

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Turner RB, Bauer R, Woelkart K, Hulsey TC, Gangemi JD. An evaluation of *Echinacea angustifolia* in experimental rhinovirus infections. *N Engl J Med* 2005;353:341-8.

SUPPORTING ONLINE MATERIAL

Biologic effects of the different Echinacea constituents

The pharmacologic and chemical properties of Echinacea have been extensively reviewed by Bauer¹. Unfortunately, much confusion still exists concerning the pharmacologic properties of this botanical due to inadequate standardization and the presence of contaminants in most preparations (e.g. presence of mixed Echinacea species and adulteration with *Parthenium integrifolium*). The constituents of Echinacea cover a wide range of polarity from the polar polysaccharides and glycoproteins, to the moderately polar caffeic acid derivatives and to the rather lipophilic polyacetylenes and alkamides. Two caffeic acid derivatives, echinacoside and cichoric acid are used as markers for the specific identification of Echinacea species of medicinal value. Because of their relative abundance and reported biological activity, all four types of constituents have been proposed as relevant marker compounds for the standardization of Echinacea products¹. The glycoproteins, polysaccharides, caffeic acid derivatives (cichoric acid) and alkamides have all been reported to have immunostimulatory activity²⁻⁴. In contrast, the alkamides of *E. angustifolia* and *E. purpurea* exhibit anti-inflammatory properties⁵. Thus, several substances found in Echinacea could produce a beneficial effect on common cold symptoms either by direct inhibition of virus replication or by modulation (i.e. enhancement or suppression) of the host immune response.

Antiviral activity. Echinacoside and cichoric acid exhibit a direct antiviral effect against vesicular stomatitis virus⁶. Whether the virus suppression is due to the induction of interferon or to a direct antiviral effect is not known. In addition, cichoric acid has been

shown to inhibit HIV integrase activity⁷⁻⁹. Some glycoproteins also demonstrate an antiviral effect¹⁰. The direct antiviral activity of these Echinacea constituents in rhinovirus infections has not been reported.

Immunostimulatory activity. A variety of immunostimulatory activities have been attributed to different constituents of Echinacea. Luettig et al.³ found that stimulation of macrophages with polysaccharides from *E. purpurea* resulted in production of TNF- α and β 2-interferon (IL-6). Other Echinacea preparations activate natural killer cells and macrophages in vitro^{2, 11} resulting in the elaboration of a variety of lymphocyte and macrophage-associated cytokines¹². Treatment with Echinacea polysaccharides is associated with activation of PMNs, elaboration of inflammatory cytokines from monocytes and production of acute phase reactants. Mice treated with these polysaccharides exhibit increased resistance to *C. albicans* and *L. monocytogenes*¹³⁻¹⁵.

Antiinflammatory activity. In contrast to the studies demonstrating pro-inflammatory activity (immune enhancement), other studies have found that the Echinacea polysaccharides have anti-inflammatory effects^{16, 17}. The alkamides exert an anti-inflammatory effect by inhibiting the liberation of prostaglandins and leukotrienes⁵. An alternative mechanism for immune modulation is suggested by recent observations that these alkamides are similar to anandamide (an endocannabinoid) and exhibit selectivity for CB2 receptors found on mouse spleen membranes (authors' unpublished observations). This observation suggests the possibility that, when present in the upper

respiratory track, alkamides from Echinacea preparations may act as CB ligands to exhibit immunomodulatory potential¹⁸.

Phytochemical Characterization of the Echinacea angustifolia extracts used in this study

Reagents: The following chemicals and reagents were used: acetonitrile (HPLC grade, ROTISOLV[®]) and formic acid (ROTIPURAN[®]) from Carl Roth GmbH + Co, Karlsruhe, Germany. A Barnstead[®] (EASY pure RF) compact ultrapure water purification system was used to obtain the purified water for the HPLC analysis.

