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Metabolomic Profiling of Liquid *Echinacea* Medicinal Products with *In Vitro* Inhibitory Effects on Cytochrome P450 3A4 (CYP3A4)

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Key words
- *Echinacea*
- Asteraceae (Compositae)
- metabolomics
- alkylamides
- cytochrome P450 enzymes
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Abstract

Echinacea is a popular and widely used herbal medicinal product and consequently, studies of its interactions with conventional drugs are of particular importance. We have shown that *Echinacea* preparations and some common alkylamides weakly inhibit several cytochrome P450 (CYP) isoforms, with considerable variation in potency. We now report a detailed analysis of six commercial *Echinacea* liquid preparations, with emphasis on the metabolomic characterisation of the *Echinacea* compounds responsible for inhibiting CYP3A4. We separated each preparation into its ethanol- and water-soluble components, and then used ^{1}H-NMR together with multivariate data analysis and partial least square regression analysis to investigate the nature of the compounds responsible for CYP3A4 inhibition. The results implicated alkylamides in the CYP3A4 inhibitory activity of *Echinacea*. One of the commercial preparations (Echinaforce®) was further fractionated using solid phase extraction. Analysis by ^{1}H-NMR and mass spectroscopy (LC/MS, tandem MS, accurate mass) identified dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid (alkylamide **1**) and a new compound (putative molecular formula $C_{18}H_{36}NO^+$) as major components of the inhibitory fractions. In addition, the alkylamide content of all six preparations was determined by reverse phase HPLC. Levels of alkylamides **1** and **3** (undeca-2*E*,4*E*/*Z*-diene-8,10-diynoic acid isobutylamide), correlated well with CYP3A4 inhibition. The acetylene tetradeca-8*Z*-ene-11,13-diyn-2-one was shown to be present in the *E. purpurea* as well as the *E. pallida* extracts. *E. purpurea* unlike *E. pallida* was thought to not contain significant amounts of acetylenes. Our results directly confirm the role of alkylamides in the inhibition of CYP3A4 by *Echinacea* and uncovered a new compound which may also be involved. Extensive differences in the composition of the commercially available preparations were found. This will inevitably impact on the product efficacy, safety and pharmacological effects, especially since the differences involve alkylamides, an important class of *Echinacea*'s active constituents. The metabolomic approach presented here may prove valuable as a screening or quality control tool.

Abbreviations

BfArM:	Bundesinstitut für Arzneimittel und Medizinprodukte
BFC:	7-benzyl-4-(trifluoromethyl)-coumarin
CYP3A4:	cytochrome P450 3A4
ELPs:	*Echinacea* liquid preparations
HMP:	herbal medicinal products
PC:	principal component
PCA:	principal component analysis
PLS:	partial least squares regression analysis
SPE:	solid phase extraction
TMS:	tetramethylsilane
TSP:	(trimethylsilyl)propionic acid-d_4, sodium salt

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Introduction

Echinacea has emerged as an important herbal medical product for the treatment and prevention of upper respiratory infections. Despite its increasing popularity, systematic evaluations of the interaction potential of *Echinacea* preparations with conventional medicines using phytochemically well characterised extracts has only just begun [1].

An important avenue for interactions is the cytochrome P450 (CYP) enzyme system, the major pathway for the metabolism of xenobiotics and endobiotics. Alterations of CYP activity by herbal medicinal product (HMP) constituents can have important undesirable effects by altering the pharmacokinetics of concurrently used medications (e.g., St. John's wort [*Hypericum perforatum*], which strongly effects CYP activity [2]).

For these reasons, regulatory authorities such as the German *Bundesinstitut für Arzneimittel und Medizinprodukte* (BfArM) have drafted new guidelines recommending *in vitro* screening of HMP for potential interactions with the five major drug metabolising CYP isoforms (1A2, 2D6, 2C19, 2C9 and 3A4) [3]. CYP3A4 is of particular importance as it is believed to be involved in over 30% of all drug metabolisms [4].

We have previously shown that *Echinacea* liquid preparations (ELPs) vary widely in their inhibitory activity on CYP3A4, with a > 150-fold difference between the most and least inhibitory ELP (IC_{50} range: 12.7 µg/mL – 1812 µg/mL) [1]. Although a few of these ELPs differ from one another in terms of preparation (i.e., tincture vs. pressed juice), the majority are extracts from the same species of *Echinacea* – *E. purpurea*, with the exception of one from *E. pallida* and another from *E. angustifolia*.

Our data showed that CYP3A4 inhibitory activity covaries with the total alkylamide content of the ELP (determined by HPLC), suggesting that the quantity and possibly the exact chemical nature of the alkylamides present is important [1]. To test this hypothesis and characterise the differences between these ELPs a metabolomic approach was chosen.

Metabolomics, in particular metabolomic fingerprinting, has been widely used as a state of the art technique for sample classification and as a diagnostic and screening tool [5]. NMR spectroscopy is a key analytical technique for the metabolomic fingerprinting of plant extracts [6] due to its high sensitivity, non-selectivity, speed, high-throughput and relatively uncomplicated sample preparation. NMR in metabolomics is often used in conjunction with multivariate data analysis, such as principal component analysis (PCA). The effectiveness of this combination in characterising differences between herbal medicinal products is well established [7–10].

