Recent developments in the rapid analysis of plants and tracking their bioactive constituents

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Abstract Natural products chemistry has witnessed many new developments in the last 5 years like extractions with subcritical water and ionic liquids, LC/HRMS and LC/SPE/cryo-NMR, UHPLC, TLC/ MS, MS-based preparative HPLC, comprehensive chromatography (GC × GC, LC × LC), high-throughput screening, introduction of monolithic columns, miniaturisation, and automated structure identification. Nevertheless identifying bioactive constituents in complex plant extracts remains a tedious process. The classical approach of bioassay guided fractionation is time-consuming while off-line screening of extracts does not provide information on individual compounds and sometimes suffers from false positives or negatives. One way out of this is by coupling chromatography with chemical or biochemical assays, so called high resolution screening. An example is the development of HPLC on-line assays for antioxidants. By the post-column addition of a relatively stable coloured radical like DPPH$^\bullet$ or ABTS$^{\bullet+}$, radical scavengers are detected as negative peaks because in a reaction coil they reduce the model radical to its reduced, non-coloured form. When combined with LC/DAD/MS and LC/SPE/ NMR, reliable identification of active constituents becomes possible without the necessity of ever isolating them in a classical sense. Also for finding leads for new drugs, combining HPLC with biochemical assays is interesting but technically more difficult. Most enzymes do not work at the organic modifier concentrations commonly encountered in RP-HPLC and the reaction time is often longer requiring dilution and lengthy coils respectively. Therefore, new techniques have to be implemented to gain the required sensitivity for on-line enzyme

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assays. For stable analytes, high temperature LC offers a solution to the organic modifier problem. When enzymes are highly expensive, like those used in the screening for Cytochrome P450 inhibitors, miniaturisation to chip format may offer a way out. Microreactors (chips) are not only useful for miniaturising larger assays but also offer completely new prospects in phytochemical analysis. One such application is in the sample clean-up of acids and bases like alkaloids. In a lay-out of three parallel channels of 100 μm width with the middle one containing organic phase and the two outer ones water of high pH (feed phase) and low pH (trapping phase) such a chip replaces two classical LLE steps but is much faster and requires less solvents and less manpower input.

Abbreviations

ABTS$^{\bullet+}$	2, 2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical cation
CE	Capillary electrophoresis
CYP450	Cytochrome P450
DAD	Diode array detection
DESI	Desorption electrospray ionisation
DPPH$^{\bullet}$	Diphenylpicrylhydrazyl radical
GC	Gas chromatography
GC × GC	Two-dimensional gas chromatography
HPLC	High pressure liquid chromatography
HPTLC	High performance thin layer chromatography
HRMS	High resolution mass spectrometry
HT	High temperature
LC	Liquid chromatography
LC × LC	Two-dimensional HPLC
LLE	Liquid–liquid extraction
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
ROS	Reactive oxygen species
RP	Reversed phase
SPE	Solid phase extraction
UHPLC	Ultra high pressure liquid chromatography
UTLC	Ultra thin layer chromatography

Introduction

The search for bioactive constituents from plants, microbial sources and marine sources has been a challenging but at the same time rewarding task which has led to many new drugs and other potent natural products. In its most classical form this is performed by bioassay guided fractionation. Usually in this line of research there is a cooperation between biologists or medical doctors who are responsible for the biochemical or pharmacological testing, and phytochemists or natural product chemists who take care of the extraction, purification and structure elucidation. The isolation of pure plant constituents started about 200 years ago and 150 years ago the term "phytochemistry" was coined (Rochleder 1854). Since then we have come a long way and many new techniques have been introduced, most noticeably HPLC and various powerful spectroscopic techniques making the identification of new compounds ever faster. However not all of these developments are always obvious in recent papers. Many extractions, purifications, separations and bioassays are still slow and labour-intensive. After giving an overview of the current exciting developments in this field, the paper zooms in on three of them. It is hoped that the paper will stimulate modern phytochemists or natural product chemists to implement some of the new techniques in their own labs.

In Table 1 a number of recent developments in natural products analysis have been summarised. In the field of extraction the replacement of organic solvents by water is environmentally benign and thus interesting. At high temperature the dielectric constant of water drops considerably, making water less polar. Of course this methodology is only suitable and useful for thermostable phytochemicals which are not soluble in water at 100°C or lower. The technique requires high pressure vessels (Ibañez et al. 2003). The higher temperatures can also lead to improved extraction and thus higher yields (Lang and Wai 2003). Ionic liquids are special organic nonvolatile solvents (liquid salts). They are expensive but on the other hand can be recycled and have special dissolving properties. An example of their application in phytochemistry is the extraction of the antimalarial drug artemisinin (Lapkin et al. 2006). Another powerful development is the large scale introduction of

Table 1 New developments in natural products screening, isolation, purification and identification

