GRAPHICAL ABSTRACT

Metabolomic analysis of *Strychnos nux-vomica, S. icaja* and *S. ignatii* extracts by ^{1}H nuclear magnetic resonance spectrometry and multivariate analysis techniques

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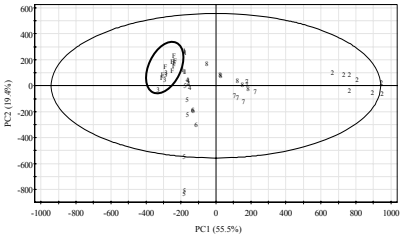

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Abstract

^{1}H Nuclear magnetic resonance spectrometry and multivariate analysis techniques were applied for the metabolic profiling of three *Strychnos* species: *S. nux-vomica* (seeds, stem bark, root bark), *S. ignatii* (seeds), and *S. icaja* (leaves, stem bark, root bark, collar bark). The principal component analysis (PCA) of the ^{1}H NMR spectra showed a clear discrimination between all samples, using the three first components. The key compounds responsible for the discrimination were brucine, loganin, fatty acids, and *Strychnos icaja* alkaloids such as icajine and sungucine. The method was then applied to the classification of several "false angostura" samples. These samples were, as expected, identified as *S. nux-vomica* by PCA, but could not be clearly discriminated as root bark or stem bark samples after further statistical analysis.

Keywords

Loganiaceae, *Strychnos nux-vomica, Strychnos ignatii, Strychnos icaja*, strychnine, brucine, metabolomic, metabolic profiling, ^{1}H-NMR, multivariate analysis.

1. Introduction

Strychnos is a genus of small trees and climbing shrubs, members of the family *Loganiaceae*, distributed throughout Asia, Australia, Africa and South-America. In fact, among about one hundred and ninety species, only seven would contain strychnine (*Strychnos nux-vomica* L. (Baser and Bisset, 1982; Bisset, 1976; Gadi-Biala et al., 1996; Sefcovic et al., 1968)*, ignatii* P. Bergius (Bisset and Walker, 1974; Bratati & Bisset, 1990) *and wallichiana* Steud ex DC. (Bisset, 1976; Bisset and Choudhury, 1974) from Asia, *Strychnos lucida* R. Br. (Bisset, 1976; Shaw and de la Lande, 1948) from Australia, *Strychnos icaja* Baillon (Denoel, 1950; Kambu et al., 1980) from Africa, *Strychnos tabescana* and *Strychnos panamensis* (Krukoff et al., 1972; Marini-Bettolo et al., 1972) from South-America). In this paper we have investigated the three most common species: *S. nux-vomica, S. ignatii* and *S. icaja* (Table 1)*. Strychnos nux-vomica* L. (Loganiaceae) is a tree from south-east Asia whose seeds have been used for the extraction of strychnine (**1**) and brucine (**2**) (Figure 1), as well as in the preparation of galenicals. Strychnine (**1**) is a particularly toxic bulbar and medullar stimulant (LD_{50} 0.2 mg/kg, parenteral) that has been intensively used both as a rat poison, as well as a tonic. The extracts of *S. nux-vomica* seeds are still used in allopathy (essentially in China and India) for a wide variety of disorders, including epilepsy, digestive disorders and debility (Ambasta, 1986; Dymock et al., 1891) and in homeopathy (in Western countries). *Strychnos ignatii* P. Bergius is a large shrub, up to 20-m high, found in Thailand, South-China, and the Philippines. The plant is used as a remedy for a number of ailments (Ambasta, 1986; Bisset, 1974; Dymock et al., 1891). *S. icaja* is a shrub that can become an important liana, up to 100-meters long, and which is found all over central Africa. Its roots have a characteristic red outer bark. The plant is mainly known for its use as a hunting or ordeal (trial) poison. Nevertheless, the use of this *Strychnos* in traditional medicine, in particular, to treat chronic malaria, has also been reported (Iwu, 1993; Neuwinger, 1996). Recently, several antiplasmodial bisindole alkaloids have been isolated from *S. icaja* roots, again drawing interest to this plant (Frederich et al., 2000, 2001; Philippe et al., 2003).

