higher the ratios of CaO to fresh pepper, the shorter the drying needed. Chemoreaction drying can be used to substitute or to reduce the sun drying process. Chemoreaction drying had no effect on the volatile oil content of pepper produced compared to the fresh pepper. This drying process resulted black pepper with volatile oil content between 2.70% [dry basis]. The color of the oil was clear greenish yellow with good flavor quality.

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References
QUALITY CONTROL OF HERBAL MATERIAL WITH NM R METABOLOMICS AND MULTIVARIATE DATA ANALYSIS

The term metabolome represents the collective metabolites in a biological organism as the end product of expression (van der Kooi et al., 2009). One of the first applications of nuclear magnetic resonance spectroscopy (NMR) for fingerprinting extracts was conducted in our lab, that was for characterizing cell cultures and quantitative analysis of sugars in the cell extracts (Schriepsena and Verpoorte, 1991). Although the NMR is lower than other methods such as mass spectrometry (MS) has some unique advantages since it is a quick non-destructive method which simultaneously detects diverse groups of metabolites in a single run (Choi et al., 2004). It is able to detect molecules containing NMR-active nuclei and no selective labelling (like in MS) or the presence of chromatophores (such as in UV spectroscopy) are required (van der Kooi et al., 2009). The hot set resulting from NMR measurement need multivariate data analysis (MVA) methods for interpretation. The most commonly used method for unsupervised principal component analysis (PCA) the data are transformed into a new coordinate system so the biggest variance by any projection of the data lie on the first coordinate (PC1), the second coordinate (PC2), and so on. Dimensionality of the data is reduced while the characteristic data set that contribute most to it are preserved (Eriksson et al., 2006; van der Kooi et al., 2009). By using PCA it is possible to reduce the data when only little information about the data is a given (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. Here with we give three examples of those studies.

1. Metabolic Fingerprinting of Ephedra Species Using Nuclear Magnetic Resonance Spectroscopy and PCA (Kim et al., 2005)

Ephedra or Ma Huang, with ephedrine type alkaloids (ephedrine, pseudoephedrine, methylephedrine, norpseudoephedrine) as the primary active ingredients, is a weight loss agent and to boost performance of athletes...
is not always true); diminishing trust to modern medicine due to side effects; or lack of access to modern medicine (due to socio-economic or geographical factors) (Calhoun, 2000; Elvin Lewis and Mosher, 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 tution with other plant which may not contain the reported principles or even worse, contain toxic substances. As an example, in 1992 many young women in Belgium suffered from massive permanent kidney damage after consuming a Chinese Hong herbal which consisted of Stephania tetrandra and Trion ion. It is supposed that Stephania tetrandra was cut with Aristolochia fangchi which contains aristolochic acid, rototoxin and carcinogen (van Herwegh, 1998).

To address such issues, chemical standardization on the basis of compounds or other marker compounds in case the active units are unknown, is necessary. Chemical standardization relies on the use of chromatographic or spectroscopic methods to identify active marker compounds (Ong, 2004). Many analytical tools such as TLC, HPLC, LC/MS, and GC/MS can be used for this purpose. However, sometimes only targeting the marker compound is not sufficient to detect adulterants/substitutes, especially when marker compound is spiked into adulterants or substitutes. The adulterants substitute may contain toxic instead. In such case, information concerning a wide range of metabolites in the herbal preparation becomes necessary. This could be a possible since plants have a large number of compounds present in plant material. A wide spectrum of analytical techniques are rapid, reproducible and stable over time without an active sample preparation is required (Choi et al., 2005). The mentioned analytical methods have limitations to provide information about all metabolites due to the wide structural diversity. As well as physical properties and chemical characteristics of the 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 dimensional 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. Herein we give three examples of those studies.

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

Ephedra or Ma Huang, with ephedrine type alkaloids (e.g. ephedrine, pseudoephedrine, methylephedrine and noephedrine) 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 1H 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.
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, intermedius, E. distachya var. distachya) and nine commercial Ephedra samples was shown.
outside of the 95% Hotelling T² confidence ellipse. Aqueous fractions was also performed since there was a separation between commercial Ephedra samples. From D, it is obvious that all commercial samples clustered close together except sample number 5. Analysis of the area showed typical signals of benzoic acid analogues at 1.72 Hz, 7.70 (t, J=7.5 Hz), and 7.56 (t, J=7.9 Hz) mono-substituted phenolic compounds like phenylalanine at 7.46 ppm (m) which were higher in E. intermedius. Sample 6 was between E. sinica and E. intermedius. PCA analysis of a mix of E. sinica and E. intermedius (Fig. 1E) confirmed this finding of the mixture of both species.

