[84] Copyright (2006) Elsevier.

De Jong et al. developed an acetylcholinesterase (AChE) assay for the screening of AChE inhibitors in natural extracts after HPLC-MS^n.[84] The effluent of the HPLC column was mixed with AChE solution in the first reaction coil allowing putative inhibitors to bind to AChE. The substrate solution (30 µM acetylcholine dissolved in 97.5% 10 mM ammonium bicarbonate, pH 7.8, and 2.5% methanol) was delivered by a superloop, and allowed to react in the second reaction coil. In the absence of an inhibitor, the substrate was converted to acetic acid and product (choline). The reactants were subsequently analyzed by ESI-MS. A 10 µM uracil solution was used as a system monitoring compound (SMC) to detect any ion suppression. A significant decrease in the substrate and a matching increase in the product trace were observed by starting the AChE pump. A positive peak in the substrate trace (Ach) and a negative peak in the product trace (Ch) matched the peak observed in the galanthamine (AChE inhibitor) trace (Fig. 16). Thus this method could successfully detect galanthamine as the active AChE-inhibiting peak in the extract of *Narcissus* cv "Bridal Crown" bulbs. Narcissus species are used in TCMs.[85]

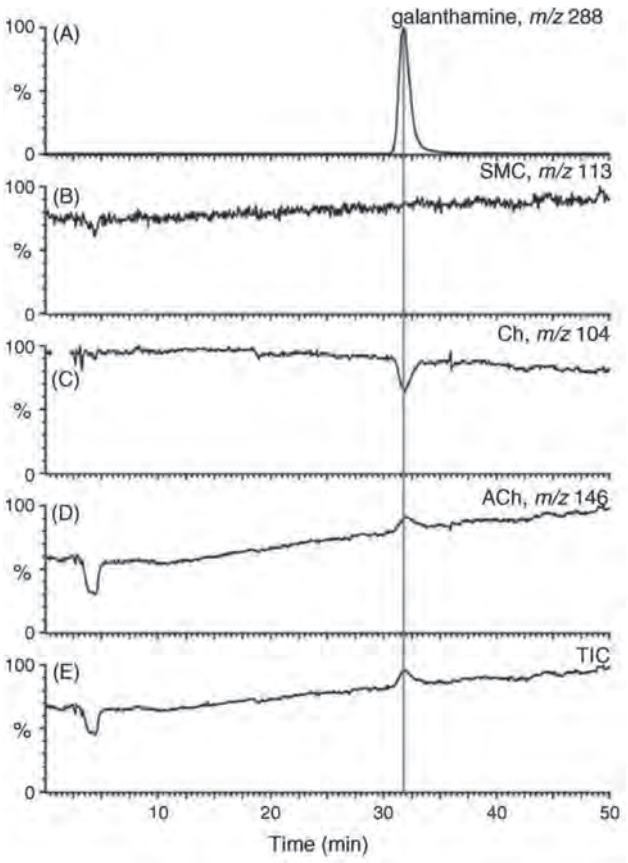


Fig. 16. Coupling of a gradient HPLC system to an MS-based AChE inhibitory assay. Trace A: *m/z* 288 (galanthamine in Narcissus extract); Trace B: system monitoring compound (SMC) detected at *m/z* 113; Trace C: product trace (choline) *m/z* 104; Trace D: substrate trace (acetylcholine) *m/z* 146; Trace E: total ion current (TIC). Reprinted with permission from Elsevier.[84] Copyright (2006) Elsevier.

4. Conclusions

From the examples presented in the previous sections, it is evident that HPLC was, is and will remain in the foreseeable future the key technique for quality control, lead-finding and metabolomics of herbs and phytopharmaceuticals in general and TCMs in particular. The main advantage of HPLC over other chromatographic techniques like gas chromatography (GC), thin layer chromatography (TLC), centrifugal partition chromatography (CPC) and capillary electrophoresis (CE) is its universal scope in terms of analytes, high separation power, reproducibility,

sensitive and flexible detection (refractive index (RI), UV, ELSD, fluorescence, electrochemical, MS, NMR) and easiness of hyphenation (bioassays). However this is not equal to stating that everything is perfect and no more progress or developments can be expected. What are currently the shortcomings in TCMs analysis by HPLC, which developments can we expect in the coming years?