Plant material: Freshly harvested roots from two-year-old plants of *E. angustifolia* were obtained from Heilpflanzen SANDFORT GmbH & Co KG, Olfen, Germany. The plant material was identified at the Institute of Pharmaceutical Sciences, Department of Pharmacognosy, Karl-Franzens-University Graz. A voucher specimen (No. 2030817) is deposited at this Department of Pharmacognosy. The roots have been extracted with 1) 60 % ethanol, 2) 20 % ethanol and 3) supercritical CO₂ (drug extract ratio 77:1, yield 1.30 %) by Finzelberg, Andernach, Germany.

ANALYSIS OF ALKAMIDES: HPLC analysis was carried out using a Thermo Finnigan Surveyor liquid chromatograph and a ZORBAX[®] 150 mm x 2.1 mm, 3.5 µm RP-18 Narrow Bore column (Agilent Technologies, Waldbronn, Germany). The column was protected by a OPTI-GUARD[®] C₁₈ 1 mm guard column (OPTIMIZE Technologies, INC). A gradient elution with acetonitrile + 0.1 % formic acid and water + 0.1 % formic acid 40/60 (v/v) to 80/20 (v/v) in 30 min was carried out at a flow rate of 300 µl/min. The

PDA detector monitored the eluent at 260 nm. The injection volume was 3 μ l for the 60 % ethanol and CO₂ extract and for the 20 % ethanol extract 5 μ l. Separations were performed at room temperature. The separation was then detected by MS using a Finnigan LCQ Deca XP Plus ion trap mass spectrometer equipped with an electrospray ionization (ESI) source run by XCALIBUR software. Operating conditions for the ESI source, used in the positive ionization mode, were optimized by constantly injecting dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamide in methanol (0.10 mg/ml) by a syringe pump in the infusion mode. The signal was optimized on the total ion current in MS mode, leading to a transfer capillary temperature of 350°C, a spray voltage of 5.00 kV, and a sheath gas flow of 70 units (units refer to arbitrary values set by the Excalibur software). At the same time, the selection of ions and the collision voltages were optimized using Xcalibur software.

Preparation of stock solutions and calibration curves: The main alkamides were isolated by semipreparative HPLC using a MERCK HITACHI L-6200A Intelligent Pump, MERCK HITACHI L-4500 Diode Array Detector with UV detection at 254 nm and fitted with a LiChroCART[®] 250-10, 10 μ m RP-18 LiChrospher[®] 100 column. The compounds were eluted with a gradient from 60/40 to 90/10 acetonitrile/water in 40 min (2.0 ml/min). They were identified by LC-MS and comparison with published data ^{1,19}

Primary stock solution of the six alkamides with different concentrations were prepared in methanol and stored at -80°C. This primary stock solution was diluted in methanol to produce a concentration of **2** 8.44 ng/ml, **3** 11.78 ng/ml, **4** 5.60 ng/ml, **7** 31.62 ng/ml, **9** 4.45 ng/ml and **11** 5.71 ng/ml.

The correlation coefficient for all calibration curves was above $R^2 = 0.98$, proving repeatability and intermediate precision of the assays over the concentration range. For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the lower limit of quantitation. (see Table 1.)

Table 1. Calibration data of alkamides from the roots of *E. angustifolia*.

Substance	Standard curve	R²	Linear range (ng·10μl⁻¹)
Undeca 2 <i>E</i> / <i>Z</i> -ene-8,10-diynoic acid isobutylamides (2)	$y = 1,939024e+006x$	0.9938	0.008 – 0.422
Dodeca 2 <i>E</i> ,4 <i>Z</i> -diene-8,10-diynoic acid isobutylamide (3)	$y = 716947,5x$	0.9955	0.012 – 0.589
Dodeca 2 <i>E</i> -ene-8,10-diynoic acid isobutylamide (4)	$y = 1,842518e+006x$	0.9952	0.006 – 0.280
Dodeca 2 <i>E</i> ,4 <i>E</i> ,8 <i>Z</i> ,10 <i>E</i> / <i>Z</i> -tetraenoic acid isobutylamides (7)	$y = 630553,5x$	0.9985	0.032 – 1.581
Dodeca 2 <i>E</i> ,4 <i>E</i> ,8 <i>Z</i> -trienoic acid isobutylamide (9)	$y = 347655,33x$	0.9837	0.004 – 0.222
Dodeca 2 <i>E</i> ,4 <i>E</i> -dienoic acid isobutylamide (11)	$y = 164378,717x$	0.9956	0.006 – 0.286

