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HPLC Method Validated for the Simultaneous Analysis of Cichoric Acid and Alkamides in *Echinacea purpurea* Plants and Products

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A reversed-phase high-performance liquid chromatography (HPLC) method has been developed to determine caffeic acid derivatives, for example, cichoric acid, and alkamides in plant parts and herbal products of *Echinacea purpurea*. The method consists of an extraction procedure whereby the hydrophilic phenolics as well as the lipophilic alkamides are released from the samples, followed by the analytical HPLC procedure for quantitative determination of these compounds. The method is the first one validated for the determination of these two groups of compounds in the same procedure. Naringenin has been used as an internal standard, as no other flavanones are present in the extract and it does not interfere with any of the compounds under investigation. Analysis of Danish-grown plant material shows that it is possible to raise plants of a very high chemical quality in Denmark. A selection of international herbal products available on the Danish market show surprisingly variable quality, not necessarily reflecting the product information given on the labels.

KEYWORDS: ***Echinacea purpurea*; Asteraceae; cichoric acid; caffeoyltartaric acid; alkamides; alkyl amides; HPLC quantification; validation; herbal medicine**

1. INTRODUCTION

Herbal remedies composed of extracts of *Echinacea purpurea* have become extremely popular in North America as well as in Europe, with obscure but increasing sales statistics. The products are used to treat the common cold (*1*−*4*), and a great number of investigations have proved the immunostimulating effectiveness of *Echinacea* preparations (*2*, *5*−*10*) as well as the antioxidant activity of extracts (*11*, *12*). Besides, they have been shown to be effective in wound healing (*13*) and against photodamage of the skin (*14*). The safe use of *Echinacea* products has been confirmed in several studies (e.g., ref *15*) and reviewed in the Cochrane report (*4*). The major active compounds in the *Echinacea* species are alkamides and caffeic acid derivatives, that is, cichoric acid or echinacoside (**Figure 1**), together with polysaccharides (*16*). Although the safe use of *Echinacea* was recently confirmed in a randomized, controlled trial, no benefit from *Echinacea* to students having the common cold could be verified (*17*).

Analytical investigation of *Echinacea* plants and products was pioneered by the important work of Rudolf Bauer and coworkers (*16*, *18*−*23*) and has recently been taken much further, with regard to the evaluation of herbal products (*24*, *25*) as well as the phytochemical variation within the genus (*26*−*29*). However, there is still a need for a rapid, reliable analytical method for plant material and herbal products, as was also concluded in the recently released review from the Cochrane Library (*4*), comprising 16 trials with *Echinacea* products, stating that the severe lack of chemically well-defined preparations made such trials difficult to compare. The pharmaceutical preparations of *Echinacea* are primarily based on extracts of the subterranean parts (root and rootstock), extract of or juice from aerial parts (leaves, stem, and flowerheads), or a mixture of these. The presence and preservation of alkamides and cichoric acid in recommended therapeutic amounts in the pharmaceuticals depend on the quality of plant material and preparation procedures (*30*, *31*) and on the formulation and storage conditions (*32*). A first step toward more standardized products is the access to a validated analytical procedure, and on this background we have developed the present method for the extraction and quantification of two of the main groups of active constituents in preparations containing parts or extracts of *Echinacea* species.

So far, alkamides and caffeic acid derivatives have mainly been quantified by means of two different HPLC systems, in general, one method for the hydrophilic compounds using cichoric acid as standard (*18*, *19*, *22*, *33*, *34*) and another one for lipophilic compounds using a mixture of two alkamide isomers as standards (*20*, *26*, *35*, *36*). In more recent developments a single extraction procedure has been applied followed by two HPLC methods as outlined above (*25*, *28*, *29*, *31*). The method by Laasonen et al. (*37*) used a single extraction and a single HPLC method for the simultaneous determination of both

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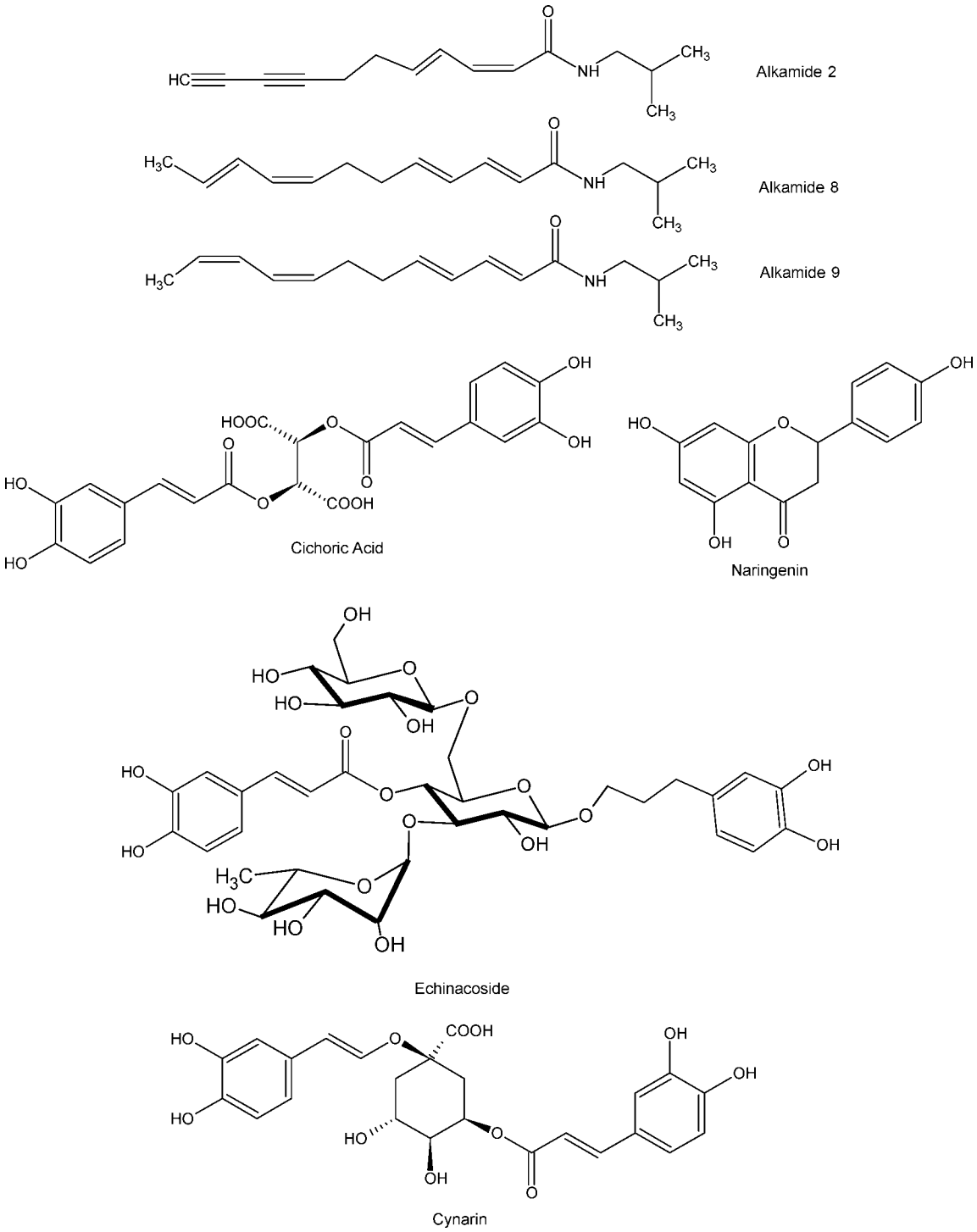