In general the HPLC as applied to plant analysis is still rather traditional, traditional meaning here 60 min analyses on 5 μm 250 × 4.6 mm columns at 1 mL/min. In spite of the fact that TCM samples are often complex, both time and solvent use can and will decrease through the application of smaller diameter, shorter columns with smaller particles (UHPLC). Short ~2.5 μm core shell HPLC columns might even provide UHPLC resolution while running on standard 400 bar HPLC equipment. Modern HPLC column technology will make the analyses also "greener", another aspect that will receive more attention as also analytical chemists are responsible for a better environment. However as greener usually also means cheaper, money is another incentive. Extractions should also become more environmentally friendly by using smaller sample sizes, smaller solvent volumes and by replacing halogenated solvents like chloroform by less toxic alternatives. Possibly reusable ionic liquids will see more use.

Even if LC separations are modern and fast, phytochemical extractions and sample pretreatment often are not, requiring long extraction times and many human handlings. Enormous gains in time and money can still be achieved by automation of sample clean-up and hyphenation thereof with the extraction step and/or the LC step. At the same time this will remove a large source of errors (caused by human handlings) and thus improve reproducibility. We will also witness more miniaturisation of sample pretreatment, not only to cut down on solvent use but also because a chip format allows intricate prefabricated leak-free fluid connections. If LLE chips (section 2.2) can be made robust and integrated with LC or SIPTE (section 2.3) carried out on-chip, this could prove to be a big step forward. Having said this, it is wise to realize that the best sample pretreatment is no sample pretreatment and to some extent techniques like fingerprinting and the metabolomics field actively invite this. Normally sample pretreatment eliminates whole classes of compounds and thus chemical information is lost. Effectively this means that different questions may require different analytical solutions.

Another aspect that deserves continued attention is proper validation. Validation is tedious and expensive but if neglected the outcome is meaningless. Two striking examples[86,87] have been reported[88,89] for *Ginkgo biloba* leaf analysis.

Key issues, which should always be checked, and thus also for TCMs analyses, are: absolute purity of reference substances, extraction efficiency, absolute and relative recovery and precision. We expect further improvements in validation of TCMs analyses in the coming years.

Proper data processing (chemometrics) also deserves more consideration. For instance, currently the Chinese Pharmacopoeia only uses relative retention times and sometimes relative ratios (i.e., qualitative information) to evaluate LC profiles. However the absolute concentrations of components are clearly also of importance for the quality of TCMs. Thus the Pharmacopoeia methodology (chemometric evaluation software) should be adapted in such a way that also such facets are taken into account. With LC-DAD-MS becoming more affordable, multi-detector fingerprints will also become more common and proper but integrated evaluation of the two chromatograms for QC again requires smart software. The same holds for the application of multi-dimensional chromatography. LC×LC will become more common but the huge amount of sometimes 4-dimensional data (time×time×concentration×mass spectrum) requires not only automated software for evaluation but also software for converting hundreds of individual chromatograms into a 4-dimensional space. On the other hand greater complexity is not always better and carries a bigger risk of increased downtime and black box analyses so it is good to consider carefully if LC×LC is really needed for QC.

TCMs are a fruitful library for new drug leads and thus mining of TCMs is on the rise. In section 3.3 various LC approaches that allow directly obtaining bioactivity and spectroscopic data on individual compounds were highlighted. Certainly high-resolution screening (HRS) will expand in the coming 10 years but it is good to realize that on-line assays also have disadvantages. Slow or large assays requiring e.g., bacteria or whole experimental animals cannot be hyphenated to HPLC and the organic modifier necessary for RP-HPLC is difficult to combine with fragile enzymes.

Finally, especially for TCMs HRS has the intrinsic drawback that it provides no information on synergism. Thus small scale high throughput off-line assays, e.g., after parking an entire HPLC run in a 96 or 384 well plate, will remain popular as well. Often the used assay will provide the first information on the mode of action of active TCMs constituents. In turn there will be a spin-off towards QC. If the pharmacologically active constituents are known, they should be included in multi-compound HPLC quantitations. A lot of development is expected in this field in the coming decennium.

The last remark to make is that no matter how fantastic the sample pretreatment and the ensuing HPLC-MS separation and quantitation, QC only controls, i.e., it can only reject a poor drug and not remedy it. A good drug starts with the correctly identified, high-quality plants, properly grown, harvested and stored (GAP = Good Agricultural Practice), which are blended and processed in the right fashion (GMP = Good Manufacturing Practice).