Technique	Application	Reference
Subcritical water extraction	Rosemary; Ginkgo	Ibañez et al., 2003; Lang and Wai, 2003
Ionic liquids for extraction	Artemisinin	Lapkin et al., 2006
LC/MS/HRMS	Ginseng	Angelova et al., 2008; Wang et al., 2009
LC/MS/SPE/cryoNMR	Oregano flavonoids	Exarchou et al., 2003
Capillary NMR	*Thapsia garganica* fruits; cyanobacteria	Lambert et al., 2007; Glauser et al., 2008; Lin et al., 2008
UHPLC	Corn oil; *Arabidopsis thaliana* extract	van der Klift et al., 2008; Grata et al., 2008
GCxGC/TOF-MS	General; Eucalyptus essential oil	Shellie and Marriott, 2003; von Mühlen et al., 2008
LCxLC/MS	Corn triacylglycerols; lemon oil coumarins	van der Klift et al., 2008; Dugo et al., 2004
MS-based prepHPLC	Oxylipins in *Arabidopsis thaliana*	Glauser et al., 2008; Thiocone et al., 2008
HPTLC/IR-MALDI-MS	Glycosphingolipids	Distler et al., 2008
DESI-MS	Atropa, Datura and Conium alkaloids	Takáts et al., 2004; Talaty et al., 2005
Monolithic stationary phases	General; affinity chromatography of lectins	Maruška and Kornyšova, 2006; Tetala et al., 2007
Automated identification	General; flavonoids	Steinbeck, 2004; Moco et al., 2006
Structure elucidation at ng scale	Volatile semiochemicals	van Beek et al., 2005
High Throughput Screening	Ubiquitin-proteasome pathway Inhibitors	Ausseil et al., 2007
High resolution screening	Antioxidants, CYP450 inhibitors	*vide infra* for details
High temperature HPLC	As part of high resolution screening set-up	*vide infra* for details
Chip-based sample clean-up	Strychnos alkaloids	*vide infra* for details

high-resolution MS (HRMS). Fourier Transform MS with 1 ppm or better mass accuracy provides directly the elemental composition of analytes and especially for unknown compounds this can save a lot of time (Angelova et al. 2008; Wang et al. 2009). Developments in NMR continue to take place. Hyphenation with HPLC via an SPE trap and cooling of the measuring coils to ultra low temperatures (so called "cryo probes") leads to cleaner samples and an increase of the concentration sensitivity with a factor 10 to 100 depending on the HPLC and LC/NMR probe parameters (Exarchou et al. 2003). Additionally the SPE step allows the use of normal non-deuterated HPLC eluents and the recording of NMR spectra in 100% deuterated solvents. Combining LC/SPE with off-line capillary NMR is not trivial but ideally leads to a much improved mass sensitivity allowing the recording of the 2D spectra of as little as 25 µg and 1D spectra of sub µg quantities (Lambert et al. 2007; Glauser et al. 2008). Interestingly Lin et al. (2008) do not use SPE in their LC/UV/MS/NMR system but collect the fractions in 96 well

plates. After evaporation the samples are redissolved in 2–4 µL of NMR solvent which is then transferred to the microcoil NMR probe by an immiscible fluorocarbon-segmented flow with little dispersion. In this way they were able to record 1D spectra of 300 ng of paclitaxel and identify several indole alkaloids after injecting 30 µg of a bioactive cyanobacterial extract (Lin et al. 2008).

Ultra high pressure LC (UHPLC) enables the use of sub 2 µm particles and thus very high chromatographic efficiencies (small H value). This leads to two interesting possibilities: either very fast separations (1 min) by using short columns at high flow rates or ultra high resolutions (high plate numbers) by combining two 15 cm columns at a standard flow rate with a flat gradient. An example of the latter can be seen in Fig. 1 which shows a 5 h separation of an extract of wounded *Arabidopsis thaliana* leaves (Grata et al. 2008). An example of the former is depicted in Fig. 2 which shows a RP-HPLC separation of several corn oil triacylglycerols within 1 min on a 3 cm 1.8 µm C18 column (van der Klift et al.
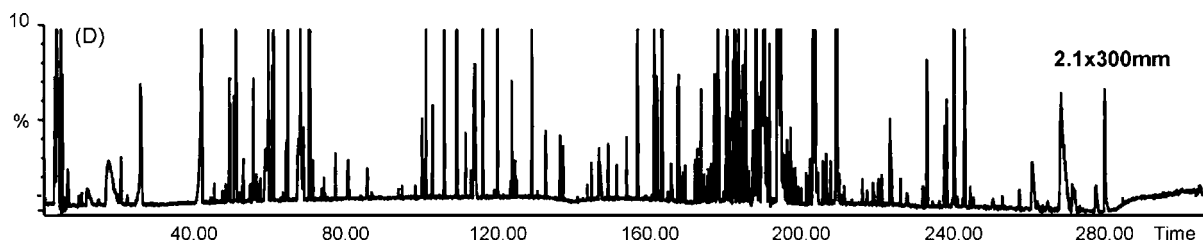
Fig. 1 UHPLC profile of a wounded pooled extract of *Arabidopsis thaliana* on a 1.7 μm C18 column of 300 mm × 2.1 mm. Eluent: gradient from water-acetonitrile (95:5) containing 0.1% formic acid to water-acetonitrile (2:98) containing 0.1% formic acid at 0.2 mL/min. Detection: TOF-MS in negative ionisation mode (Reprinted from J Chromatogr B, vol. 871, Grata E, Boccarda J, Guillarme D, Glauser G, Carrupt P-A, Farmerd EE, Wolfender J-L, Rudaz S, UPLC–TOF-MS for plant metabolomics: a sequential approach for wound marker analysis in *Arabidopsis thaliana*, 261–270. Copyright (2008), with permission from Elsevier.)
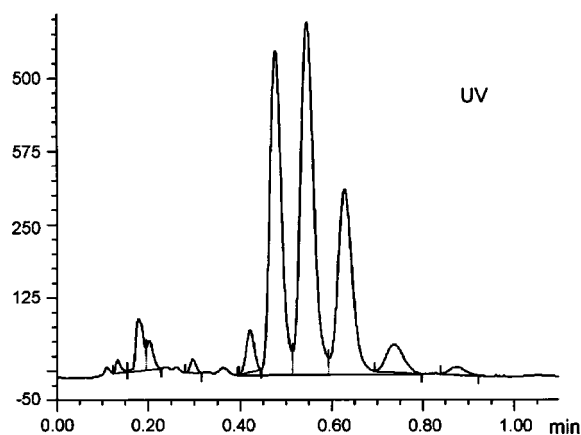

