



The potential of metabolic fingerprinting as a tool for the modernisation of TCM preparations

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ABSTRACT

A vast majority Chinese herbal medicines (CHM) are traditionally administered as individually prepared water decoctions (*tang*) which are rather complicated in practice and their dry extracts show technological problems that hamper straight production of more convenient application forms. Modernised extraction procedures may overcome these difficulties but there is lack of clinical evidence supporting their therapeutic equivalence to traditional decoctions and their quality can often not solely be attributed to the single marker compounds that are usually used for chemical extract optimisation. As demonstrated by the example of the rather simple traditional TCM formula Danggui Buxue Tang, both the chemical composition and the biological activity of extracts resulting from traditional water decoction are influenced by details of the extraction procedure and especially involve pharmacokinetic synergism based on co-extraction. Hence, a more detailed knowledge about the traditional extracts' chemical profiles and their impact on biological activity is desirable in order to allow the development of modernised extracts that factually contain the whole range of compounds relevant for the efficacy of the traditional application. We propose that these compounds can be identified by metabolomics based on comprehensive fingerprint analysis of different extracts with known biological activity. TCM offers a huge variety of traditional products of the same botanical origin but with distinct therapeutic properties, like differentially processed drugs and special *daodi* qualities. Through this variety, TCM gives an ideal field for the application of metabolomic techniques aiming at the identification of active constituents.

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1. Introduction

There is an increasing interest in the use and application of CHM throughout the Western hemisphere. Notwithstanding the intake of powdered herbal drugs in different delivery forms, the typical preparation of CHM involves some kind of extraction of herbal drug material. Traditionally, the most important delivery form is the

Abbreviations: AR, Astragali Radix; ASR, Angelicae sinensis Radix; CHM, Chinese herbal medicine; CMM, Chinese Materia Medica; DBT, Danggui Buxue Tang; FP, fingerprint analysis; PCA, principal component analysis; PLS-DA, partial least square discriminant analysis; SCO, supercritical carbon dioxide; SFE, Supercritical Fluid Extraction; TCM, traditional Chinese medicine.

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water decoction of a mixture containing typically 2–12 different herbal materials (Yi and Chang, 2004). It should be explicitly stated that the item which is finally administered to a patient is an extract which is not just represented by the botanical origin of its herbal ingredients but also influenced by any procedure that is applied to the herbal material. Any such procedure may severely impact upon the extract's chemical composition and hence the products' quality with regard to its therapeutic efficacy. This is especially true for complex TCM formulae where we still lack a comprehensive understanding of the interactions between the thousands of chemical compounds that constitute the herbal metabolome(s). Besides showing synergistic effects on a pharmacodynamic level, chemical compounds of a TCM formula or even an individual herbal material can also interact with each other (i) on a pharmacokinetic level, influencing the solubility, stability or resorption of compounds or (ii) on a biochemical level, where residual herbal

enzymes may impact the chemical composition; (iii) a mixture of herbal drugs could even be shown to contain metabolites that none of the individual herbal ingredients displayed (Nüsslein et al., 2000; Spinella, 2002; Gu et al., 2004; Nualkaew et al., 2004; Ma et al., 2009; Nahrstedt and Butterweck, 2010). Facing this complexity, the recent approaches for modernisation of CHM, aiming at the development of more convenient and better standardised products, bear the risk that such features are lost by turning away from the traditional products and their traditional drug processing and extraction procedures. Extract optimisation is frequently guided by the analysis of single compounds which can often be regarded as the active substance (Yan et al., 2010). Yet, lacking the appropriate knowledge, the activity of many herbal preparations cannot be clearly linked to single chemical compounds (Vlietinck et al., 2009). Even very well established herbal products like extracts of *Salicis cortex* have shown efficacy beyond that which is explicable by their content of salicin derivatives which have been considered to solely constitute the extract's active substance for decades (Schmid et al., 2001). With regard to CHM, it seems desirable that the traditional procedures like the unique *pàozhì* drug processing and the extraction of specific combinations of herbal drugs are systematically investigated for their impact on the herbal extracts' therapeutic qualities and metabolomes (Yi and Chang, 2004; Zhao et al., 2010). In this review we will describe the current situation and problems connected to the extraction and compound analysis of CHM and show possible research approaches to gain the chemical information required for a rational extract modernisation that gives consideration to the complex composition of traditional preparations.

2. Traditional extraction of CHM

The majority of CHM for oral use is applied as water decoctions (*tang*). Other oral preparations include macerates in aqueous ethanol, the intake of powdered drugs suspended in water or prepared in pills, e.g. with honey, water or rice gruel as excipients (Li et al., 2007; Martin and Stöger, 2008). Decoctions, macerates and suspensions are very simple to prepare and allow a high flexibility of the recipes. A factor which fundamentally influences the chemical composition of extracts is the *pàozhì* processing of the crude drug prior to extraction. This is of special importance for the detoxification of toxic materials like *Aconitum* drugs (Singhuber et al., 2010) but also for the preservation of active constituents, ease of administration, flavour correction or cleansing (Zhu, 1998). Many drugs can be processed by various methods like stir-frying, steaming or calcining in order to gain a product with altered therapeutic properties and – implicitly – altered chemical composition (Zhao et al., 2010; Zhan et al., 2011).

