Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

Research Article

METABOLOMIC ANALYSIS OF *ECHINACEA* SPP. BY ^{1}H NUCLEAR MAGNETIC RESONANCE SPECTROMETRY AND MULTIVARIATE DATA ANALYSIS TECHNIQUE

Michel Frédérich*

Laboratory of Pharmacognosy, Natural and Synthetic Drug Research Center, University of Liège, Avenue de l'Hôpital, 1, B36, B-4000 Liège, Belgium.

Céline Jansen

Laboratory of Pharmacognosy, Natural and Synthetic Drug Research Center, University of Liège, Avenue de l'Hôpital, 1, B36, B-4000 Liège, Belgium.

Pascal de Tullio

Laboratory of Pharmacognosy, Natural and Synthetic Drug Research Center, University of Liège, Avenue de l'Hôpital, 1, B36, B-4000 Liège, Belgium.

Monique Tits

Laboratory of Pharmacognosy, Natural and Synthetic Drug Research Center, University of Liège, Avenue de l'Hôpital, 1, B36, B-4000 Liège, Belgium.

Vincent Demoulin

Laboratory of Pharmacognosy, Natural and Synthetic Drug Research Center, University of Liège, Avenue de l'Hôpital, 1, B36, B-4000 Liège, Belgium.

Luc Angenot

Laboratory of Pharmacognosy, Natural and Synthetic Drug Research Center, University of Liège, Avenue de l'Hôpital, 1, B36, B-4000 Liège, Belgium.

Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

ABSTRACT:
Introduction – The genus *Echinacea* (*Asteraceae*) comprises about 10 species originally distributed in North America. Three species are very well known as they are used worldwide as medicinal plants: *Echinacea purpurea*, *E. pallida*, *E. angustifolia*.
Objective – To discriminate between these three *Echinacea* species and *E. simulata* by ^{1}H NMR-based metabolomics.
Methodology – ^{1}H NMR and multivariate analysis techniques were applied to diverse *Echinacea* plants including roots and aerial parts, authentic plants, commercial plants and commercial dry extracts.
Results – Using the ^{1}H NMR metabolomics, it was possible, without previous evaporation or separation steps, to obtain a metabolic fingerprint to distinguish between species.
Conclusion – A clear distinction between the three pharmaceutical species was possible and some useful metabolites were identified.

Keywords: Metabolomics; ^{1}H NMR; *Echinacea purpurea*; *Echinacea pallida*; *Echinacea angustifolia*; *Echinacea simulata*; Asteraceae

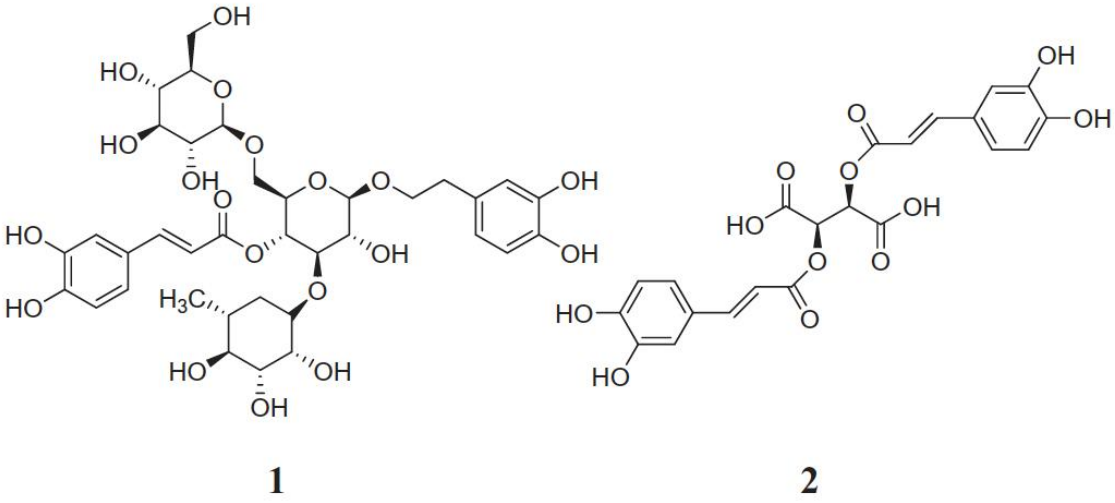
Introduction

In recent years, metabolomics, metabolite profiling or metabolic finger printing have been increasingly used to provide information in a number of areas and particularly in the fi eld of plant sciences. Among these applications, the chemical fingerprinting of species and plant organs has proved especially useful for quality control or taxonomic purposes (Choi *et al*., 2005; Fiehn, 2002; Frédérich *et al*., 2004; Kim *et al*., 2005). NMR-based methods have the advantages of requiring relatively little sample preparation, being non-destructive and allowing the determination of molecular structures of individual compounds, even in mixtures. It therefore has a great potential as a method for the quality control of phytopharmaceuticals (Verpoorte *et al*., 2007, 2008).