Fig. 2 UHPLC profile of corn oil triacylglycerols on a 1.8 μm C18 column of 30 mm × 4.6 mm. Eluent: methanol-methyl *tert*-butyl ether (70:30) at 3.0 mL/min. Detection: UV 210 nm (Reprinted from J Chromatogr A, vol. 1112, van der Klift EJC, Vivó-Truyols G, Claassen FW, van Holthoon FL, van Beek TA, Comprehensive two-dimensional liquid chromatography with ultraviolet, evaporative light scattering and mass spectrometric detection of triacylglycerols in corn oil, 43–55. Copyright (2008), with permission from Elsevier.)

2008). Another way to obtain very high resolution is 2-dimensional HPLC, also called comprehensive LC or LC × LC. After a slow high-resolution separation in the first dimension, 1 min portions of analytes are rapidly separated in real-time in the second dimension on a short column of different selectivity. While this happens, a new 1 min portion is collected in a loop waiting to be analysed. After proper chemometric treatment more peaks can be observed than with either of the two columns when used alone (Dugo et al. 2004; van der Klift et al. 2008). Multiple detectors can be used including MS. Two-dimensional GC (GC × GC) is even more powerful than

LC × LC and is theoretically capable of separating > 10,000 volatile constituents. This technique is well adapted to essential oil analysis where overlap between peaks is frequent (Shellie and Marriott 2003; van Mühlen et al. 2008). Data processing remains a bottleneck. In preparative HPLC, MS-based instead of UV-based fraction collection is being introduced and makes it possible to selectively collect sub mg quantities of the desired metabolite even if larger amounts of undesired compounds coelute (Glauser et al. 2008; Thiocone et al. 2008).

Thin layer chromatography has been an all-time favourite of phytochemists and is still much used. Direct interfacing of HPTLC with MS has always been difficult but nowadays this becomes possible. A natural products example is the analysis of an HPTLC separation of glycosphingolipids by IR-MALDI-MS (Distler et al. 2008). DESI-MS is a relatively new technique that can also be combined with HPTLC (Takáts et al. 2004; Van Berkel et al. 2005). Additionally DESI-MS allows for the first time, the direct probing of analytes in intact living plants by MS under ambient conditions without any sample preparation. Examples include alkaloids in *Datura stramonium* roots, *Atropa belladonna* seeds, and various parts of *Conium maculatum* and lycopenes in tomato (Takáts et al. 2004; Talaty et al. 2005). Ultra-thin layer chromatography (UTLC) can also be combined with MS. UTLC is performed on 10 μm non-particulate planar stationary phases. Claimed benefits encompass faster development (1–6 min) and less solvent consumption (1–4 mL) at a similar or slightly lower resolution than with HPTLC (Salo 2007; Salo et al. 2007). Although—as far as we

know—this technique has not yet been applied to natural products, it seems worthwhile to explore its usefulness in phytochemical analysis.

Monolithic materials as a stationary phase are being introduced as HPLC columns or as a flexible matrix to attach specific groups for affinity chromatography. A claimed advantage of monolithic materials is their lower back pressure at higher flow rates resulting in significant time gain. Maruška and Kornyšova (2006) reviewed their application in phytochemical analysis. Similar to UHPLC phases, either multiple columns can be linked to obtain very high plate numbers for fingerprints of complex mixtures (metabolomics) or very short columns can generate 1 min separations. Because they can be made in situ from many different components and can be easily modified afterwards, especially polymeric monoliths in capillaries are useful for affinity chromatography. An example is the attachment of different sugars to acrylate-based monoliths to selectively capture lectins (Tetala et al. 2007).

Automated identification of natural products has been a long-standing wish and work in this field has been on-going since the 1970s. Problems include variable HPLC retention times, lack of reference substances, poor ionization in MS, variable field strength of NMR spectrometers, impurities in spectra, and lack of proper software to combine all the data sets. Still some of these problems are being solved and building large NMR data sets for certain classes of compounds seems the way to go (Steinbeck 2004; Moco et al. 2006). When metabolites are only present in the low nanogram range, NMR is impossible but even then structure elucidation by GC/MS after derivatisation is possible (van Beek et al. 2005). High throughput screening allows the automated screening of an ever greater number of plant extracts in a short amount of time and is especially important for the lead finding in the pharmaceutical industry (Ausseil et al. 2007). Another powerful new tool for speeding up lead-finding is high resolution screening.

This technique together with high temperature HPLC and chip-based sample preparation of crude alkaloid extracts is discussed in greater detail below.