Figure 1. Structures of some of the alkamides and caffeic acid derivatives present in *Echinacea* spp. plants and products together with naringenin used as internal standard in the analyses.

groups of compounds; however, their method was for qualitative and not quantitative determination. Most recently, two qualitative HPLC methods and one capillary electrophoretic method have been developed using one extraction and one elution (*38*−*40*). However, none of these methods make use of internal standards; the extraction procedure (*39*) is still not justified and the concentration of alkamides only relative. The method by Luo et al. (*40*) requires very expensive equipment, which may not be standard in many analytical laboratories. To meet the demand for a time-saving, general purpose method, we have developed, and thoroughly validated, a combined extraction procedure and HPLC method using pure compounds as standards. Besides Danish-grown plant material, a selection of international *Echinacea* preparations available on the Danish market have been analyzed for the content of cichoric acid and alkamides.

2. EXPERIMENTAL PROCEDURES

2.1. Samples under Investigation. *2.1.1. Plant Material.* Fresh plant material of *E. purpurea* (L.) Moench cv. Magnus was harvested weekly during the flowering period in 2001. The plants were grown on heavy clay soil in eastern Zealand in Denmark. They were propagated from seeds in a greenhouse and planted 2 years before harvest. At harvest

Table 1. Preparations with Content of *E. purpurea* Included in This Investigation

no.[a]	producer	name of preparation	declaration
		Tinctures	
1	Vogel/BioForce	Echinamin5	*E. purpurea* 38 mg/g, *Equisetum arvense* 25 mg/g, *Achillea millefolium* 7 mg/g
2	Vogel	EchinaMild	*E. purpurea* herba, flos
3	Vogel	EchinaForce	*E. purpurea*, herba 72 mg/mL, root 4 mg/mL
4	Tjellesen	Echina	*E. pallida/angustifolia*
5	Tjellesen	Echina + timian	*E. pallida/angustifolia*, *Thymus vulgaris*
6	BIOSYM/MediHerb	Optima 2	*Uncaria tomentosa*, *E. angustifolia/purpurea*, *Eleutherococcus senticosus*
7	Mezina	Biotox	*E. purpurea* flos 40 mL/100 mL, root 30 mL/100 mL, propolis 30 mL/100 mL
8	Natures Sunshine	Super Solhatt[b]	*E. purpurea/angustifolia/pallida* root extract
9	Madaus/Tjellesen	EchinaGard[d]	*E. purpurea* dried press juice from herba, 800 mg/mL
10	Winther Medico	Alsøgaard	*E. purpurea* L. herba
11	Winther Medico	Echinacea	*E. purpurea* extract
12	Winther Medico	Echinacea and thyme	*E. purpurea* extract and *Thymus vulgaris* extract
13	BIOSYM/MediHerb	Optima Echinaform	*E. purpurea* 500 mg/mL
		Tablets	
14	Eurovita Scand.	IMIMAX	*Parthenium integrifolium* extract 18.3%, *E. purpurea* extract 6.1%
15	The Herbalist[c]	Echinacea	none
16	Mezina	Echina Max	*E. purpurea* herba, 250 mg of press juice powder, minimum 3% cichoric acid
17	Solaray	Echinacea	*E. angustifolia*: root extract 125 mg, root powder 43 mg, >4% echinacoside
18	Solaray	Echinacea	*E. purpurea* extract of herba 380 mg
19	Madaus/Tjellesen	EchinaGard[d]	*E. purpurea* herb 88.5 mg of dried press juice per tablet

[a] Numbers in bold (**1**, **3**, **9**, **11**, **19**) highlight products approved as herbal medicine by the Danish Food and Drug Administration. [b] Manufactured in Norway. [c] Zimbabwean product purchased in Harare. [d] Has been withdrawn from the market.

time the plants were in full bloom on the main stems. They were dug up and dried instantly after separation into five parts: root, rootstock, stem, leaves, and inflorescence.

2.1.2. Commercial Products. Most of the *Echinacea* products under investigation were purchased in Danish shops and are representative for the Danish market; however, a number of them are internationally available. They are listed in **Table 1** along with comments from the labels regarding the producer, content of plant part, and procedure for manufacturing. The majority of the products are categorized as food supplements, and Danish authorities have approved only a small number as herbal medicines. One product (no. 16) is still under development for approval and is not yet commercially available. One of the samples (no. 15) has been obtained in a health shop in Harare in Zimbabwe (**Table 1**). Most products are liquid, either fresh plant press juice or extracts in a standardized alcoholic tincture.

2.1.3. Chemicals and Reagents. The solvents used for chromatography were MeCN of HPLC-UV−vis quality from Scanlab (code 2503) and water purified locally on Milli-Q equipment. For pH adjustment of the eluent was used trifluoroacetic acid (TFA) of reagent quality (GC: 99%, Fluka). For the extraction of commercial products was used MeOH of HPLC quality (code 2517).

2.1.4. Standards. Cichoric acid was purified from dried fresh plant press juice (Paninkret, Sonnenberg, Westerhorn, Germany) using a preparative HPLC column Supelco Discovery C18, 5 μm (250 mm × 20.1 i.d.). The purity was determined to 98% by HPLC and confirmed by NMR. Alkamides **2** and **8**/**9** were purified from a concentrated alkamide mixture on a semiquantitative Knaur Vertex column packed with Nucleosil 100, C18, 5 μm (250 mm × 16 mm i.d.) and determined to 98% purity on HPLC. The internal standard, naringenin, was from Sigma with a declared purity of >95%. The stock solutions were made by weighing the standards before they were dissolved in the liquid containing the internal standard, 0.20 mg/mL naringenin in MeOH/water (70:30).

2.1.5. Equipment. The HPLC analyses have been carried out on Shimadzu equipment: pump LC-10AT; autoinjector SIL-10Axl; column oven CTO-10A; two detectors, an SPD-10A UV−vis detector for the quantitative part and an SPD-M10A detector for the verification of UV spectra for wavelengths of 200−400 nm. Data collection and computation were carried out with Shimadzu Class LC-10 software. Standard graphs and analyses were carried out on a Merck LiChroCART Superspher 100 RP-18 column, 5 μm (125 mm × 4.1 mm i.d.). Examples of chromatograms are shown in **Figures 2** and **3**.