Here, we obtain the extract's NMR metabolomic profile and correlate it with *in vitro* CYP3A4 inhibition. The six ELPs were separated into their ethanol- and water-soluble components (ethanol and water fractions). PCA and partial least squares regression (PLS) analysis were used to identify regions in the NMR spectra that are associated with CYP3A4 inhibitory activity. In parallel we analysed the alkylamide content of all ELPs to investigate whether particular alkylamides were associated with the more potent ELP.

To extend the search for the inhibitory components we analysed the Echinaforce® ethanol fraction in greater detail, using LC-MS, in order to identify individual compounds that could be responsible for the inhibition. Previously we have determined the CYP3A4 inhibitory activity of two alkylamides dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamide (**1**) and dodeca-2*E*,4*E*-dienoic acid isobutylamide (**2**) isolated from other samples of *Echinacea* [1]. We now report the results of assaying the alkylamides undeca-2*E/Z*-ene-8,10-diynoic acid isobutylamides (**3**) and dodeca-2-ene-8,10-diynoic acid isobutylamide (**4**), as well as selected caffeic acid derivatives (identified in the extracts under investigation) for their CYP3A4 inhibitory potential.

Materials and Methods

Echinacea liquid preparations

The ELPs used in this study were Madaus (A), Viridian (B), Salus (C), Echinaforce® (D), Holland & Barrett (E) and Echinagold (F) (see **Table 1**).

Chemicals

Deuterated ethanol, tetramethylsilane (TMS) and deuterium oxide were from Goss Scientific Instruments Ltd. The alkylamides dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamide (**1**) and dodeca-2*E*,4*E*-dienoic acid isobutylamide (**2**) were a kind gift from Dr. Juerg Gertsch [1]. Undeca-2*E/Z*-ene-8,10-diynoic acid isobutylamides (**3**) and dodec-2-ene-8,10-diynoic acid isobutylamide (**4**) were purchased from Phytolab GmbH & Co. KG. The alkylamides were shown to be > 95% pure by ^{1}H-NMR. Cichoric acid and echinacoside were from Chromadex, Inc. SPE silica C_{18} (10 g/60 mL) giga tubes were purchased from Strata Phenomenex. The human supersome CYP3A4 (with cytochrome b5) and its substrate 7-benzyl-4-(trifluoromethyl)-coumarin (BFC) were purchased from Gentest. Ketoconazole (> 98% purity), 3-(trimethylsilyl)propionic acid-d_4, sodium salt (TSP) and all other chemicals and solvents were purchased from Sigma-Aldrich.

Analytical data

The total dry mass (g/100 g m/m) and the concentration (mg/mL) of each extract were determined as described previously [1].

Extraction of ethanol-soluble compounds and NMR

The solvent was removed from 50 mL of ELP with a rotary evaporator and lyophilised. The dry residue was washed three times with 5 mL of ethanol. All ethanol washes were pooled, dried in a rotary evaporator and freeze dried (the "ethanol fraction"). The undissolved residue remaining after the ethanol washes was again dried and lyophilised (the "water fraction", as the residue was freely soluble in water). The whole process was performed twice for each extract.

The ethanol fraction (10 mg) was reconstituted with 1 mL of deuterated ethanol. For the NMR analysis, 0.01% TMS was used as the internal standard. The water fractions were processed in a similar fashion using deuterium oxide and 0.01% TSP instead of ethanol and TMS. ^{1}H-NMR spectra (128 scans) of each fraction were processed using the 400 MHz Bruker Avance spectrometer.

CYP3A4 supersome assay

CYP3A4 inhibitory activity was assayed with the previously described supersome assay [1], using BFC as a substrate. Appropriate controls for intrinsic and quenching fluorescence effects as well as corrections for solvent effects were included as described by Modarai et al. [1]. Ketoconazole was used as the positive control for CYP3A4 inhibition. The samples were reconstituted as described above (without the internal NMR standard).

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Table 1 IC_{50} values of the commercial preparations, and their respective ethanol and water fractions. Upper and lower 95% confidence limits (C.L.) are depicted in brackets.

ELP	ELP name and batch no.	Purchased	ELP ethanol content (%)	Echinacea species (tincture of fresh plant/fresh pressed juice)	ELP total alkylamide content (µg/mL)**	ELP IC_{50} value* (µg/mL)	Ethanol fraction IC_{50} value (µg/mL)	Water fraction IC_{50} value (µg/mL)
A	Madaus, F0500227	Pharmacy, Germany	22	*E. purpurea* (juice)	1	1812 (1343–2447)	N.I. – *400*	N.I. – *875*
B	Viridian, 1881	Health Food Shop, U.K.	22	*E. purpurea* (tincture)	55.9	83.71 (66.09–106.0)	20.07 (16.66–24.19)	N.I.– *400*
C	Salus, U0O114/4/3	Pharmacy, Germany	50	*E. pallida* radix (juice)	19.1	66.08 (61.89–70.55)	N.I. – *500*	169.6 (130.8–219.8)
D	Echinaforce®, 018451	Bioforce, Switzerland	65	*E. purpurea* herb and root (tincture)	67.7	27.81 (25.52–30.31)	2.49 (2.330–2.65)	132.6 (111.3–158.0)
E	Holland & Barrett, 18827	Health Food Shop, U.K.	65	*E. purpurea* (tincture)	98.9	22.18 (21.40–23.0)	1.97 (1.83–2.13)	293.6 (166.8–516.8)
F	Echinagold, 221601–2	Health Food Shop, Denmark	50	*E. purpurea* (tincture)	1384.1	12.71 (11.18–14.46)	0.73 (0.65–0.84)	89.82 (70.09–115.1)