To prepare a typical traditional decoction, a complex TCM formula is macerated with water for a period of time before a first boiling step follows. After filtration, the herbal material is re-extracted a second time (usually with less water) and the combined extracts are ingested and/or kept for further use. The time spans for soaking and cooking depend on the drugs as well as on the indication. As a rule of thumb, decoctions for acute conditions are cooked for a shorter period of time as compared to preparations for chronic diseases. Over thousands of years numerous special instructions for the boiling process have been developed in the use of CHM. These methods seem to be related to the different physical, chemical and pharmacological characteristics of the active compounds (Martin and Stöger, 2008; Körfers and Sun, 2009).

Herbal drugs containing volatile or temperature-sensitive substances are added only a few minutes before the end of the first boiling period to avoid losses or decomposition (*hòuxià*). Expensive drugs, e.g. *Ginseng Radix* or *Panacis quinquefolii Radix* might

be cooked separately to optimise the yield and avoid adsorption of active compounds to other ingredients within the prescription (*lingjiān, lingdūn*). In some instances drugs have to be added to the mixture wrapped in a thin cloth (*bāojiān*), this occurs with drugs like *Typhae Pollen*, which might lead to turbid decoctions or *Inulae Flos* which contains drug particles which could cause intestinal irritations. For toxic drugs such as *Aconiti radix*, which are only used orally after *pàozhì* processing, additional cooking steps or longer boiling times for the preparation are recommended. In preparations containing *Acori Rhizoma*, an extended decoction process (in summary 3 h) has been proven to be very efficient for the reduction of genotoxic β -asarone (Chen et al., 2009).

3. The example of *Danggui Buxue Tang* (DBT)

In order to evaluate the significance of traditional procedures for the therapeutic value of a CHM, the rather simple two-herb formula DBT has been chosen as a model to investigate the influence of numerous extraction parameters on the chemical composition and pharmacological efficacies of a water decoction. The traditional composition of DBT as established over the centuries is reported as five parts *Astragali Radix* (AR) and one part *Angelicae sinensis Radix* (ASR). An investigation of several mixtures with different ratios of the two drugs has shown the best pharmacological effects as well as the highest yields of the (active) markers astragaloside IV, calycosin, formononetin, ferulic acid, total saponins, total flavonoids and total polysaccharides for the traditional composition, while the undesired compound ligustilide was found to be least concentrated with this ratio. The concentrations of these compounds varied over the examined drug ratios (AR:ASR from 1:1 to 10:1) by a factor of ~2, demonstrating a significant impact of the drug ratio on the extraction yield (Dong et al., 2006; Po et al., 2007). Systematic investigations compared different durations of boiling, drug-solvent-ratios and numbers of extractions for DBT and also examined the chemical properties of both herbal ingredients' individual extracts. It could be shown that the treatment with DBT was up to twice as effective as individual extracts of its herbal ingredients at the same concentration. Interestingly, the extraction corresponding most closely to the traditional prescription (twofold extraction of the drug mixture with the eightfold amount of water and an entire boiling time of 2 h) resulted in the best extraction of active markers and the best effect in two bio-assays (Song et al., 2004; Gao et al., 2006). A mixture of individually prepared extracts of AR and ASR showed an inferior pharmacological effect when compared to the traditional co-extraction of the drugs as well as a lower concentration of active markers. It is interesting to note that the investigated markers from ASR (ligustilide and ferulic acid) showed an astonishing increase of concentration of more than 25 fold when co-extracted with ASR, indicating a massive influence of AR on the extraction of these compounds (Mak et al., 2006). Another study of DBT yielded similar results with respect to biological activity, however, the chromatographic fingerprints did not show such a huge influence of co-extraction on the ASR markers. The authors concluded that other, non-observed compounds influenced by co-extraction would impact the biological activity (Choi et al., 2011). The *pàozhì* processing of ASR with rice wine prior to extraction was also examined and resulted in higher extraction yields of astragaloside IV, isoflavones and polysaccharides (Dong et al., 2006). As the processing of ASR with rice wine results in a decreased concentration of ligustilide in the processed product, it was examined if the co-extraction of AR with pure ligustilide would impact the extraction yields of AR markers; in fact, ligustilide lowered the yields of these markers in a dose-dependent manner, indicating that the *pàozhì* processing of ASR influences the activity of DBT by altering its properties with regard to co-extraction with AR (Zheng

et al., 2010). Further, the exchange of ASR with another drug, Radix Chuanxiong, which shares the occurrence of ligustilide and ferulic acid with ASR, was examined, resulting in both an inferior pharmacological efficacy and lower concentrations of the AR markers when compared to a DBT extract, indicating that other, non-observed compounds of ASR may impact the quality of DBT (Li et al., 2009).

It must be stated that the activities in the above mentioned studies were assessed by completely different methods. Nevertheless, the example of DBT impressively demonstrates that the subject of extraction is not a trivial one and that the chemical composition and pharmacological efficacies of the traditional water decoctions are influenced to an astonishing degree by a multitude of factors which apparently have found their optimum in the traditional DBT prescription.

4. Modernisation of CHM extracts

The development of modernised extracts and application forms is desired for numerous reasons: (i) individually prepared water decoctions are more likely to entail quality shortcomings caused by improper herbal drugs when compared to herbal medicines produced in industrial scale under best controllable conditions. (ii) Water decoctions are probably the worst possible preparation in terms of stability and may give rise to microbial contaminations, decomposition of constituents by hydrolytic or oxidative reactions or precipitations that may impact the product's quality. (iii) TCM water decoctions are infamous for their unpleasant organoleptic properties. (iv) The rather complicated and time consuming preparation and storage of water decoctions may cause compliance problems while modernised application forms based on dry extracts (granules, capsules, tablets, etc.) are easily manageable for both patients and practitioners. (v) The possibility for standardisation of large-scale extracts is a prerequisite for future evidence-based research. (vi) The blinding of clinical studies employing traditional water extracts is hampered by their strong organoleptic properties (Martin and Stöger, 2008; Flower et al., 2011).

