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CURRENT ISSUES AND CHALLENGES IN FOOD SAFETY:

science - based approach for food safety management

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SEAFAST CENTER

Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center
Bogor Agricultural University

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'Current Issues and Challenges in Food Safety:
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APPLICATION OF NMR BASED METABOLOMICS AND MULTIVARIATE DATA ANALYSIS FOR QUALITY CONTROL OF HERBAL MATERIAL

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ABSTRACT

Despite of lack of solid scientific proof for its efficacy and safety, up to now herbal medicines are widely used both in developed and developing countries. Standardization by focusing on active constituents or marker compounds is important but sometimes it is not sufficient to guarantee the quality of herbal preparations. NMR based metabolomics is a reliable tool to provide a wealth of information concerning plant metabolites since it is able to comprehensively detect a diverse groups of plant metabolite in a single run. In combination with multivariate data analysis, the obtained NMR data can be applied to determine any adulterants or substitutes in commercial herbal preparation which cannot be detected by other chromatographic methods. Here we reviewed three projects of our lab in Leiden, focusing on the application of NMR based metabolomics and multivariate data analysis, particularly principal component analysis for quality control of herbal medicines.

INTRODUCTION

Herbal medicine has been used since ancient times to treat a wide range of diseases and to improve the health status of human beings. Despite the advanced development of modern medicine, herbal preparations are still widely used both in developed and developing countries. The reasons are various; the belief that herbals are made from natural sources therefore safer than chemical drugs

(which is not always true); diminishing trust to modern medicine due to its side effects; or lack of access to modern medicine (due to poverty or geographical factors) (Calixto, 2000; Elvin Lewis and Steve, 2005). Two important issues concerning the use of herbal medicine are proof of efficacy and safety. The last issue is the focus of this paper, highlighting on the possibility of adulteration or substitution with other plant which may not contain the reported active principles or even worse, contain toxic substances. As an example; in 1992 many young women in Belgium suffered from progressive permanent kidney damage after consuming a Chinese slimming herbal which consisted of *Stephania tetrandra* and *Magnolia officinalis*. It is supposed that *Stephania tetrandra* was substituted with *Aristolochia fangchi* which contains aristolochic acid, a nephrotoxin and carcinogen (van Herweghem, 1998).

To address such issues, chemical standardization on the basis of active compounds or other marker compounds in case the active compounds are unknown, is necessary. Chemical standardization involves the use of chromatographic or spectroscopic methods to identify active/marker compounds (Ong, 2004). Many analytical methods such as TLC, HPLC, LC/MS, and GC/MS can be used for that purpose. However, sometimes only targeting the marker compound is not sufficient to detect adulterants/substitutes, especially when chemical marker compounds are spiked into adulterants/substitutes while the adulterants/substitute may contain toxicant instead. In such a case, information concerning a wide range of metabolites present in the herbal preparation becomes necessary. This could be a hurdle since plants have a large number and wide variety of compounds, and herbal medicine may consist of two or more different plant materials. A wide spectrum of analytical techniques which are rapid, reproducible, and stable over time without an elaborative sample preparation is required (Choi *et al.*, 2005). The aforementioned analytical methods have limitations to provide information about all metabolites due to the wide structural variation, as well as physical properties and chemical characteristics of compounds present in plant material (Jahangir *et al.*, 2008).

