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South African Journal of Botany 82 (2012) 21 – 32

SOUTH AFRICAN JOURNAL OF BOTANY

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Review

NMR-based metabolomics as a quality control tool for herbal products

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Available online 16 May 2012

Abstract

The full potential of the herbal market is mainly not realised due to the lack of knowledge of the chemical composition of most herbal products. The growth potential of the herbal medicine industry can only be achieved if the composition of herbal medicine is standardised to ensure proper quality control and accountability. Plant-based nuclear magnetic resonance metabolomics is one such way of ensuring quick and reliable quality control and metabolite profiling to ensure quality and reproducibility of herbal medicine. Nuclear magnetic resonance-based metabolomics is robust and relatively easy to use, thus ensuring that herbal medicine can be verified and quality controlled much quicker and more accurate than is currently the case. Although nuclear magnetic resonance is not as sensitive as other analytical techniques such as liquid chromatography and gas chromatography–mass spectroscopy, it is far more reproducible, non-destructive, covers a much wider dynamic range and sample preparation is simpler and quicker to use. Economical development of herbal medicine and the use of nuclear magnetic resonance-based metabolomics should go hand in hand for a better future for herbal medicine. In this review an introduction is given to herbal extracts as therapeutic agents and to the quality control aspects of herbal medicine by means of metabolomics. The experimental methodology for plant metabolomics which covers extraction, nuclear magnetic resonance analysis and multivariate data analysis is also discussed. Some examples are given on the possible applications of nuclear magnetic resonance-based metabolomics in the industry and finally the future of nuclear magnetic resonance-based metabolomics is discussed regarding advances in research and development.
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Keywords: Herbal medicine; Metabolomics; Nuclear magnetic resonance spectroscopy; Quality control

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doi:10.1016/j.sajb.2012.04.001

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1. Introduction

1.1. Herbal extracts as therapeutic agents

Traditional herbal medicine has been used over millennia in many different forms and is still being used as primary health care in many underdeveloped and developing countries. In 1983, Bannerman et al., found that up to 70% of the world's population was using plants for medicinal purposes and mainly as their primary health care. In 1994, Farnsworth reported that approximately 60% of the world was at that point using plants for medicinal purposes. This is still probably the case today and is given as such in the WHO fact file on Traditional Medicine (WHO, 2008). It is also stated in this document that in many African and Asian countries up to 80% of the population is still using medicinal plants in primary health care. It is not only the developing countries that are using plants for medicinal purposes, but the use of herbal medicine (HM) in developed countries, especially the USA and Germany, is on the increase. Increases in the market showed growth rates of between 5 and 18% per year from 1985 to 1995 (Grünwald, 1995). The average imports into the USA and Hong Kong reached more than \$220 million and \$167 million respectively in the period from 2004 to 2008. The leading exporters in the same period were China and India with a value of more than \$348 million and \$93 million, respectively (Lubbe and Verpoorte, 2011).

In the document of the WHO (2000), "General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine", the following statement was issued concerning traditional medicine: "Despite its existence and continued use over many centuries, and its popularity and extensive use during the last decade, traditional medicine has not been officially recognised in most countries. Consequently, education, training and research in this area have not been accorded due attention and support. The quantity and quality of the safety and efficacy data on traditional medicine are far from sufficient to meet the criteria needed to support its use world-wide. The reasons for the lack of research data are due to not only health care policies, but also to lack of adequate or accepted research methodology for evaluating traditional medicine".

The usual assessment of HM on the basis of identifying one or two biomarkers of pharmacologically active constituents in the herbal product does not give a holistic view of the product as the activity is usually due to multiple constituents working together to give the activity of the product. It is therefore necessary that HM be investigated to determine most of the phytochemical constituents contained in the product, ensuring that the bioactivities are better understood. This will also help to identify and understand the possible side effects of the active constituents and thus finally enhancing quality control of the HM product. Good quality control practices will confirm the reliability and repeatability of pharmacological and clinical research and finally more credible interest in the HM product (Bauer, 1998; Raven et al., 1999; Yan et al., 1999).

When comparing conventional drugs to herbal medicine, it is necessary to distinguish between these two forms of therapeutic agents as well as how these are administered. The administering of a pure chemical and that of a plant extract containing the same chemical entity is essentially different. The difference is mainly due to the complexity of a plant extract that introduces many variables to conventional phyto-medicinal research, which could possibly contribute to chemical complexity and bioactivity. This is very clearly illustrated in the study of Weathers et al. (2011), in which the administration of plant material (e.g. *Artemisia annua*) versus pure drug (e.g. artemisinin) revealed that the bioavailability from the leaves was 45 times more than that of the pure drug. Thus the complexity of the plant extract could have contributed to the increased bioavailability and thus the bioactivity.

As our analytical techniques as well diagnostic techniques are being improved we are once again faced with the reality that living organisms and life in general is complex. The fact that science is only starting to comprehend the different subtle, varied and complex mechanisms in many of the biochemical systems in organisms, is a confirmation that we can not ignore the possibility of complex herbal medicine contributing greatly as therapeutic agents (Wills et al., 2000).

New therapeutic herbal agents have delivered variability in the efficacy in treating especially recurrent and chronic infections. It is also noteworthy to consider that a number of plant extracts containing a number of bioactive compounds may be used not only to provide important combination therapies which affect multiple pharmacological targets but also at the same time to provide clinical efficacies which are normally beyond single compound-based drugs capabilities (Schmidt et al., 2007; Williamson, 2001).

1.2. Quality control and quality assurance of herbal medicine

Quality control (QC) and the standardisation of herbal extracts are very important to protect the integrity of the herbal extracts for pharmaceutical quality. It also forms part of a prerequisite for the reproducibility of the effect of the active ingredients from one batch to another (Saller and Reichling, 2002).