* The ELP IC_{50} results are from Modarai et al., 2007 [1]. The median inhibitory concentrations (IC_{50}) were estimated by using nonlinear regression analysis – four parameter Hill model [1]. Regression models were based on three independent experiments each with at least 8 different concentrations run in duplicate N.I. indicates no inhibition, with the number italicised being the maximum concentration tested. For comparison the IC_{50} of ketoconazole, a reference inhibitor for CYP3A4, was 0.12 (0.10–0.14 µM) [1]; ** to one decimal place

Data processing

^{1}H-NMR spectra (**Fig. 1**) were corrected for phase and baseline distortions using the TOPSPIN Bruker software (version 5). The spectral region between δ = 0.02 and 9.98 ppm was divided ("bucketed") into 250 regions of 0.04 ppm (AMIX Viewer software version 3.5; Bruker Bio Spin GmbH), and the signal intensity in each region was integrated. Regions corresponding to solvent peaks and internal standard were removed.

The spectra were normalised to the integral of the entire processed spectrum in Microsoft Excel before exporting to The Unscrambler® v 7.6 software (CAMO, Inc.). The data were mean centred and scaled prior to principal component analysis (PCA) and partial least squares (PLS) regression analysis. PLS analysis was carried out with respect to either the total alkylamide content of the unprocessed ELP or the CYP3A4 IC_{50} of the ethanol fraction. For extracts A and C, which did not inhibit CYP3A4 the maximum concentration tested (400 µg/mL) was used instead of the IC_{50} value.

Solid phase extraction for the identification of inhibitory constituents

A reverse phase SPE cartridge (Phenomenex strata silica C_{18}, 10 g/60 mL giga tubes) was conditioned with 40 mL of ethanol then equilibrated twice with 40 mL water, prior to loading 400 µL (200 mg) of reconstituted Echinaforce® ethanol fraction. A step gradient (100% water to 100% ethanol in 10% increments) was used for elution. Eleven fractions (F1–F11) were collected, dried in a rotary evaporator and lyophilised. Each fraction was reconstituted (10 mg/mL) with the appropriate solvent (deuterium oxide or deuterated ethanol – see Results) for NMR analysis and the supersome assay.

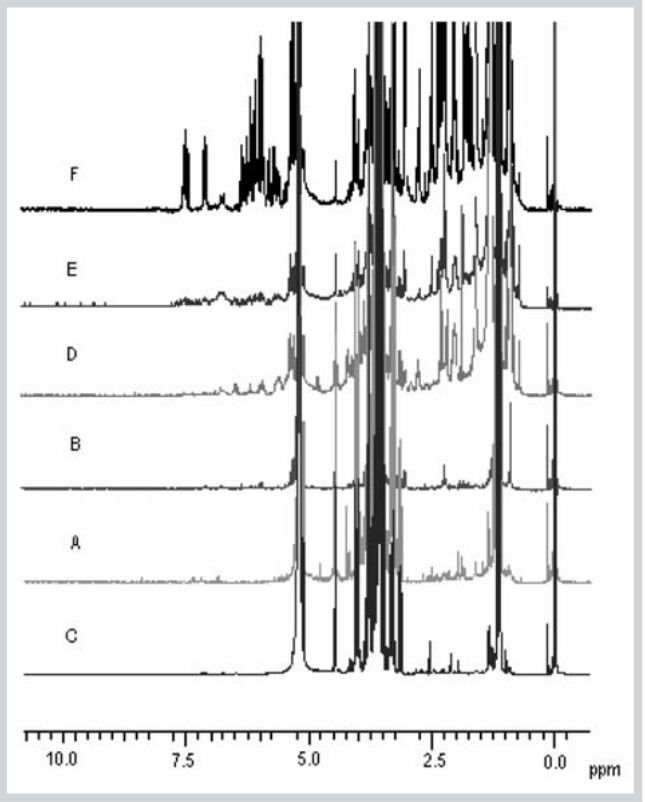

Fig. 1 ^{1}H-NMR spectra of ethanol fractions from all six commercial ELPs (in decreasing order of CYP3A4 inhibitory potency). All fractions were dissolved in deuterated ethanol with TMS (0.01%) as internal standard (δ = 0 ppm).

LC-MS

The fractions of interest were analysed on a Waters 2690 HPLC separation system with a reverse phase column (Atlantis C18, 100 mm × 2.1 mm, 3 µM, 110 Å) coupled to a quadrupole mass spectrometer (Thermo Navigator 400; Micromass), and a Z-spray electrospray ionisation source. Samples were diluted to 50 µg/mL in 20% acetonitrile, 0.1% formic acid and 80% water before inject-

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ing 50 µL. Elution conditions were based on those reported by Cech et al. (2006) [11], using (A) water and (B) 0.1% formic acid in acetonitrile (20% B to 100% B over 30.2 min). Three blank runs of methanol were injected in between samples to clean the column. The mass spectrometer was operated in the positive ion mode, with a scan range of 207–900 *m/z*, a cone voltage of 25 V and with a nitrogen gas flow of 398.4 L/h. Chromatograms of each fraction were obtained using the Mass Lab software (version 3.2).