High-resolution screening

In this chapter we will mainly focus on on-line activity screening or as it is also called "high resolution screening". It involves the coupling of a chromatographic separation with a chemical or biochemical assay. As each separated compound is measured individually, in many cases it allows the rapid pinpointing of active constituents in complex mixtures. This in turn saves a lot of time as not every compound needs to be purified to homogeneity. One can focus exclusively on the active constituents. A well-known example of a biochemical assay coupled to GC is GC/EAD (electroantennographic detection). In this case the antenna of an insect is used as detector. It can detect for instance sex pheromones very sensitively (Roelofs 1984; Drijfhout et al. 2002). Coupling to HPLC is however more common and of wider applicability.

A typical example developed in our group is on-line radical scavenging detection (Dapkevicius et al. 1999; Koleva et al. 2000; Koleva et al. 2001; Dapkevicius et al. 2001; Niederländer et al. 2008). Phenolic antioxidants can terminate radical chain processes in lipid rich foods by reacting with reactive oxygen species (ROS) forming more stable resonance-stabilised radicals or quinone-like structures (Fig. 3). Although they can be used, ROS themselves are not very practical and stable model radicals are easier to work with and lead to more robust on-line systems. The most used model radicals are DPPH$^{\bullet}$ and ABTS$^{\bullet+}$. In Fig. 4 a scheme of an HPLC on-line radical scavenging assay is presented. In such an assay a relatively stable coloured model radical replaces for instance a peroxyl radical. When an antioxidant is present, mostly a phenolic, the model
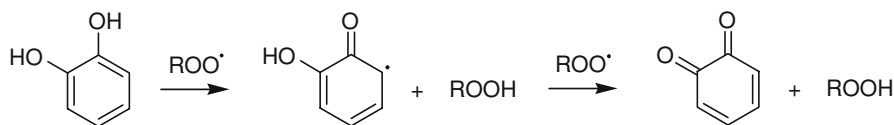
Fig. 3 Consecutive radical scavenging of two peroxyl radicals by a diphenol leading first to an intermediate stabilized radical and then to a non-radical ortho-quinone molecule
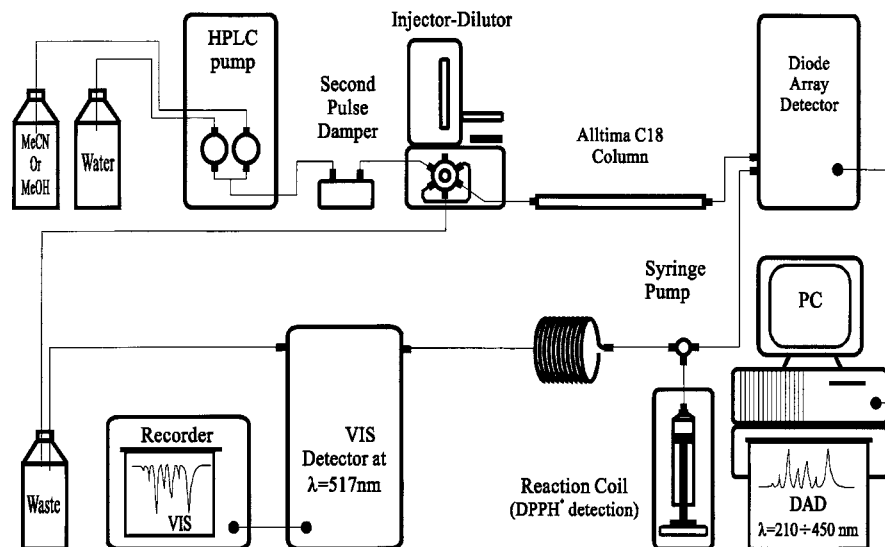
Fig. 4 Lay-out of high-resolution screening assay for radical scavengers in complex mixtures. After the DAD, a pump adds a coloured radical (here: purple DPPH•) which is only reduced to non-coloured DPPHH when radical scavengers elute from the column. Thus a negative peak indicates the presence of a radical scavenger (Reprinted in slightly modified form from J

Chromatogr A, vol. 912, Dapkevicius A, van Beek TA, Niederländer HAG, Evaluation and comparison of two improved techniques for the on-line detection of antioxidants in liquid chromatography eluates, 73–82. Copyright (2001), with permission from Elsevier.)

radical is scavenged (reduced) under the formation of an oxidised antioxidant (e.g. a quinone-like structure as in Fig. 3). As the reduced model radical is not coloured, this leads to disappearance of colour in an off-line assay, on a TLC plate with phenolics when sprayed with model radical or in an on-line HPLC assay as in Fig. 4. Thus with one injection two HPLC profiles are obtained: the "normal" one with positive peaks (e.g. by UV or MS detection) and a "negative" one where negative peaks indicate disappearance of colour and thus the presence of radical scavengers. In this way it is easy to rapidly pinpoint antioxidants in a complex mixture. This saves not only expensive chemicals and time but also avoids possible decomposition of relatively instable antioxidants. An application is the identification of rosmarinic acid and a new caffeic acid trimer in a methanolic extract of thyme leaves (Dapkevicius et al. 2002).