QUALITY CONTROL OF HERBAL MATERIAL WITH NMR-BASED METABOLOMICS AND MULTIVARIATE DATA ANALYSIS

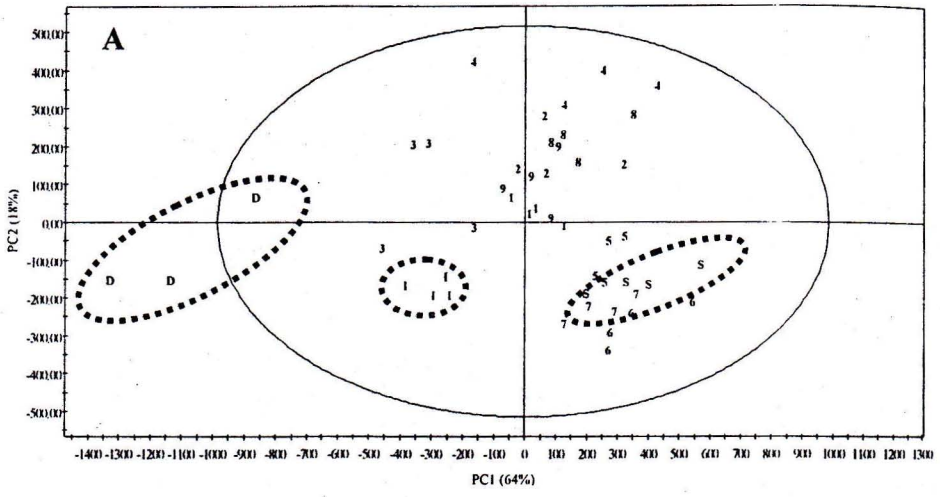
The term metabolome represents the collection of all metabolites in a biological organism as the end product of their gene expression (van der Kooy *et al.*, 2009). One of the first application of nuclear magnetic spectroscopy (NMR) for fingerprinting of plant extracts was conducted in our lab, that was for characterizing plant cell cultures and quantitative analysis of sugars in the cell culture extracts (Schripsema and Verpoorte, 1991). Although the sensitivity is lower than other methods such as mass spectrometry (MS), NMR has some unique advantages since it is a quick non-destructive method which simultaneously detects diverse groups of plant metabolites in a single run (Choi *et al.*, 2004). It is able to detect all molecules containing NMR-active nuclei and no selective ionization (like in MS) or the presence of chromophores (such as in ultraviolet spectroscopy) are required (van der Kooy *et al.*, 2009). The huge data set resulting from NMR measurement need multivariate data analysis (MVA) methods for interpretation. The most commonly used MVA method is unsupervised principal component analysis (PCA). In PCA, the data are transformed into a new coordinate system so that the biggest variance by any projections of the data lie on the first coordinate (PC1), the second coordinate (PC2), and so on. The dimensionality of the data is reduced while the characteristics of the data set that contribute most to it are preserved (Eriksson *et al.*, 2006; van der Kooy *et al.*, 2009). By using PCA it is possible to classify the data when only little information about the data is available (Eriksson *et al.*, 2006).

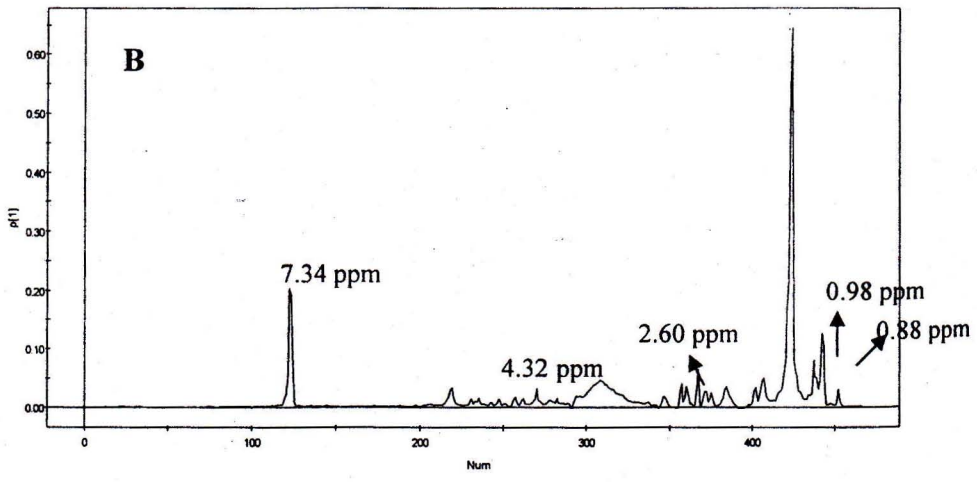
In our lab, several studies related to quality control of botanicals by using NMR based metabolomics coupled with multivariate data analysis, in particular PCA, have been conducted. Herewith we give three examples of those studies.