Other mass spectrometry experiments

MS/MS (MS²): MS/MS data was obtained on Q-TOF Global Ultima mass spectrometer (Micromass/Waters). The samples were ionised using a Z-spray nano-ES interface, with metal coated borosilicate tips (Proxeon Biosystems A/S). Further fragmentation of selected molecular ions (MH^+) was accomplished by argon gas collision, with a capillary voltage of 1.8 kV and a cone voltage of 100 V, collecting centroid data. All other settings were according to the standard operating procedures. The collision energy for the fragmentation ranged between 10–50 eV.

MS³: Detailed fragmentation (MS^n, n = 1, 2 or 3) was performed on a Finnigan LCQ-Orbitrap (Thermo Electron Corp.) analyser equipped with an electrospray ionisation source, using a spray voltage of 1.00 kV and capillary temperature of 200 °C. The scan range was *m/z* = 50–500 and centroid data were collected. The most intense ions were selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 45%. The data were recorded on Mass Lab software (version 3.2).

Accurate mass: Accurate mass analysis was carried out in a Q-TOF Global Ultima mass spectrometer (Micromass/Waters). The settings were as described above. The internal standard was cortisone $[M + H]^+ = 361.2010$.

Determination of alkylamide content

For the six *Echinacea* liquid preparations (A – F), an in depth analytical HPLC determination of individual alkylamide contents was performed according to the validated *Prüfvorschrift PV0988.d07* protocol from Bioforce, as described previously [1]. Briefly the measurements were carried out, using a reverse phase C-18 (RP18) column with a gradient of acetonitrile and water : phosphoric acid. The alkylamides were determined against an external working standard at 260 nm.

Supporting information

Half maximal CYP3A4 inhibitory concentrations (IC_{50} values) of the Echinaforce® SPE fractions, summary of retention times (min) and molecular ions (depicted in brackets) in SPE fractions 2, 7 and 8, ^{1}H-NMR spectra of SPE fractions 1–11, MS^2 spectra of fraction 2, scores plot of PC1 vs. PC2 for the PLS regression analysis and regression analysis of the contribution of individual alkylamides to IC_{50} are available as Supporting Information.

Results and Discussion

Individual *Echinacea* constituents were assayed in an attempt to identify candidate CYP3A4 inhibitors. We have previously shown that alkylamides **1** and **2** inhibit [1], so testing was extended to two more alkylamides (**3** and **4**), revealing that both inhibit CYP3A4 with IC_{50} values of 0.47 µM (95% confidence interval: 0.44–0.51 µM) and 3.76 µM (3.17–4.44 µM), respectively. In contrast echinacoside, chlorogenic acid and cichoric acid showed no inhibitory activity, though quenching effects limited the maximum concentration that could be tested to 6.3 µM, 4.65 µM and 0.5 µM, respectively. Caffeic acid did not inhibit CYP3A4 up to a concentration of 2.2 mM. All alkylamides were less potent than the CYP3A4 reference inhibitor ketoconazole which had an IC_{50} of 0.12 µM (0.10–0.14 µM) (**Table 1**).

Extraction of the ELP with ethanol successfully separated the ethanol- and water-soluble components (ethanol fraction and water fraction). Testing the two fractions with the supersome assay (**Table 1**) revealed that the inhibitory components of *Echinacea* reside mostly in the ethanol fraction, except for ELP C. This is expected as ELP C is an *E. pallida* extract and is therefore different from the *E. purpurea* extracts. Thus, ELP C may contain distinct inhibitory compounds that are not found in the other ELPs.

The spectra for both fractions for each ELP were further analysed by PCA, to determine if unique NMR regions associated with the more inhibitory extracts could be found. The spectra of the water fractions did not produce any clear pattern and their inhibitory activity was very low. Therefore, they were not analysed further (data not shown). The ethanol fractions are well separated along the first principal component according to their inhibitory potency (**Fig. 2a**). The ethanol fractions of A and C which did not inhibit are grouped together on the left, while those of D, E and F are grouped together on the right, while B lies between the two clusters (**Fig. 2a**).

The NMR regions with the greatest impact on computing PC1 (**Fig. 2b**) were 0.9 ppm, 1.3 ppm, 1.34 ppm for the more inhibitory ELPs. For the less inhibitory ELPs they were 3.3 ppm, 3.66 ppm and 3.78 ppm. Signals at 0.9 ppm and 1.3–1.34 ppm are associated with CH_2 and CH_3 groups and therefore it is likely that the main inhibitory compounds are aliphatic (such as alkylamides). This was confirmed by obtaining the spectra of alkylamides **1** and **2** and processing them in the same way (data not shown).