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

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

Ilex paraguariensis var. paraguariensis, or yerba mate, is a popular beverage in South America, besides it is also use as tonic, diuretic, to reduce fatigue and to improve gastric fluid flow (González et al., 1993). This study was conducted to study 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. brevifolia, I. dumosa var. dumosa, I. dumosa var. guaranae, I. integerrima, I. microdonta, I. pseudobuxus, I. taubertiana, chSpinosa). Moreover, the difference between the 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 1H-NMR spectra (Fig. 2), Ilex paraguariensis var. paraguariensis was observed to contain caffeine and theobromine. This was confirmed by score plots.
The solvent mixture of CHCl₃–MeOH–H₂O–NH₄OH was used to extract metabolites from *Ephedra* species. Organic and aqueous fractions were measured by NMR separately. The most important alkaloids were present in the organic solvent phase. The PCA plots and loading plots for each fraction are presented in Figure 1A-D. Figure 1A shows that the three species of *Ephedra* are separated from each other. The loading plot of PC1 was analyzed and since it is the main PC for the separation, it was found to have characteristic NMR signals for ephedrine alkaloids such as at δ 0.9–0.8 ppm, N–CH₃ at δ 2.60 ppm, and the aromatic al at δ 7.3 ppm were responsible for the separation. These loids are higher in *E. sinica* while ephedrine was not detected in *distachya* var. *distachya*, therefore this species was located outside of the 95% Hotelling T² confidence ellipse. Analysis of aqueous fractions was also performed since there was no clear separation between commercial *Ephedra* samples. From Fig. 1C-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, 1.72 Hz), 7.70 (t, 1.75 Hz), and 7.56 (t, 1.78 Hz) and monosubstituted phenolic compounds like phenylalanine at δ 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. 1E) 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 the Species Based on Metabolomic Fingerprinting Using NMR and Multivariate Data Analysis (Civi et al., 2005)

*H. paraguayensis* var. *paraguayensis*, 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 *H. paraguayensis* var. *paraguayensis* and some other *H. species* which are commonly used as adulterants or substitutes, (i.e. *H. argentina*, *H. brasiensis*, *H. brevicuspis*, *H. durensis* var. *durensis*, *H. durensis* var. *guaranina*, *H. integerrima*, *H. microdonita*, *H. pseudobuxus*, *H. tenuifolia*, and *H. theassinis*). 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 1H-NMR spectra (Fig. 2), only *H. paraguayensis* var. *paraguayensis* 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.

The use of NMR and MVA made it possible to further analyze the difference between 10 other species beside *paraguariensis* by using the same data. For this purpose, *paraguariensis* was omitted from the score plot (Fig. 4A). *Ilex dumosa* var. *dumosa* (4) and *I. tauberiana* (10) were separated from other species since *I. dumosa* var. *dumosa* contains triterpenoids while *I. tauberiana* contains more fatty (Fig. 4B). Further analysis of the aqueous fractions performed by adding another principal component (Fig. 5A). As a result, it was shown that *Ilex paraguariensis* is separated from other species since it has a high amount of phenylpropanoids and sucrose (Fig. 5B-C).

Figure 3. Score plot of organic fractions of *Ilex* species (1) *Ilex argentina*; 2= I. brasiliensis; 3= I. brevicuspis; 4= I. dumosa var. *dumosa*; 5= I. dumosa var. *guarenei*; 6= I. integer; 7= I. microdonta; 8= I. paraguariensis var. *paraguariensis*; 9= I. pseudobuxus; 10= I. *tauberiana*; 11= I. *thee* (reproduced from Cho et al., 2005, with permission)
the organic fractions (Fig. 3.) which shows that *Ilex paraguariensis* var. *paraguariensis* is clearly separated from other species.

The use of NMR and MVA made it possible to further analyze the difference between 10 other species beside *Ilex paraguariensis* by using the same data. For this purpose, *Ilex paraguariensis* was omitted from the score plot (Fig. 4.A.). *Ilex dumosa* var. *dumosa* (4) and *I. tauberiana* (10) were separated from other specially since *I. dumosa* var. *dumosa* contains more triterpenoids while *I. tauberiana* contains more fatty acids (Fig. 4.B.). Further analysis of the aqueous fractions was performed by adding another principal component (PC3) (Fig. 5.A.). As a result, it was shown that *Ilex paraguariensis* was separated from other species since it has a high amount of phenylpropanoids and sucrose (Fig. 5.B-C).

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Figure 3. Score plot of organic fractions of *Ilex* species (1= *I. argentina*; 2= *I. brasilensis*; 3= *I. brevicuspis*; 4= *I. dumosa* var. *dumosa*; 5= *I. dumosa* var. *guaraniana*; 6= *I. integerrima*; 7= *I. microdonta*; 8= *I. paraguariensis* var. *paraguariensis*; 9= *I. pseudobuxus*; 10= *I. tauberiana*; 11= *I. theezans*) (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* sp b. Loading plot of PC2 c. Loading plot of PC3
1. (reproduced from Choi et al., 2005, with permission) a. Score plot of organic solvent fractions of 10 species of Jlex (without L. paraguariensis) b. Loading plot of organic solvent fractions of 10 species of Jlex (without L. paraguariensis)

Figure 5. (reproduced from Choi et al., 2005, with permission) a. Score plot of PC1, PC2, PC3 of aqueous fractions of Jlex 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)

*Artemisia 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 derivatives (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 transferred to NMR tubes. The 1H 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*.

**CONCLUSION**

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

**References**


**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.

**References**


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