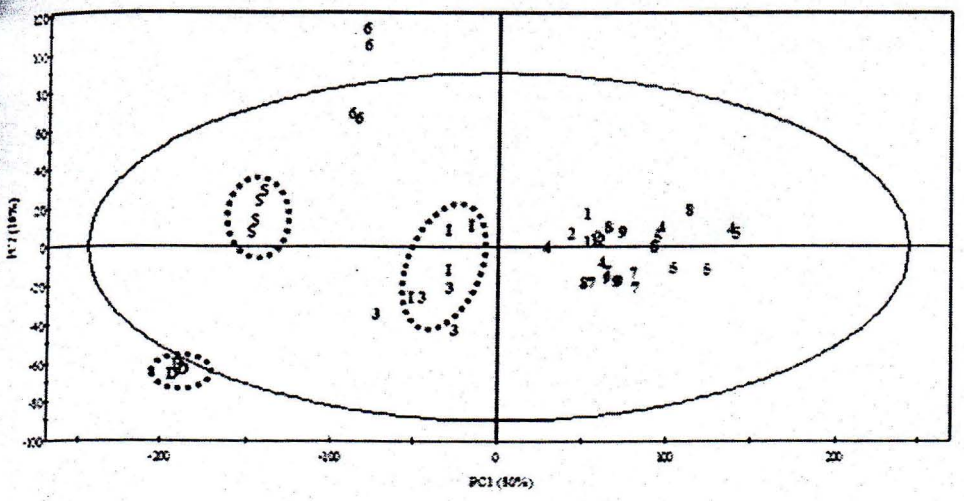
1. Metabolic Fingerprinting of *Ephedra* Species Using ^{1}H-NMR Spectroscopy and PCA (Kim *et al.*, 2005)

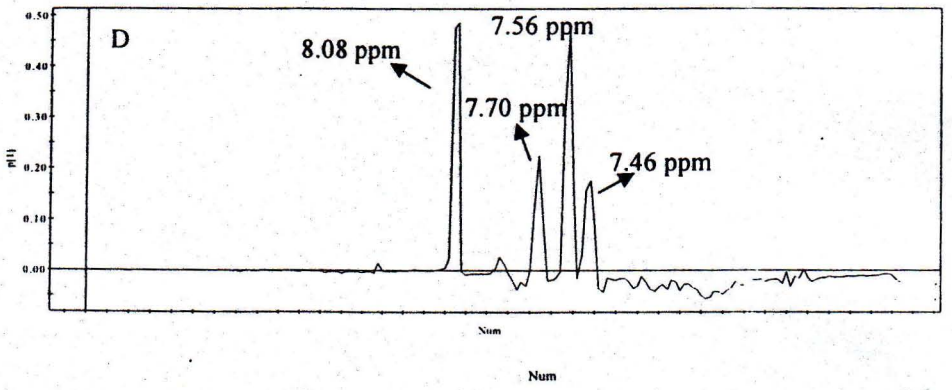
   Ephedra or Ma Huang, with ephedrine type alkaloids (e.g. ephedrine, pseudoephedrine, methylephedrine and norephedrine) as the primary active ingredients, is used as a weight loss agent and to boost performance of athletes (Haller

and Benowitz, 2000; Calfee and Fadale, 2006). *Ephedra sinica* is considered as the main source of the active ingredients the ephedrine alkaloids, although other *Ephedra* species may also contain these alkaloids though in different concentrations. In this paper, the use of ^{1}H NMR coupled with PCA for the metabolite fingerprinting of *Ephedra* species was reported. The discrimination between 3 different species of *Ephedra* (*E. sinica*, *E. intermedia*, *E. distachya* var. *distachya*) and nine commercial *Ephedra* samples was shown.






PC1 (80%)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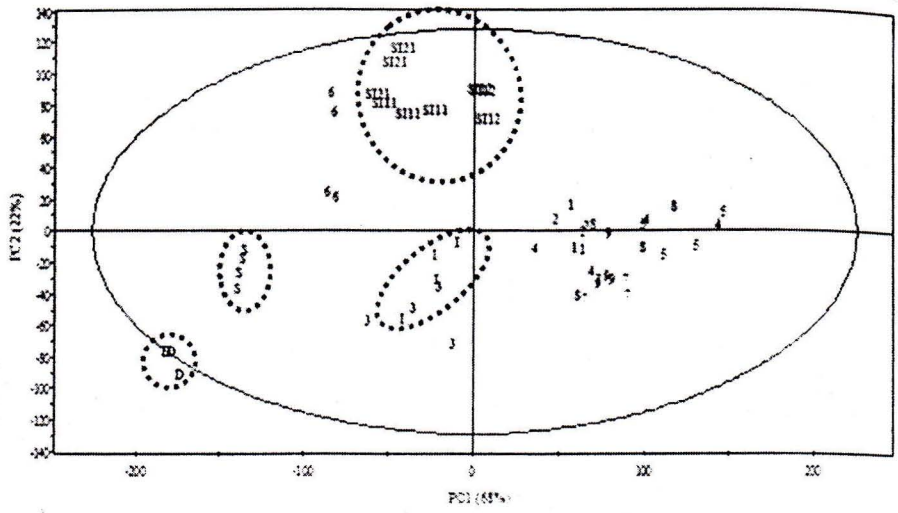