In order to comply with legal conditions in Europe with respect to effectiveness, quality and safety, it is required to follow certain steps of standardisation (Ulrich-Merzenich et al., 2007). The five

steps for phytopreparation quality assurance can briefly be described as:

(1) Definitive authentication and taxonomic assignment e.g. through DNA-fingerprinting and DNA bar-coding.
(2) Isolation and structural elucidation of all major constituents of the herbal drug.
(3) Identification of the true bioactive constituents.
(4) Multi-extract mixtures; standardisation of the single extracts or 3D HPLC fingerprint analyses of the multi-compound extracts.
(5) Global harmonisation of standardisation criteria under the umbrella of the International Federation of Pharmaceutical Manufacturers Associations (IFPMA).

It is thus clear that it is very important to standardise the HM products to comply with international standards. Standardisation of HM products is very difficult to achieve as there are numerous variables that will influence the standardisation process of HM products. It is therefore necessary to optimise all aspects of cultivation, harvesting, sample preparation and sample processing to ensure reproducibility and eventually standardisation of the HM product.

Chromatographic and spectroscopic techniques should be incorporated to ensure that sufficient quality control measures are implemented. By using chromatography and spectroscopy it is possible to analyse the full HM product and thus generate a standardised "metabolic fingerprint" of the specific HM product and thus the HM as a whole can be regarded as the "active constituent" with such a characteristic "metabolic fingerprint". Metabolic profiling can then be incorporated to identify the constituents that make up the "metabolic fingerprint" (Bailey et al., 2002; Tyler, 1999). In compliance with the five steps of the European herbal standardisation regulation and the German concept of phyto-equivalence, chromatographic and spectroscopic techniques are ideal components in standardising HM products as well as comparing the chemical profile of HM products to clinically proven reference products. This will ensure much more accurate, reliable and repeatable analyses of the HM products on the market (Tyler, 1999).

It should globally be considered to evaluate HM products not only on a few biomarkers, but also on multiple constituents in HM products (Liang et al., 2004). It is also necessary to note that these chemical fingerprints obtained from chromatography or spectroscopy contribute significantly in determining the similarities and differences between various samples (Valentão et al., 1999; Xie, 2001).

Recent developments have led to chemometric approaches being used together with data from spectroscopy and chromatography. When these are used in combination with chemometrics even more accurate data is obtained to establish the integrity of the HM product and observations concerning the similarities and differences of the HM data. The power of chemometrics lies in the different dimensional observations that are used to explain the similarities and differences of the data and subsequently presenting it in a user-friendly graphical manner (Liang et al., 2004).

1.3. Metabolomic techniques

Metabolomics can best be described as a comprehensive quantitative and qualitative analysis of 'all' metabolites present in a specific cell, tissue or organism. Metabolomics can thus be defined as "the systematic study of the distinctive chemical fingerprints that specific cellular processes leave behind" — and even more specifically, the study of the metabolite profile of 'all' the small molecules in an organism (Daviss, 2005). Thus, the metabolome is a collective representation of all the metabolites in a biological organism, which are the end-products of its gene expression (Van der Kooy et al., 2009).

Unlike the other '-omics' approaches like genomics, transcriptomics and proteomics, a one-step analysis and display of all metabolites in a metabolome is not possible due to the enormous complexity in biological systems, especially in that of plant systems (Sumner et al., 2003). Metabolic analysis can be divided into four major areas:

(1) Targeted compound analysis — quantification of specific metabolites.
(2) Metabolic profiling — quantitative and qualitative determination of a group of related compounds or of specific metabolic pathways.
(3) Metabolomic fingerprinting — sample classification by rapid global analysis.
(4) Metabolomic analysis — this involves the quantitative and qualitative analysis of 'all' metabolites (which is not possible yet).

The techniques used in all four areas are multidisciplinary and make use of many different complementary analytical methods, some rely on chromatographic separation and others do not comprise any separation step and represent a global view of the sample (Ulrich-Merzenich et al., 2007).

The two major approaches in metabolomics are targeted (biased) and global (unbiased) metabolite analyses. Targeted metabolite analysis or metabolite profiling targets a subset of metabolites in a sample instead of analysing the complete metabolome. Metabolome analyses use a particular set of analytical techniques (Fig. 1) such as Fourier transformed infrared spectroscopy (FT-IR), gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), nuclear magnetic resonance (NMR), capillary electrophoresis–mass spectrometry (CE–MS), and thin layer chromatography (TLC) (Shyur and Yang, 2008). Recent advances made in analytical chemistry for small mass compound detection and characterisation, such as MS and high-field NMR, coupled with modern multivariate statistics have led to a highly efficient system for comprehensive analysis of the metabolite data matrices generated by metabolomic experiments (Lindon et al., 2007). A comparison of metabolomic techniques is illustrated in Fig. 2 and these need to be taken into account when a new metabolomic investigation is being considered. In this review the focus is on the use of the NMR technique because of its rapid and robust characteristics that are necessary for industrial quality control purposes.