Although high resolution screening speeds up the identification of active constituents as no time is wasted on the purification of inactive constituents, still much time is lost if active constituents need to be isolated for the determination of their structure. Hyphenation with MS and/or NMR is also possible and allows on-line structure elucidation. A nice example is the investigation of a Lonicera extract

(Exarchou et al. 2006). For this investigation a two-way valve in between the diode-array detector and the DPPH syringe pump was mounted. In run 1 the valve caused the flow to go to the reaction coil, i.e. exactly as in Fig. 4. This run yielded information on which peaks contain the active radical scavengers (Fig. 5). In run 2 the flow was switched with the flow going to an SPE/NMR unit. Only peak L1 and L2 were measured and for instance no time was spent on studying the main UV-active peak in between L1 and L2 (Fig. 5). After recording of the NMR spectra, the content of the LC/NMR probe was collected and infusion ESI-MS and MS/MS was performed yielding molecular weights and fragmentation patterns. With this information the two peaks could be identified as the known chlorogenic acid and 3′-caffeoyl-chlorogenic acid respectively (Exarchou et al. 2006). In the whole process no preparative separation step occurred. The same technique was used to detect the radical scavengers present in *Rhaponticum carthamoides* (Miliauskas et al. 2005). A new flavonoid was identified.

Thus one can conclude that the on-line detection of radical scavenging antioxidants is a simple and robust tool rapidly providing information about the activity of individual compounds. However proper evaluation
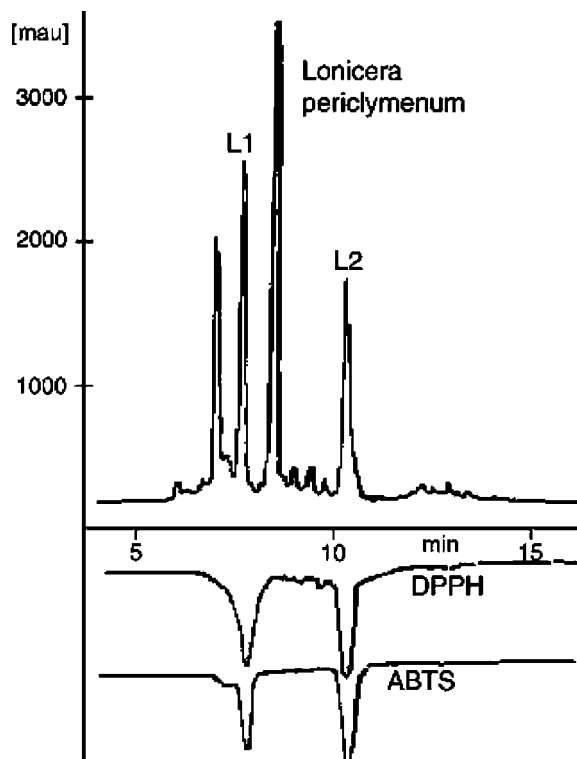
Fig. 5 HPLC-UV (*upper*) and on-line DPPH• and ABTS•+ radical scavenging profiles of a crude methanolic extract of *Lonicera periclymenum*. L1 and L2 were identified on-line as chlorogenic acid and 3′-caffeoyl-chlorogenic acid respectively (Reprinted from J Chromatogr A, vol. 1112, Exarchou V, Fiamegos YC, van Beek TA, Nanos C, Vervoort J. (2006) Chromatographic hyphenated techniques for the rapid screening and identification of antioxidants in traditional pharmaceutical plant extracts, 293–302. Copyright (2006), with permission from Elsevier.)

(Exarchou et al. 2005) especially in combination with high-resolution screening have the future. They allow rapid dereplication of known active compounds which is of vital importance for the pharmaceutical industry.

Also more complicated high-resolution assays making use of enzymes exist. Such biochemical assays are of more interest to the pharmaceutical industry as many drugs target enzymes as part of their mode of action (van Elswijk and Irth 2003; Ingkaninan et al. 2000; Schenk et al. 2003; Schobel et al. 2001). Within our group in cooperation with the Department of Toxicology of Wageningen University, we have developed an on-line HPLC system for detection of Cytochrome P450 (CYP450) 1A2 inhibitors. CYP450 1A2 is involved in the bioactivation of various human procarcinogens such as the herbal constituents methyleugenol and estragole. These compounds occur in low concentrations in certain plants like basil and tarragon and thus enter the human food chain via nowadays popular products like pesto. Via a CYP450 mediated pathway these compounds are metabolically activated and can eventually bind covalently to DNA which in turn could lead to cancer (Fig. 6). Therefore, we were interested whether these herbal products also contain other constituents which, through inhibition of CYP450 1A2, could reduce the metabolic activation and thus toxicity (Fig. 6). The classic approach would be to perform bioassay guided fractionation. A potentially faster method is high resolution screening with the set-up as depicted in Fig. 7.