A. Score Plot of PC1 and PC2 of organic fraction of *Ephedra* species
B. Loading plot of PC1 of organic fraction of *Ephedra* species
C. Score plot of PC1 and PC2 of aqueous fraction of *Ephedra* species
D. Loading plot of PC1 of aqueous fraction of *Ephedra* species
E. Score Plot of PC1 and PC2 of aqueous fraction for *Ephedra* including mixture of *E. sinica* and *E. intermedia*

(S=*E. sinica*; I=*E. intermedia*; D=*E. distachya* var. *distachya*. 1—9, commercial *Ephedra* herbs, SI12=mixture of *E. sinica* and *E. intermedia* (1 : 2, w/w), SI11=mixture of *E. sinica* and *E. intermedia* (1 : 1, w/w), SI21=mixture of *E. sinica* and *E. intermedia* (2 : 1, w/w)

Figure 1. (adapted from (Kim *et al.*, 2005), with permission)

The solvent mixture of $CHCl_3$–MeOH–H_2O–NH_4OH was used to extract metabolites from *Ephedra* species. Organic and aqueous fractions were measured by NMR separately. The most alkaloids were present in the organic solvent phase. The PCA score plots and loading plots for each fractions are presented in Fig. 1.A-D. Figure 1.A. shows that the three species of *Ephedra* are separated from each other. The loading plot of PC1 was analyzed further since it is the main PC for the separation. It was found that characteristic NMR signals for ephedrine alkaloids such as CH_3 at δ 0.9—0.8 ppm, N–CH_3 at δ 2.60 ppm, and the aromatic signal at δ 7.3 ppm were responsible for the separation. These alkaloids are higher in *E. sinica* while ephedrine was not detected in *E. distachya* var. *distachya,* therefore this species was located

outside of the 95% Hotelling T2 confidence eclipse. Analysis of aqueous fractions was also performed since there was no clear separation between commercial *Ephedra* samples. From fig. 1.C-D, it is obvious that all commercial samples clustered close to *E. intermedia* except sample number 6. Analysis of the aromatic area showed typical signals of benzoic acid analogues at 8.08 (d, *J*_7.2 Hz), 7.70 (t, *J*_7.5 Hz), and 7.56 (t, *J*_7.8 Hz) and monosubstituted phenolic compounds like phenylalanine at d 7.46 (m) which are higher in *E. intermedia*. Sample 6 was located between *E. sinica* and *E. intermedia.* PCA analysis of a mixture of *E. sinica* and *E. intermedia* (fig.1.E.) confirmed that sample 6 was indeed the mixture of both species.

This study showed that standardization by focusing on alkaloids content alone is not efficient and it is not possible to detect if synthetic ephedrine alkaloids were added to the commercial herbs. This is the advantage of applying NMR spectroscopy coupled to MVA since the analysis is based on total metabolic fingerprinting.

2. Classification of *Ilex* Species Based on Metabolomic Fingerprinting Using NMR and Multivariate Data Analysis (Choi *et al.*, 2005)

Ilex paraguariensis var. *paraguariensis*, or yerba mate, is a popular beverage in South America, besides it is also used as tonic, diuretic, to reduce fatigue and to improve gastric function (González *et al.*, 1993). This study was conducted to see the differentiation between *Ilex paraguariensis* var. *paraguariensis.* and some other *Ilex* species which are commonly used as adulterants or substitutes, (i.e. *I. argentina, I. brasiliensis, I. brevicuspis, I. dumosa var. dumosa, I. dumosa var. guaranina, I. integerrima, I. microdonta, I. pseudobuxus, I. taubertiana*, and *I. theezans*). Moreover, the difference between these 10 adulterants/substitutes was analyzed as well. An Extraction method similar to the one used in the *Ephedra* study was performed and NMR analysis was carried out for both organic and aqueous fractions

From visual observation of the ^{1}H-NMR spectra (Fig. 2), only *Ilex paraguariensis* var. *paraguariensis* was observed to contain caffeine and theobromine. This was confirmed by score plot of

the organic fractions (Fig.3.) which shows that *Ilex paraguariensis* var. *paraguariensis* is clearly separated from other species.