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Chapter 9

General Discussion

The research in this Thesis aimed at making phytochemical analyses simpler, faster, greener, cheaper or more environmentally benign. To achieve these aims, various techniques were developed to miniaturize, omit or simplify the extraction or sample pretreatment steps or to use greener solvents for analytical and preparative LC separations. In the following, the outcome of this work will be compared with the five aims formulated at the beginning of this thesis. Then these results will be discussed in the context of the general setting of the screening and quality control of Traditional Chinese Medicines, and recommendations for further research will be presented.

Aim 1. Improve an existing 3-phase LLE chip and convert macroscale induced phase separation extraction (IPSE, an existing 2-phase LLE sample pretreatment technique) to a microfluidic chip-based format by optimizing parameters, evaluating efficiency, and hyphenating with commercial LC. Both chips in their optimized format will be tested with TCMs samples.

Achieved: Microfluidic techniques give us opportunities to reduce the solvent and time consumption. In Chapter 2 an existing 3-phase LLE chip was optimized in terms of the solvent used to make it greener (the halogenated chloroform was replaced by butyl acetate), faster (25 sec for extraction and back-extraction) and more efficient (extraction of alkaloids > 90%). In addition, a versatile IPSE chip separating uniform acetonitrile – water (1:1) sample solutions into organic and aqueous phases was developed and optimized in Chapter 3. In such a device analytes in a sample solution are separated based on their affinity for both phases, which is correlated to their log D values. Like in traditional LLE, selective extraction of bases by pH modification worked well in these two chips. Model compounds were used for providing proof of principle (around 93% extraction efficiency for suitable analytes and RSD≈ 3.5%). The flow of the needed dichloromethane could be reduced to 40 nL/min. The hyphenation of the 3-phase chip with nanoLC was successful and could ultimately lead to an automatic and integrated chip. However, the hyphenation of the IPSE chip could not be realized due to time constraints. The 3-phase chip was applied to real samples including *Strychnos nux-vomica* seeds, *Cephaelis ipecacuanha* roots, *Atropa belladonna* leaves, and *Vinca minor* leaves, and the IPSE chip was applied to *Scutellaria baicalensis* roots. Several of these plants are relevant given their use in TCMs.

Aim 2. Evaluate if ambient mass spectrometry like DART or direct plant spray MS can be used for quick authentication of Chinese star anise and Japanese star anise or for distinguishing between Scutellaria (important TCM ingredient) samples from different origins in China without any extraction or sample pretreatment.
Achieved: Differentiation of similar plants in their raw formats is always tricky for customers and even for quality control supervisors who are not botanists. Chapter 4 and 5 give an example of quickly differentiating Chinese star anise from toxic Japanese star anise by ambient mass spectrometry techniques e.g., DART and direct spray, without sample pretreatment. Direct analysis of toxic anisatin showed a huge and clearly distinguishable difference (> 1000 times in signal intensity) between Japanese star anise and Chinese star anise in both positive and negative modes ($[M+NH_4]^+$ at *m/z* 346.1496, $C_{15}H_{24}NO_8$ for DART positive mode, $[M+K]^+$ at *m/z* 367.079, $C_{15}H_{20}KO_8$ for direct spray positive mode, and $[M-H]^-$ at *m/z* 327.1074, $C_{15}H_{19}O_8$ for both DART and direct spray in negative mode). Adulteration of Chinese star anise tea bags with Japanese star anise was carried out in a visually unrecognisable format, a hot tea was made from this, and a glass rod with adhering tea was analyzed by DART-HRMS. Two ambient mass spectrometry techniques (DART and direct plant spray ionization) were compared with each other for this aim. Both approaches require a high-resolution mass spectrometer (a mass resolution of at least 27,000), to distinguish the compounds of interest from the plethora of other compounds present. DART has the advantage of a slightly higher selectivity, the absence of high voltages or the need of adding solvents, and places no requirements on the shape of the sample. Direct plant spray ionization has the advantage of low cost, simplicity, room temperature and lower standard deviations. However, neither method is very suitable for quantitative measurements of solid samples like star anise fruits.
Many Scutellaria samples were analyzed by DART-HRMS (data not shown), but due to a lack of time and sample size issues, their chemometric analysis could not be completed. A preliminary interpretation of the data showed that different plant parts could be easily distinguished, but distinction by their geographic origin failed in these preliminary trials. A disadvantage, compared to an HPLC analysis of the same samples, was the inability to detect flavonoid glycosides.