A constraint when using enzymes is the fact that most enzymes are not well compatible with organic solvents. As the use of organic solvents in both normal- and reversed phase chromatography is inevitable for most analytes, a solution to reduce the percentage organic solvent in the post-column reaction coil must be found. At concentrations >15%,
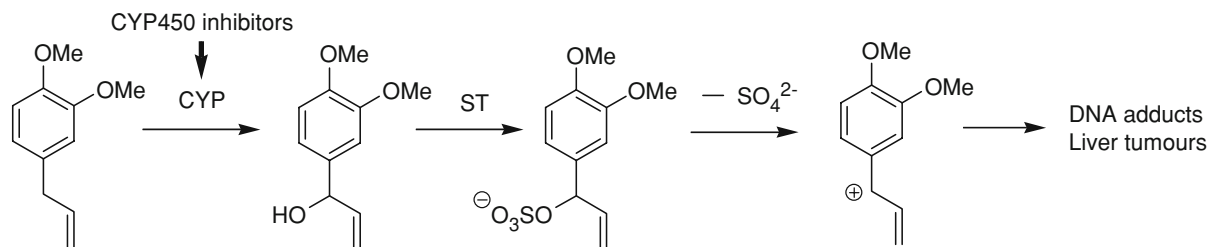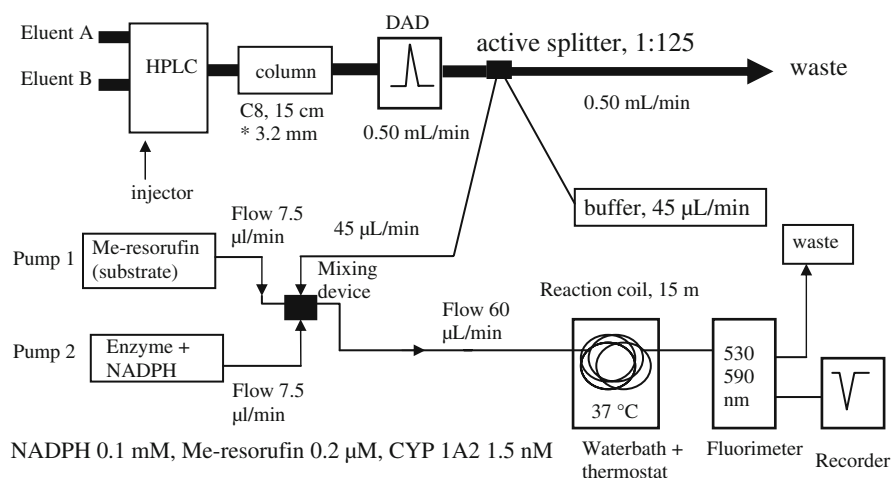
of promising extracts and compounds remains necessary (efficacy in real-life food systems, taste, odour, thermal stability, toxicity). The on-line detection of radical scavenging activity should only be considered a screening tool. And finally on-line identification techniques like LC/DAD/MS and LC/UV/SPE/NMR

Fig. 6 Bioactivation pathway of methyleugenol. ST = sulfotransferases

CYP1A2 activity dropped due to enzyme inhibition
and/or denaturation, whereas with concentrations of
5–15% methanol, a substrate dependent decrease in
sensitivity of the assay was observed (Jeurissen et al.
2007). Acetonitrile was less well tolerated. As the
analytes in plant extracts are unknown, a maximum
methanol percentage should be set, e.g. 5%. The
simplest way to realise this, is a 15-fold dilution and
the use of a C8 instead of C18 column as less
methanol is required to elute analytes from a C8
column. The exact configuration is shown in Fig. 7.
After a split and 12-fold dilution, the column effluent
is mixed with CYP450 1A2, NADPH (co-factor) and

a model substrate for CYP450 1A2 (methoxyresoru-
fin). When no inhibitors are present the enzyme
converts the model substrate to the fluorescent
resorufin which can be detected by its fluorescence.
When an inhibitor elutes this will cause a decrease in
the formation of resorufin and thus a decrease in
fluorescence (negative peak in HPLC profile) similar
to the decrease in absorbance with the radical
scavenging assay (*vide supra*). Proof-of-principle
was obtained with the set-up shown in Fig. 7 and
CYP450 inhibitors were shown to be present in a
kava kava and a basil extract. However, further
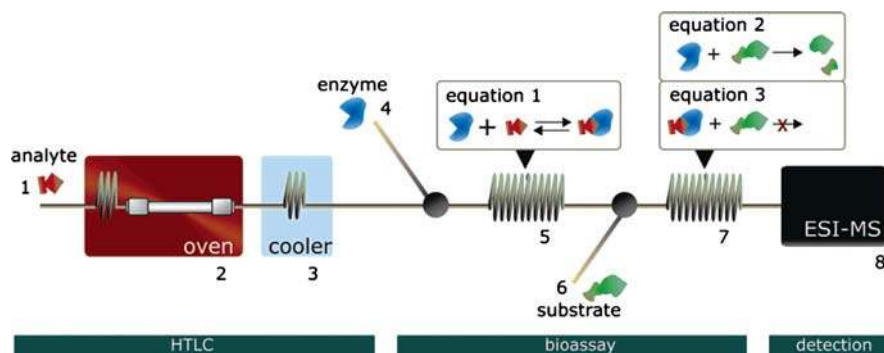optimization is required before the high resolution

Fig. 8 Scheme of high resolution screening assay for enzyme
inhibitors with high-temperature HPLC. Key: 1, sample
introduction; 2, HTLC oven containing the heating coil and
column; 3, cooler; 4, superloop with enzyme solution; 5,
bioreactor A; 6, superloop with substrate solution; 7, bioreactor
B; 8, ESI-MS. The enzyme continuously converts the substrate
into products (*eq 2*) if no bioactive compounds are eluting
from the column. Bioactive compounds present in the effluent
bind to the enzyme (*eq 1*) resulting in a decrease of product