2.2. Sample Preparation. *2.2.1. Plant Material.* The plant parts were analyzed immediately after drying, using 400 mg of dried plant material

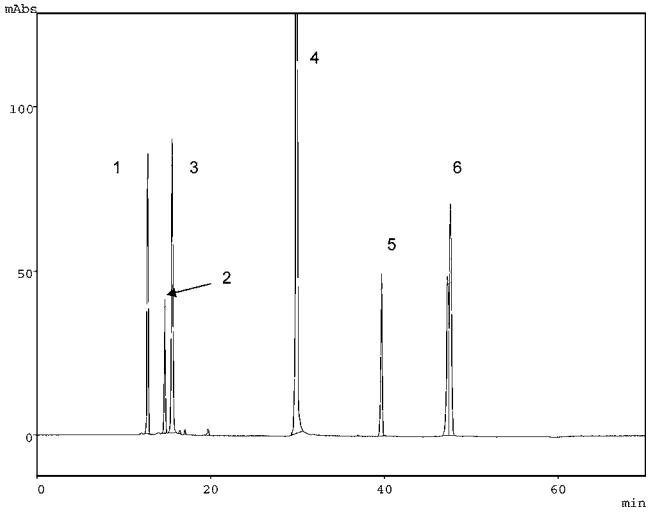


Figure 2. Standard chromatogram of reference compounds and internal standard as described under Experimental Procedures. Peak sequence in the elution is shown on the chromatogram. Peaks: 1, cynarine; 2, echinacoside; 3, cichoric acid; 4, naringenin; 5, alkamide **2**; 6, alkamides **8**/**9**.

of each category for extraction with 10.00 mL of solvent, that is, MeOH/water (70:30), with a known content of the internal standard, naringenin. The extraction was carried out on an ultrasonic bath for 30 min followed by 120 min on a blood turner. This procedure repeatedly gave the highest concentration in comparison with other time combinations (see validation in the Appendix).

2.2.2. Capsules. From each product one capsule (300−400 mg) was extracted as above with 10.00 mL of the extraction medium, that is, MeOH/water (70:30), with known content of the internal standard, naringenin.

2.2.3. Extracts and Tinctures. The liquid preparations were diluted directly 1:1 with the naringenin standard solution. When outside the range of the calibration curve, they were further diluted, and before application to the HPLC system, the samples were centrifuged at 3000 rpm for 5 min or filtered. No products were analyzed later than the date for latest application indicated on the label. All analyses were carried out as triple determinations, and the results are given as means of triplicates.

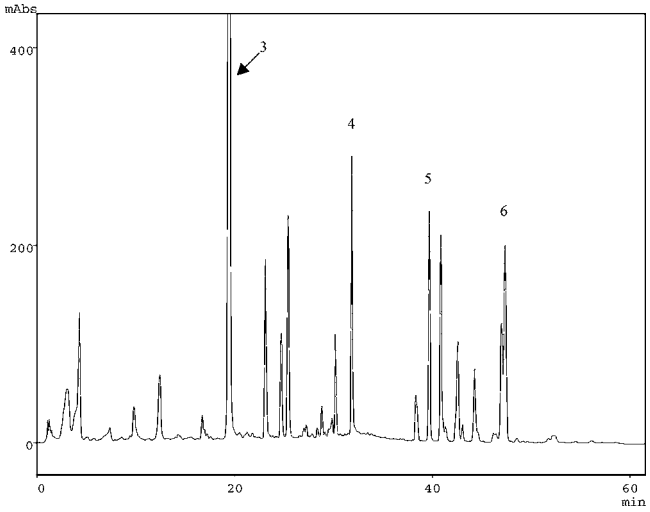


Figure 3. Chromatogram of sample 13 with internal standard as described under Experimental Procedures. Peak sequence in the elution is shown on the chromatogram. Peaks: 3, cichoric acid; 4, naringenin; 5, alkamide **2**; 6, alkamides **8/9**.

Table 2. Gradient Program for HPLC Eluent

time (min)	A (%)	B (%)	C (%)	UV detection (nm)
0.01	75	5	20	290
4.00	75	5	20	
4.00, curve –2[a]				
7.00	68	12	20	
19.00	60	20	20	
19.00, curve –2[a]				
30.00	41	39	20	
30.00, curve –2[a]				
35.00				260[b]
55.00		27	53	20
equilibration	75	5	20	

[a] Convex/concave curves as described in Shimadzu's Class-LC-10 software using the formula $(e^{2(1-t/T)} - 1)/(e^2 - 1)$, where T is time lapse from beginning of curve to end of curve, and time beginning of curve < t < time end of curve. [b] The UV–vis detector shifts the wavelength at 35 min, but the time is dependent on the applied LC system and is for comparison only.

2.3. Statistics. Data were processed and visualized by means of analytical procedures using Graph PAD Prism version 3.0 for Windows, GraphPad Software, San Diego, CA (www.graphpad.com), copyright 1994−1999 by GraphPad Software. Calibration curves and graphs are presented in the figures.

2.4. HPLC Parameters. Standard solutions and extracts of plants and products were chromatographed according to the program in **Table 2** by applying a flow rate of 1.3 mL/min and a column oven temperature of $T = 40$ °C. Injection volumes were 10 μL for standards and 5 μL for extracts. Eluents were A = MeCN/water (5:95), B = MeCN/water (95:5), and C = MeCN/water (5:95) containing 0.1% v/v TFA. The liquids were vacuum filtered, and during elution He was bubbled through the flasks. Chromatograms are shown in **Figure 2** for standards and in **Figures 3** and **6** for analyses.

3. RESULTS

3.1. Plant Analyses. As no significant variation was seen during the growing season, the results are presented as means for the whole period (**Table 3**). Selection of results from a single week would bias the results as any one week would give high results for some of the constituents in one of the plant parts analyzed. However, as seen from the values of standard deviation, there is only little variation during the season. Cichoric acid is high in all parts but the stem, and the rootstock contains a higher content than the root. It is composed of the reddish basal part of the stem and the top of the root, which is normally not harvested on its own but included with the root when the plants are dug up after several years of production of above-ground material. Alkamides are contained only in the root and stem material; however, alkamides **8**/**9** are also contained in the flowerheads.

Table 3. Contents of Cichoric Acid and Alkamides in Dried Material of Danish-Grown *E. purpurea*

plant part	cichoric acid, mg/g	alkamides **8/9**, mg/g	alkamide **2**, mg/g	n[a]
root	24.3 (8.0)[b]	1.20 (1.0)	0.77 (0.31)	30
rootstock	35.7 (9.0)	0.70 (0.4)	0.70 (0.37)	26
stem	9.3 (4.1)	0.35 (0.2)	nd[c]	26
leaves	42.4 (9.1)	nq[d]	nd	30
flowerheads	26.7 (11.4)	0.81 (0.7)	nd	24

[a] Plant material was harvested weekly during the growing season of 2001. [b] Standard deviations (SD) are given in parentheses. [c] nd, not detected. [d] nq, not quantifiable.