Aim 3. Develop a process in which magnetic nanoparticles can substitute for a heptane LLE or preparative gravity column chromatography for the purification of ginkgolic acids from *Ginkgo biloba* leaves.

Achieved: Three simple steps: (1) extraction; (2) selective purification by MNPs; (3) preparative HPLC on a C_8 column, were introduced in Chapter 6 to purify individual ginkgolic acids to a purity >95% from raw plant material (*G. biloba*, 0.15% - 0.60% of individual GAs) without using tedious (gravity) column chromatography (CC) or halogenated solvents. The high selectivity, stability and loadability of the MNPs open up more possibilities for phytochemical applications in the field of GAs. Even the substitution of MNPs for CC to purify GAs was successful.

Aim 4. Determine if the three most commonly used organic modifiers (methanol, acetonitrile and tetrahydrofuran) for (preparative) reversed phase (RP) HPLC can be replaced by less toxic and greener modifier solvents (ethanol, acetone and ethyl acetate) without loss of resolution. Terpene trilactones from *G. biloba* will serve as test case.
Achieved: By a two-step chromatographic optimization procedure consisting of (1) a simplex triangle design and (2) an HPLC simulation software step, a gradient using acetone, ethyl acetate, ethanol and water was developed, which gave a much higher minimal resolution than the benchmark methanol-THF-water eluent. The gradient could be successfully scaled-up to a 250×22 mm reversed phase preparative HPLC column and allowed for a baseline separation of the five TTLs when 0.5 g was injected.

Aim 5. Prepare an overview on the use, recent developments and prospects of HPLC in TCMs QC and drug discovery.
Achieved: A review focusing on applications and developments of HPLC-based methods for TCMs research and the current situation regarding quality control of TCMs in China (Chinese Pharmacopoeia 2010) was published. It convincingly shows that HPLC remains the key technique in TCMs analysis as well as in drug discovery programmes based on TCMs.

Perspectives and outlook

1) From a technical point of view, even though the miniaturization steps for LLE and IPSE have been successfully achieved as demonstrated in Chapter 2 and 3 in this Thesis, further optimization is still desirable. Aspects for further

attention include preferably lower production costs of the chip, the design of the microchannel (with the aim to reduce any turbulence as much as possible), and development of more hyphenation, e.g., miniaturized extraction cells (upstream), or HPLC, UV or MS for automated on-line analysis (both downstream). These hyphenated extraction and analysis set-ups will enable a fully automatic system for detailed analysis of the composition of raw plant materials. While desirable, it should be considered that even such a multi-functional extraction and separation chip will not be able to provide the full metabolome of one plant, due to the complexity and variety of all primary and secondary metabolites present.

In spite of all its advantages, also ambient mass spectrometry is not perfect (see Chapter 4 and 5). This is largely related to the absence of selectivity. The power of ambient mass spectrometry can therefore be optimally unleashed, if some, even partially, selective step is used prior to MS analysis. An example of the latter, as introduced in this thesis for the purification of ginkgolic acids, is comprised of metal-containing nanoparticles (MNPs; see Chapter 6). The selective adsorption of phytochemicals onto the surfaces of MNPs that are easy to handle, followed by a truly quick desorption and detection by ambient mass spectrometry could combine the best of both worlds for phytochemical-related research.

Although MNPs clearly have potential to improve upon current LLE or CC techniques (see Chapter 6), further investigations are in order. These should be directed towards further optimization of the technique, in which an optimized solvent miscibility is a major aspect to avoid evaporation and redissolution steps and increase the adsorption capacity. In addition, further mechanistic studies are in order, in which it would be of specific interest to study how MNPs react with analytes. For example, the ubiquity of salicyclic acid binding via chelation may be investigated for a wide range of phytochemicals. It is via such more detailed studies that further application of this approach will come within reach, both in the life sciences (e.g. study of phosphorylated peptides) and in the area of food safety (citrinin study).

2) From an application point of view, TCMs are very commonly used in China, and have great export potential to other markets. Following the successes of a scientific approach to medicine as developed in western medicine, great efforts are currently undertaken by (predominantly) Chinese scientists, and generously sponsored by the Chinese government, to study TCMs in an internationally acceptable scientific way.