PLS analysis of the spectra with respect to either the CYP3A4 IC_{50} (PLS_{IC50}) of the ethanol fractions, or the total alkylamide content ($PLS_{alkylamide}$) of the whole ELP revealed that both the IC_{50} on CY-

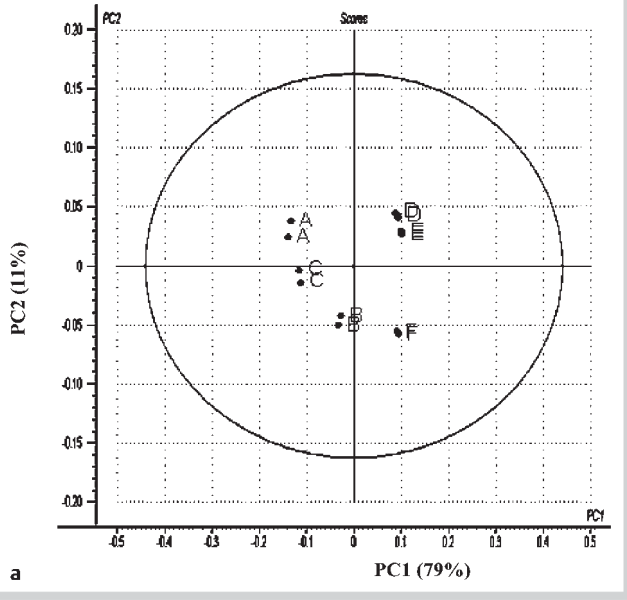

Fig. 2a Scores plot for PC1 vs. PC2 generated by PCA of the ethanol fractions. The ellipse represents the "Hotelling T2", i.e., 95% confidence ellipse in the score plots.

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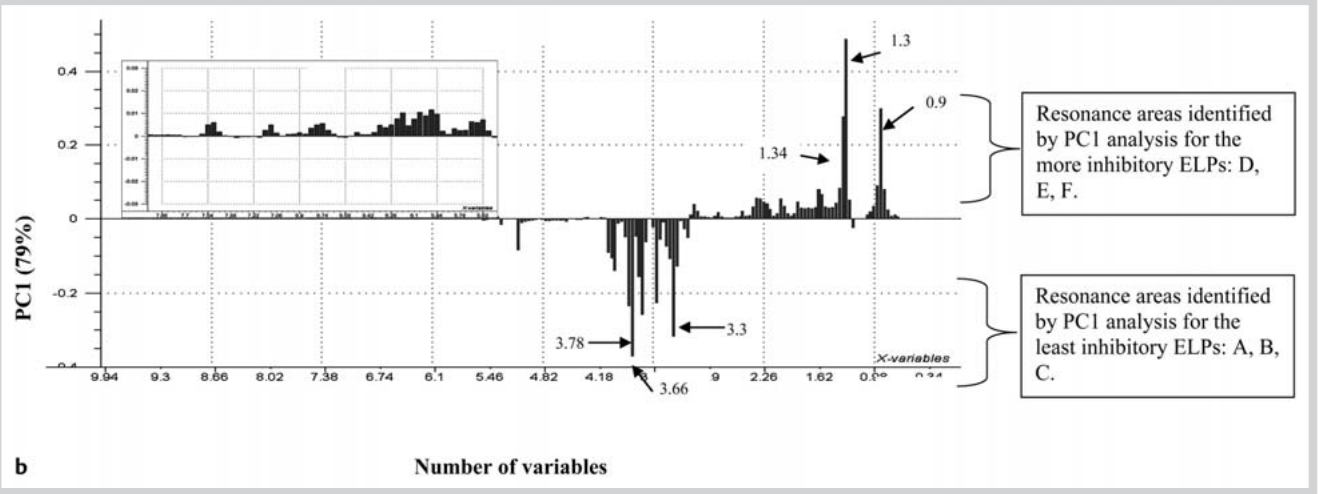

Fig. 2 b Loadings plot for PC1 generated by PCA of the ethanol fractions, explaining 79% of the total variability in the ^{1}H-NMR spectra. The same regions were obtained for both the PLS (alkylamide content) and PLS (IC_{50}) analysis. Also shown is an expansion of regions 5.6–7.8 ppm. Number of variables refers to chemical shifts binned at interval of 0.04 ppm from 0.02–9.98 ppm.

P3A4 and the alkylamide content are strongly associated with the variability in the spectra (**Fig. 3S**, Supporting Information). The first principal component for PLS_{IC50} could account for 97% of the variation in IC_{50} values, while for $PLS_{alkylamide}$ it could explain 81% of the variation in alkylamide content. Remarkably, the NMR regions with the greatest impact on computing PC1 for both the PLS and the PCA analyses were essentially the same, with mostly small variations in the scores (**Fig. 2 b** and **Fig. 3S**, Supporting Information). Overall, this metabolomic analysis, despite the limited number of samples analysed, could clearly separate the extracts according to their activity.

Since the inhibitory compounds appeared to be concentrated in the ethanol fraction, we focused on a single ELP (Echinaforce® – D), by further fractionating its ethanol-soluble compounds with SPE. ELP F is enriched with alkylamides, so it was not chosen for further analysis despite being the extract with the highest inhibition.

All SPE fractions were assayed for CYP3A4 inhibition and ^{1}H-NMR spectra were obtained (**Table 1S** and **Fig. 1S**, Supporting Information). Most of the inhibitory activity concentrated in fractions 5–11 (40–100% ethanol). Fractions 7 and 8 had the lowest IC_{50} and both contained a unique triplet signal at ~7 ppm (**Figs. 1Sb** and **1Sc**, Supporting Information), which matches the H-3 proton signal (a triplet at ~7 ppm) in the spectrum of the pure alkylamide **1** (**Figs. 1Sc** and **1Sd,** Supporting Information) [12]. Fraction 2, unlike the other low ethanol SPE fractions (1, 3 and 4), had comparable inhibitory activity to the higher ethanol SPE fractions (5–11) (**Table 1S**, Supporting Information) and may contain different inhibitory species.