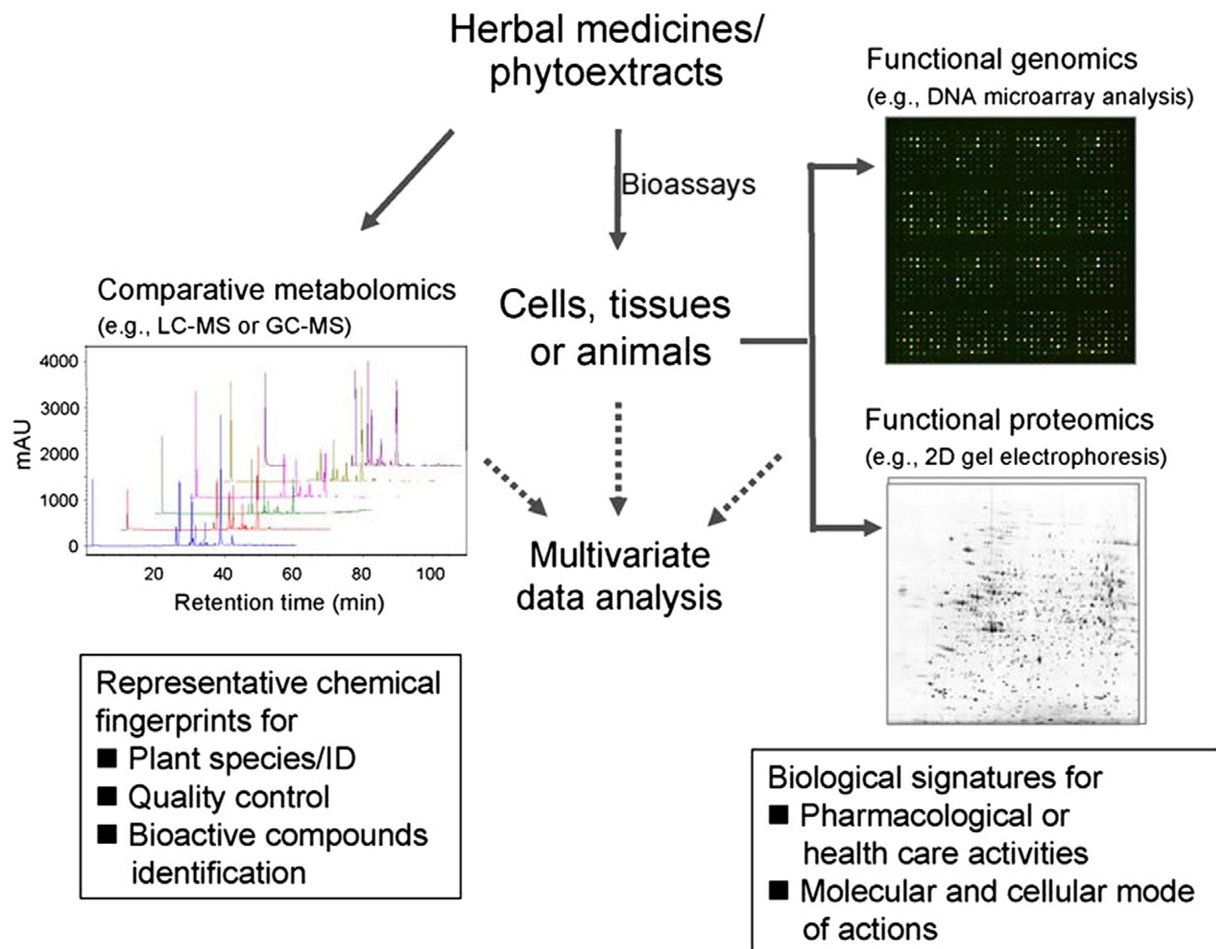

Fig. 1. Key features of the technologies used in metabolomics for herbal medicine research (Shyur and Yang, 2008).

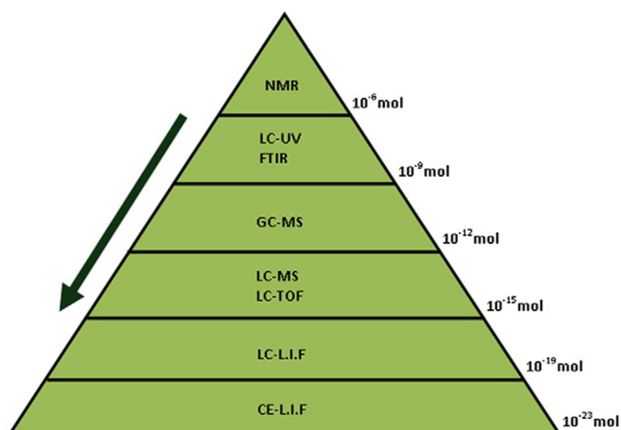

Fig. 2. A comparison of the relative sensitivities of various metabolomic techniques. NMR has rapid analysis time but suffers from lower sensitivity thus allowing visualisation only of the more concentrated metabolites. GC–MS and HPLC–MS provide good selectivity and sensitivity. CE-LIF (laser induced fluorescence) provides very high sensitivity but lower selectivity.
Based on work from Sumner et al., 2003.

2. Experimental methodology for plant (herbal) metabolomics

2.1. Collection and extraction of the herbal product

Conventional collection of plant material has to take many factors into account that may have a significant influence on the integrity of the sample (e.g. collection time, season, method, soil, weather, etc.). Preparation of herbal plant material differs from the conventional preparation for metabolomics analysis as the HM material is already processed and samples are taken from the production line as a processed HM product. It is still very important to collect a good representation of the herbal plant material that will be used as the herbal product. Good manufacturing practices (GMP) as well as good laboratory practices (GLP) need to be adhered to as not to introduce any unwanted variation in the data.

The extraction procedure may lead to biochemical reactions taking place in the plant material that will result in a change in the metabolome of the sample. Thus it is important to take into account the preparation method of the selected HM. Preparation of the sample for analysis should follow the preparation method of the selected HM as it would be prepared for consumption by the

patient. The collected HM product has to be ground and extracted to release the metabolites from the cells, this is best done at low temperature and/or in the presence of a solvent. Making use of ultrasonication has been found to be the best method to use for the efficient breakage of cells of the plant material and thus yielding the largest quantity and range of metabolites to be analysed (Jaki et al., 2006).

To date there is no single extraction method that can extract all the metabolites in an organism. Due to the polarity and pH of the solvents used a limited range of metabolites can be extracted. This can be avoided to a certain degree by applying a two-phase solvent system consisting of chloroform:methanol:water (2:1:1) to extract compounds from the plant material both of polar and non-polar nature in a single extraction (Choi et al., 2004a, 2004b, 2005; Suhartono et al., 2005).

The two-phase solvent extraction system is very complex and it could affect the composition of the samples and result in the loss and degradation of the sample due to the longer preparation time. Using this method it makes it more challenging when faced with large number of samples to be analysed. This prompted the use of a two-phase deuterated solvent system that is quicker and reduces the sampling handling time. This method provides much better results and NMR spectra however, it is much more expensive (Hendrawati et al., 2006; Le Gall et al., 2004; Liang et al., 2006; Ward et al., 2007; Widarto et al., 2006).