Table 4. Contents of Cichoric Acid and Alkamides in Tinctures/Extracts of *E. purpurea*

sample[a]	cichoric acid, μg/mL	alkamides **8/9**, μg/mL	alkamide **2**, μg/mL
1	197	33	nd[b]
2	76	21	nd
3	375	45	11
4	296[c]	22	nd
5	1149[c]	23	nd
6	516	85	40
7	132	51	nq[d]
8	783[c]	73	24
9	nd	nd	nd
10	279	32	nd
11	299	26	nd
12	851	23	nd
13	3891	358	187

[a] Numbers in bold (**1**, **3**, **9**, **11**) highlight products approved as herbal medicine by the Danish Food and Drug Administration. [b] nd, not detected. [c] These products also contain a large amount of echinacoside. [d] nq, not quantifiable.

Table 5. Contents of Cichoric Acid and Alkamides in Capsules of *E. purpurea*

sample[a]	cichoric acid, mg/g	alkamides **8/9**, μg/g	alkamide **2**, μg/g
14	0.700	nd[b]	nd
15	26.200	673	373
16	34.600	nd	nd
17	0.039[c]	572	nd
18	9.100	274	nd
19	nd	nd	nd

[a] Number in bold (**19**) highlights a product approved as herbal medicine by the Danish Food and Drug Administration. [b] nd, not detected. [c] This product contains in addition a large amount of echinacoside, estimated at 30 mg/g.

3.2. Herbal Product Analyses. The products under investigation show a great variation in the content of cichoric acid and alkamides, especially in the capsules and tablets (**Table 5**; **Figures 4** and **5**), whereas tinctures (with the exception of no. 13) are more uniform and generally at a lower content (**Table 4**). As expected, the content of alkamides is low in preparations primarily consisting of above-ground plant parts (herba). In some cases (no. 9, 16, and 19) it is not possible to detect alkamides at all in the chromatogram (**Figure 6**). The only product made

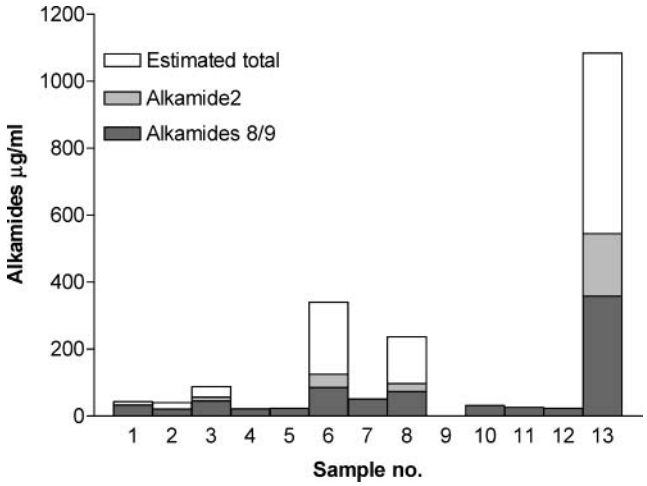


Figure 4. Tinctures of *E. purpurea* analyzed for alkamide content and pattern. Alkamides **2** and **8/9** are determined by the internal standard method, whereas the total is estimated on an average relative content of all alkamides.

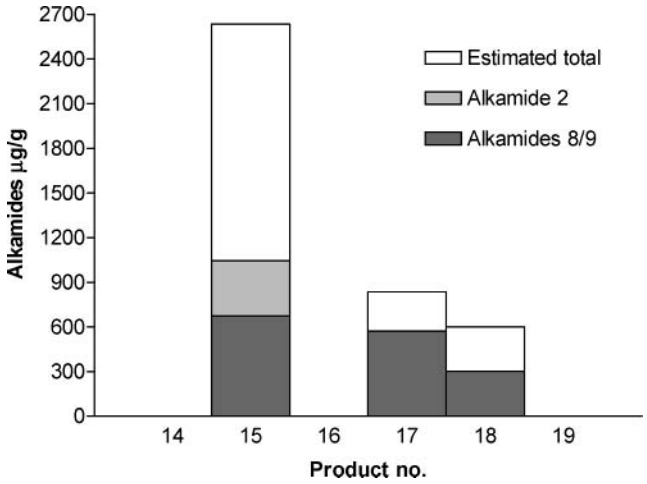


Figure 5. Capsules with *E. purpurea* extract or powder analyzed for alkamide content and pattern. Alkamides **2** and **8/9** are determined by the internal standard method, whereas the total is estimated on an average relative content of all alkamides.

of Danish-grown plants (no. 10) contains both cichoric acid and alkamides, although it is made of above-ground plant parts only and of a different cultivar (Verbesserte Leuchtstern) from the one we have used (Magnus).

It is remarkable that the products for sale on the Danish market are so different in quality, even more than seen for 35 products from the American market (*45*), and no clue to the quality can de deduced from the label. The variation range expands from what should be expected as good quality to what is very close to false or fraudulent products. Five of the products are approved by the Danish Food and Drug Administration and can be marketed as herbal remedies with full advertisement (*41*). However, these five (no. 1, 3, 9, 11, and 19) are no better than the other products on the list.

Two preparations (no. 9 and 19) from the same producer, EchinaGard as tincture and tablet, respectively, did not contain any of the compounds under investigation, although a reasonable content of *E. purpurea* was declared in both cases. EchinaGard has in the meantime been withdrawn from the Danish market. This seems a strange decision as the product has been tested in a double-blind, placebo-controlled trail with good evidence for activity against early symptoms of the common cold (*42*). As

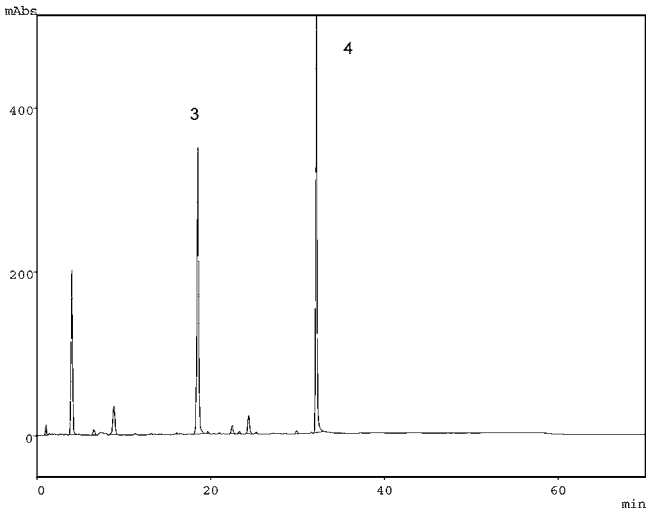


Figure 6. Chromatogram of sample 16 with internal standard as described under Experimental Procedures. Note the missing peaks for alkamides late in the elution. The product contains press juice, which should contain at least small amounts of alkamide **2** (cf. **Table 3**). 3, cichoric acid; 4, naringenin.

our results do not indicate activity of cichoric acid or alkamides, the activity must be ascribed to a content of polysaccharides (*43*).