As evident from the studies on DBT, turning away from traditional procedures bears the risk of altering the product's therapeutic properties. Therefore some modernisation approaches, directly based on water decoctions, have been developed to overcome the above mentioned problems. One such approach involves the delivery of water decoctions in sealed, separately packaged, daily dosages thus increasing the compliance and stability of the product. Also an unpleasantly tasting herbal placebo composed of nine culinary herbs was designed (Flower et al., 2011).

4.1. Pressurised hot water

Pressurised hot water extraction at high temperature is sometimes applied and can be regarded as a kind of modernisation. This method has been shown to decrease the extractant's polarity and thus provides the extraction of a wider range of compounds (Deng et al., 2007). Nevertheless, it is reported that the use of pressure cookers for the preparation of decoctions to shorten cooking times leads to less active preparations (Martin and Stöger, 2008).

4.2. Formation of granules

A major approach to modernise and facilitate the application of CHM was the introduction of granules, which are usually prepared from decoctions by fluid-bed granulation or spray-drying. As the technological properties of such extracts are impaired by high amounts of hydrophilic constituents, especially carbohydrates which result in hygroscopic, sticky and hence hardly processable extracts. Thus, excipients are added to the decoction of the herbal

material (one step procedure) or mixed with the spray-dried product (two step procedure) (Martin and Stöger, 2008; Ai et al., 2008). Rather high amounts of additives are required (Wang et al., 2011) and these additives further add on to the extract dose which is already quite large due to the presence of polar "bulk material". Another method to improve the technological properties is the removal of highly polar compounds from the extract by ethanol precipitation prior to drying (Tan et al., 2006). Such preparations can be instantly applied by suspension in hot water or can be used to produce single dosage forms like capsules. An advantage is that these products can be applied quickly avoiding the time consuming preparation of a decoction.

4.3. Use of less polar extractants

Other modernisation approaches employ less polar extractants and hence can yield a dry extract with superior technological properties when compared to dried water extracts. These approaches comprise the extraction with (hydro)alcoholic or organic extractants performed by different extraction techniques like maceration, Soxhlet extraction, microwave-assisted extraction, ultrasonic extraction or accelerated solvent extraction. Though these different extraction techniques show clear distinct properties (e.g. thermal decomposition during Soxhlet extraction, solvent limitations for microwave-assisted extraction), the differences can mainly be attributed to extraction speed while the chemical composition of the resulting extracts after establishment of equilibration is mainly influenced by the kind of extractant used and its temperature (Yan et al., 2010). From a general view, the differences in extract composition between these techniques can be regarded as negligible when compared to the differences between any of these techniques employing (hydro)alcoholic or organic solvents and a traditional water decoction. A study comparing hydroalcoholic extracts of more than 30 drugs with their respective water decoctions clearly showed that decoction in most cases allows an extraction of phenolics which is similarly efficient as maceration with 50% ethanol. The yields after extraction with 80% methanol, a very common method for analytical determination of phenolics and their testing on antioxidant activity, was much lower. In this study, most of the decoctions, among them drugs from *Rehmannia glutinosa* Libosch, *Astragalus membranaceus* (Fisch.) Bge. or *Atractylodes macrocephala* Koidz., showed better or similar antioxidant activity as compared to the hydroethanolic macerates. The activity of the 80% methanolic extracts was worse for almost all tested drugs (Li et al., 2007). In a comparison of aqueous and ethanolic extracts from different drugs a more pronounced effect on various CYP enzymes was proven for the water extracts (Tang et al., 2006).

4.4. Supercritical Fluid Extraction (SFE)

A completely different extraction technique that by its technical properties is attractive for large scale extraction, is Supercritical Fluid Extraction (SFE). This is an extraction technique which takes advantage of the enhanced solvating and penetrating capacity of gases or liquids in their critical phases (ASTM, 2006). The unique properties of supercritical fluids were observed more than a century ago, but only in the last four decades SFE has emerged as an extraction technique (Khosravi-Darani, 2010). Advantages of SFE in contrast to conventional extraction are (i) superior extraction efficiency and selectivity for low polar phytochemicals. The extraction efficiency and selectivity can be tuned by optimising pressure and temperature (Wang et al., 2008; Liu et al., 2008a). (ii) Among the many applicable solvents for SFE, the most commonly used for extraction of CHM is supercritical carbon dioxide (SCO) which is inert, easily available, inexpensive, odourless, environmental

friendly and has mild critical point properties. To increase the solubility of compounds from CHM in SCO, small amounts of polar modifiers, e.g. ethanol, may be added, usually not more than 10% of the amount required for conventional extraction techniques (Chen and Ling, 2000; Silva et al., 2009; Chen et al., 2011). (iii) SCO is easily removable from the extract by depressurization, and thus no solvent residue is left in the extract (Vagi et al., 2002). (iv) Preferable product stability. The extraction is conducted at oxygen and light free operating conditions which prevent oxidation and light dependent changes, for example, a SCO extract of tomatoes could be stored for more than half a year at -20°C without lycopene loss (Lenucci et al., 2010). Furthermore, the low temperatures minimize thermal degradation of sensitive materials, e.g. volatiles. As a green separation technique, SFE has a promising future in its application in the fields of TCM and natural products (Martinez, 2008).