The genus *Echinacea* (Asteraceae) comprises about 10 species originally distributed in North America. The genus *Echinacea* has a long history of medicinal use for a wide variety of diseases or disorders. Current interest in the medicinal use of *Echinacea* is focused on its immunostimulant effects, particularly in the treatment and prevention of common colds, influenza and other upper respiratory tract infections (Barett, 2003; Woelkart *et al*., 2008). Three species are used worldwide as medicinal plants: *Echinacea purpurea*, *E. pallida* and *E. angustifolia*. The phytochemical composition of these species is relatively well known, and includes phenylpropanoids (caffeic acid derivatives such as cichoric acid and echinacoside) (Fig. 1), alkamides and heterogeneous polysaccharides as principal secondary metabolites (Barnes et al., 2007). In spite of some differences in the phytochemical constituents of these three species, their identification is not easy, often leading to some confusions (Mistrikova and Vaverkova, 2006). This could have implications in the efficacy and safety of *Echinacea* preparations. Recently the etabolomic profiling of various *Echinacea* species on the basis of lipophilic metabolites from roots, HPLC and GC-MS was conducted (Wu *et al*., 2009). Based on these metabolites, *E. pallida* proved to be very close to *E. simulata*. In our study, ^{1}H nuclear magnetic resonance

Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

spectrometry (NMR) and multivariate analysis technique were applied to the discrimination of the three medicinal *Echinacea* species and the common *E. simulata* (roots and aerial parts, plant and commercial dry extracts were analysed) but focusing this time on polar metabolites.



1 **2**

Figure 1. Chemical structures of echinacoside (**1**) and cichoric acid (**2**).

Experimental

SOLVENTS AND CHEMICALS

Methanol-*d4* (99.98% D), trimethylsilyl-3-propionic acid-d_4(TMSP) and deuterium oxide (99.96% D) were purchased from Eurisotop (Gif-sur-Yvette, France). Phos phate buff er (P7994) was purchased from Sigma-Aldrich (Steinheim, Germany). Echinacoside (reference compound) was obtained from ChromaDex (Irvine, CA, USA).

PLANT MATERIAL

All information on collected samples and their origin is recorded in Table 1. The first eight samples were identified at the Missouri Botanical Garden (St Louis, MO, USA).

Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

Table 1. *Echinacea* samples used for chemical fingerprinting by ^{1}H NMR

Species	Organ	Origin	Voucher or batch no.
E. purpurea	Roots	Missouri Botanical Garden	20050712a
E. purpurea	Aerial parts	Missouri Botanical Garden	20050712a
E. pallida	Roots	Missouri Botanical Garden	20050708
E. pallida	Aerial parts	Missouri Botanical Garden	20050708
E. angustifolia	Roots	Missouri Botanical Garden	20050712b
E. angustifolia	Aerial parts	Missouri Botanical Garden	20050712b
E. simulata	Roots	Missouri Botanical Garden	20050712c
E. simulata	Aerial parts	Missouri Botanical Garden	20050712c
E. purpurea	Roots	Louveigné Nursery, Belgium	20050615a
E. purpurea	Aerial parts	Louveigné Nursery, Belgium	20050615a
E. purpurea	Roots	Louveigné Nursery, Belgium	20050615b
E. purpurea[a]	Aerial parts	Louveigné Nursery, Belgium	20050615b
E. purpurea	Roots	Dry extract, commercial	Ortis 01040994
E. purpurea	Roots	Dry extract, commercial	Dolisos 03C12
E. pallida[b]	Roots	Dry extract, commercial	Dolisos 02L31
E. pallida[b]	Roots	Dry extract, commercial	Dolisos 02L12
E. pallida[b]	Roots	Dry extract, commercial	Dolisos 03F24
E. purpurea	Aerial parts	commercial	Ortis 01041007
E. purpurea	Aerial parts	commercial	Agrimed 02158757

[a] This plant was labelled as *E. pallida*, but, according to *European Pharmacopoeia* was in fact *E. purpurea*.
[b] This extract was labelled *E. angustifolia* but, according to *European Pharmacopoeia* was in fact *E. pallida*.