The components of the metabolome can be viewed as the end products of gene expression and define the biochemical phenotype of a cell or tissue. Metabolic profiling could be used in a number of areas to provide biological information beyond the simple identification of plant constituents (Sumner et al., 2003). Among these areas is the chemical fingerprinting of species and plant organs for identification or taxonomic purposes. The method commonly

used by industry for quality control of plant material is analysing the product for the presence of chemical markers known to be present in the plant. However, the presence of specific chemical makers does not always guarantee the identity of the plant material, especially if the product has been spiked with chemical markers. A better authentication of the plant material could be possible by a chemical fingerprinting of the botanical metabolites, implying a general overview of all plant constituents, and should be included into quality control methods (Bailey et al., 2002; Schaneberg et al., 2003). NMR associated to PCA could then also be used for chemotaxonomic analysis of plants, microbes and worms (Bundy et al., 2002).

In this work, high-resolution NMR spectroscopy was used to characterize the overall composition of three strychnine-containing species of *Strychnos*, aiming at identifying a wide range of metabolites. The principal aim was to explore the potential of ^{1}H NMR spectroscopy method, coupled with multivariate analysis to enable an efficient identification (metabolic fingerprinting) of the nature of *Strychnos* samples (species, organs). Secondarily, the method was also applied to a series of samples of "false angostura bark". The angostura bark (cortex Angusturae, *Galipea officinalis* St. Hiliare, Rutaceae) comes from a northern South American tropical tree, and is used in traditional medicine to treat dysenteries, paralytic infections, and as a tonic and to make bitter liquors (Jacquemond-Collet et al., 1999; Tschirch, 1923). At first appearance, this bark resembles the poisonous bark of the *nux-vomica* tree. So it was not previously uncommon (early 19th century) to find portions of the latter mixed with angostura bark. This proved to be a most dangerous adulteration, causing several deaths between 1804 and 1806 in Germany, Switzerland and Hungary. As a result, *true* angostura fell into disfavour, (the *nux-vomica* being called *false* angostura) (Flückiger and Hanbury, 1878; Grieve, 1971; Tschirch, 1923). We have analysed here four different samples of false angostura bark, to check if they were classified amongst *S. nux-vomica* samples.

2. Results and discussion

2.1. Extraction and analysis of plants

The extraction method used was very simple, requiring direct extraction of powdered, freeze-dried material by methanol-d_4 for NMR. The use of a deuterated solvent for extraction eliminated the need for evaporation and re-dissolution of extracts, which has the associated potential problem of loss of material and risk of chemical modifications. Trifluoracetic acid was added just before NMR measurements to control the acidity and minimise the risks of signal shifting for acidic or basic compounds (alkaloids). Minimal shifts of signals between replicates are indeed essential to realise metabolic profiling. Three replicates were measured for each plant material studied.

2.2. Visual inspection of ^{1}H NMR spectra

Some differences were observed between the spectra of the various samples. In general, the ^{1}H NMR spectra obtained showed a dominance of signals in the carbohydrate (δ 2.5 to δ 4.5) and, for seeds, in the aliphatic region (δ 0.5 to δ 1.5) of the spectra. In addition to these signals, well-defined (but smaller) signals were present in the aromatic region of the spectra, and essentially attributed to alkaloids, caffeic acid, and loganin (seeds). Some differences could be visually observed between the different spectra. Strychnine was present in all extracts except *S. icaja* leaves, but the amount of the compound was extremely variable according to the species and batches: major alkaloids present in *S. nux-vomica* seeds or in *S. ignatii* seeds, but only present in traces in *S. icaja* leaves (unidentified in crude extract). Brucine was absent from *S. icaja* and very abundant in *S. nux-vomica* roots and in "false angostura" bark. Different substances were identified in the extracts. These identified substances are listed in Table 2. Signals were assigned using reference compounds and various 2D-NMR techniques (COSY, TOCSY, HSQC, and HMBC).

As chlorogenic acid is generally described as present in *S. nux-vomica*, we then compared our extracts with an authentic sample of chlorogenic acid by NMR, TLC and HPLC. The presence of a close compound, showing the same fluorescence and close retention factor on TLC (probably an isomer of chlorogenic acid) was observed, but chlorogenic acid itself was not present. It was impossible to clearly identify this compound because of the lack of reference compounds and the crowded NMR spectra in the sugar part. Although there were clear visual

differences between the spectra, for easier and nonbiased interpretation of the results, and to reduce the dimensionality of the multivariate data obtained with the NMR results, we analysed the samples using principal component analysis (PCA).