The two Echina products (no. 4 and 5) contain *Echinacea pallida* and *Echinacea angustifolia*, but not *E. purpurea*, but it is not stated what plant parts have been used. The content of cichoric acid must derive from *E. pallida* herb, which can be distinguished from *E. angustifolia* on the content of cichoric acid in roots but not in the herb (*11*). According to Speroni et al. (*44*), *E. pallida* contains only traces of cichoric acid in the roots but substantial amounts of echinacoside. Both *E. pallida* and *E. angustifolia* contain alkamides **8**/**9**, and these two alkamides are in fact present in the products, but alkamide **2**, which is characteristic for *E. purpurea*, is not. It may be deduced from our investigation that both of the preparations contain above-ground material (herba) from the two plant species, but adulterations with *E. purpurea* cannot be ruled out.

In sample 17 (**Table 5**) cichoric acid was present only in a very small amount (39 μg/g), but a neighboring peak was identified as echinacoside (**Figure 1**); on the basis of one-point calibration, the concentration was estimated to be 30 mg/g, that is, 3%, low in comparison to the declared 4% (**Table 1**). One of the preparations, no. 13 of Australian origin, was exceptional in having a very high content of both alkamides and cichoric acid (3.8 mg/mL) compared to the rest of the samples. The declaration says 500 mg/mL of dried root, which is a very high proportion in the extraction, probably requiring a special technique.

In products in which *Echinacea* extracts are combined with other drugs, there may be difficulties in the determination of specific compounds in the case of cochromatography with constituents from these other drugs. The content of propolis in no. 7 makes it difficult to identify the alkamides, as a series of propolis compounds coelute with alkamides in the chromatogram. However, alkamides **8**/**9** could be identified, but not alkamide **2**, although the declaration claims a content of 30% root material, which should lead to the presence of alkamide **2** (cf. **Table 3**). The product information of no. 14 declares 18.3% *Parthenium integrifolium* extract together with 6.1% *E. purpurea* extract. *P. integrifolium* is known as an adulterant to *E. purpurea* (*16*, *45*). However, the cinnamoyl sesquiterpenes, a group of

compounds characteristic for *P. integrifolium*, are easily recognizable from their UV spectra ($\lambda_{max} = 280$ nm). Although in a very low concentration, we have been able to detect as well alkamides as four different cinnamoyl sesquiterpenes in the extract together with small amounts of cichoric acid (**Table 5**).

4. DISCUSSION

4.1. Plant Analyses. The content of cichoric acid and alkamides in roots of *E. purpurea* grown in Denmark may be compared with the results from Europe (*16*, *46*) and from New Zealand (*34*). The content of cichoric acid in the Danish root material (24 mg/g) is very similar to their results of 23 and 17−22 mg/g, respectively, but much higher than the corresponding figures from the United States (*29*), which are between 5 and 8 mg/g. None of these authors give information on the content in the rootstock, where we find the highest concentration of cichoric acid (36 mg/g). The content in leaves, 42 mg/g, is 10 times higher than in leaves from plants grown in central Europe (*46*). The content in inflorescences, 27 mg/g, compares well to other determinations (*29*, *46*), although it is much lower than seen for inflorescences from other *Echinacea* species (*29*).

The content of alkamides in Danish-grown plants is generally lower than in plants grown in New Zealand (*35*, *36*). These have especially high alkamide levels in the vegetative stem, 14 mg/g, where we find only 0.35 mg/g of alkamides **8**/**9** in Danish-grown plants. As the extracts were made immediately after drying of the plant material, decomposition of alkamides can be ruled out. Similarly, the root content of alkamides in Danish-grown plants was low compared to the American figures (*29*). These differences may be due to genetic difference between cultivars and not only to the growth conditions. For alkamides we find the highest values in the roots, whereas for cichoric acid the rootstock gave the highest values. This stresses the importance of discriminating between plant parts for chemical analysis. From our results it should be recommended to select the rootstock for enhancing the cichoric acid content in herbal products. The results are encouraging for cultivation of *E. purpurea* under Danish conditions.

4.2. Herbal Products. The chemical evaluation of these products should not be taken as a recommendation of which product to prefer in the treatment of infections in the respiratory tract. One major group of compounds, the polysaccharides, has not been dealt with, although they are also involved in immunostimulating activity (*6*, *47*) and wound healing (*13*) and are known for their effect on the complement system (*48*). The recommended daily intake must be taken into account, as a wide interval is generally given with the declaration. The present product evaluation has shown the importance of a proper analytical method to quantify the constituents believed to be responsible for the pharmacological activity when assessing the efficacy of *E. purpurea* preparations in clinical trials. We hope that our method will serve as another step toward standardization of such products, knowing that the initiative has been taken to include *Echinacea* in the European Pharmacopoeia (*49*).

The producers are probably not aware of using raw material of poor quality in their products, and for their benefit a reliable analytical method for quality control is important. Genetic variation, the quality of selected cultivars, abiotic and biotic conditions during the growing season, that is, rainfall, nutrition, attack by insects or microorganisms, and of course the treatment of plant material during harvest and storage may influence the quality (*27*−*29*, *45*, *50*). Determination of the stability of active compounds in the dried plant material, in extracts, and in the final products is important. Handling of the plant material is crucial, as cichoric acid is sensitive to heat, UV irradiation, and enzymatic and oxidative decomposition (*30*). We recommend monitoring the content of cichoric acid closely by analyses of the product during processing. The alkamides deteriorate even more rapidly than cichoric acid in dried, powdered material but keep well in alcoholic extractions (*36*, *51*).

4.3. Method Evaluation. The method has proved to be valuable for determining the content of alkamides and cichoric acid in plant parts and preparations of *E. purpurea*. The method has the advantage, compared to previously published methods, that it is possible to determine the content of cichoric acid and the alkamides (**2** and **8**/**9**) in the same analytical procedure. Both the extraction procedure and the HPLC method have been shown to be reliable and robust with a very good reproducibility and repeatability. Especially for the analysis of plant parts and crude drugs the method is fast due to the possibility of only one extraction and one HPLC elution for each sample (**Figures 2** and **3**). In this respect it is an improvement compared to previously known analytical procedures with one method for alkamides and another one for cichoric acid (*18*, *22*, *23*, *26*, *28*, *29*, *34*, *35*, *37*).

The method is selective to parts and products of *E. purpurea* as it can be used for quantitative determination of the characteristic content of cichoric acid and alkamides **2** and **8**/**9** from this species (*16*). The method has been validated according to the guidelines given under the International Committee for Harmonisation (ICH 1994 section Q2B) as documented in the Appendix. As internal standard we have used the flavanone naringenin (the aglycon of naringin) with a retention time between those of cichoric acid and the alkamides. According to the known literature on *E. purpurea* it does not contain flavanones and the UV− spectrum of naringenin differs substantially from that of cichoric acid as well as those of alkamides.