EXTRACTION OF PLANT SAMPLES

The dry powdered plant material (200.0 mg) was fi rst vortexed with 1.30 mL of a mixture of methanol-d_4 and phosphate buff er in D_2O (1 : 1, pH 7.4) and then ultrasonicated for 1 h at room temperature. The mixture was then centrifuged at 13000 rpm for 10 min and 600 µL of the supernatant were distributed in 5 mm tubes for NMR measurements. The same procedure was applied for dry extracts, using only 50.0 mg of material and 1.0 mL (1 : 1) of solvent.

NMR MEASUREMENTS

All spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. For each sample, 128 scans were recorded with the following parameters: pulse width (PW) = 30° (2.57 µs), and relaxation delay (RD) = 1.0 s. The acquisition time was 3.17 s. FIDs were Fourier transformed with LB = 0.3 Hz. The spectra were referenced to TMSP at δ 0.00. Two replicates were measured for each plant material studied.

DATA ANALYSIS

The ^{1}H-NMR spectra were automatically reduced to ASCII fi les using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.40– 10.00 or of δ 5.8–10 (aromatic region of the spectrum). The regions of δ 4.50–5.24 of HDO and δ 3.30–3.35 of methanol-d_4 were removed for further analysis. The matrix size was then of 220 variables. Principal component analysis (PCA) was performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) using Pareto scaling method.

Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

ASSIGNMENT OF PEAKS

To assign selected peaks, various two-dimensional NMR methods such as ^{1}H ^{1}H correlated spectroscopy (COSY), *J*-resolved, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) were applied (Bruker standard parameters were applied).

HPLC AND TLC ANALYSIS

HPLC and TLC analysis were performed following the procedures described in the *European Phar macopoeia* (2009). The TLC analysis was carried on silica gel plates with a mobile phase consisting of anhydrous formic acid, water, methyl ethyl ketone and ethyl acetate (3 : 3 : 9 : 15, v/v/v/v). For HPLC, a C_{18} column (250 × 4.6 mm; 5 µm particle size) was used and samples were eluted with a gradient of 90% of phosphoric acid 0.1% (solvent A) and 10% acetonitrile (solvent B) to 22% B in 13 min, then to 40% in 14 min and then 40% until 20 min. The injection volume was 10 µL and separation was carried out at 35°C with a fl ow rate of 1.5 ml/min. Detection wavelength was 330 nm and the total run 20 min.

Results and Discussion

The extraction method used in this study proved to be very simple, directly providing an extract of the powdered, dried material in a mixture of phosphate buff er in D_2O (pH 7.4)–methanol–d_4 (1 : 1) for NMR. The use of a deuterated solvent for extraction eliminated the need for evaporation and re-dissolution of extracts, reducing the risk of loss of material and chemical alterations.

A visual inspection of the whole NMR spectra allowed the identifi cation of common primary metabolites, such as sucrose, *α*- and *β*-glucose (free or conjugated) or amino acids (alanine, valine), and showed the presence of low to medium amounts of caff eic acid derivatives in the aromatic part of the spectra. A zoom on this aromatic part of the NMR spectra (Fig. 2) allowed the identifi cation of echinacoside in *E*. *pallida*, *E*. *angustifolia* and *E*. *simulata* and of cichoric acid in *E*. *purpurea* on the basis of comparison with authentic samples.

Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

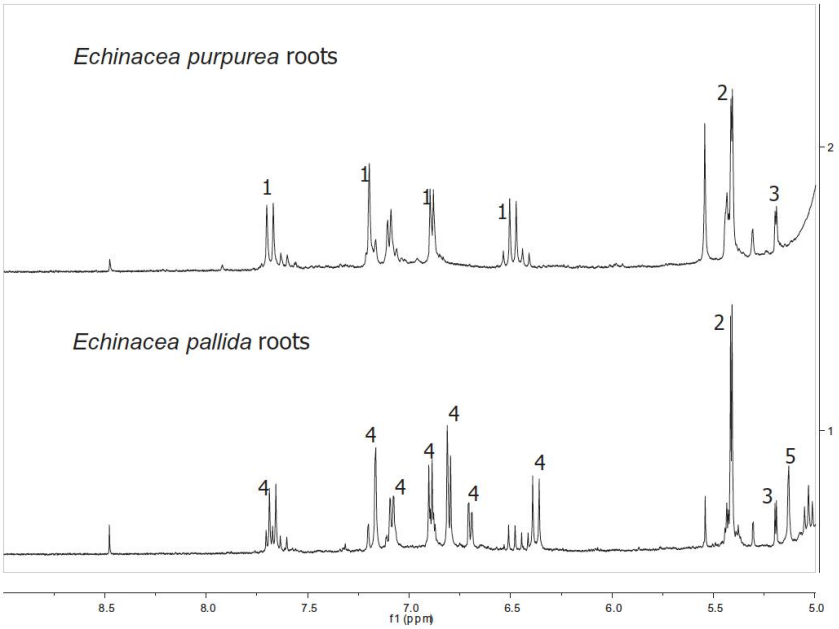


Figure 2. ^{1}H NMR spectra of *Echinacea pallida* and *Echinacea purpurea* roots in aromatic region (δ 5.0–9.0). **1**, Cichoric acid; **2**, sucrose; **3**, α-glucose; **4**, echinacoside; **5**, rhamnose moiety of echinacoside.

Multivariate data analysis of the NMR spectra was then used to try to discriminate the four species. Using the whole spectra, no complete discrimination between the species could be achieved. However, a good discrimination between aerial parts and roots could very easily be obtained when using only the fi rst principal component (PC1) from PCA analysis (Fig. 3). The metabolites allowing this discrimination were clearly sugars and fatty compounds, present in higher quantities in roots and aerial parts, respectively. This is an indication that the primary metabolite content of all four *Echinacea* species is quite similar.

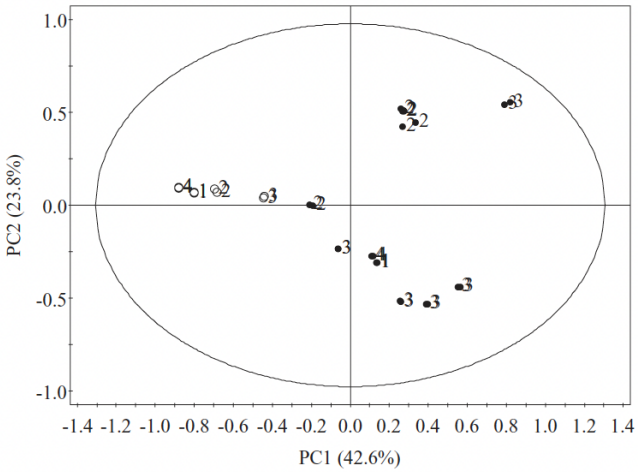


Figure 3. Score plot (PC1 vs PC2) of PCA results obtained from whole range of ^{1}H NMR spectra (δ 0.4–10.0) of *Echinacea* samples. **1**, *Echinacea angustifolia*; **2**, *Echinacea pallida*,; **3**, *Echinacea purpurea*, roots; **4**, *Echinacea simulata*. (•) Roots; (◦) aerial parts. The ellipse represents the Hotelling T2 with 95% confidence in score plots.

Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

It was however possible to discriminate the three medicinal *Echinacea* species among the four species employed in this study using only the aromatic part (δ 5.8–10.0) of the NMR spectra. A good separation could be obtained between *E*. *purpurea* and *E*. *pallida* (roots) using PC1 and PC2 of the loading plot (Fig. 4), while a complete separation of the three medicinal species could be achieved using PC1 and PC4 (Fig. 5A). The metabolites contributing to this discrimination were different caffeic acid derivatives and particularly echinacoside and cichoric acid (Fig. 5B). *E*. *simulata* was nevertheless impossible to discriminate from *E*. *pallida* (and *E*. *purpurea* for roots). The similarity of the metabolite content between *E*. *simulata* and *E*. *pallida* has already been pointed out by Wu and co-workers, who focused on roots and lipophilic metabolites (Wu *et al*., 2009). *E*. *simulata* could therefore be used as a substitution of *E*. *pallida* in the pharmaceutical market.