60 % ethanol extract

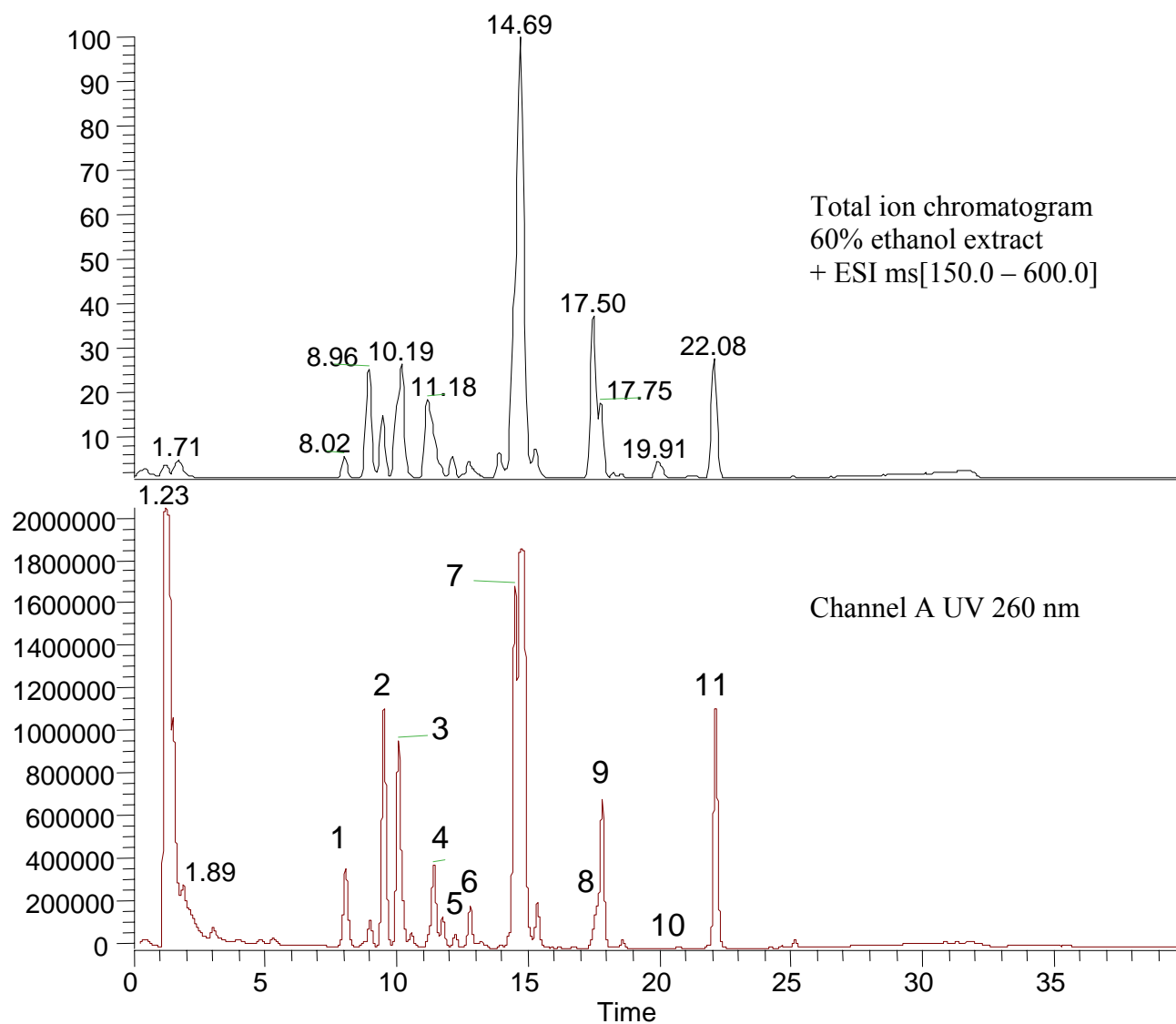
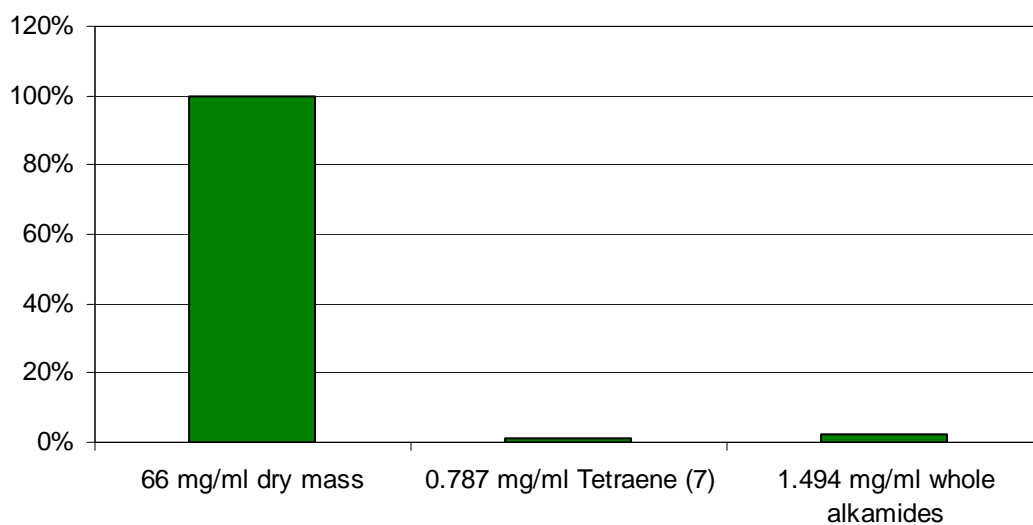