turnover (*eq 3*). The reaction products are continuously
detected by ESI-MS (Reprinted from Anal Chem, vol. 77, de
Boer AR, Alcaide-Hidalgo JM, Krabbe JG, Kolkman J, van
Emde Boas CN, Niessen WMA, Lingeman H, Irth H, High-
temperature liquid chromatography coupled on-line to a
continuous-flow biochemical screening assay with electrospray
ionization mass spectrometric detection, 7894–7900. Copyright
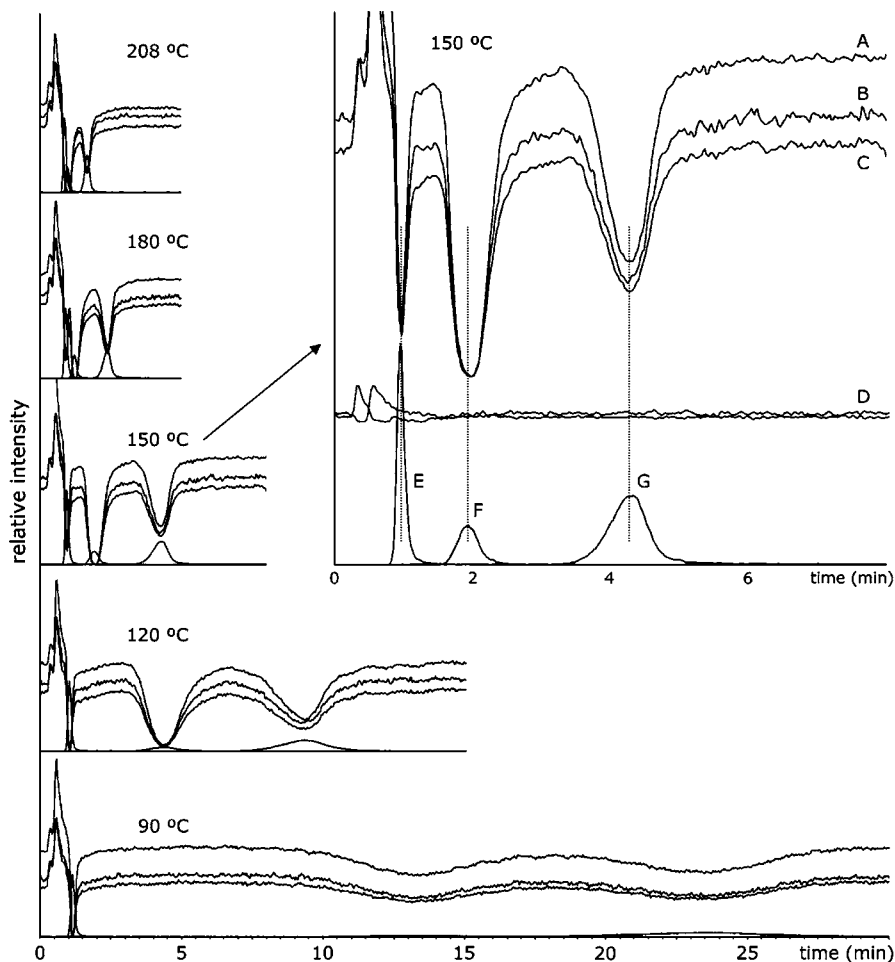(2005), with permission from the American Chemical Society.)

Fig. 9 Screening of 3 inhibitors (55–65 µmol/L) of cathepsin B at various column temperatures by means of the system depicted in Fig. 8. **a–c** mass chromatograms of three products **d** control mass chromatograms; **e–g** three analytes. Injection volume, 0.5 µL; column, PRP-1; flow rate LC, 300 µL/min water-methanol (90–10) (Reprinted from Anal Chem, vol. 77, de Boer AR, Alcaide-Hidalgo JM, Krabbe JG, Kolkman J, van Emde Boas CN, Niessen WMA, Lingeman H, Irth H, High-temperature liquid chromatography coupled on-line to a continuous-flow biochemical screening assay with electrospray ionization mass spectrometric detection, 7894–7900. Copyright (2005), with permission from the American Chemical Society.)

screening of herbal extracts for CYP450 inhibitors can be routinely used. The main problems included peak broadening in the reaction coil due to the necessary long residence time of 4 min and poor sensitivity due to the 15-fold dilution step. Down-scaling to a 0.15 mm i.d. LC column with a flow of 1 µL/min may solve some of these issues. The mixing and post-column reaction coil can be constructed on a chip (de Boer et al. 2005a). In addition, the much lower total flow will allow higher enzyme concentrations to be used at reduced costs. To further increase the sensitivity capillary laser-induced fluorescence detection can be considered.

High-temperature HPLC

An elegant option to reduce the percentage organic modifier and thus the dilution step is high temperature HPLC. As mentioned before, at higher temperatures water becomes appreciably more non-polar by itself so at temperatures of 150–200°C much less organic modifier is necessary and correspondingly less dilution is required (de Boer et al. 2005b). Of course this demands some adaptations of the technical part and the method only works for natural products which are stable in water at the chosen temperature. The technical changes consist of a larger

column oven, a preheater for the mobile phase before the column and a cooler for the mobile phase after the column (Fig. 8). Standard C18 columns cannot be used at such elevated temperatures and they have to be replaced by more rugged but less efficient columns. De Boer et al. showed nevertheless that the technique works and retention times with the eluent methanol–water (10:90) decreased from 24 to 2 min when the temperature was increased from 90 to 205°C (Fig. 9). The high temperature LC did not affect the biochemical high-resolution assay for cathepsin B inhibitors (de Boer et al. 2005b). An overview of the pros and cons of HT-HPLC was presented by Heinisch and Rocca (2009).