The use of single solvent systems to extract metabolites out of plant material has been done in some studies, but using a combination of solvents has now become the preferred method for extraction. Using for example a combination of D_2O and CD_3OD in different ratios (aligned with the aims of the study) has shown to extract more diverse metabolites from plant material. The majority of studies use a combination of CD_3OD and KH_2PO_4 buffer (to avoid possible fluctuations in chemical shifts of signals in the NMR spectra) in D_2O with a pH of 6 in order to extract an extensive range of metabolites which might include, phenolics, terpenoids, fatty acids, organic acids, carbohydrates and amino acids, in only one extraction step. The direct extraction method with deuterated solvents saves time and makes it feasible to analyse a large number of samples (Hendrawati et al., 2006; Kim et al., 2005; Liang et al., 2006; Van der Kooy et al., 2009; Widarto et al., 2006).

As discussed above, when focussing on a more specific group of metabolites, for example in commercial herbal preparations, a more targeted approach and extraction procedure could be followed to be able to use the spectral data to evaluate the herbal products' content with the utmost accuracy.

A general guide for the two-phase solvent extraction of plant material would be to start with the grinding of dried plant material, extraction in appropriate two-phase solvent system (e.g. chloroform:methanol:water), after which the plant material can be further homogenised (e.g. vortexing, sonication, etc.). The extraction procedure should be repeated at least twice, to increase the yield of extraction. After the extraction and filtration the organic and aqueous layers will need to be separated and be dried under vacuum. The samples can then be re-dissolved in the deuterated solvents (0.7–1.0 ml), after which ^1H NMR analysis can be conducted (Kim et al., 2005).

If deuterated solvents have been used, the extracts will be centrifuged and the supernatant be transferred to NMR tubes and analysed directly. In most cases a mixture of CD_3OD and KH_2PO_4 buffer in D_2O (pH 6) is used, which as mentioned previously will extract a large range of metabolites (Hendrawati et al., 2006; Kim et al., 2005; Liang et al., 2006; Van der Kooy et al., 2009; Widarto et al., 2006).

2.2. NMR analysis

After the extracts have been prepared, NMR analysis is carried out to obtain a ^1H NMR spectrum. The ^1H NMR spectra are a wealthy source of information of the content of metabolites in plant samples due to the relatively high sensitivity and widespread occurrence of protons in organic metabolites (Kim et al., 2006).

It is not important to have large quantities of plant material for NMR metabolomics. Using 10–50 mg of plant material is sufficient to generate a ^1H NMR spectrum within 10 min. From this NMR spectrum that could contain approximately 50–100 metabolites, it is usually possible to identify in the region of 10–20 known compounds (Kim et al., 2006).

The NMR spectra (Fig. 3) are recorded in deuterated solvents on an NMR spectrometer (preferably 400 MHz or higher) that is operated at a proton NMR frequency corresponding to the specific spectrometer. For each sample a number of scans are run which can range for high-quality spectra from 64 to 256. The number of scans will depend on the magnet strength of the NMR spectrometer, thus with the increase in magnet strength fewer scans will be required. Two other factors to be considered to obtain high-quality spectra are the (1) relaxation delay and (2) pulse width parameters. The optimum parameters were previously discussed in detail in the review of Pauli et al. (2005) and focussed on important factors like locking, tuning and shimming which are non-negotiable steps when it comes to reproducible metabolomics analysis. It is important that the NMR spectra are corrected for any baseline and phase distortions. The spectra need to be normalised and correctly scaled and this is done by using either an internal standard (e.g. trimethylsilyl propionic acid, tetramethylsilane, etc.)

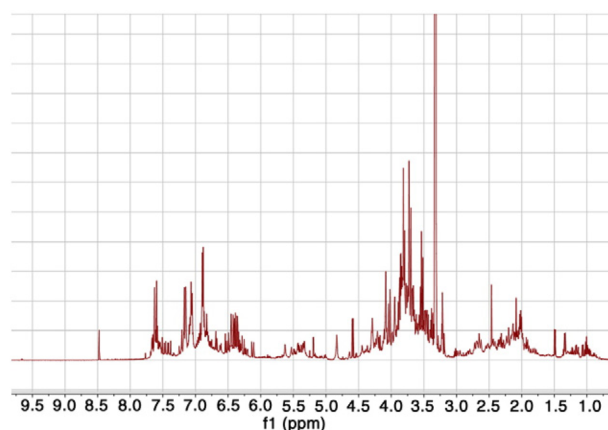

Fig. 3. 600 MHz ^1H NMR spectrum of 2:1:1 ($CHCl_3$:CH_3OH:H_2O) extract of air-dried *Helichrysum adenocarpum* subs. *adenocarpum* DC in D_2O (Heyman et al., 2009).

or the residual solvent (e.g. $CHCl_3$, CH_3OH, etc.) as reference peak. The normalisation of the spectral areas is done to compensate for differences in the overall concentration due to variation during extraction. Multivariate analysis is then performed on the normalised NMR spectra (Wang et al., 2004).

When working with samples that have a residual water peak, it is very important to suppress this signal to allow for proper spectral analysis. Pre-saturation of the residual water peak is achieved by either (1) pre-saturating with an additional pulse, or (2) addition of paramagnetic ions (e.g. Mg^{2+}). The most common method used is pre-saturation using an additional pulse, but this has the disadvantage of an unwanted reduction of the signal intensity close to the suppressed water peak (Beckwith-Hall et al., 2003).

NMR data manipulation starts with splitting the data into distinct regions (also called 'bins' or 'buckets') that normally cover areas of approximately 0.01 to 0.04 ppm in width. These distinct areas in turn are converted into a list of values for each spectrum. This might seem counterproductive in that it will reduce the resolution, but it has the added advantage of not being influenced by chemical shift changes between repetitions and other samples that could occur due to pH variations of the samples. The practice of using all the data points in an NMR spectrum is starting to gain momentum and with special algorithms that align the peaks, eliminating any unwanted variation, the use of all data points will become a much more common practice (Ward and Beale, 2006).

2.3. Multivariate data analysis

One of the most important steps in metabolomics, metabolite fingerprinting and metabolic profiling is the data analysis step and the significance of bioinformatics as a tool should not be underestimated.