Table 2. Alkamides with Retention time and quantity of the main 6 compounds.

compound	retention time (min)	m/z [M+H⁺]/MS/MS	quantity/ml extract
Undeca 2 <i>E/Z</i> ,4 <i>Z/E</i> -diene-8,10-diynoic acid-isobutylamides (1)	8.02, 9.45	229 230/131	0.120 mg/ml
Undeca 2 <i>E/Z</i> -ene-8,10-diynoic acid-isobutylamides (2)	8.96, 10.19	231 232/133	0.325 mg/ml

Dodeca-2 <i>E</i> ,4 <i>Z</i> -diene-8,10-diynoic acid-isobutylamide (3)	9.93	243 244/145	0.358 mg/ml
Dodeca-2 <i>E</i> -ene-8,10-diynoic acid-isobutylamide (4)	11.18	245 246/147	0.277 mg/ml
Dodeca-2 <i>E</i> ,4 <i>Z</i> -diene-8,10-diynoic acid 2- methylbutylamide (5)	12.10	257 258/145	
Dodeca-2 <i>E</i> -ene-8,10-diynoic acid 2- methylbutylamide (6)	12.67	259 260/147	
Dodeca-2 <i>E</i> ,4 <i>E</i> ,8 <i>Z</i> ,10 <i>E</i> / <i>Z</i> -tetraenoic acid-isobutylamides (7)	14.69, 15.27	247 248/149	0.787 mg/ml
Pentadeca-2 <i>E</i> ,9 <i>Z</i> -diene-12,14-diynoic acid isobutylamide (8)	17.50	285 286/230	
Dodeca-2 <i>E</i> ,4 <i>E</i> ,8 <i>Z</i> -trienoic acid-isobutylamide (9)	17.75	249 250/167	0.191 mg/ml
Pentadeca-2 <i>E</i> ,9 <i>Z</i> -diene-12,14-diynoic acid 2-methylbutylamide (10)	19.91	299 300/185	
Dodeca-2 <i>E</i> ,4 <i>E</i> -dienoic acid-isobutylamide (11)	22.08	251 252/179	0.168 mg/ml