The representatives chosen for the two constituent groups are cichoric acid (2,3-dicaffeoyltartaric acid) for caffeic acid derivatives and both alkamide **2**, that is, undeca-2*Z*,4*E*-dien-8,10-diynisobutylamide and the stereoisomeric alkamides **8**/**9**, that is, dodeca-2*E*,4*E*−8*Z*,10*E*/*Z*-tetraenisobutylamide (*16*), for alkamides. Cichoric acid is the most important caffeic acid derivative present in *E. purpurea*, whereas *E. angustifolia* and *E. pallida* have echinacoside and no cichoric acid (*16*, *29*, *34*). Minor amounts of other derivatives are seen also in our investigations, primarily caftaric acid (in *E. purpurea*), cynarin (in *E. angustifolia*), and chlorogenic acid (*24*, *29*, *34*).

The alkamides have been selected not only because they make up a substantial part of the total alkamide content but also because they are well separated in the developed chromatographic system. Selection of alkamide **2** as representative of the alkamide group is based on the following criteria: Extracts of the below-ground plant parts contain a much more complex mixture of alkamides than the above-ground parts (*20*, *24*, *26*, *29*, *35*). Alkamide **2** has until now been found only in below-ground plant parts (cf. **Table 3**), and it is therefore a very good marker for the presence of any root material in a commercial product. Besides, during the development of this method, we have realized that alkamide **2** has no coeluting compounds, not even at high concentrations of alkamides. This gives a resolution of >1.5, which is desirable under quantitative determination (cf. the general requirements to internationally accepted chromatographic principles). Until now extracts of above-ground plant parts have been shown to contain only alkamides **1**, **3**,

8/**9**, **10**, and **11** (*18*), and of these only alkamides **1** and **8**/**9** (and probably also **11**) may be present in substantial amounts (*29*, *35*).

During the development of this method, comprising also a fast purification procedure for alkamides from the above-ground plant parts, we have realized that the alkamide isomers, **8** and **9**, are closely eluted with two minor alkamides, not yet identified. This leads to a resolution of <1.5, which then influences the selectivity when the peaks for these compounds are integrated. As alkamides **8**/**9** are commonly used for quantification studies due to their presence in all plant parts (*16*, *18*, *35*), this information stresses the need for a better quantitative method. A method with an external standard that more or less coelutes with other components is not acceptable (*24*, *25*, *29*), as it is not selective according to the general chromatographic principles mentioned above.

In conclusion, we recommend cichoric acid and alkamide **2** be used for the evaluation of extracts of below-ground plant parts and cichoric acid and alkamides **8**/**9** be used for above-ground plant parts of *E. purpurea*.

APPENDIX: VALIDATION OF THE HPLC METHOD AND EXTRACTION PROCEDURE

The method presented here was developed for the qualitative and quantitative determination of the content of cichoric acid and alkamides **2** and **8**/**9** in preparations with content of *E. purpurea* press juice and extracts. Validation of the HPLC method has been carried out according to the guidelines given by the International Committee of Harmonisation (ICH) section Q2B (http://www.ich.org/pdfICH/Q2B.pdf).

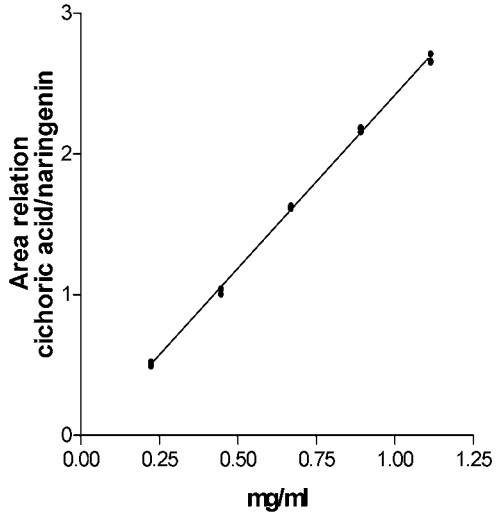
The following criteria were investigated: 1, linearity; 2, specificity; 3, measuring range of the method; 4, accuracy; 5, precision [(a) repeatability, (b) intermediate precision]; 6, detection and quantification limit; and 7, robustness.

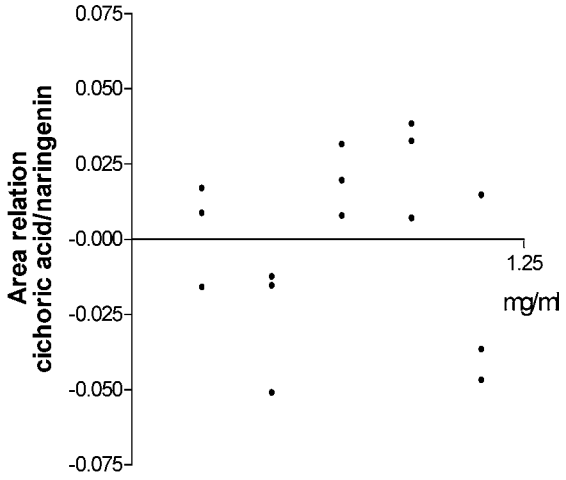
Materials and methods are as described in the main text.

HPLC Method. *Linearity.* Three stock solutions of cichoric acid, alkamide **2**, and alkamides **8**/**9**, respectively, were produced with content of the internal standard, naringenin. Five standard solutions were produced with concentrations within the expected range of concentrations in the material under investigation. The calibration curves were determined using the least-squares method, and for the independent variable (x) the concentration proportion was given as the proportion between the area of the respective peak and the peak of naringenin. For the dependent variable (Y) the detector response was determined as the area proportion using the formula

$$\text{content} = [\text{RF1}(i) \times \text{Ai/Ais} + \text{RF2}(i)] \times \text{Wis/Wspl} \times \text{Dfact}$$

where response factors RF1 are 1.0, 1.3025, 0.5326, and 0.466





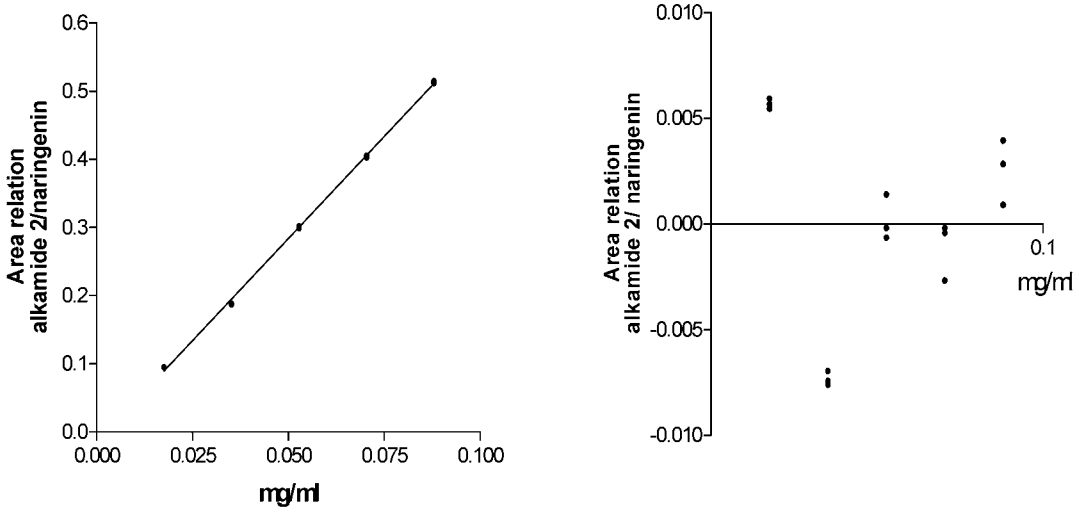
Linearity - regression parameters: Cichoric acid