In the beginning of the attempts to modernize the study of TCMs, the R&D methods developed for western medicines, i.e., isolation of active compounds and looking for their individual activity was directly applied to TCMs. This approach did produce one or two new drugs like artemisinin, a very successful antimalaria drug isolated from *Artemisia annua* (*qinghao* in Chinese) by Youyou Tu.[2] However, the limitations of this approach are also becoming increasingly obvious, as the development of novel medication seems to run out of steam for chronic and infectious diseases. The trend of western medicine is shifting from "one disease-one target-one size fits all-drug" to more personalized (combinations of) medicines in recent years.[3] The more holistic, personalized approach noted in the development of western medication, also perfectly fits a core characteristic of TCMs, namely synergism. This stimulates novel initiatives, such as the herbalome, a project with the objective of globalizing Chinese herbal medicine (CHM) by clarification of its composition, structure, and function; by establishing a standard resource library; and by interpreting the synergistic and complementary mechanisms of multi-components on multi-targets.[4] Such new TCM analyses are in progress to bridge the gap between western and Chinese medicines. These advances will certainly have to rely on proper incorporation of advanced analytical techniques, as the range of -omics, high-throughput screening, high content separation, and microfluidic chips. In addition, since multicomponent complexity is key to the functioning of TCMs, it is difficult to overestimate the role of systems biology and chemometrics.

Next to analysis of the modes of functioning of TCMs, the development of a proper and reliable quality control (QC) of TCMs is essential. QC of TCMs has gradually become a serious issue, precisely because the functioning of TCMs is still not fully understandable within modern scientific paradigms. QC methods copied from pure western medicines are not easily applied to TCMs, because of their intrinsic complexity. This shortcoming has unfortunately led to illegal manufacturing issues, which include the addition of chemical additives to TCMs, and the ignorant or wilful use of low-quality, adulterated or even fake TCMs in prescriptions. For both practical use and quality control, a quick and reliable authentication of Chinese materia medica is therefore of major importance. Given the still tremendous importance of TCMs within China, both in terms of health and of economy, a clear recommendation based on the research of this thesis is that much more analytical chemical research into this is needed, as well as serious governmental control, simply because otherwise many people will suffer unnecessarily.

One aspect that we tried but did not finish and, which remains worthy to be continued in the near future, is the analysis of TCMs by ambient mass spectrometry, and to analyze the data using chemometrics to pinpoint the useful information in the truly huge data set. *S. baicalensis*, a plant used in many TCMs constitutes an example. In principle, the same kind of TCM, like *S. baicalensis,* can be planted in different places in China, which can be thousands of kilometers away from each other, with concomitantly big differences in growing and processing conditions. However, the resulting TCMs from the different areas are used without difference in TCM prescriptions. Evidently, this may cause quality control issues, and lead to a range of medical problems upon large-scale applications. Several examples along these lines have recently been published for combining chemometrics with ambient mass spectrometry.[5-9]

The necessity to come to truly 21st century solutions for the complex systems and situations at hand, however, provides an urge to continue with these efforts. In this, it is important to continue to aim for seemingly unreachable goals: a diamond with a flaw is better than a common stone that is perfect.

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Summary

Chapter 1 provides a short introduction into the constraints of phytochemical analysis. In order to make them faster, less laborious and greener, there is a clear scope for miniaturized and simplified sample preparation, solvent-free extractions and the use of cleaner solvents in preparative HPLC. Possible modern techniques to achieve this, such as microfluidic chips, ambient mass spectrometry, selective magnetic nanoparticles, and use of less toxic but equally efficient solvents are discussed. Clear aims were formulated and research towards fulfilling these aims in the field of phytochemical analysis is carried out in this thesis.

A first version of a 3-phase liquid-liquid extraction (LLE) chip for the miniaturized sample pretreatment of alkaloids was introduced by our group in 2009. In **Chapter 2** more biodegradable and less-toxic solvents for the transport phase and a more suitable pH for the feed phase were evaluated. The extraction efficiency improved. On-line hyphenation of the 3-phase chip to nanoLC-UV/MS was also investigated. This combination saved a lot of time and solvent in comparison with traditional methods for the purification of alkaloids from plant materials.