2.2. Principal component analysis of ^{1}H NMR spectra

PCA is an unsupervised method requiring no knowledge of the data set and which acts to reduce the dimensionality of multivariate data while preserving most of the variance within it (Eriksson et al., 2001). If the data is mean-centered with no scaling, then a covariance matrix is produced. But if the data is mean-centered, and the columns of the data matrix are scaled to unit variance, a correlation matrix is produced. An advantage of the covariance matrix is that the loadings retain the scale of the original data. For the correlation method, however, a weaker signal possessing a discriminatory power can be considered at the same level as stronger signals (Ward et al., 2003). In this study, both methods were evaluated, and the covariance method showed better separation results (Figure 2). For the data set obtained from the analysis of *Strychnos* extract, a nine-component model explained 99% of the variance, with the first three components explaining 85.4%. As observed in Fig. 2A, there is a clear discrimination possible between *S. nux-vomica* seeds and other samples, particularly *S. ignatii* seeds. The extracts of *S. ignatii* seeds, however, show quite similar metabolomic patterns to those of *S. icaja* leaves. The *S. icaja* stem bark, collar bark and root bark are not easily distinguishable but the leaves present a clearly different metabolic content.

The seeds of S. *nux-vomica* are clearly separated from other samples by PC 1 (Fig. 2A). The seeds of S. *nux-vomica* are located in the higher PC1 region. Lipids play an important role in this discrimination. The discriminating metabolites are distinguishably shown in loading plot of PC1 and PC2 (Fig 3). It is obviously postulated that the seeds of *S. nux-vomica* contains higher amount of lipids and the major part of the variability of PC 1 is explained by them and also by loganin (δ 7.38), also quite common in seeds (Fig. 3A). PC2 is essentially influenced by loganin at δ 7.36 and various *Strychnos icaja* alkaloids such as icajine at δ 8.06 and sungucine at δ 8.34. For more separation, PC3 was additionally used (Fig. 4). In this case, the root extracts of *S. nux-vomica* is obviously separated from other samples by PC3 (Fig. 4A) but the differentiating metabolite was identified by loading plot of PC3 and found to be brucine almost alone (Fig. 4B). Two positive distinguishable ^{1}H NMR signals in the loading plot of PC3 were identified as H-12 and H-9 of brucine, respectively.

The data from the false angostura samples were then added to these results. They were plotted in Fig. 5. As expected, these samples were plotted close to the *S. nux-vomica* samples, but more particularly, close to the *S. nux-vomica* root bark samples (Fig. 5A). For confirmation, ANOVA test was performed to PC1, PC2, and PC3 of *S. nux-vomica* samples (roots and stem barks) and false angostura. Unfortunately, the PC scores are not significantly different. The discriminating metabolites in the plot containing false angostura samples are distinguishably shown in Fig. 5B and 5C (loading plots of PC1 and PC2) and are identical to those previously described, although *S. icaja* alkaloids have here a negative influence on PC2.

2.3. Conclusions

We showed that it was possible to discriminate three different *Strychnos* species from various origins by multivariate analysis of ^{1}H NMR spectra of crude extracts. Seeds of *S. ignatii* and *S. nux-vomica* are easily distinguishable. *S. icaja* and *S. nux-vomica* stem barks are much more similar**,** but could be distinguished using the brucine content (mostly influencing pc3). Finally, we analysed four collection samples from "false angostura", which is a falsification of angostura bark (*Galipea officinalis*). In the literature, from the 19th and early 20th century, the false angostura was generally described as "bark from *S. nux-vomica* imported from India", without more precision, the real origin of the samples remaining quite confused (Felter & Lloyd, 1898; Flückiger and Hanbury, 1878; Grieve, 1971; Tschirch, 1923). According to our PCA results, the false angostura bark should be classified as an *S. nux-vomica* root bark sample. This classification is quite plausible, as stem and root barks of *S. nux-vomica* are still used in traditional medicine in India (Ambasta, 1986). Further statistical analysis (ANOVA) could not show significant differences between PCs of false angostura samples and root bark or stem bark of *S. nux-vomica*. To confirm this point, as score plots of root and stem bark of *S. nux-vomica* were quite close, it would be useful to further analyse authenticated root bark and stem bark samples from *S. nux-vomica* (and samples from different origins). It is likely that the different false angostura samples, clearly plotted as *S. nux-vomica* in our PCA analysis do not have a homogeneous origin (some could be stem bark and others root bark).