NMR bucketed data is initially analysed using the Principal Component Analysis (PCA) multivariate statistical method or other similar statistical methods. The PCA statistical method is used to make the large datasets easier to be interpreted by making the data more visually comprehensible (Ward and Beale, 2006). PCA was designed to extract and display the methodical variation in a dataset. The partial least squares projections to latent structure (PLS) analysis method is a regression extension of PCA, thus revealing the correlations between two kinds of datasets. For the analysis of raw analytical data, both PCA and PLS as multivariate data analysis (MVA) programmes are becoming everyday processing steps. The PCA and other related multivariate analyses can be carried out by quite a few commercially available software products. One software product often used is that of Umetrics, SIMCA-P (Umetrics, Umea, Sweden) (Ward and Beale, 2006).

PCA models are best displayed as a scoring plot (Fig. 4). This illustration of a scoring plot is an example of an unsupervised comparison of samples of *Artemisia afra*, *A. annua* and a herbal remedy which differentiated on the basis of their chemical composition. The use of scoring plots is very valuable for the observation of any groupings in a dataset; it is also possible to identify outliers that could skew the plots due to errors in experimental conditions, instrumental parameters or errors in the preparation of the samples (Van der Kooy et al., 2009). To obtain these PCA scoring plot values, the original variable of the NMR/MS data is multiplied with coefficients which are referred to as loadings. The specific numerical value that the loading has will be indicative of the relationship between the original variable and the component (Massart et al., 1988). It is thus possible to say that the 'loading plots' can highlight the spectral regions responsible for the separation in the data and are thus responsible for the specific position of the scoring plots (Ward and Beale, 2006).

It is important to keep in mind that before PCA the data must be scaled appropriately. This is to insure that the spectral data obtained is represented correctly in the MVA and that the results obtained from MVA can be correlated back to the original NMR spectra or MS chromatograms. When applying unit variance scaling to NMR data, it will be difficult to interpret the loading plots obtained due to the fact that all signals will have the same variance/weight and as a result will be different from the original NMR spectra or MS chromatograms. When applying scaling to the data of plant extracts, it is necessary to use different scaling than the unit variance scaling. The signals of the secondary metabolites can easily be underestimated when compared to the high levels of primary metabolites. It will thus be more appropriate to use a scaling technique called 'Pareto scaling' (PAR). PAR scaling is becoming a common scaling method for secondary metabolites studies as PAR scaling gives each variable a variance numerically equal to its initial standard deviation instead of unit variance. PAR scaling not only gives weight to minor signals but also gives interpretable loadings (Colquhoun, 2007).

3. Application of NMR-based metabolomics in natural products

3.1. NMR-based metabolomics in QC of fruit juice

Bruker BioSpin GmbH and SGF International recently published results from an application that was developed for QC of fruit juices in industry. The major reasons for this investigation were the constant challenges that the food and lifestyle industries face in analysing the presence of specific ingredients in samples, contamination of samples and incorrect labelling of products. Bruker and SGF International introduced the combination of NMR spectroscopy and multivariate analysis to analyse fruit juices samples. This resulted in a successful application technique, "Bruker JuiceScreener™" and "SGF Profiling™" for the profiling of fruit juices (Rinke et al., 2007).

In SGF Profiling™ each fruit juice is analysed on a multitude of parameters that are related to the quality of the fruit juice on a fully automated platform. Thus from a single NMR dataset that is acquired within a few minutes numerous parameters can be evaluated simultaneously to establish the quality and authenticity of the specific fruit juice. The SGF Profiling™ NMR screening has a low cost-per-sample approach and compares very well with conventional and targeted fruit juice QC approaches (Rinke et al., 2007).

Bruker and SGF International evaluated fruit juices with a targeted as well as non-targeted approach. The targeted approach's primary interest was to focus on the classical fruit juices procedure

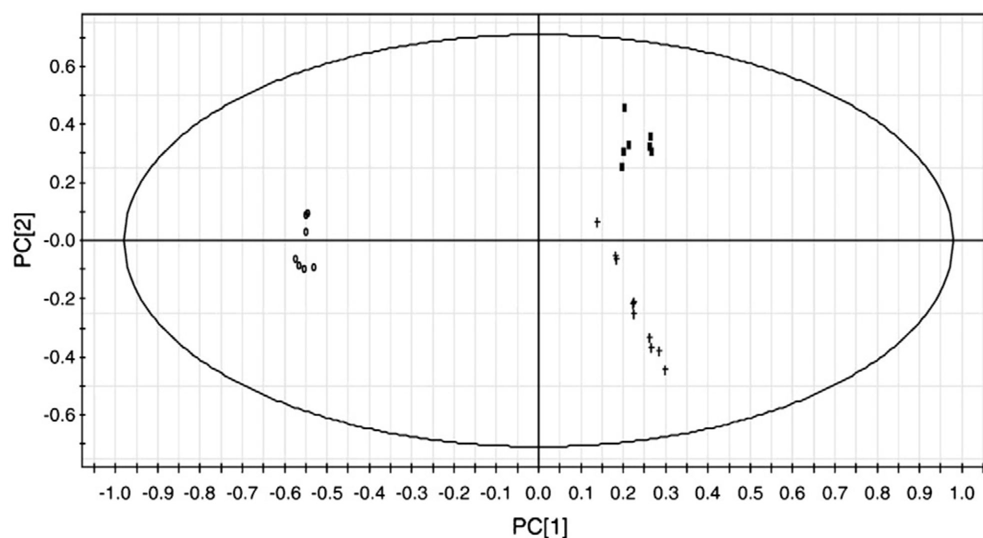

Fig. 4. PCA scoring plot illustrating the clear differentiation in PC1 between the *Artemisia annua* (circles) samples and the *A. afra* (crosses) samples and the herbal capsules (boxes) (Van der Kooy et al., 2008).

that relies mainly on the concentrations of various pre-determined specified ingredients. It is thus important to identify the presence of a specified ingredient and its concentration. The advantages that NMR spectroscopy has over classical analysis techniques were clearly shown. NMR spectroscopy allows simultaneous identification and quantification of many compounds in a mixture. The particular quantification method provided absolute concentrations for more than 28 different compounds depending on the type of fruit juice. The large amount of data obtained from one single measurement enables the detection of contaminates, adulterants, etc. in the fruit juices (Rinke et al., 2007).