Variables		95% Confidence intervals/Goodness of Fit		Data	
Slope	2.456	Slope	2.403 to 2.508	Number of X values	5
Y-intercept	-0.0398	Y-intercept	-0.0786 to 0.0011	Max. Number of Y replicates	3
X-intercept	0.0162	r^2	0.9987	Total number of values	15
1/slope	0.4072	Sy.x	0.0296	Number of missing values	0

Residuals: Cichoric acid

Residuals	
Points above line	9
Points below line	6
Number of runs	5
P value (runs test)	0.0629
Significantly nonlinear	Not significant

Figure A1. Calibration curve and residual plot for cichoric acid.



Linearity - regression parameters: Alkamide 2

Variables		95% Confidence intervals/ Goodness of Fit		Data	
Slope	5.993	Slope	5.887 to 6.100	Number of X values	5
Y-intercept	-0.0164	Y-intercept	-0.0226 to –0.0102	Max. Number of Y replicates	3
X-intercept	0.0027	R^2	0.9991	Total number of values	15
1/slope	0.1669	Sy.x	0.0047	Number of missing values	0

Residuals: Alkamide 2

Residuals	
Points above line	7
Points below line	8
Number of runs	5
P value (runs test)	0.0513
Significantly nonlinear	Not Significant

Figure A2. Calibration curve and residual plot for alkamide **2**.

and those for RF2 are 0.0, 0.0471, 0.0088, and 0.0217 for naringenin, cichoric acid, alkamide **2**, and alkamides **8**/**9**, respectively. For simplification, the concentrations shown in the three graphs in this paper are given in milligrams per milliliter. Regression analyses on the three calibration curves were made by GraphPAD Prism 3.0, and the slope, intercept with the *Y*-axis, linear correlation coefficient (r^2), and their confidence intervals were determined.

The calibration curves for cichoric acid, alkamide **2**, and alkamides **8**/**9**, all with naringenin, are shown in **Figures A1**, **A2**, and **A3**, respectively, and the distributions of the respective residuals are shown alongside. The figures show linearity between the concentration proportion and the response proportion, as all of the calibration curves are significantly linear. The correlation coefficients for all curves were >0.9995 and hence fully acceptable (see tables in **Figures A1**, **A2**, and **A3**).

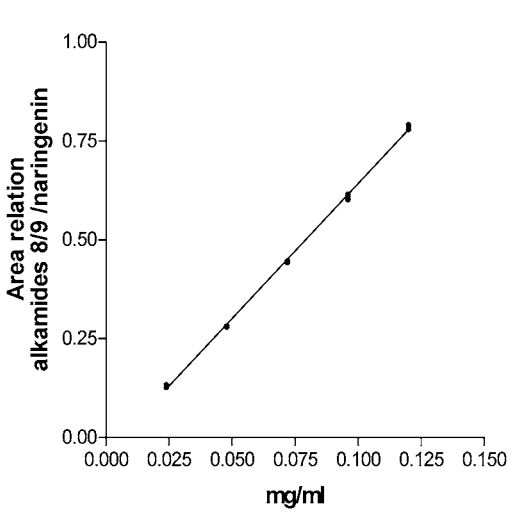
Selectivity. For the selectivity test a diode array detector was applied. The test was carried out on a naringenin standard extract of root and rootstock of *E. purpurea*. The test was carried out using peak purity judgment, which is part of the Shimadzu Class LC10 software for handling 3D chromatographic data. The principle of peak purity judgment is to obtain three spectra, one at 50% up slope, one at the apex, and one at 66% down slope of individual peaks. These three spectra are compared, aiming for a similarity index as close to 1.0000 as possible. In this case they were all between 0.999 and 1.000 for cichoric acid, alkamide **2**, and alkamides **8**/**9**.

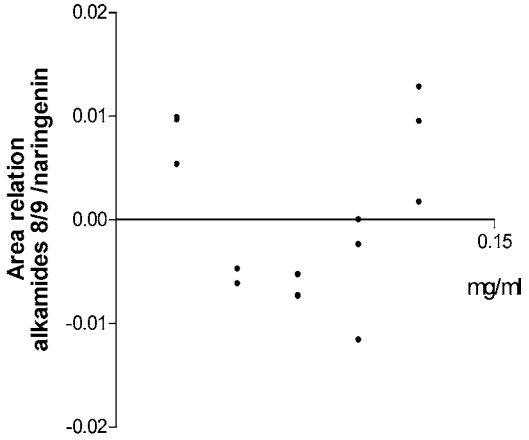
It was shown that also extracts gave high index values (0.995−0.999), indicating that the method is selective for cichoric acid as well as alkamides **2** and **8**/**9**. It should be noted that cichoric acid in this LC system is separated from an isomer eluting shortly after. In the same way alkamide **2** is completely separated from closely eluting alkamides (alkamides **1A**, **1B**, and **3**). The selectivity is shown on a chromatogram from one of the commercial preparations with a declared content of root material of *E. purpurea* (**Figure 3**).

Recovery. Three different concentrations diluted from the stock solution were added to an extract with a known content of cichoric acid, alkamide **2**, and alkamides **8**/**9**, and the recovery of, respectively, cichoric acid, alkamide **2**, and alkamides **8**/**9** was calculated. Relative standard deviation (RSD) values were determined as the difference between measured and expected values. The results of the tests were acceptable, as the recovery was very high for cichoric acid and alkamides **8**/**9**, 95 and 98%, respectively, and >80% for alkamide **2** (**Table A2**).

Precision. The precision (repeatability) is a test for the distribution of the concentrations measured. Three different concentrations of the stock solutions were exposed to triple determination. As shown in **Table A3** all RSD values are below 2%, which is considered to be acceptable.