We then showed that the major compounds responsible for the discrimination were brucine, fatty acids, loganin and several *S. icaja* alkaloids (mainly icajine and sungucine). Strychnine, though present in various amounts in all extracts analysed, was not the key compound for the discrimination of samples.

3. Experimental

3.1. Solvents and chemicals

Methanol-d_4 (99.80% D) and deutarated trifluoracetic acid were purchased from Eurisotop (Gif-sur-Yvette, France). Trifluoracetic acid, strychnine, brucine, caffeic acid, chlorogenic acid were purchased from Aldrich (Steinheim, Germany). Methanol was from Merck Biosolve Ltd. (Valkenswaard, The Netherlands).

3.2. NMR measurements

All spectra were recorded on a Bruker AV-400 NMR spectrometer operating at a proton NMR frequency of 400.13 MHz. For each sample, 256 scans were recorded with the following parameters: 0.126 Hz/point, pulse width (PW) = 30 ^{o} (4.0 μs), and relaxation delay (RD) = 1.0 sec. FIDs were Fourier transformed with LB = 0.5 HZ, GB = 0, and PC = 1.0. The spectra were referenced to solvent.

3.3 Extraction of alkaloids

The powdered plant material (100.0 mg) was freeze-dried during 24 hours and then extracted with 1.0 ml of methanol-d_4 under ultrasonication for one hour at room temperature. The mixture was then centrifuged at 13000 *g* for 10 minutes and 750 μl of supernatant were mixed with trifluoracetic acid (1%, v/v) for NMR measurement.

3.4. Plant material

Strychnos nux-vomica seeds and *Strychnos ignatii* seeds were commercial samples obtained from Denolin (Braine l'Alleud, Belgium) and Longeval (Deux Acres, Belgium). Seeds of *Strychnos nux-vomica* were authenticated according to the description in the Swiss and French Pharmacopoeia and those of *S. ignatii* following the microscopic description in Perrot and Gathercoal (Gathercoal and Wirth, 1947; Perrot, 1943). The samples of *S. icaja* were

collected near Kasongo-Lunda, Mulika and Kikwit (Congo-Zaire). Voucher specimens of the plants (Duvigneaud H787, H864 and H900) have been deposited in the herbarium of the Pharmaceutical Institute in Liège, and in the herbarium of the Belgian National Botanical Garden, in Meise. The samples of "false angostura bark" NV06 came from the laboratory collections and were identified by the late Professor Bisset (King's College, University of London.). The *S. nux-vomica* stems were collected in Madras (India) by Phn. A. Kone (NV01, stems). A voucher specimen (NV01) of this plant has been deposited in the herbarium of the Pharmaceutical Institute in Liège. *S. nux-vomica* root barks (NV03) were collected at Midnapur, West Bengal, India, and identified by the late Prof. N. G. Bisset (King's College, University of London). A voucher sample (NGB 23289) has been deposited in the Pharmaceutical Institute, University of Liège, Belgium. *S. nux-vomica* stem bark NV05 and the "false angostura" barks NV08, NV09, NV10 came from the collections of the University of Göttingen (Herbarium GOET, Germany, Pharmakognostische Sammlung 12/2001 N° 3294, 1112, 2670, 3280, respectively). All species and their origin are reported in Table 1.

3.5. Data analysis

The ^{1}H-NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to 0.01% (v/v) of HMDS and reduced to integrated regions of equal width (0.02 ppm) corresponding to the region of δ 0.40 – δ 10.00. The region of δ 4.75 - δ 5.24 of HDO, δ 3.55 - δ 3.65 of residual ethanol, δ 3.25 - δ 3.50 of residual MeOH, δ 2.14 - δ 2.15 of residual acetone, and δ 1.14 - δ 1.19 of residual ethanol were removed for further analysis. Principle component analysis (PCA) and ANOVA analysis were performed with the SIMCA-P software (Umetrics, Umeå, Sweden).