The SPE fractions of interest (2, 7 and 8) were subjected to LC-MS analysis (**Table 2S**, Supporting Information). The peak with molecular ion ($M - H^+$) 338 (at 38 min retention time) appeared in the methanol blank runs and was hence ignored. The major peaks in each fraction ($M - H^+$ 248 for fractions 7 and 8 and $M - H^+$ 292 for fraction 2) were analysed by tandem mass spectrometry.

The molecular ion 248 in fraction 7 and the pure alkylamide **1** produced identical MS/MS spectra and identical results (within 5 ppm) upon accurate mass analysis. The masses of the major MS/MS fragments were 152, 167, 100, 95, and 81.

The major ion 282 in fraction 2 had an accurate mass of 282.2791. The only possible formula, within a 5 ppm tolerance is $C_{18}H_{36}NO^+$. Tandem mass spectrometry (MS^2) revealed two major peaks at 265 and 247 (**Fig. 2Sa**, Supporting Information). The 247 peak was further fragmented (MS^3) to reveal the spectrum presented in **Fig. 2Sb** (Supporting Information). This fragmentation pattern is very similar to that of compounds investigated by Cravatt et al. (1996) [13], especially the nonconjugated diene derivatives. Based on this work we suggest a possible structure for compound 281 (**Fig. 3 a**).

The hydrogen to carbon ratio suggests two unsaturated bonds and the MS^3 spectrum indicates the presence of a long unbranched hydrocarbon chain [13]. The position of the hydroxy group, position and the stereochemistry of the NH_2 group and the first double bond can be easily assigned according to a previ-

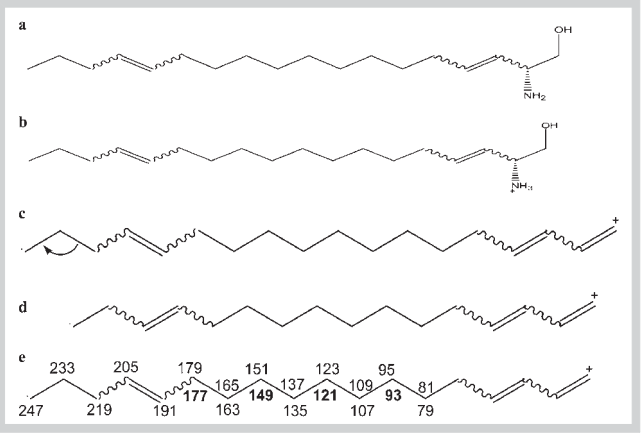

Fig. 3 Proposed structures for the molecular ion $[M - H]^+$ 282 in fraction 2. **a** Uncharged molecule (M). **b** Molecular ion $[M - H]^+$. **c** Proposed structure of fragment 247 produced by loss of water and ammonia from ion 282. **d** Fragmentation mechanism for ion 247. **e** The molecular masses of fragments produced from ion 247. Breakage at each carbon produces a fragment of the indicated molecular weight. The numbers in bold indicate the size of fragments produced after migration of the double bond between carbons 15 and 14 to the right.

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ELP	A	B	C	D	E	F
Alkylamide (µg/mL)						
1	0.01	33.87	0.30	42.79	29.28	535.47
2	nil	nil	nil	0.70	nil	nil
3a	0.46	3.97	6.33	4.94	6.26	78.30
3b	0.01	5.90	3.07	3.93	16.22	196.96
4	nil	nil	nil	0.92	nil	nil
5	nil	3.88	0.84	2.78	0.20	273.22
6	0.47	1.12	0.02	1.42	1.46	16.43
7	nil	0.86	0.88	0.88	3.44	45.65
8–9	nil	1.83	3.17	1.48	10.08	118.48
10–11	nil	0.69	1.97	0.79	1.52	15.56
12	nil	nil	0.37	nil	0.02	nil
13	nil	1.51	0.66	2.10	8.24	90.95
14	nil	0.10	0.75	0.52	1.21	3.67
15	nil	0.21	0.02	4.21	0.04	7.33
16	0.01	1.95	0.72	1.8	0.77	0.95

Table 2 Content of alkylamides (µg/mL) **1–14, 16** and of acetylene **15** in the ELPs.