However, there are limitations for any of the extraction methodologies to be considered for the production of more convenient application forms. Because of the “Lipinski rule of five” (Lipinski et al., 2001), it is generally believed that less polar extractants like alcohols, acetone or SCO are capable of extracting pharmacologically relevant analytes while excluding higher amounts of polar, technologically difficult “bulk material” like carbohydrates, proteins, amino acids etc. from the extract. However, this is a very reductionistic view which actually applies to a single active compound and especially excludes the possibility of pharmacokinetic synergism in herbal extracts, which has been shown to have a major impact on the chemical composition of CHM (e.g. Mak et al., 2006). It cannot be generally ruled out that the extraction of very polar constituents (needing water as extractant) can be essential for producing efficient extracts from specific drugs or for specific applications, polymeric carbohydrates or compatible solutes like ectoine are examples for such highly polar bioactive compounds (Lentzen and Schwarz, 2006). When relating any modernised CHM extract to the long experience of TCM it should be considered that its clinical efficacy should at least match the one of the traditional preparation it relates to. It has been shown that the single active markers that are typically used for the quality control of herbal drugs may not be able to fully explain a product’s quality, especially when the drug is used as part of a co-extracted herbal mixture (Schmid et al., 2001; Li et al., 2009; Vlietinck et al., 2009), and frequently not even an active marker is known but analytical markers without known clinical relevance are used as a surrogate. As a consequence, such single markers cannot be generally recommended to guide the optimisation of an extract. Hence, for the rational development of new products it is essential to increase our knowledge about which chemical compounds define the quality of a traditional preparation, so a production chain can be established that finally yields an improved product lacking technologically problematic “bulk material” while conserving any compound that has been shown to be relevant for the traditional product’s quality in terms of clinical efficacy. In summary, the rational modernisation of CHM requires further research to identify chemical compound that can be linked to an herbal preparation’s quality considering pharmacodynamically and pharmacokinetically relevant compounds.

5. Fingerprint analysis in activity studies of herbal extracts

A major challenge in the modernisation of CHM is the incorporation of between one and twelve herbs in a given formula, thus conferring a high degree of complexity and the potential for variation in composition and quality of a preparation. It has also been established that interactions between chemical components may take place during traditional co-extraction of complex herbal mixtures and thus impact the extract’s chemical composition. Due to the lack of knowledge about the chemical compounds that

completely constitute the quality of many CHM, the widespread practice of using single herb monographs and analysing of single compounds for the characterisation of CHM extracts tested in pharmacological or clinical studies seems to be insufficient. This is of particular issue when dealing with HM that contain multiple herbs, and can be illustrated by the fact that sometimes the same, rather unspecific metabolites are used for the quality assessment of herbal drugs with distinct clinical properties. Examples are chlorogenic acid, which is used as a marker compound for *Caulis Lonicera*, *Flos Lonicera* and *Flos Chrysantemi*, or berberine, which is used as a marker compound for *Rhizoma Coptidis* and *Cortex Phellodendri* (Zhou et al., 2008). Consequently, a more comprehensive view on the chemistry of extracts is desirable. It is obvious that the metabolomic techniques currently available for fingerprint analysis (FP) of complex biological and herbal samples and those with evolving applications in this area, have the potential to enhance the quality control of CHM and to assist with the correlation of bioactivity with composition. FP can be defined in this context as an analysis aiming at the representation of an extract’s chemical composition to a maximum possible degree; while being largely untargeted, FP can also be used for or as an addition to the quantification of single compounds.

Chromatographic FP and the simultaneous determination of multiple compounds is becoming an important trend (Liang et al., 2010). However an analysis of 97 original papers¹ assessing biological effects of CHM extracts revealed that only 16 provided – exclusively chromatographic – FP data while 24 characterised the extracts by quantifying relevant single compounds and 57 did not chemically characterise the tested extract at all. We consider the presentation of FP data within activity studies of herbal extracts as fundamental information for chemical characterisation. Considering the possible variability of an extract’s chemical profile that can be caused by differences in the herbal materials (growth conditions, post-harvest treatment, *pào zhì* processing, etc.) or manufacturing (extraction conditions, drying, etc.) it must be stated that the examined item in such studies is literally unknown without at least some basic chemical characterisation.

Different methods are used for FP which may be roughly categorised as (i) low resolution techniques like TLC or IR-based methods which are typically applied for assessing the identity or origin of an herbal material by visual or – in case of NIR – computer aided comparison of signal patterns, mostly without (detailed) assignment of signals to chemical compounds (Xie et al., 2006; Sun et al., 2010). (ii) High resolution techniques like GC, HPLC, MS, NMR or hyphenated techniques allow for a detailed assignment of signals to the detected chemical compounds and are commonly accepted as well suited for FP aiming at a comprehensive extract characterisation as well as for metabolomic approaches. The advantages and disadvantages of these methods with regard to suitable target metabolites, reproducibility of signal intensities and positions, sensitivity, resolution and sample preparation efforts have been extensively discussed (Verpoorte et al., 2008). It must be stated that no available analytical method is capable of fulfilling the demand for a complete qualitative and quantitative assessment of a biological sample’s whole chemical composition.

¹ The literature research was performed with either Scopus or Pubmed using an OR-conjoined combination of any botanical identifiers given by the consortium’s priority list of species (CP2005 Latin binominal species name, taxonomically accepted Latin binominal species name, Latin drug names, Pinyin names, Latin binominal synonyms). The search result was then refined by limiting the hits to the topics “Extraction” and “Chemistry” or adding these terms to the general search term with “AND” conjunctions for Pubmed, respectively. The 97 papers mentioned here were chosen from the results by the fact that they assessed the biological activity of an herbal extract.