3.6. Assignment of peaks

To assign selected peaks, various two dimensional NMR methods such as ^{1}H ^{1}H COSY, ^{1}H ^{13}C COSY (Hetcor), and ^{1}H ^{13}C long range coupling (HMBC) were applied.

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Table 1. *Strychnos* species and organs used for chemical fingerprinting by ^{1}H NMR

Species	Organ	Origin	Batch Code	Number of samples
S. nux-vomica	Seeds	Commercial	NV02	4
S. nux-vomica	Seeds	Commercial	NV04	4
S. nux-vomica	Stem bark	Madras, India	NV01	3
S. nux-vomica	Stem bark	University Götingen collections	NV05	3
S. nux-vomica	Bark	Falsification of angostura bark University Götingen collections	NV08	3
S. nux-vomica	Bark	Falsification of Angostura bark University Götingen collections	NV09	3
S. nux-vomica	Bark	Falsification of Angostura bark University Götingen collections	NV10	3
S. nux-vomica	Root bark	West Bengal, India	NV03	4
S. ignatii	Seeds	Commercial	IGN02	3
S. ignatii	Seeds	Commercial	IGN03	3
S. icaja	Leaves	Kikwit, Congo	ICA12	3
S. icaja	Leaves	Mukila, Congo	ICA13	3
S. icaja	Stem bark	Kikwit, Congo	ICA05	4
S. icaja	Collar bark	Kasongo-Lunda, Congo	ICA04	3
S. icaja	Root bark	Mukila, Congo	ICA03	3
S. icaja	Root bark	Kikwit, Congo	ICA14	3

Table 2. Metabolites detected and identified from the ^{1}H NMR spectra of various crude extracts

Compounds	*S. nux-vomica* seeds	*S. nux-vomica* stem bark	*S. nux-vomica* "false angostura"	*S. nux-vomica* root bark	*S. ignatii* seeds	*S. icaja* stem bark	*S. icaja* root bark	*S. icaja* collar bark	*S. icaja* leaves
α-Colubrine		+							
β-Colubrine	+	+	+	+					
Brucine	+	+	+	+	+				
Caffeic acid ester	+	+	+	+	+	+	+	+	
Epoxy-novacine						+	+	+	+
Fatty acids	+	+	+	+	+	+	+	+	+
Fatty acids (Unsaturated)	+				+				
Hydroxy-epoxy-novacine						+	+	+	+
Icajine						+	+	+	
Isosungucine							+	+	
Loganin	+				+				
Novacine	+					+			+
Strychnine	+	+	+	+	+	+	+	+	
Sucrose	+	+	+	+	+	+	+		
Sungucine							+	+	
Vomicine	+					+	+	+	

Table 3. Important chemical shifts influencing the PCA discrimination.

PC	Chemical shift	Corresponding compound and signal
PC1	0.88	Fatty acids CH3
PC1	0.90	Fatty acids CH3
PC1	1.10	Loganin H10
PC1	1.32	Fatty acids CH2
PC1	1.60	Fatty acids *CH_2-CH_2-CO
PC1	2.02	Insaturated Fatty acids (*CH_2-CH=CH)
PC1	2.26	Fatty acids CH_2-*CH_2-CO
PC1, PC2, PC3	3.84	Brucine methoxy
PC1	4.18	Unidentified signal
PC1	5.34	Insaturated Fatty acids (CH_2-*CH=CH)
PC1, PC2, PC3	7.02	Brucine H9
PC2	7.36	Sungucine + Isosungucine + Strychnine
PC1	7.38	Loganin H3
PC1, PC2, PC3	7.76	Brucine H12
PC2	8.06	Icajine H12
PC2	8.34	Sungucine H12´

Fig. 1. Chemical structure of strychnine and Brucine.