A novel Induced Phase Separation Extraction (IPSE) chip was introduced in **Chapter 3** for efficient sample pretreatment. The acetonitrile – water (1:1) sample solutions were separated in organic and aqueous phases in this IPSE chip based on their affinity for both phases. In turn this could be correlated with the log D values of the analytes. Some optimization regarding design, operation, flows and solvents was carried out. Extraction efficiencies of several model compounds were determined. A real sample application with a plant used in Traditional Chinese Medicines (TCMs) was carried out to show the usefulness of the IPSE chip in dealing with complex matrixes.

Chapter 4 presented an unambiguous distinction between toxic Japanese star anise and non-toxic Chinese star anise fruits within seconds without any sample pretreatment by DART-orbitrap MS technology. Both positive and negative mode gave the same result, although the latter mode is preferred because of its higher sensitivity and cleaner spectra. Not only raw plant materials but also a herbal tea containing both Chinese and Japanese star anise could be quickly and accurately distinguished by DART-HRMS.

In **Chapter 5**, direct plant spray in combination with orbitrap HRMS allowed, like DART-HRMS, for an unambiguous distinction between toxic Japanese star anise and non-toxic Chinese star anise fruits within seconds without any sample pretreatment in both positive and negative mode. Direct plant spray ionization

has the advantage of low cost, simplicity, room temperature and low standard deviations. Neither the DART nor the direct spray method is very suitable for quantitative measurements of solid samples like star anise fruits.

Chapter 6 describes the purification of eight ginkgolic acids (GAs) from raw plant material (*Ginkgo biloba)* by using only three steps, namely (1) extraction; (2) selective purification by cheap Fe_3O_4 magnetic nanoparticles (MNPs); (3) preparative HPLC on a C_8 column. The three main constituents occurring at concentrations of 0.15% - 0.60% were enriched to >95% absolute purity without using tedious (gravity) column chromatography with halogenated solvents.

Preparative RP-HPLC is an efficient but not very green technique for the final purification of fine chemicals and natural products as large volumes of acetonitrile, methanol and tetrahydrofuran (THF) are consumed. In **Chapter 7** it was investigated whether less toxic organic solvents could replace them. As a test case the preparative separation of Ginkgo terpene trilactones (TTLs) was selected. By a two-step chromatographic optimization procedure a 30 min gradient using only water, ethanol, acetone and ethyl acetate was developed, which gave a baseline separation of 480 mg of an injected TTLs mixture. All five individual TTLs were > 95% pure.

Traditional Chinese Medicines (TCMs) are one of the oldest and most used traditional drugs in the world. Many plants are used for their preparation. An overview of HPLC-related methods such as: multicomponent quantitation, fingerprinting, bioaffinity chromatography and on-flow assays for screening and quality control of TCMs was presented and discussed in **Chapter 8**.

The final **Chapter 9** discusses the major findings of this work and gives further perspectives.

摘 要

第一章简要介绍了目前在植物化学分析中存在的非绿色、高耗费问题，如：分析周期长，步骤繁冗，溶剂使用量大等。阐述了分析方法中微型化、简易化的样品前处理，无溶剂萃取以及绿色化、低成本化的制备分离对绿色化学的贡献。重点介绍了与本研究相关的现代分析技术的研究现状，包括：微流控芯片技术、敞开式质谱技术、选择性磁性纳米萃取技术、以及绿色溶剂技术（使用低毒或无毒溶剂）。并对本论文的目的及其相关研究内容进行了简要归纳。

三相微流控萃取芯片于 2009 年被本组博士提出，并用于生物碱成分微量萃取。承接前期研究基础，论文**第二章**采用生物降解性更好、毒性更小的溶剂取代了前期三相萃取芯片中的氯仿，重点考察、优化了水相溶剂的 pH 值对萃取效率的影响，使其更加绿色和高效。并成功的将芯片和纳升液相/质谱联机使用，使得该芯片有可能成为终端分析设备的在线净化装置。相较传统的液-液-液萃取，不仅大幅度提高了分析速度而且显著降低了溶剂的消耗量。

第三章介绍了用于样品前处理的诱导相分离萃取芯片研究。50%的乙腈水溶液在芯片中被诱导溶剂诱导分成水相和有机相，并从芯片中选择性流出，相应化合物依据其极性大小被高效分离，目标化合物的选择性可根据化合物的 logD 值，通过调节溶液的 pH 来实现。此诱导萃取芯片在复杂基质中的选择性通过对中药黄芩中黄酮类化合物糖苷和苷元的快速分离得以验证。