In order to find a recommendable procedure for FP based on current practice we analysed 157 original papers² for the methodology used for the general analysis of herbal extracts from Chinese drugs and found that a majority of 64% used HPLC-based techniques, 18% used other separation techniques and 18% used spectroscopic techniques. Restricted to studies involving the assessment of an extract's biological activity, the proportion of HPLC-based techniques raises to 94%, indicating a preference of HPLC for studies where FP would be advisable. With regard to its analytical properties and its wide availability, HPLC seems to be an ideal standard methodology for FP of many herbal extracts, if some aspects are considered. (i) A precise reproduction of even relative retention behaviour is not expectable to a degree that would justify recommending a specific global method for, e.g. direct data comparison between different labs or building up a public FP database. Furthermore, the separation requirements are usually variable for different extracts and research focuses. (ii) To guarantee a fundamental comparability and reproducibility of FP, we recommend the use of conventional equipment whenever possible, e.g. a most conventional RP-18 column based on type-B silica that allows for an unproblematic separation of basic compounds. Full binary gradient elution using water and acetonitrile each with 0.1% formic acid will display a maximum possible range of compounds, is LC-MS-ready and can be adapted to specific separation problems by flattening the gradient slope or including isocratic steps for regions of special interest, e.g. for the quantification of known active markers. As most HPLC systems are not capable of actively cooling the column compartment, it is recommended to consider a column temperature that is higher than usual room temperatures, e.g. 40 °C. (iv) Facing the difficulties with the exact reproducibility of separation conditions, it is of importance to offer some orientation within a presented FP chromatogram. Fundamentally, this can be achieved by presenting a couple of UV-chromatograms of the examined extract recorded at different wavelengths (230 nm, 254 nm, 280 nm and 340 nm are suitable for many plant secondary metabolites); the ratios of a peak's signal intensities at different wavelengths can be used as simple qualifiers and can be read out from the presented chromatogram at least for major compounds. UV chromatograms can also provide rather reliable relative quantitative information when compared to, e.g. MS-based TICs, though a TIC or an ELSD chromatogram complements an UV-chromatographic FP with regard to compounds lacking a chromophore. Assignment of unidentified peaks to their UV-spectra and/or MS-spectra or the presentation of 2D contour plots may give further orientation. Whenever possible, chromatographic peaks should be assigned to compounds that could be unambiguously identified.

The presentation of such a FP consisting of several chromatograms allows the assessment and basic comparison of extract characteristics beyond the quantities of a few markers by number, position and characteristics of unidentified peaks and their peak-to-peak ratios (Liang et al., 2010). For example, we could compare quantitative data of one paper about DBT (Mak et al., 2006) with another paper that did no quantification but presented a suitable FP (Choi et al., 2011).

However, there are clear limitations: (i) difficult analytes like extremely lipophobic or lipophilic substances, polymers or complex compounds like tannins will not be well assessable by such a system (ii) the chromatographic selectivity and hence the relative position or even succession of peaks may be influenced by, e.g. the brand and age of the applied column, temperature, gradient slope or the instrument's dwell volume. (iii) When comparing

peak intensities within a chromatogram or between different chromatograms care must be taken, e.g. the signal intensities in any MS-based chromatogram are strongly biased by complex and partly instrument-specific parameters settings, unattended contamination or aging of instrumental components and other factors which are hardly controllable or reproducible; furthermore, signal intensities may be biased by any detector's linear range. These limitations could be overcome by the additional presentation of an ¹H NMR spectrum-as NMR is a method solely based on physical characteristics with rather limited experimental factors influencing signal positions and intensities, the reproducibility is excellent and hence such data are well suited for the desired establishment of a public database of FP data, taken that a standardised protocol can be agreed on. Limitations of ¹H NMR fingerprinting are a rather low sensitivity and the representation of chemical compounds as rather complex signal groups which hamper a direct perception of single chemical entities when compared to well separated chromatographic peaks (Verpoorte et al., 2007). Because of the excellent reproducibility, the increasing use of NMR for metabolomic studies and the efforts towards an establishment of public data platforms make NMR a feasible alternative also for the FP of extracts in activity studies.