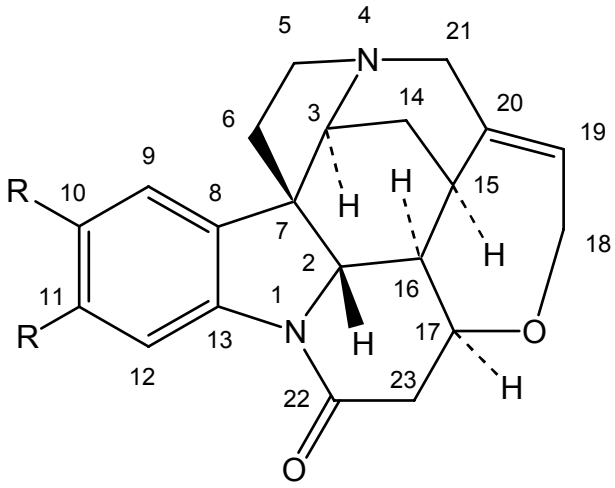
Fig. 2. Score plot of principal component analysis of *Strychnos* extracts obtained by covariance (A) and correlation (B) method using PC1 and PC2. The ellipse represents the Hotelling T2 with 95% confidence in score plots. 1; stem barks of *Strychnos nux-vomica*, 2; seeds of *Strychnos nux-vomica*, 3; roots of *Strychnos nux-vomica*, 4; stem barks of *Strychnos icaja*, 5; root barks of *Strychnos icaja*, 6; collars of *Strychnos icaja*, 7; leaves of *Strychnos icaja*, 8; seeds of *Strychnos ignatii*.

Fig. 3. Loading plot of principal component analysis of *Strychnos* extracts obtained by covariance method. A; PC1, B; PC2.

Fig. 4. Score plot of principal component analysis of *Strychnos* extracts obtained by covariance method using PC1 and PC3 (A) and loading plot of PC3 (B). The ellipse represents the Hotelling T2 with 95% confidence in score plots. 1; stem barks of *Strychnos nux-vomica*, 2; seeds of *Strychnos nux-vomica*, 3; roots of *Strychnos nux-vomica*, 4; stem barks of *Strychnos icaja*, 5; root barks of *Strychnos icaja*, 6; collars of *Strychnos icaja*, 7; leaves of *Strychnos icaja*, 8; seeds of *Strychnos ignatii*.

Fig. 5. Score plot of principal component analysis of *Strychnos* and "False angostura" extracts obtained by covariance method using PC1 and PC2 (A), and loading plot of PC1 (B) and PC2 (C). The ellipse represents the Hotelling T2 with 95% confidence in score plots. 1; stem barks of *Strychnos nux-vomica*, 2; seeds of *Strychnos nux-vomica*, 3; roots of *Strychnos nux-vomica*, 4; stem barks of *Strychnos icaja*, 5; root barks of *Strychnos icaja*, 6; collars of *Strychnos*

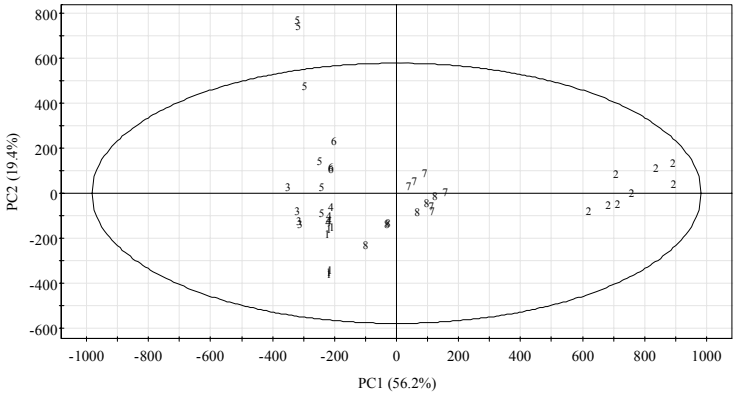
icaja, 7; leaves of *Strychnos icaja*, 8; seeds of *Strychnos ignatii*, F; stem barks of "False angostura" stems.