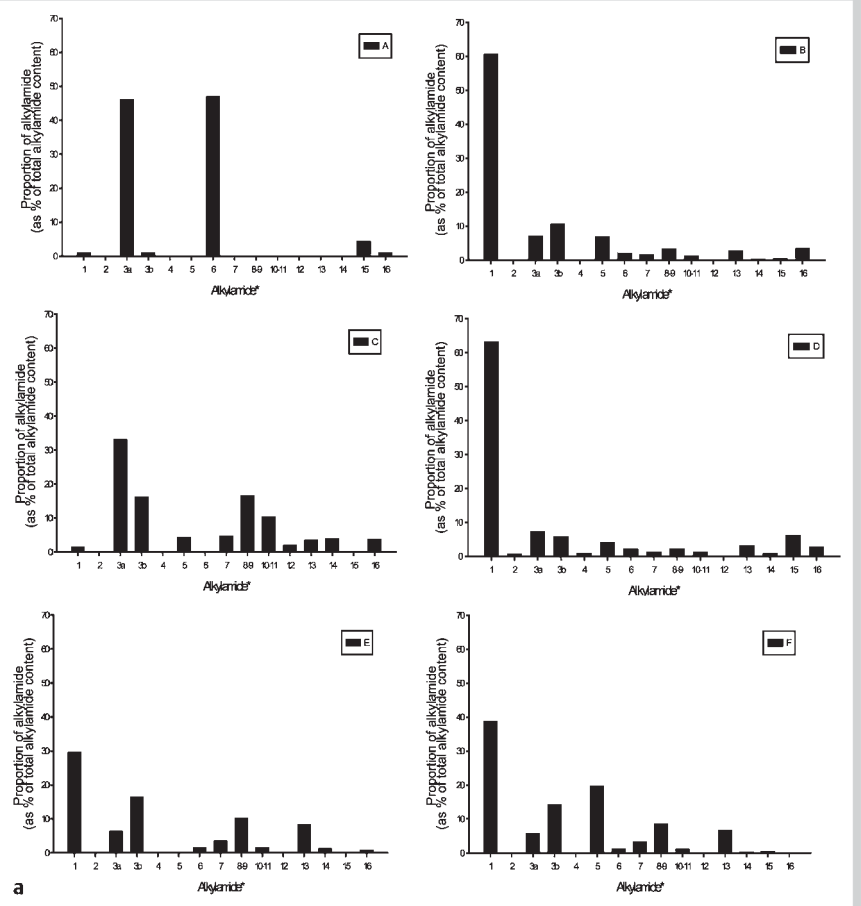

Fig. 4 a Content of the alkylamides **1–14**, **16** and of acetylene **15*** in the ELPs. Results for each alkylamide are reported as a percentage of the total alkylamide content in the respective preparation.

ously described natural product [13]. A reasonable prediction of the second double bond's position can be made, though the stereochemistry is unclear. As seen in **Fig. 2Sb** (Supporting Information), there are two overlapping series of peaks, one beginning at 219 and another at 179, the difference between subsequent fragments in both series is always 14 due to continuous loss of ethylene units. The second series begins at 179, suggesting that there is a choice between losing an ethyne group (CH≡CH) producing the 179 fragment or an ethylene group (CH_2=CH_2) producing the 177 fragment. This leads to assignment of the double bond to carbon 14 (▶ **Fig. 3**). ▶ **Fig. 3 e** shows the structures of all the major peaks.

Although the structure requires final verification by NMR, it is clear that this compound has not been previously identified in *Echinacea*. The presence of this compound in all 3 inhibitory fractions, its structural similarity to alkylamides and its aliphatic character, make it a strong candidate for one of the *Echinacea*'s CYP3A4 inhibiting components.

Given the apparent importance of alkylamides in CYP3A4 inhibition, we characterised the alkylamide content of all six ELPs in more detail to investigate the correlation between individual alkylamides and the inhibitory activity (▶ **Table 2** and **Fig. 4**). Com-

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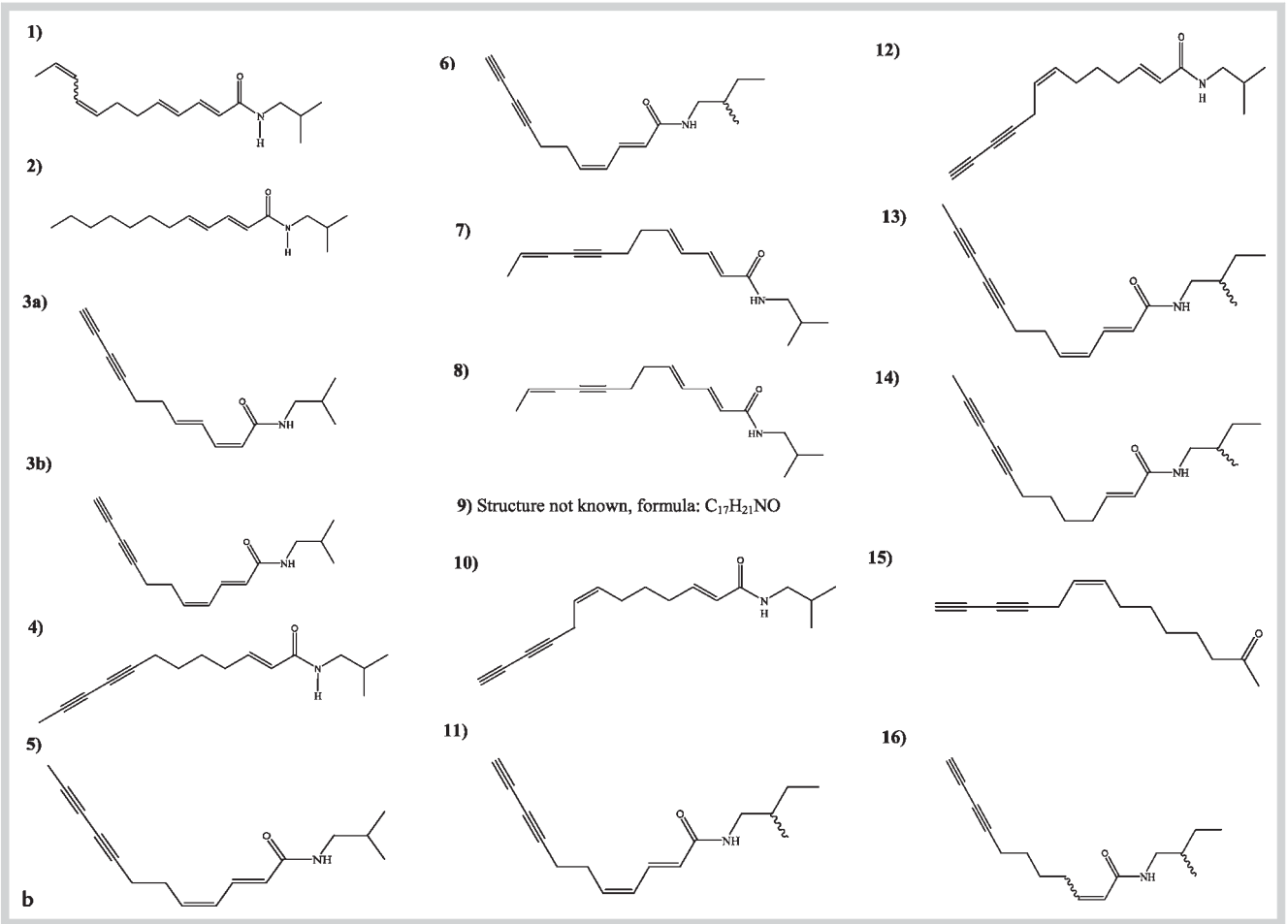