The non-targeted approach was used in cooperation with a large reference database of more than 5 000 samples of more than 30 different fruit juice samples from more than 50 countries. The NMR predicting model was able to determine the specific type of fruit, product type (pure fruit juice or juice made from concentrate), fruit content as well as the country of origin of the juice. In Fig. 5, the verification analysis is demonstrated which compared an apple juice sample with the distribution of the spectra from the database of apple juice. The deviations from normality as shown in Fig. 5B indicated an unknown contaminant by comparing the test sample with high phlorin concentrations to that of the database spectra with a non-targeted analysis approach (Spraul et al., 2008).

3.2. Analysis of complex pharmaceutical preparations using NMR metabolomics

In the commercial industry of herbal medicine, the preparations are typically very complex and have a multitude of compounds. The relationship of the content and the pharmacological effect of herbal medicine is also usually unclear, but these herbal medicine formulations are usually standardised using single marker compounds or a group of related compounds which does not include information on other seemingly unimportant abundant constituents present in the herbal preparation (Rasmussen et al., 2006).

The work done by Rasmussen et al. (2006), explored the complex pharmaceutical preparation of St. John's Wort (*Hypericum perforatum*). Their investigation showed the possibility of NMR spectroscopy and multivariate analysis being used to distinguish between different variations in batches from the same supplier. No clustering was observed between the tablets and capsules thus indicating that variability between samples is mainly contributed by plant extract variability and not the manufacturing processes. NMR metabolomics was also able to show major variations in the content of the flavonoids linked to the antidepressant activity of the *H. perforatum* extract.

The study also investigated the use of two NMR solvents (methanol-d_4 and DMSO-d_6) which showed to be very similar and revealed complementary datasets, thus making it possible to link various aspects of the samples' composition from separate PCA models. The analysis was also done on both integrated (+/ −200 variables) and full resolution (+/−30 000 variables) NMR data. The full resolution data produced loading plots with more precise information of the compounds in the extract responsible for the clustering and possibly for the therapeutic effect, thus indicating that full resolution NMR data could be better for PCA analysis of complex plant extracts and HM (Rasmussen et al., 2006).

3.3. Analysing complex Artemisia herbal medicine with NMR metabolomics

In a study conducted by Van der Kooy et al. (2008), the use of NMR spectroscopy in combination with PCA showed to be very promising as a method to detect the presence of a particular constituent in herbal extracts that was claimed to be in the extract preparation. In this study the claim of the active anti-malarial, artemisinin, being present in capsules made from *A. afra* was investigated. The investigation included *A. afra* extracts, *A. annua* extracts and capsules of the product which were all analysed by NMR. Analyses were done on a 500 MHz Bruker

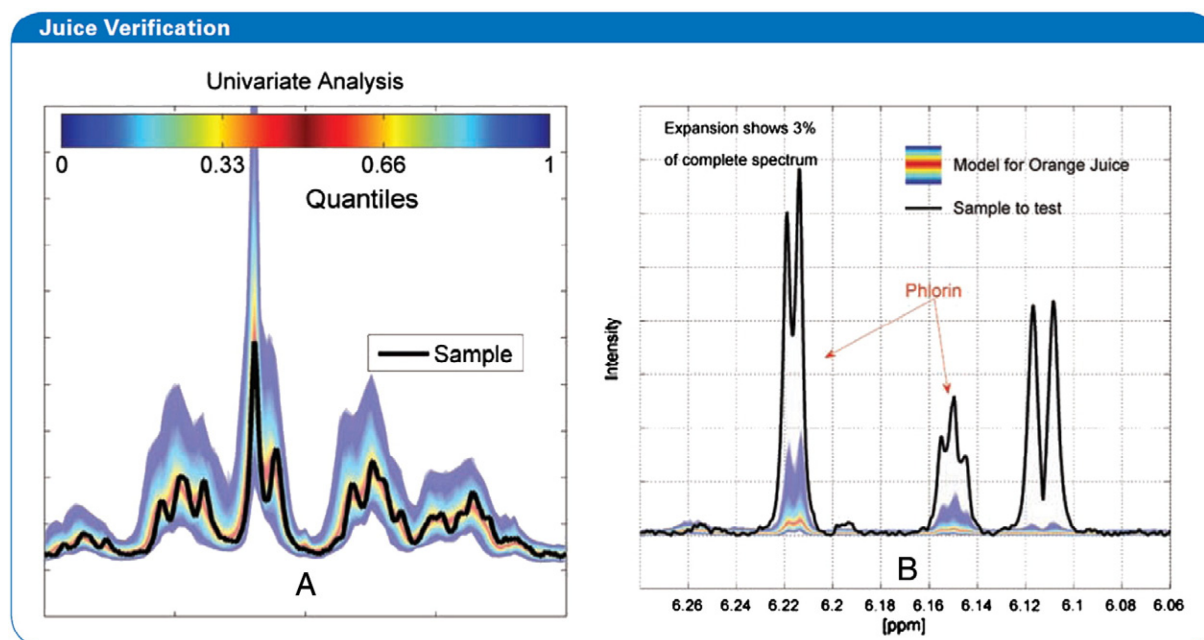

Fig. 5. (A) Non-targeted verification of the sample (black line): apple juice (~1% of spectrum), (B) orange juice with high phlorin concentration (black line — indicating over extraction) compared to model for orange juice (Bruker BioSpin GmbH and SGF International).

NMR spectrometer and for each sample 128 scans were performed. Spectra were referenced to the residual chloroform peak with a total of 6 min analysis time per sample. NMR data was processed and files were transferred to SIMCA-P (10.0 Umetrics, Umea, Sweden) for PCA analysis.