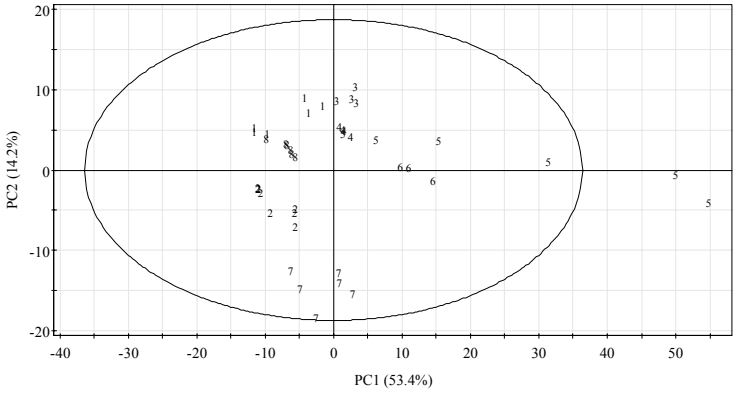
1 R = H, Strychnine

2 R = OCH_3, Brucine

Figure 1.

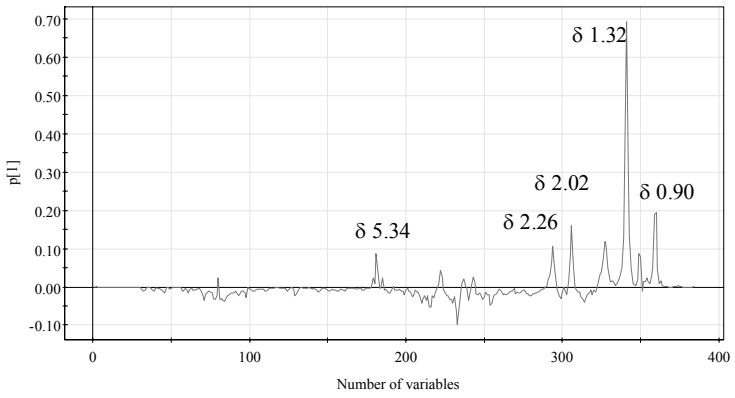


A

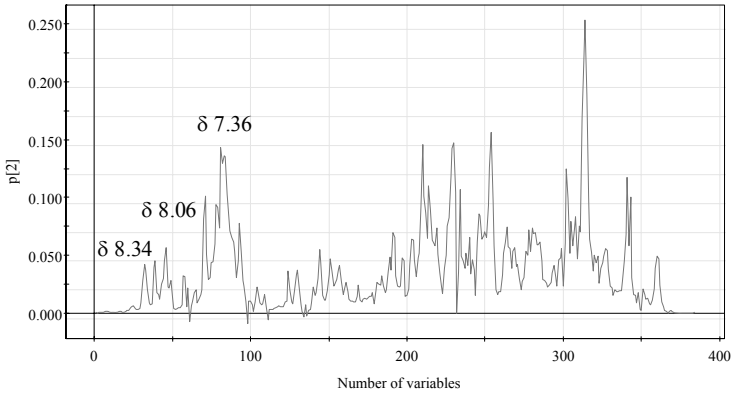


B

Fig. 2

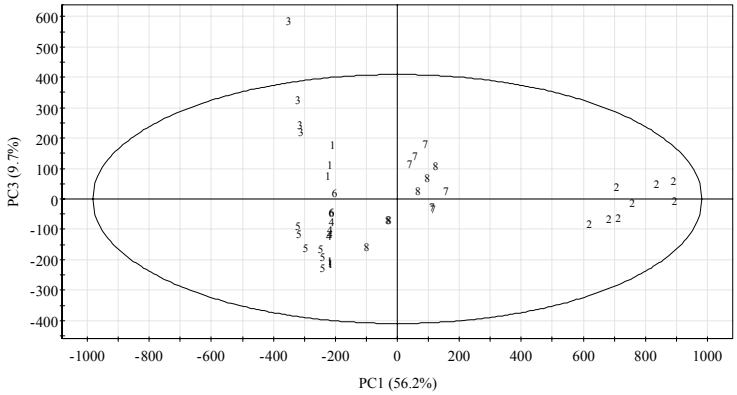

δ 1.32
δ 2.02
δ 0.90
δ 2.26
δ 5.34
p[1]
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
0
100
200
300
400
Number of variables