Repeatability and Intermediate Precision. Another analyst, using the same equipment, carried out this test on a different





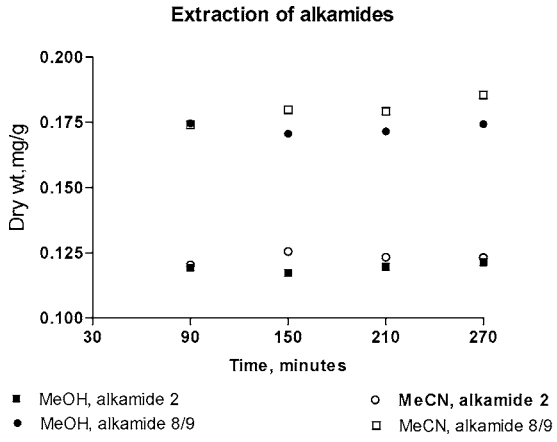
Linearity – regression parameters: Alkamides 8/9

Variables		95% Confidence intervals/ Goodness of Fit		Data	
Slope	6.821	Slope	6.690 to 6.953	Number of X values	5
Y-intercept	-0.0407	Y-intercept	-0.0512 to –0.0302	Max. Number of Y replicates	3
X-intercept	0.0060	R^2	0.9990	Total number of values	15
1/slope	0.1466	Sy.x	0.0080	Number of missing values	0

Residuals: Alkamides 8/9

Residuals	
Points above line	7
Points below line	8
Number of runs	5
P value (runs test)	0.0513
Significantly nonlinear	Not Significant

Figure A3. Calibration curve and residual plot for alkamides **8/9**.



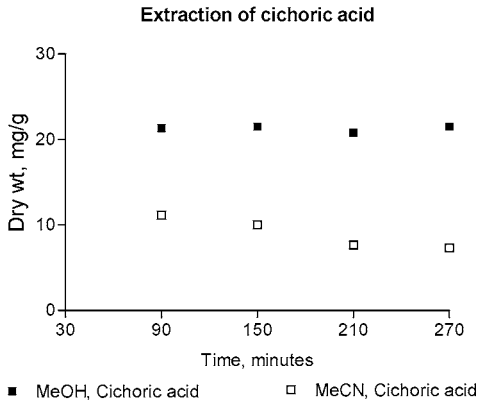


Figure A4. Concentration of alkamides (left) and cichoric acid (right) in response to extraction time and media, for example, MeOH compared to MeCN.

day on three different samples run in triplicate. The results are presented in **Table A3**. As shown from the figures almost all RSD values are below 1%, and the results are considered to be acceptable.

Robustness. Different flow rates (1.0, 1.15, and 1.3 mL/min) and column oven temperatures (35, 37, and 40 °C) were used to perform this test. From previous experience it was known that the eluent pH should be <2.5. A root extract with known content of cichoric acid, alkamide **2**, and alkamides **8**/**9** was used for the test. The deviations are calculated on the basis of peak areas with parameters 1.3 mL/min and 40 °C as reference values, that is, the lower right corner in **Table A4**. Mean deviation is <5% and should not be problematic.

Limits of Detection/Quantification (LOD/LOQ). Using the linearity curve (**Figure A1**) for determining LOD and LOQ, the following equations were applied: LOD = $3S_{y,x}/b$ and LOQ = $10S_{y,x}/b$, where $S_{y,x}$ is the standard deviation of the *Y*-value distribution around the regression line and *b* is the slope of the calibration curve.

Table A1. Selectivity of the Method for Determination of Cichoric Acid, Alkamide **2**, and Alkamides **8/9** with Naringenin as Internal Standard

compound	similarity index	separation factor	resolution
naringenin	0.9999	1.05	5.70
cichoric acid	0.9997	1.11	2.85
alkamide **2**	0.9979	1.04	3.96
alkamides **8/9**	0.9994	1.04/1.01	*a*

[a] The peaks for two unknown, neighboring isomers are too narrow to determine the resolution.

Table A2. Recovery of Cichoric Acid, Alkamide **2**, and Alkamides **8/9** after Addition of Known Amounts to Standard Extracts of *E. purpurea*

content, μg, in 500 μL	content, g, in added standard solution (50 μL)	expected concn, μg/mL	determined concn, μg/mL	recovery, %
		Cichoric Acid		
184.6	22.3	207	201	95.8
184.6	33.45	218	204	93.6
184.6	44.6	229	218	95.1
		Alkamide **2**		
2.35	1.76	4.11	3.3	80.3
2.35	2.64	4.99	4.0	80.2
2.35	3.52	5.87	4.9	83.5
		Alkamides **8/9**		
20.8	2.4	23.2	22.9	99.0
20.8	3.6	24.4	23.6	96.7
20.8	4.8	25.6	25.2	98.4

Table A3. Repeatability and Intermediate Precision[a] Measured by the Concentrations of Cichoric Acid, Alkamide **2**, and Alkamides **8/9** in Standard Extracts of *E. purpurea*

repeatability			intermediate precision		
content, mg/mL	SD	RSD %	content, mg/mL	SD	RSD %
		Cichoric Acid			
0.44	0.00075	0.17	1.40		
0.66	0.00601	0.90	1.38	0.021	1.52
0.89	0.01020	1.15	1.37		
		Alkamide **2**			
0.035	0.00006	0.17	0.093		
0.053	0.00015	0.29	0.096	0.0026	2.67
0.070	0.00023	0.33	0.098		
		Alkamides **8/9**			
0.048	0.00031	0.57	0.207		
0.072	0.00012	0.22	0.215	0.0086	4.0
0.096	0.00093	0.91	0.225		

[a] Different days, same equipment and location.

From these equations we determined LODs for cichoric acid, alkamide **2**, and alkamides **8**/**9** to be 0.011, 0.000074, and 0.0011 mg/mL, respectively, and LOQs were in the same way determined as 0.038, 0.00025, and 0.0037 mg/mL, respectively.

Extraction Procedure. The extraction procedure was carried out in triplicate using methanol/water (70:30) and compared to acetonitrile.water (50:50), in both cases with naringenin added as internal standard. From previous experiments we knew that MeCN was most efficient with the alkamides. **Figure A4** shows the time dependency of both extraction media, and **Table A5** shows the standard deviation for each of the entries. As we have used an extraction time of 150 min, we have obtained a full extraction, and the standard deviations for triplicate analyses are within an acceptable limit.

Table A4. Robustness of the Method, As Measured by the Relative Peak Area (Index Values)[a] for Cichoric Acid, Alkamide **2**, and Alkamides **8/9** in Standard Extracts of *E. purpurea*, by Using Different Flow Rates and Column Oven Temperatures

	column oven temp		
flow, mL/min	35 °C	37 °C	40 °C
	Cichoric Acid		
1.00	1.01	0.98	1.06
1.15	1.06	1.02	1.04
1.30	1.01	1.08	1.00
	Alkamide **2**		
1.00	1.08	1.02	1.06
1.15	1.08	1.04	1.04
1.30	1.01	1.08	1.00
	Alkamides **8/9**		
1.00	1.07	1.01	1.07
1.15	1.06	1.03	1.05
1.30	0.96	1.07	1.00[b]

[a] Index values corrected for peak area variation due to variation in flow. [b] Flow of 1.3 mL/min used as reference.

Table A5. Standard Deviation on Triple Determination of Cichoric Acid and Alkamides in Extracts of *Echinacea* Powder

cxtraction time, min	cichoric acid	alkamide **2**	alkamides **8/9**
90	8.83	2.76	3.49
150	1.34	1.19	2.23
210	2.51	1.09	5.01
270	2.68	1.40	1.84

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