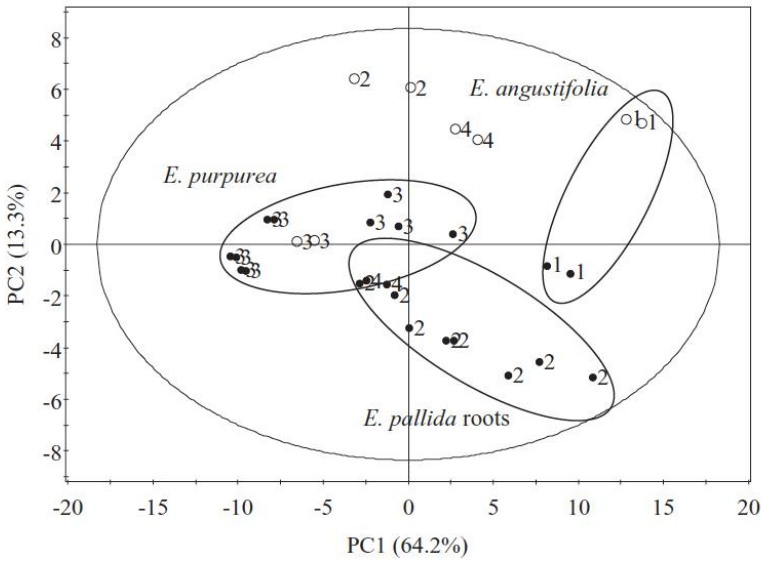


Figure 4. Score plot (PC1 vs PC2) of PCA results obtained from aromatic part (δ 5.8–10.0) of ^{1}H NMR spectra of *Echinacea* samples. **1**, *Echinacea angustifolia*; **2**, *Echinacea pallida*; **3**, *Echinacea purpurea*, roots; **4**, *Echinacea simulata*. (•) Roots; (○) aerial parts. The ellipse represents the Hotelling T2 with 95% confidence in score plots.

Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

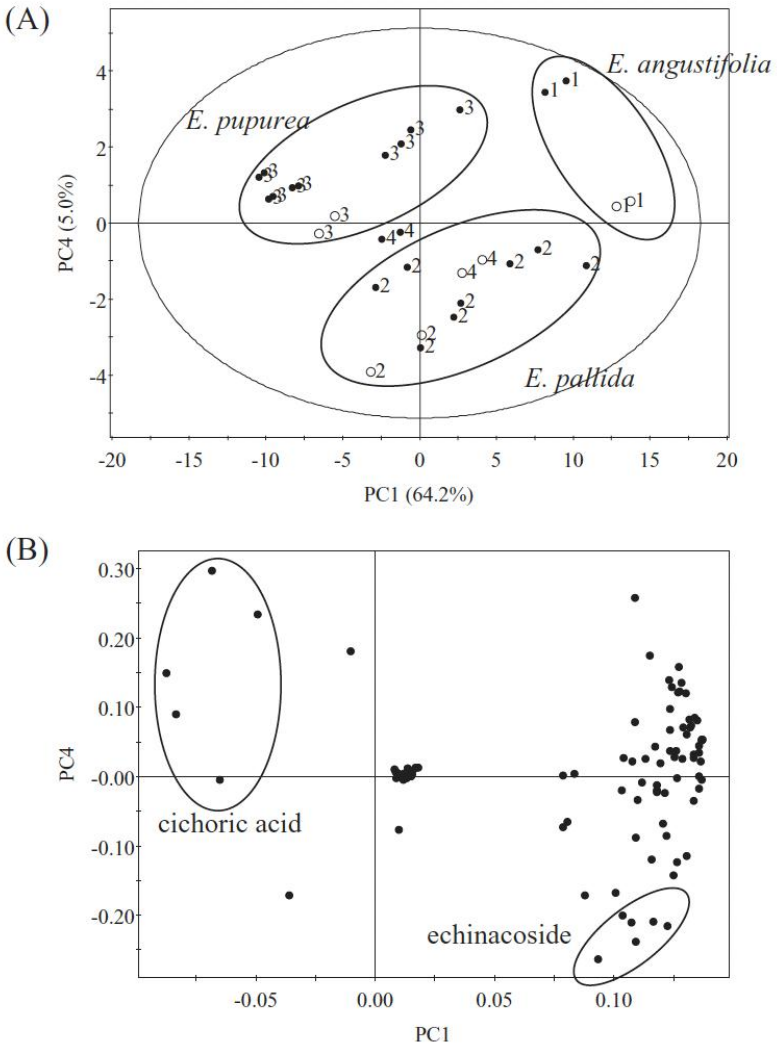


Figure 5. Score (A) and loading (B) plot (PC1 vs PC4) of PCA results obtained from aromatic part (δ 5.8–10.0) of ^{1}H NMR spectra of *Echinacea* samples. **1**, *Echinacea angustifolia*; **2**, *Echinacea pallida*; **3**, *Echinacea purpurea*, roots; **4**, *Echinacea simulata*. (•) Roots; (○) aerial parts. The ellipse represents the Hotelling T2 with 95% confidence in score plots.