6. NMR for fingerprinting and metabolomics

In the last decade the power of ¹H NMR spectroscopy for high throughput metabolomic analysis of complex mixtures including plant extracts and traditional medicines has been well established (Lindon et al., 2000, 2001; Feng and Zhao, 2009; Lao et al., 2009; Agnolet et al., 2010; Gilard et al., 2010; Novoa-Carballal et al., 2011). ¹H NMR analysis has been used for quality control (Tarachiwin et al., 2008; Lee et al., 2009; Frédéricich et al., 2011), identification of compounds, impurities and adulterants (Peng et al., 2004; Balayssac et al., 2009a,b; Holzgrabe and Malet-Martino, 2011), quantification (Pauli et al., 2005), determination of species origin (Kang et al., 2008) and correlation of a plant's metabolome with biological activity (Tarrago et al., 2006; Kim et al., 2011). Hyphenated techniques such as LC-NMR have found application in the analysis of plant extracts, wherein metabolites are separated and enter the NMR instrument directly from the HPLC column so that no isolation and sample manipulation is required (Wolfender et al., 2001). In metabolomic analysis ¹H NMR has the advantage of having simple sample preparation, being fast, non-selective and a non-destructive technique, providing global information in a single analysis but also can allow structural characterisation (Gilard et al., 2010). New advances in instrumentation such as use of cryogenic probes (Kovacs et al., 2005; Kang et al., 2008), capillary probes (Hu et al., 2008) and preamplifier components (Huan et al., 2010) together with the use of one and two dimensional strategies enable the identification and quantification of multiple samples at molar concentrations in far shorter times (>15 min) (Lewis et al., 2007). However, despite these advances one main drawback with NMR is the limit of sensitivity when compared to techniques such as mass spectrometry (10^{-9} and 10^{-11} mol in comparison with 10^{-19}) (Pan and Raftery, 2007). One and two dimensional ¹H NMR spectra of mixtures and extracts can be very complex making normal methods of analysis, such as peak resonance assignment and peak integration very difficult. However, comprehensive and representative metabolite profiles or fingerprints can be generated (Malet-Martino and Holzgrabe, 2011). The main problems arise from peak overlap which does not interfere with reproducibility (Dumas et al., 2006) but it does with quantification. Such complex samples generate an enormous amount of data that needs to be refined to make it amenable to statistical analysis (Lewis et al., 2007). The combination of ¹H NMR and multivariate

² Same literature research as indicated above. The 157 papers mentioned here were selected because they documented the chemical characterisation of an herbal extract.

analysis is quite often used in metabolomics. Numerous resources are available to assist with principal component analysis, group clustering and pattern recognition based on spectral differences (Malet-Martino and Holzgrabe, 2011). These forms of analysis generate fingerprints that can be used to distinguish between classes of compound and subsequently different metabolites (Frédérich et al., 2011). PCA also facilitates the analysis of sample composition, determination of sample origin, and can be used for quality control as demonstrated in a study of *Panax ginseng* (Yang et al., 2006), and recently in *Verbascum* species when the ^1H NMR datasets generated for five species were subjected to PCA and hierarchical cluster analysis showing qualitative and quantitative differences between species (Georgiev et al., 2011).

Several new NMR techniques have been applied to the analysis of complex mixtures including Diffusion Ordered Spectroscopy (DOSY), a ^1H NMR method which can be regarded as a “virtual chromatography” allowing for the separation of small molecules from larger ones. It has been applied to the screening of herbal medicines (Peng et al., 2004; Balayssac et al., 2009a,b; Novoa-Carballal et al., 2011). In a recent DOSY NMR study, Kang et al. (2008) successfully differentiated between the origin and age of Korean and Chinese *Panax* samples using a cryogenic probe and PCA analysis. The cryogenic probe reduced acquisition times to circa 1 min.

In addition the statistical total correlation spectroscopy (STOCSY) method (Sands et al., 2011) is a well-established NMR approach to enhancing information recovery from a complex mixture and a valuable method in the elucidation of both inter- and intra-metabolite correlations in NMR metabolomic data sets. A modified cryogenic probe ‘on flow’ LC–NMR approach using a STOCSY variation was used by Cloarec et al. (2007). Cryoflow technology enables sensitive and efficient NMR detection of metabolites on flow. This approach, although applied to drug metabolites in urine in this study, has wider potential in metabolomics analysis (Cloarec et al., 2007).

The ability of NMR metabolomic profiling to predict biological activity is an important development that may contribute significantly to the correlation between composition of traditional medicines and their activity. ^{19}F NMR was used by Tarrago et al. (2006), to identify traditional Chinese medicines possessing prolyl oligopeptidase inhibitory activity. Frutos et al. (2006) have also used ^{19}F NMR for the evaluation of the inhibitory activity of new compounds against HIV-1 protease. More recently, analysis of ^1H NMR data sets by PCA and partial least square discriminant analysis (PLS-DA) have shown clear correlations between the free radical scavenging activity of *Psidium guajava* L. (Kim et al., 2011) and the anxiolytic and sedative activity of *Galphimia glauca* Cav. with their respective metabolomes (Cardoso-Taketa et al., 2008).

The increasing number of research publications, detailing the numerous and varied applications of NMR in the metabolomic analysis of plant extracts and traditional medicines, support the important and evolving role of NMR as a complementary or stand-alone technique in the analysis of herbal extracts. Hence the application of NMR seems also reasonable for FP in activity studies of CHM. A protocol for NMR-based metabolomic analysis of plant materials has been published (Kim et al., 2010) and could generally be recommended for extract characterisation as well. However, a difference has to be considered: While plant metabolomics typically looks at plant metabolomes and hence tries to avoid the generation of artefacts during sample preparation, an activity study of CHM should look at the “extract metabolome”, namely at the chemical composition of the very same item that was also used for activity assessment including any possible artefacts and decomposition products that may result from post-harvest treatment, *pào zhì* processing or the extraction process and that may contribute to an extract’s efficacy (Xie et al., 2009). A general consideration of any kind of FP of an herbal extract is that the composition of the sample

for FP should be exactly identical to the composition of the sample that was used for activity assessment. This means that sample discrimination, e.g. by incomplete dissolution, different methods of filtration etc. should be avoided; for example, if a sample for bioactivity assessment is dissolved in water with a minor amount of DMSO, the same solution (or even better the very same solution) should also be used for analysis; if a filtration step is found to be necessary before analysis, the sample for activity assessment should be subjected to the same filtration procedure. However, this approach may be impeded by the different technical requirements of the biological assays and FP techniques to be used so that compromising may become inevitable.