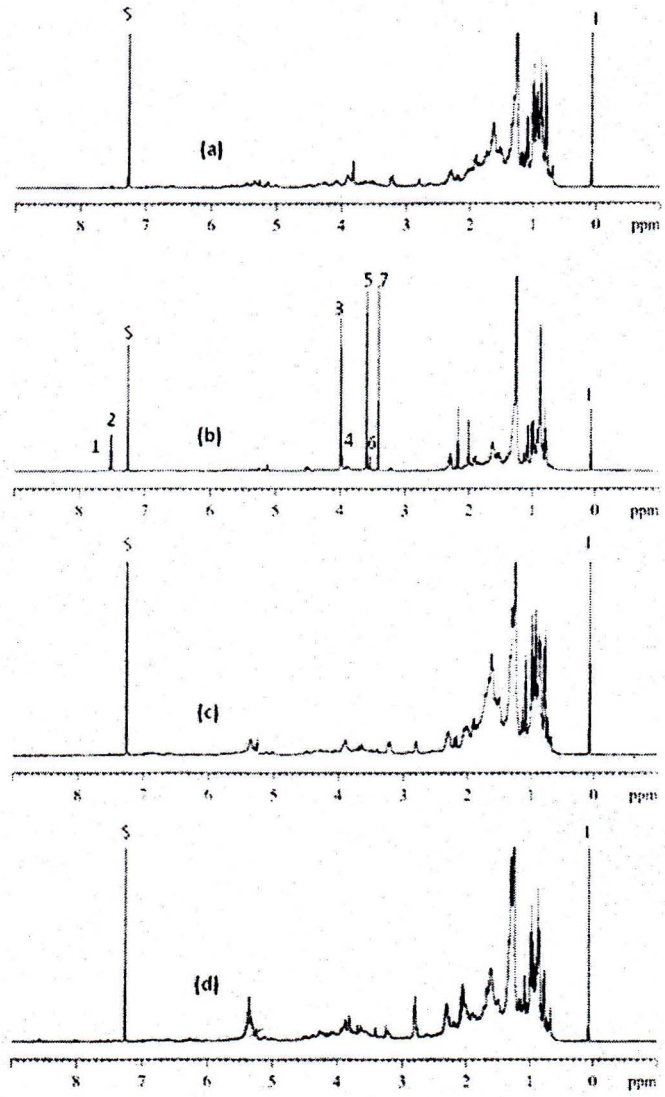

Figure 2. ^{1}H spectra of organic fractions of *Ilex argentina* leaves (**a**), *I. paraguariensis* leaves (**b**), *I. pseudobuxus* leaves (**c**), *I. tauberiana*leaves (**d**). Peaks: 1= H-8 of theobromine; 2= H-8 of caffeine (Reproduced from (Choi *et al.*, 2005), with permission)