Amongst the commercial samples analysed, dry extracts labelled as *E*. *angustifolia* roots were actually identified as *E*. *pallida* roots when submitted to the phytochemical analysis described by the European Pharmacopoeia (based on cichoric acid, echinacoside and other caffeic acid derivatives content). This classification was confirmed by our NMR method (Fig. 4). Similarly, a plant sample from a nursery, labelled as *E*. *pallida* was in fact *E*. *purpurea* (probably a cultivar), confirming a high degree of mislabelling between the three officinal *Echinacea* species sold in the Belgian phytomedicines market.

Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

In conclusion, our work confirmed that there is a great deal of confusion among different *Echinacea* species, up to the point that all *E*. *angustifolia* commercial samples found in the Belgian pharmaceutical market were in fact *E*. *pallida*. It also proved that the *European Pharmacopoeia* method for discriminating between *E*. *pallida* and *E*. *purpurea* focusing on caffeic acid derivatives and especially echinacoside is adequate since these are indeed discriminating metabolites. In our study, *E*. *angustifolia* (roots and aerial parts) also appeared to be clearly diff erent from the other species, although we only analysed two samples of this species.

ACKNOWLEDGEMENTS

This research was supported by the Belgian National Fund for Scientifi c Research (FNRS—grant no. 3452005). M. Frédérich. is a senior research associate from the FNRS. We thank the Missouri Botanical Garden (St. Louis, MO, USA) for the identifi cation of *Echinacea* samples. We thank warmly Dr. Young Hae Choi (Leiden University) for his help in the preparation of this manuscript.

References

Barett B. 2003. Medicinal properties of echinacea: a critical review. *Phytomedicine* **10**: 66–86.

Barnes J, Anderson LA, Phillipson JD. 2007. *Herbal Medicines*. Pharmaceutical Press: London.

Choi YH, Sertic S, Kim HK, Wilson EG, Michopoulos F, Lefeber AWM, Erkelens C, Kricun SDP, Verpoorte R. 2005. Classification of *Ilex* species based on metabolomic fingerprinting using nuclear magnetic resonance and multivariate data analysis. *J Agric Food Chem* **53**: 1237–1245.

European Pharmacopoeia. 2009. 6th edn. European Directorate for the Quality of Medicines and Health Care.

Fiehn O. 2002. Metabolomics: the link between genotypes and phenotypes. *Plant Mol Biol* **48**: 155–171.

Frédérich M, Choi YH, Angenot L, Harnischfeger G, Lefeber AWM, Verpoorte R. 2004. Metabolomic analysis of *Strychnos nux-vomica*, *Strychnos icaj*a and *Strychnos ignati*i extracts by ^{1}H nuclear magnetic resonance spectrometry and multivariate analysis tectiniques. *Phytochemistry* **65**: 1993–2001.Metabolomic Analysis of *Echinacea spp.*

Kim HK, Choi YH, Erkelens C, Lefeber AWM, Verpoorte R. 2005. Metabolic fi ngerprinting of *Ephedra* species using ^{1}H-NMR spectroscopy and principal component analysis. *Chem Pharm Bull* **53**: 105–109.

Mistrikova I, Vaverkova S. 2006. Echinacea—chemical composition, immunostimulatory activities and uses. *Thaiszia J Bot* **16**: 11–26.

Verpoorte R, Choi YH, Kim HK. 2007. NMR-based metabolomics at work in phytochemistry. *Phytochem Rev* **6**: 3–14.

Verpoorte R, Choi YH, Mustafa RN, Kim HK. 2008. Metabolomics: back to basics. *Phytochem Rev* **7**: 525–537.

Woelkart K, Linde K, Bauer R. 2008. Echinacea for preventing and treating the common cold. *Planta Med* **74**: 633–637.

Published in : Phytochemical Analysis (2010), vol.21, pp. 61–65
DOI: 10.1002/pca.1156
Status : Postprint (Author's version)

Wu L, Dixon PM, Nikolau BJ, Kraus GA, Widrlechner MP, Wurtele ES. 2009. Metabolic profiling of Echinacea genotypes and a test of alternative taxonomic treatments. *Planta Med* **75**: 178–183.