Miniaturisation of liquid–liquid extraction (LLE)

LLE is one of the oldest and most used sample preparation techniques. It is especially valuable for the isolation of organic acids and bases. Natural bases like alkaloids can be purified to a great extent by two separate LLE steps. When the pH is alkaline the
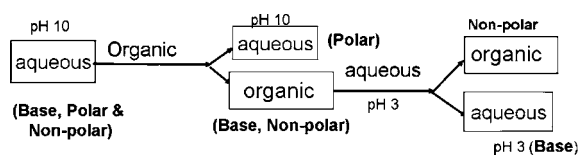

Fig. 10 General purification scheme for alkaloids (base) from neutral polar and non-polar impurities

alkaloids are non-polar and dissolve readily in organic solvents leaving behind polar impurities like sugars, salts and amino acids. When the organic layer is subsequently extracted with acidified water the alkaloids are protonated, become water-soluble and partition into the aqueous layer leaving behind non-polar neutral impurities like sterols, waxes and other fats (Fig. 10). Unfortunately this method is labour-intensive, solvent intensive, slow, and if emulsions are formed it becomes even more tedious. Therefore we made an attempt to miniaturise and combine these two separate LLE steps by carrying them out on a microfluidic glass chip. The general three-phase microfluidic chip design is shown in Fig. 11. The three solvents (basic aqueous phase with crude alkaloids, organic phase in the middle, and acidic aqueous phase, all enter the chip on one side) form parallel streams in the core part of the chip. Due to a selective non-polar coating of the middle channel, some small pillars and the curvature of the channels, the three phases remain each in their own channel, i.e. the flows remain laminar. In Fig. 12 a photo of the three flows can be seen. For greater clarity the dye Sudan red has been added to the chloroform phase in the middle channel. After some optimisation of various parameters with strychnine as model compound, an extract of Strychnos seeds was infused into the chip. All three phases were collected and investigated by off-line HPLC. At flow rates of 0.25 μL/min for both basic and acidic aqueous phases and 0.5 μL/min for the organic phase more than 90%
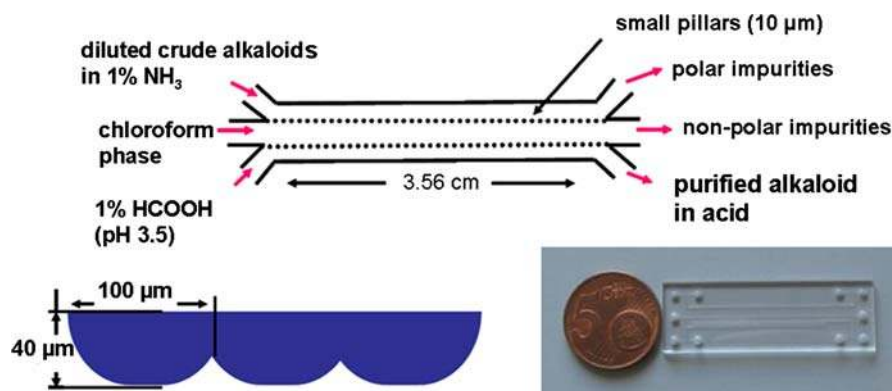

Fig. 11 Top middle: general lay-out of 3-phase microreactor capable of separating a crude alkaloid fraction into three separate fractions; bottom left: lay-out (*side view*) with dimensions of the three parallel channels, the solvents in the channels make direct contact; bottom right: real image of 5 × 2 cm glass chip, the holes at the side are for the fluid connections. Five Euro cents coin is for size comparison only
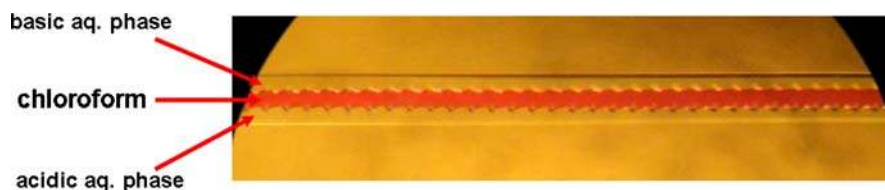
Fig. 12 Photo of 3-phase chip showing three laminar parallel flows. The middle chloroform containing channel has been made hydrophobic to maintain a stable phase separation. For better visual clarity (*for photo only*) the dye Sudan red has been added to the chloroform phase

of the present strychnine and brucine was extracted in 25 sec into the acidic aqueous phase showing rapid transport in the 3.5 cm chip. The transport is so fast because diffusion is fast at these small distances. According to HPLC, polar impurities remained in the basic aqueous phase (Tetala et al. 2009). Thus proof of principle was obtained that this lab-on-a-chip device can combine two separate LLE steps. In future the wider applicability will be investigated. Most interesting would be hyphenation with an extraction cell, a separation step (nanoLC or CE) or spectroscopic devices (capillary DAD or nanoMS).

Conclusion

Phytochemical analysis is still a vibrant field with many new and exciting developments. As highlighted above, high resolution screening, especially when combined with DAD, MS and/or NMR can really lead to not only on-line detection of bioactive compounds but also to their identification without prior isolation. This can speed up the finding of new leads from plants, marine organisms and microbes in pharmaceutical industry. A prerequisite is of course that the bioassay is amenable for post-column application. Other developments focus on improved sample clean-up and especially further hyphenation of often still separate extraction—sample prep—separation—detection steps. Potentially this leads to a huge decrease in necessary time, effort and use of chemicals.

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