第四章介绍了利用敞开式离子化高分辨质谱技术（DART-HRMS）快速鉴别植物品种的研究。以有毒的日本八角以及无毒的中国八角为模型，DART-HRMS 技术在几秒内能快速、准确分辨易混淆的植物品种。方法在正离子和负离子模式下都能得到可靠的结果，相比而言负离子模式有着更高的灵敏度和更低的干扰。方法不仅能用于八角植物原料的准确分辨，还能对中国八角茶包中是否掺杂有毒的日本八角进行准确的鉴别。

接续上一章的工作，**第五章**介绍了直接植物喷雾离子化-高分辨质谱分辨有毒日本八角以及无毒中国八角的研究。直接施加高电压在药材上，产生电喷雾，质量分析器对电喷雾产生的离子进行分析，可在几秒内快速、准确的分辨日本八角和中国八角。研究表明在正、负离子模式下都能得到可靠的结果。通过 DART 和直接植物喷雾离子化方法的比较表明，直接植物加压喷雾离子化方式更简单、且无需加热、成本低、结果的标准偏差更小。但是无论是 DART 还是直接加压喷雾离子化方式都不太适合精确定量分析。

第六章介绍了“三步法”Fe_3O_4 磁性纳米粒子纯化银杏叶中 8 种银杏酸的研究。“三步”包括：(1)银杏叶的萃取；(2) Fe_3O_4 磁性纳米粒子选择性吸附萃取液中的银杏酸；(3) C8 制备柱分离纯化银杏酸。创新点在于第二步，即高度结构选择性的富集、纯化银杏酸，为第三步的制备色谱提供了成分相对简单且含量高的样品。第二步的创新还有着绿色的含义，即：能耗低，只用磁铁即可完成富集、纯化过程。

反相制备色谱制备在天然产物纯化过程中是一个高效且不可缺少的步骤，传统制备色谱常用的有机试剂主要有乙腈、甲醇以及四氢呋喃。论文的**第七章**介绍了制备色谱绿色化的研究。考察了三种相对绿色环保的候选替代溶剂（乙醇、丙酮以及乙酸乙酯）用作制备色谱流动相时的分离能力；并以五种银杏内酯为模型化合物，通过两步色谱优化成功将 480 mg 的五个银杏内酯用水、乙醇、丙酮和乙酸乙酯作为流动相在 30 分钟内分离制备。制备的五个三萜类化合物的纯度都在 95%以上。

第八章对中药质量控制方法中高效液相色谱及相关现代分析技术，如：多成分分析、中药指纹图谱、生物亲和色谱以及在线筛选等，进行了介绍和讨论。

论文的**第九章**总结了本论文研究的主要成果，并对未来的研究提出了自己的想法。

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Curriculum Vitae

Yao Shen was born in Changde, a small city in Hunan, China. She obtained her bachelor and master degrees in chemistry at Hunan Normal University in Changsha, China. In 2010, she obtained a Sandwich PhD grant from Wageningen University, the Netherlands. Early 2011, she went to Wageningen to start her PhD studies. During those years, she partially worked in Hunan Normal University, supervised by Prof. Bo Chen and partially worked in Wageningen University, supervised by Dr. Teris A. van Beek and Prof. Han Zuilhof. Yao mainly worked on analytical method development using microfluidic, chromatographic and mass spectrometric techniques. Most of the analytes of interest were constituents in herbals, especially traditional Chinese medicines. This Thesis describes what she did those years and what she added to science.

List of Publications

In peer-reviewed journals

1. Guozhu Liu, Zhonghai Xu, Jitao Chen, Gesangsuo Lang, Qingqing Tian, **Yao Shen**, Bo Chen, Shouzhuo Yao. On-line strategies for the identification of unknown flavone glycosides in *Dracocephalum tanguticum* Maxim. ***Journal of Chromatography B***, 877 (2009) 2545-2550.

2. Yingzhuang Chen, Guozhu Liu, **Yao Shen**, Bo Chen, Jianguo Zeng. Analysis of alkaloids in *Macleaya cordata* (Willd.) R. Br. using high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry. ***Journal of Chromatography A***, 1216 (2009) 2104-2110.

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4. **Yao Shen**, Yumin Hu, Ke Huang, Shi'an Yin, Bo Chen, Shouzhuo Yao. Solid-phase extraction of carotenoids. ***Journal of Chromatography A***, 1216 (2009) 5763-5768.