7. Metabolomic approaches for extract optimisation

On its way from the plant to a Chinese Materia Medica (CMM), a herbal material has to undergo numerous and partly TCM-specific production steps that can include storing, washing, rinsing, drying, remoistening and cutting and eventually the unique *pào zhì* processing techniques. All these steps should guarantee that a preparation made of such a CMM can develop its full range of effects and avoid unwanted side effects. Each of these manipulations represents a step in the production chain that can be expected to influence the drug’s chemical profile. For example, the drying of herbal material results in a disorganization of cellular compartmentalisation and an oxidative burst both of which are capable to change plant secondary metabolites by oxidation (e.g. the generation of sennosides in leaves of *Cassia angustifolia* Vahl or generation of primary amide glucosides in *Prunus laurocerasus* L.) or direct enzymatic activity (e.g. the generation of secondary glycosides in *Digitalis purpurea* L.) until further loss of water stabilises the material (Lemli and Cuveele, 1978; Sendker and Nahrstedt, 2009). A very interesting aspect can be found with regard to *pào zhì* processing: there are several examples where the same raw drug material is processed by different methods to yield products that are claimed to have improved pharmacological and/or toxicological properties. Examples for this are *Coptidis Rhizoma*, *Angelicae sinensis Radix* and *Aconiti lateralis Radix* (Stöger, 1999; Zhao et al., 2010). Another aspect that is claimed to impact an herbal drug’s therapeutic properties is the occurrence of presumably superior *daodi* qualities which is attributed to herbs grown in a specific region. Taken these claims for granted, the huge variety of CHM should give an ideal sample set to link these different pharmacological or toxicological properties to differences in the products’ chemical profiles by metabolomics. A major challenge is that Chinese herbal medicines are usually administered as a mixture of several herbal drugs, whereupon the mixture may show synergetic effects that at least partly can rely on co-extraction of highly specific herbal materials. Even the generation of new metabolites during co-extraction cannot be ruled out (Mak et al., 2006; Zheng et al., 2010). This means that the adulterated properties of, e.g. a *pào zhì* product could not become visible before the processed product is co-extracted as part of an herbal mixture following a traditional protocol.

It is desirable, also from a regulatory point of view, that the above mentioned impacts on the herbal metabolomes are well characterised and that the state of an herbal drug material within the production chain can be traced by suitable analytical methods. For example, the *pào zhì* processing of TCM drugs is currently controlled by experienced, long studied masters called *Yi Shi*, but without objective endpoint definitions (Zhu and Woerdenbag, 1995; Zhao et al., 2010). A processing method’s chemical impact on the product can be assessed by FP of a variety of processed and unprocessed samples and their subsequent analysis by multivariate statistics may lead to the identification of specific markers that could aid suitable objective endpoints definitions. The same applies for any

other step within the production chain. However, a prerequisite is that the extracts of (intermediate) products that are to be analysed are well documented with regard to the production steps they had been subjected to. Bioactive compounds may be identified if it is possible to achieve or generate extracts of different materials from the same plant species that are highly variable in bioactivity. A PCA may then discriminate the chemical fingerprints in a way that separates the extracts, e.g. by activity or by spatial origin and relevant chemical compounds can subsequently be deduced from their contribution to the respective discrimination (Tanaka et al., 2008; Okada et al., 2010). Yet, prominent chemical differences must not necessarily be linked to activity. Supervised methods like PLS-DA may overcome this problem by relating chemical fingerprints to a dependent variable, e.g. the results of a bioactivity assay. Examples for this procedure have been published for anxiolytic and sedative activity of *Galphimia glauca* (Cardoso-Taketa et al., 2008) and antioxidant activity of *Psidium guajaval* (Kim et al., 2011).

However, there are limitations to this approach: (i) As multivariate statistics cannot distinguish between correlation and causality, the result must be regarded as a hypothesis and the factual meaning of any identified compounds or sets of compounds should be subjected to further examinations in order to prove the hypothesis. (ii) The results of any approach that is supervised by bioactivity data can only be as meaningful as the biological test system.

8. New emerging technologies for the purification of active compounds

Once active or synergistically relevant components have been identified using the metabolomic methods described above, it will still be a challenge to isolate sufficient material for identification, toxicology studies and combining with other identified actives to model potentially simplified Chinese medicines that still include any component relevant to their traditional efficacy. The isolation of pure compounds has always been difficult as any natural product and especially complex products as CHM are complicated systems constituted of hundreds and thousands of chemical entities, often with unknown structures and low abundances. Modern HPLC based technologies allow for a high level of automation and hence a high throughput also for the preparative separation, concentration, drying and weighing of natural product fractions and pure compounds in a scale that is sufficient for building natural product libraries (Abel et al., 2002; Eldrige et al., 2002; Koehn, 2008). Also orthogonal, two-dimensional separation systems employing novel stationary phases with distinct chromatographic selectivity have been used for the isolation of active constituents from natural products (Liu et al., 2008b, 2010; Guo et al., 2009). However, as the loading capacity for well resolved preparative HPLC is suitable for structure elucidation by NMR (e.g. Glauser et al., 2008) and high-throughput-screening (Tu et al., 2010), the amounts needed for experimental work verifying the results of a metabolomic approach may exceed the amounts that can be reasonably achieved by HPLC.