Fig. 4 b Structures of the alkylamides (and compound **15**) detected by HPLC: Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (**1**), dodeca-2*E*,4*E*-dienoic acid isobutylamide (**2**), undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (**3a**), undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide (**3b**), dodeca-2-ene-8,10-diynoic acid isobutylamide (**4**), dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (**5**), undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2 methylbutylamide (**6**), *E*/*Z* forms of dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide (**7**), *E*/*Z* forms of dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (**8**), unknown structure – formula is $C_{17}H_{21}NO$ (**9**), *E*/*Z* isomer mixture of trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide (**10**), *E*/*Z* isomer mixture of trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide (**11**), trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide (**12**), dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (**13**), dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide (**14**), tetradeca-8*Z*-ene-11,13-diyn-2-one (**15**) and undeca-2*E*/*Z*-ene-8,10-diynoic acid 2-methylbutylamide (**16**).

pound **15**, which is an acetylene and not an alkylamide was also determined.

It is immediately apparent from the data in **Fig. 4** and **Table 2** that alkylamides **1** and **3** (i.e., **3a** and **3b**) are the major alkylamide components and the greatest source of variability in alkylamide content. All active extracts have significant amounts of either alkylamide **1** or **3** or both. Other alkylamides do not seem to be consistently present in active extracts and consistently absent from inactive ones. Regression calculations verify these results (**Fig. 4S**, Supporting Information). Only alkylamides **1** and **3**, individually have correlation coefficients (R^2) greater than 0.8 with CYP3A4 IC_{50}. In addition a combination of these two alkylamides behaves in a remarkably similar way to the total alkylamide content. This is not observed with other combinations such as alkylamides **1** and **5** (**Fig. 4S**, Supporting Information).

It appears therefore that alkylamides **1**, and **3** (i.e., the isomeric alkylamides **3a** and **3b**) are better predictors of inhibitory potency than the other alkylamides. It is interesting to note that alkylamide **1** is overall the most abundant alkylamide, while alkylamide **3** is the most potent of those tested.

The detailed HPLC analysis of the ELPs also produced some unexpected results. ELP C was found to contain alkylamides, which was surprising because *E. pallida* roots are not known to contain alkylamides [14]. Furthermore, the acetylene compound **15** was more abundant in the *E. purpurea* preparations (B, D, F) than in ELP C, despite the fact that acetylenes were believed to be more prevalent in *E. pallida*.

Previously we had shown that the inhibitory activity of *Echinacea* covaried with alkylamide content, suggesting that alkylamides may be important inhibitory constituents. The NMR-based, metabolomic analysis presented here confirmed the importance of alkylamides: The first principal component from both the PCA could successfully separate the ethanol fractions of the extracts in the order of inhibitory potency, strongly suggesting that inhibitory potency is indeed related to the major NMR spectral differences. The PLS analysis confirmed the relationship between the spectral differences and IC_{50}. It also showed that the spectral differences are strongly associated with differences in alkylamide content. In addition the regions contributing to PC1 were consistent with alkylamides as evidenced by the spectra presented for

alkylamides **1** and **2**. Positive proof of the central role of alkylamides was provided by isolating highly active fractions of Echinaforce® and showing that their major constituent is indeed an alkylamide. Additionally, a compound, related to alkylamides was identified in an active Echinaforce® fraction. It is conceivable that this and similar compounds may be responsible for the activity found in the water fraction of *Echinacea* as their structure gives them greater water solubility.

Overall, it was possible to correlate differences in inhibitory activity on CYP3A4 with alkylamide content offering opportunities for novel approaches in quality control of ELPs and in screening herbal medical products for CYP inhibition [15]. In particular it appears that alkylamides **1** and **3** may be useful in this respect, especially for the *E. purpurea* extracts.

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