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6. **Yao Shen**, Teris A. van Beek, Frank W. Claassen, Han Zuilhof, Bo Chen, Michel W. F. Nielen. Rapid control of Chinese star anise fruits and teas for neurotoxic anisatin by direct analysis in real time (DART) high resolution mass spectrometry. ***Journal of Chromatography A***, 1259 (2012) 179-186. (Chapter 4 in this Thesis)

7. Xiyue Xiong, Zihui Yang, Yongbin Huang, Linbo Jiang, Yingzhuang Chen, **Yao Shen**, Bo Chen. Organic-inorganic hybrid fluorous monolithic capillary column for selective solid-phase microextraction of perfluorinated persistent organic pollutants. ***Journal of Separation Science,*** 36 (2013) 923-931.

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11. Renkai Li, **Yao Shen**, Xiaojuan Zhang, Ming Ma, Bo Chen, Teris A. van Beek. Efficient purification of ginkgolic acids from *Ginkgo biloba* leaves by selective adsorption on Fe3O4 magnetic nanoparticles. ***Journal of Natural Products***, 77 (2014) 571-575. (Chapter 6 in this Thesis)

12. Tingting Wu, Yaru Liu, **Yao Shen**, Ming Ma, Bo Chen, Teris A. van Beek. Rapid analysis of ginkgolic acids in *Ginkgo biloba* tissues and their extracts by direct spray mass spectrometry. (submitted to ***Journal of Agriculture and Food Chemistry***, under revision)

13. **Yao Shen**, Bo Chen, Han Zuilhof, Teris A. van Beek. Conversion of macroscale induced phase separation extraction to a chip-based version. (submitted to ***Lab on a chip***, Chapter 3 in this Thesis)

14. **Yao Shen**, Bo Chen, Teris A. van Beek. Alternative solvents can make preparative liquid chromatography greener. (submitted to ***Green Chemistry***, Chapter 7 in this Thesis)

Other publications

1. Teris A. van Beek, **Yao Shen**, Radostina Manova. Extractionless analyses by direct analysis in real time (DART) MS. ***Separation Science***, 6 (2014) 2-7.

2. **Yao Shen**, Teris A. van Beek, Frank W. Claassen, Han Zuilhof, Bo Chen. 3-phase sample preparation chip hyphenated to nano-HPLC/ESI-MS for rapid miniaturised analysis of alkaloids. ***Planta Medica***, 78 (2012) 1252.

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4. Teris A van Beek, **Yao Shen**, Tijmen Verweij, Alexandre Villela, Frank W. Claassen. Ambient mass spectrometry for extractionless analyses of plants: Holy Grail, useful tool or hoax? ***Planta Medica***, 78 (2012) 1055.

Overview of completed training and education activities

Discipline specific activities

Courses

Advanced Organic Chemistry, Wageningen, 2011
Modern Chromatographic Analysis, Changsha, 2012
Natural Products Chemistry, Changsha, 2012

Conferences

Trends in Natural Products Research, Olomouc, Czech Public, June 23-25, 2014 (The Best Lecturer Award)

The 15th Beijing Conference and Exhibition on Instrumental Analysis (BCEIA), Beijing, China, October 23-26, 2013

The 19th Chinese Symposium on Chromatography, Fuzhou, China, March 31-April 3, 2013 (The Best Poster Award)

International Congress on Natural Products Research (ICNPR), New York, US, July 28-August 1, 2012 (Poster, Abstract published in Planta Med., 2012, 78: PJ13)

European Lab Automation, Hamburg, Germany, June 30 - July 1, 2011 (Poster)

General courses

VLAG Ph.D. week, Baarlo, VLAG, 2011
Techniques for writing and presenting a scientific paper, Wageningen, VLAG, 2014
Validation and Verification of Analytical Procedures, USP, Milan, Italy, 2014

Optionals

Group meetings, Laboratory of Organic Chemistry, Wageningen, 2011-2014
Group meetings, Key Laboratory of Phytochemical R&D of Hunan Province, Changsha, 2011-2015
Colloquia, Laboratory of Organic Chemistry, Wageningen, 2011-2014
Colloquia, Key Laboratory of Phytochemical R&D of Hunan Province, Changsha, 2011-2015
Ph.D. trip, Laboratory of Organic Chemistry, UK, 2011

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