In this study it was clear to observe the differences between the three different samples. Based on the PCA data (Fig. 4), the two plant species *A. afra* and *A. annua* can easily be differentiated from each other, separating very well in the first PC. In PC1 the capsules clustered together with *A. afra* and separated from *A. annua*, thus confirming that the content of the capsules are indeed *A. afra*. In PC2 the *A. afra* and capsule samples separated very distinctively, with the separation being explained by the difference in compound concentration between them. In the same study they also showed with LC–MS data that the anti-malarial compound, artemisinin was only detected in the *A. annua* samples and not in the *A. afra* at all. Thus, the claimed reports of the anti-malarial compound artemisinin being present in the capsules from *A. afra* were proven to be incorrect using NMR-based metabolomics (Van der Kooy et al., 2008).

3.4. Chemical profiling of HM

Most of the current traditional methods of QC of whole extracts are not sufficient for application on HM. This has led to large variation between batches of products encountered in the market. Due to the fact that many factors can have a significant influence on the chemical composition of phytomedicine (plant growth environment, collection/harvesting season, preparation and extraction process, etc.), it is vital to make sure that these factors are considered in the QC of phytomedicine/herbal medicine (Abe and Kamo, 2003; Bailey et al., 2002).

High resolution ^{1}H NMR can play a vital role in this regard and combined with chemometric analysis it offers a way to assess the whole plant extract. It provides a way to view 'all' the chemical components present in a plant extract concurrently as a "metabolic fingerprint" (Bailey et al., 2002). By applying PCA techniques to NMR data, the differences and similarities between economical important samples can be visualised in 2D of 3D plots very easily and quickly (Bailey et al., 2002), thus also adding to the high-throughput need for QC purposes.

In a study conducted by Wang et al. (2004), NMR-based metabolomics was used to determine the global composition of three chamomile (*Matricaria recutita* L.) samples gathered from three different countries. The aim was to differentiate these samples on (1) the origin, (2) herb quality in terms of percentage of desired plant parts and (3) extraction method. The study investigated the development of a new robust QC method with the use of NMR-based metabolomics and the development of methods to also chemically profile phytomedicine like chamomile.

This study clearly indicated that there are significant differences between samples from different geographical distributions which is clearly separated in PC1 (t[1]) (Fig. 6). It was also possible to determine the quality of the herb in terms of the percentage of stalk contamination in the samples. When the average scores were used as a function of the percentage of stalks it showed that the linear relationship was an excellent fit ($r^2=0.98$). The final objective was to determine the effect of the preparation and extraction. Both preparation and extraction indicated large influences on the chemical composition of the chamomile phytomedicine preparation. When dried and wet plant material was compared, large reductions in the methoxy resonance groups were experienced in the wet extract and significant increases of the sugar resonance peaks in the dried extract were observed. Lastly the

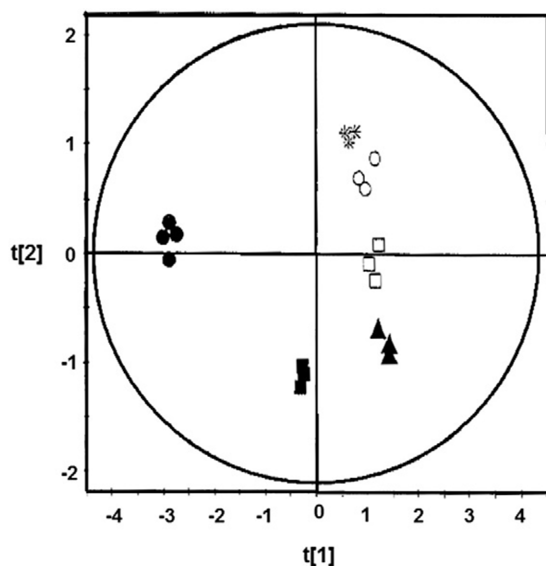

Fig. 6. PCA scoring plot (t[1] vs. t[2]) of ^{1}H NMR spectra from different chamomile extracts (▲) — Slovakia, (●) — Egypt, (■) — Hungary, (*) — 100% stalk, (○) — 70% stalk, (□) — 30% stalk (Wang et al., 2004).

influence of water extract compared to 50% ethanol extracts was also shown to be significant with large separation in PC1 (Wang et al., 2004).

In the field of plant-derived medicine conventional analytical methods are not sufficient to cope with the complexity of such extracts. This study on the other hand has clearly indicated the potential of NMR-based metabolomics as an innovative QC technique for phytomedicine and herbal medicine in general (Wang et al., 2004).

3.5. NMR-based metabolic fingerprinting of Ephedra

A study conducted by Kim et al. (2005) on the metabolic fingerprinting of the *Ephedra genus*, one of the oldest medicinal herbs known to mankind. *Ephedra sinica* is known to be the main source of the ephedrine alkaloids, but there are other 14 species that are known to also contain ephedrine alkaloids. Thus, the need for identification of the different species is a necessity and the study showed that this is possible with NMR-based metabolomics. As highlighted by this study, previous chemotaxonomical studies focused mainly on the small group of metabolites i.e. ephedrine alkaloids which may be misleading and thus a broader chemical fingerprint analysis is needed.

In the study (Kim et al., 2005) three *Ephedra* species (*E. sinica*, *Ephedra intermedia* and *Ephedra distachya* var. *distachya*) were analysed with NMR and compared with nine commercially available *Ephedra* herbal plant samples purchased from a Taiwanese market. The three known *Ephedra* species separated very well in PC1, with *E. distachya* var. *distachya* separating completely from the other two species and this was determined to be due to no ephedrine being detected in it. Separation was best in the aqueous extracts and thus PCA analysis was done on the aqueous extracts only. Most of the commercial samples clustered close to the *E. intermedia* except for one species that group between *E. sinica* and *E. intermedia* which was determined to be a mixture of these two.

Thus, from this study it was clear that NMR-based metabolomics can easily and quite conveniently be used to verify if herbal plant material is authentic. As a large part of the world is still making use of traditional herbal markets to obtain their primary medicine it can be very useful to authenticate these supplies of herbal medicine. Some herbal medicine companies also rely on wild harvested plant material for their herbal production and thus it is also important to validate the supplies that are received before using it in production.