One emerging new technology with high loading capacity that has been extensively used in China for the isolation and identification of new active ingredients is counter-current chromatography (Chen et al., 2007; Sutherland and Fisher, 2009; Zhang et al., 2009; Qi et al., 2010; Zhang et al., 2010). This is an entirely liquid flow process with immiscible liquid mobile and stationary phases where compounds are separated on their differential solubility in these two liquids. This technology was introduced in 1966 by Ito who invented the coil planet centrifuge to demonstrate how compounds could be separated by the counter-current motion of two immiscible liquids in a coil/column undergoing a variable force field (Ito et al., 1966). This became known as counter-current chromatography (CCC), a continuous form of Craig's counter-current distribution (Craig and Post, 1949). CCC with a coil planet

centrifuge is a hydrodynamic process, where, if the flow stops the two phases move to opposite ends of the column. Later hydrostatic process known as centrifugal partition chromatography was developed. Hydrodynamic CCC occurs in a long continuous tube that has been wound to form a coil (Ito, 2005). In chromatographic terms this coil is the column, which is rotated in a planetary (epi-cyclic) motion retaining one solvent phase (stationary) while allowing another solvent phase to be pumped through the column. Within the column there are numerous alternating zones where the solvent phases are mixed together and then settled into separate layers. These alternating zones are the extraction stages and there are many within the column. Today, there can be found high-speed CCC (HSCCC) and high-performance CCC (HPCCC) (Sutherland et al., 2011). The difference is the separation efficiency of the equipment used (HPCCC works at higher g-level or force field) and has the ability to purify products more rapidly with a higher throughput and without losing purity.

The second form, hydrostatic CCC is commonly known as centrifugal partition chromatography (CPC) which is a separation technique made up of a series of interlinked cells located around a rotor – each cell being an extraction stage (Foucault, 1994). One phase is held in the cell (stationary) while the mobile phase flows through allowing extraction to occur between the phases. CPC instruments have only one rotational axis and therefore, centrifugal force field is uniform and if the flow stops the stationary phase does not redistribute hence its hydrostatic notation. Historically, HSCCC and HPCCC are more widespread within China than CPC.

The CCC/CPC process is more like a continuous liquid–liquid extraction process than a chromatography one, which makes it very attractive for Chinese herbs as there is no loss of sample ingredients and the process is scalable (Sutherland et al., 2011). Extracts can be dissolved in either phase and injected into the column with the mobile phase – compounds elute from the column at various times according to their affinity to the stationary phase. This new technology has proved itself very useful for fingerprinting also (Zhang et al., 2010). The big advantage is that it can focus on a particular compound and even concentrate it in the column using an intermittent continuous counter-current extraction mode where hydrophobic impurities elute from one end of the column and hydrophilic ones from the other while the target compound itself is being concentrated in the column (Hewitson et al., 2009).

9. Summary and conclusions

Chinese herbal medicines have been used for the treatment of numerous ailments for millennia and their use is gaining increasing popularity across the northern hemisphere. However, the traditional water decoction is a disadvantageous formulation especially for reasons of stability and compliance; furthermore the highly polar extracts obtained from these decoctions bear technological difficulties that hamper the development of modernised application forms. The move toward the use of less polar solvent extracts of herbs, rather than decoctions may overcome these problems but may also cause complications by distorting the composition and activity of CHM's prepared in this fashion when compared with traditional decoctions. It has also been established that interactions between chemical components may take place during traditional co-extraction of complex herbal mixtures and thus impact the extract's chemical composition. The move towards new formulations including the use of *Granules* represents a significant modernisation. However these preparations, with the inclusion of large quantities of excipients, deviate significantly from the traditional decoctions used in CHM. The use of such modern dispensing systems also opens up the legal status of these formulations when being viewed by legislators.

It is obvious that the techniques currently being used for the metabolic fingerprinting of complex biological and herbal samples have the potential to help enhancing the quality, safety and efficacy of CHM by providing a comprehensive view on a multitude of quality-determining compounds. However, we believe that we are not yet at a point where modern metabolic fingerprinting can fully unfold this potential. This is because of the special features and complex issues that obviously exist especially with the use of complex herbal medicines and that have to date impeded a comprehensive knowledge about their quality determining components. Currently, we still rely on monographs for single herbs with their associated chemical markers when the quality analysis is actually being carried out on a multicomponent medicine.

But then it is this variety of chemical and also therapeutic properties that makes an essential prerequisite for the successful identification of active components and analytical markers by metabolomics, so that the same complexity that often hampers an understanding of CHM by reductionist means may turn out to be the key for a successful metabolomic approach. Researching and understanding this variation is a huge task, especially when considering that both chemical and therapeutic properties of a specific drug and its constituents may only become visible after co-extraction within a complex TCM formula. The ability to assess information relating to FP and activity testing between different research groups and laboratories will depend on having standardised approaches and methodologies. Though such standardisation is not yet in place, efforts have been begun in this direction (e.g. Jenkins et al., 2004; Fiehn et al., 2007) and comprehensive protocols have been published (e.g. de Vos et al., 2007; Kim et al., 2010). Its limitations notwithstanding, NMR is a suitable method for FP that offers the required reproducibility with only few parameters to be agreed on (Verpoorte et al., 2007), and hence would complement the widely used but less comparable chromatographic FP for extracts in bioactivity studies. Still, the rational modernisation of CHM requires coordinated studies on extracts' chemical profiles and pharmacological properties identifying the active and auxiliary components in order to conserve the traditional experience in modernised application forms. Current methodologies result in the majority of the medicine and its various synergisms not being fully represented in QC practices. It seems realistic to approach the modernisation of CHM by firstly providing a solid basis for QC methods that will reflect its special features.

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