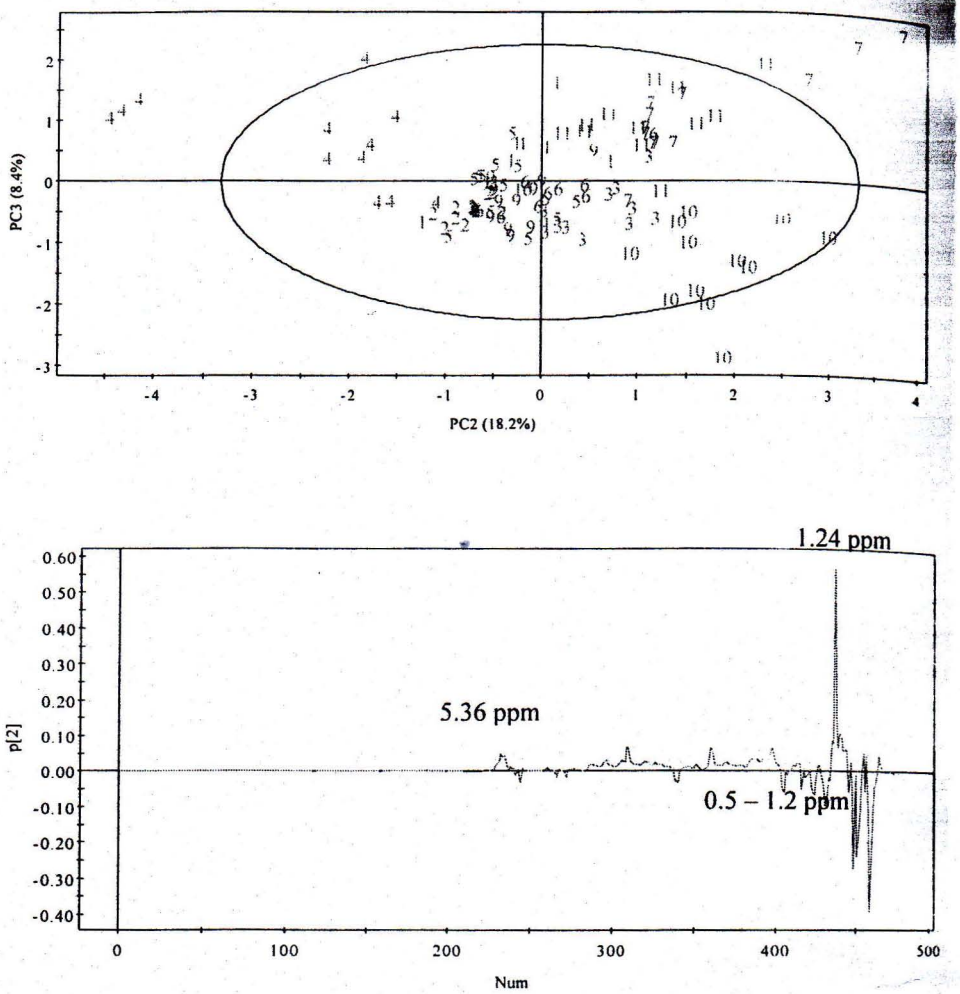

Figure 4. (reproduced from (Choi *et al.*, 2005), with permission) a. Score plot of organic solvent fractions of 10 species of *Ilex* (without *I. paraguariensis*) b. Loading plot of organic solvent fractions of 10 species of *Ilex* (without *I. paraguariensis*)