4. Future perspective of NMR-based metabolomics

4.1. Economical outlook

NMR-based metabolomics can also be used in many other applications, i.e. food authenticity, functional genomics and also in the substantial equivalence testing of GMO's.

NMR is robust, reliable and non-destructive and thus an ideal companion for QC of economical important samples. NMR spectroscopy combined with multivariate statistical analysis software provides new opportunities for conducting sound and reliable QC analyses on botanical samples. These samples can range from botanical, pharmaceutical to everyday fruit juice QC samples. In future the standardisation of HM will probably play an important role for approval of complex HM as a treatment and require robust and rapid analytical methods for the QC of these products without the tedious preparation normally necessary for QC on complex mixtures. So in the future QC analyses will probably not be done on a few selected constituents anymore, but will be done on the whole composition of the sample.

In combination with functional genomics, NMR metabolomics can assist in investigating the deeper understanding of the complex nature of the networks operating in plants and how these networks change due to genetic manipulation. Not only is it possible to determine the genetic changes but also establish the fundamental nature of the plant phenotypes in relation to development, physiology and environment. In studies that have been conducted it was possible to use metabolomics in determining the effects of environmental stress on root and on specific enzyme function (Moing et al., 2004). It is also becoming more and more the trend to use different 'omics' datasets and to cross-correlate these datasets to extract as much information out of these information rich matrices (Ward et al., 2007).

Determining the unintended effects of genetic modification is a very important area of research and with regulatory bodies placing more and more emphasis on safety issues of GMO's it is of utmost importance to use the best methods available to us. NMR-based metabolomics is starting to gain momentum in this field. NMR fingerprinting with the use of multivariate analysis is a very powerful tool and has been used to identify and classify seeds of maize, wheat etc. obtained from transgenic plants into different classes on the basis of different metabolite compositions. Even with the use of relatively small models it is possible to easily explain up to 90% of the variability and the

causes of the variability in the specific datasets (Manetti et al., 2004).

NMR-based metabolomics will in future contribute significantly to research in many biological fields and also to the economical development of these fields. In the field of drug discovery and development NMR-based metabolomics will contribute to achieve rapid and efficient lead identification and might in some cases be able to replace the tedious practice of bio-guided fractionation. It can also be incorporated in the early stages of drug discovery to rapidly identify known compounds in the plant under investigation and not to waste any valuable time on these known compounds, thus quickly narrowing down the search.

4.2. Future developments in perspective

Recent advances that have been made with NMR methods make it an appropriate technique with unique characteristics for the analysis of plant metabolites. In spite of all the above mentioned positive attributes of NMR-based metabolomics it is necessary to take into account that there are a few disadvantages with NMR as a technique. NMR's greatest weakness lies in the resolution and sensitivity capabilities. Compared to MS, NMR is lacking significantly in this area and it limits NMR-based metabolomics to compete at low compound levels. MS has the added advantage to be joined easily to a chromatography unit to do combinational analysis which is not the case with NMR where it is a more cumbersome task to combine it with chromatography techniques. With MS analysis compounds can be detected at very low concentrations and even at trace element level, thus enriching the data to be used for chemometric analysis. Chromatographic techniques assist MS detection to separate the large amount of constituents in plant samples to assist the increased number of constituents being analysed and identified. With NMR the complex plant extracts cause huge overlap in most of the spectral regions thus making it difficult to extract the necessary information from the spectra (Kim et al., 2011).

Recent developments and trends in NMR analysis have been highlighted in a review done by Kim et al. (2011). Developments in hardware (e.g. high-resolution NMR, cryoprobes, microprobes) as well as new developments in the protocols of pulse sequence and spectral acquisition have been reviewed regarding improvements in sensitivity. Kim et al. (2011) also discussed recent approaches like 2D ^{1}H J-resolved NMR and heteronuclear single quantum coherence (HSQC) spectroscopy that spreads the spectral content over a 2D plane. This makes analysis of plant samples much easier and more informative (but more time consuming). It therefore simplifies QC techniques and helps to identify the common constituents in herbal samples and the quantification of them for routine analysis.

Another improvement that has come under the spotlight is magical angle spinning (MAS)-NMR spectroscopy. This technique requires very simple sample preparation (e.g. direct insertions of lyophilised tissue on a MAS 4-mm zirconium rotor) with very little deuterated solvent (20 μl), thus having a dual benefit of no sample preparation problems and small amounts of expensive solvents are consumed. Samples are then analysed on a 54.7° angle (the magic angle) which reduces line broadening significantly and a high resolution spectrum is obtained. This has the benefit that samples do not run the danger of being altered due to chemical reactions during sample preparation (Kim et al., 2011).

5. Conclusion

Taking all of the above into account, NMR-based metabolomics has some limitations which are the topic of intense current research, but it is evident that it could contribute quite significantly to the QC of HM. The added advantage that can be gained from NMR-based metabolomics above other traditional QC methods is that firstly it analyses all the compounds in the sample thus giving a holistic view of the HM. It has the capability to differentiate between samples from different origins, harvested in different seasons, grown in different soil types, with different plant parts composition, with added adulterance etc. and therefore is more reliable than the normal narrowly focused TLC or HPLC QC methods.

The regulative environment is becoming increasingly more stringent and will in future require a much better and more in-depth analysis of what is being put on the HM market. Traditional QC methods give a restricted view of the contents of the product, but with NMR-based metabolomics much more detail will be available, thereby improving reliability.

In South Africa the regulative environment of the HM market is due for a revision as there are very limited regulative conditions for HM to reach the market. The South African market has a potential to be developed to an international level. From a review of Van Wyk (2008), it is very clear that southern Africa does not lack any potential candidates that can be used and are already used commercially. Having economical species like round leaf buchu (*Agathosma betulina*), rooibos (*Aspalathus linearis*), wild wormwood (*A. afra*), Cape aloe (*Aloe ferox*), fever tea (*Lippia javanica*) just to mention a few, gives southern Africa a huge advantage to compete in the HM industry. It is now up to the HM industry to regulate the HM cultivation and production and with the correct QC capabilities to ensure that these authentic HM products reach the market.

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Edited by AM Viljoen