A

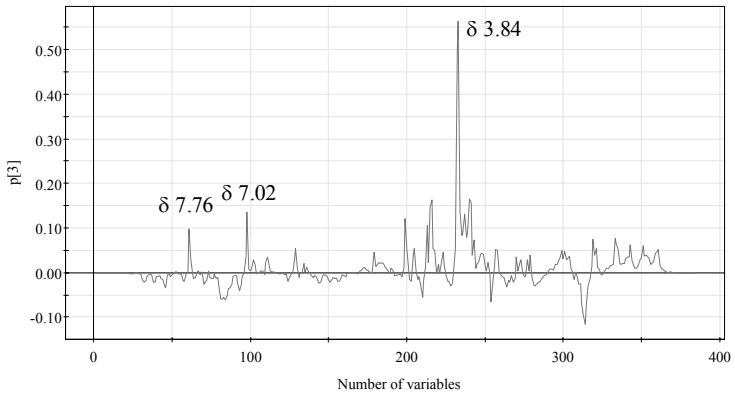

δ 7.36
δ 8.06
δ 8.34
p[2]
0.250
0.200
0.150
0.100
0.050
0.000
0
100
200
300
400
Number of variables

B

Fig. 3

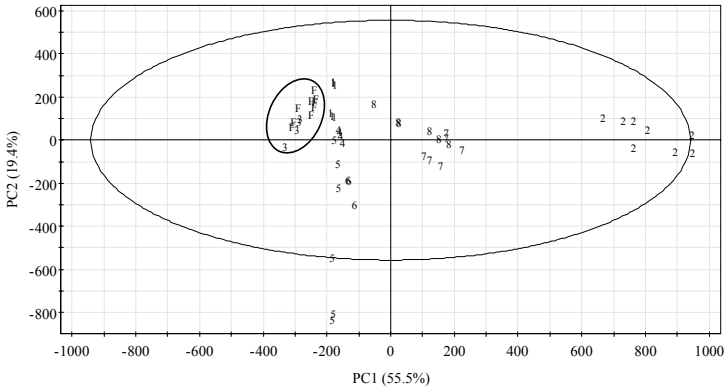


A

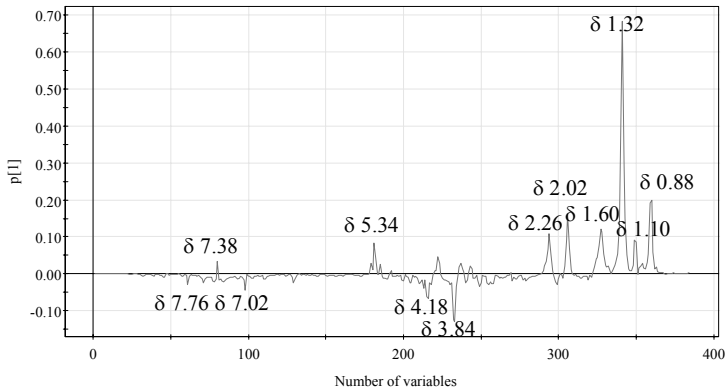


B

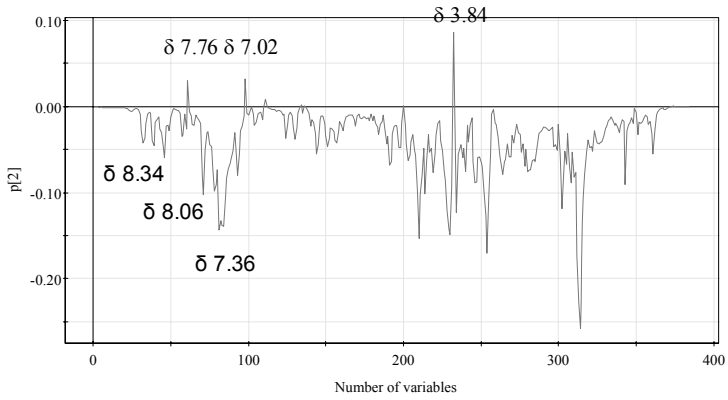
Fig. 4


PC2 (19.4%)
PC1 (55.5%)

A


p[1]
δ 1.32
δ 2.02
δ 0.88
δ 2.26
δ 1.60
δ 1.10
δ 5.34
δ 7.38
δ 7.76 δ 7.02
δ 4.18
δ 3.84
Number of variables

B


p[2]
δ 3.84
δ 7.76 δ 7.02
δ 8.34
δ 8.06
δ 7.36
Number of variables

C

Fig. 5