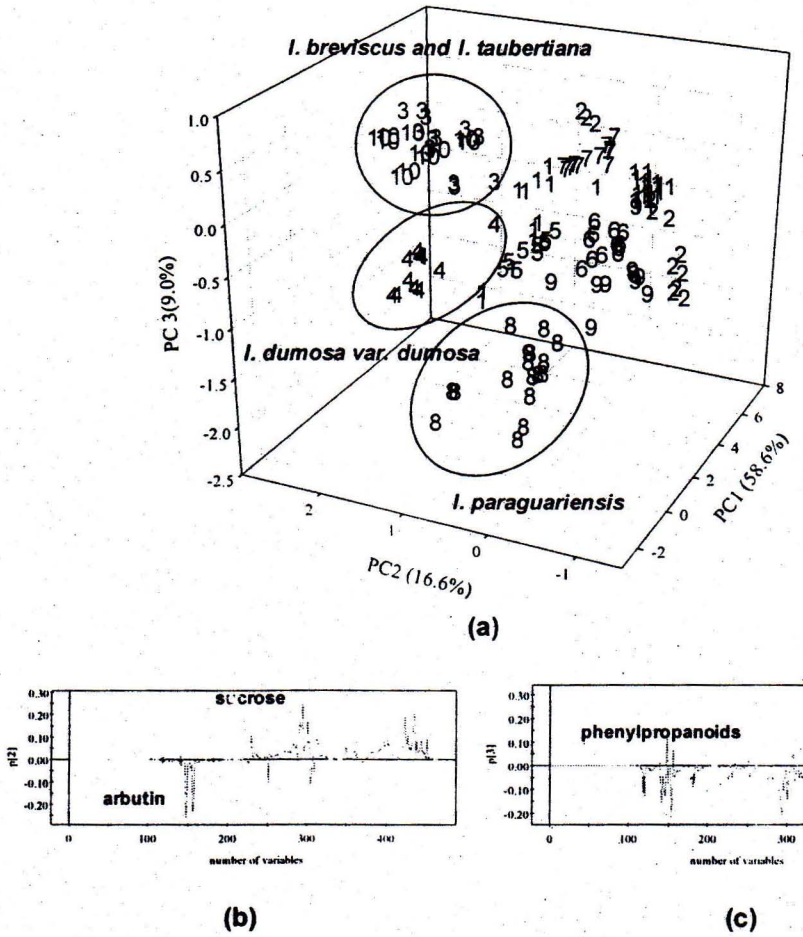

Figure 5. (reproduced from (Choi *et al.*, 2005), with permission)
a. Score plot of PC1, PC2, PC3 of aqueous fractions of *Ilex* species
b. Loading plot of PC2
c. Loading plot of PC3

3. Metabolomic quality control of claimed anti-malarial *Artemisia afra* herbal remedy and *A.afra* and *A. annua* plant extract (van der Kooy *et al.*, 2008)

Artemesia annua L. has been used for centuries in traditional Chinese medicine to treat malaria and fever with artemisinin as active constituent (Klayman, 1985). *Artemisia afra* jacq. Ex Willd. is originally from South Africa and commonly used as a medicine for a wide range of illness such as cough, colds, fever, loss of appetite, and also malaria, although there is no report that *A. afra* contains artemisinin or its derivetives (van der Kooy *et al.*, 2008). In this study, a commercial anti-malaria capsule, which was claimed to contain 400 mg of pure *A. afra* without any additives or excipients, was investigated. The producer claimed that artemisinin is the active ingredient in these capsules. For NMR analysis, 100 g of each sample (plant material removed from capsules, powder of *A. annua*, powder of *A. afra*) were extracted with *deuterated* chloroform, sonicated, filtered, then transefered to NMR tubes. The ^{1}H NMR data was subjected to PCA analysis, to determine whether the capsule contained *A. annua*, *A. afra*, or a combination of the two.

From the PCA score plot it is clear that *A. annua* and *A. afra* are separated from each other in PC1, but the commercial sample could not be differentiated as *A. annua* or *A. afra* The loading plot revealed that artemisinin is an important marker for the differentiation in PC1 (methyl signals at 0.99 ppm, 1.21 ppm, 1.44 ppm). LC-MS analysis of three samples, confirmed that *A. annua* contains 0.078 – 0.84% artemisinin but it is not present in *A. afra* or the commercial sample. It could be concluded that the commercial capsules contain *A. afra*.

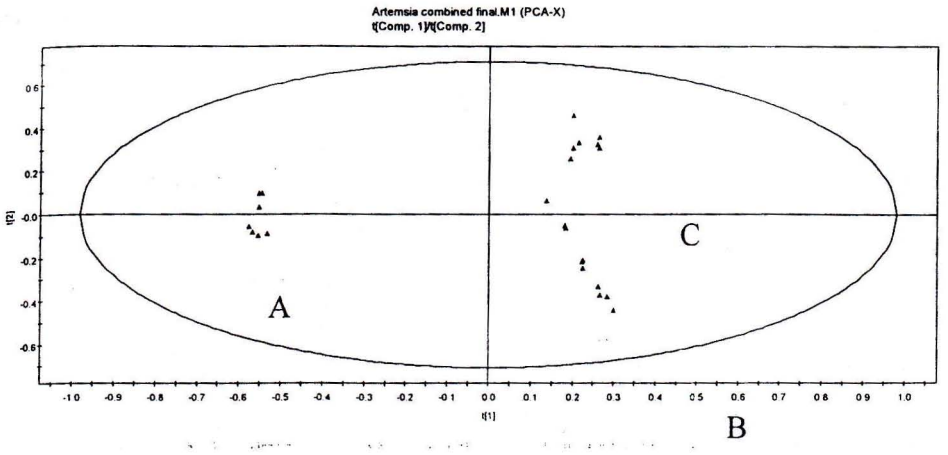

Figure. 6. Score plot of the *Artemisia* samples showing the clear differentiation in PC 1 between the *A. annua* (A) samples and the *A. afra* (B) and commercial herb (C) (reproduced from (van der Kooy *et al.*, 2008), with permission)

CONCLUSION

NMR based metabolomics in combination with multivariate data analysis can be applied as a finger printing tool for the quality control of herbal medicine. Adulterants or substitutes in commercial herbal preparations can be identified very quickly with a simple sample preparation.

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