Phytochemical analyses of this 60 % ethanol extract revealed that there are 2.26 % alkamides. The content of the main constituent Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid-isobutylamide (**7**) is 0.787 mg/ml from the whole 1.494 mg/ml alkamides.



20 % ethanol extract

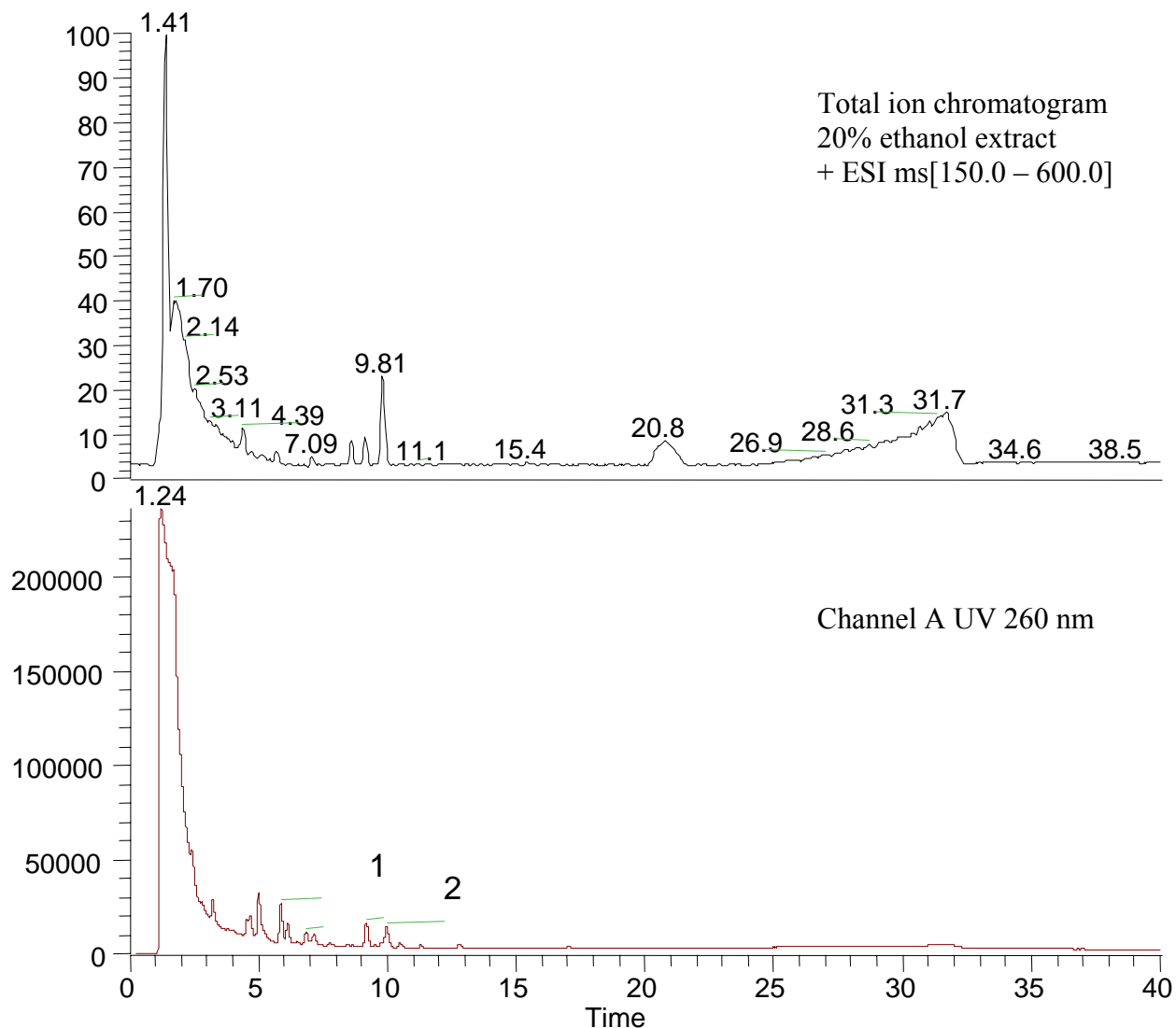
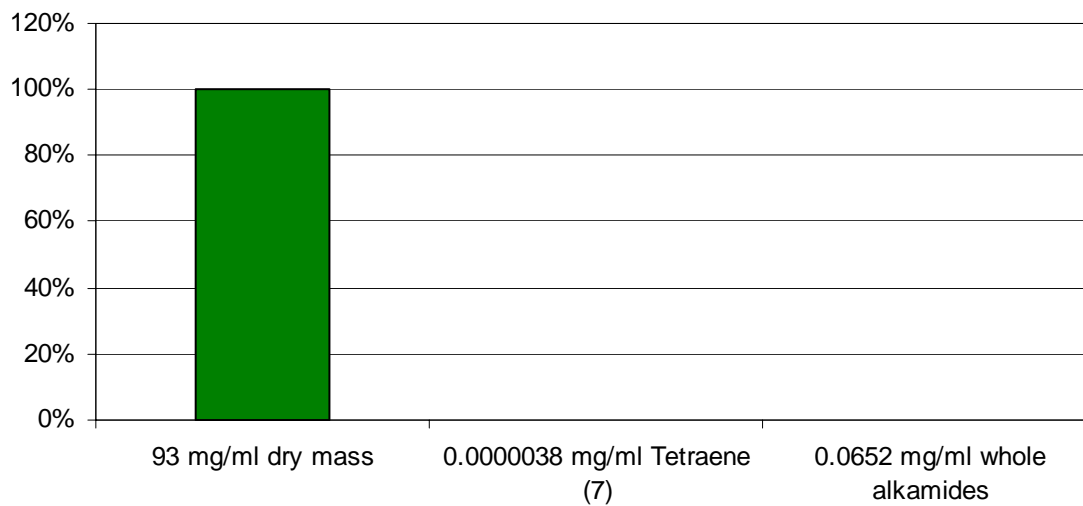


Table 3. Alkamides with Retention time and quantity per ml extract.

compound	retention time (min)	m/z [M+H ⁺]/MS/MS	quantity/ml extract
Undeca 2 <i>E</i> /Z,4 <i>Z</i> / <i>E</i> -diene-8,10-diynoic acid-isobutylamides (1)	9.16	229 230/131	2.234 µg/ml
Undeca 2 <i>E</i> /Z-ene-8,10-diynoic acid-isobutylamides (2)	8.62, 9.81	231 232/133	5.672 µg/ml
Dodeca-2 <i>E</i> ,4 <i>E</i> ,8 <i>Z</i> ,10 <i>E</i> / <i>Z</i> -tetraenoic acid-isobutylamides (7)	15.40	247 248/149	0.0038 µg/ml

Phytochemical analyses of this 20% ethanol extract revealed that there are only 0.07 % alkamides. The content of the main constituent Dodeca-2E,4E,8Z,10E/Z-tetraenoic acid-isobutylamide (**7**) is 0.0038 $\mu\text{g/ml}$ from the whole 65.2 $\mu\text{g/ml}$ alkamides.



Supercritical CO₂ extract

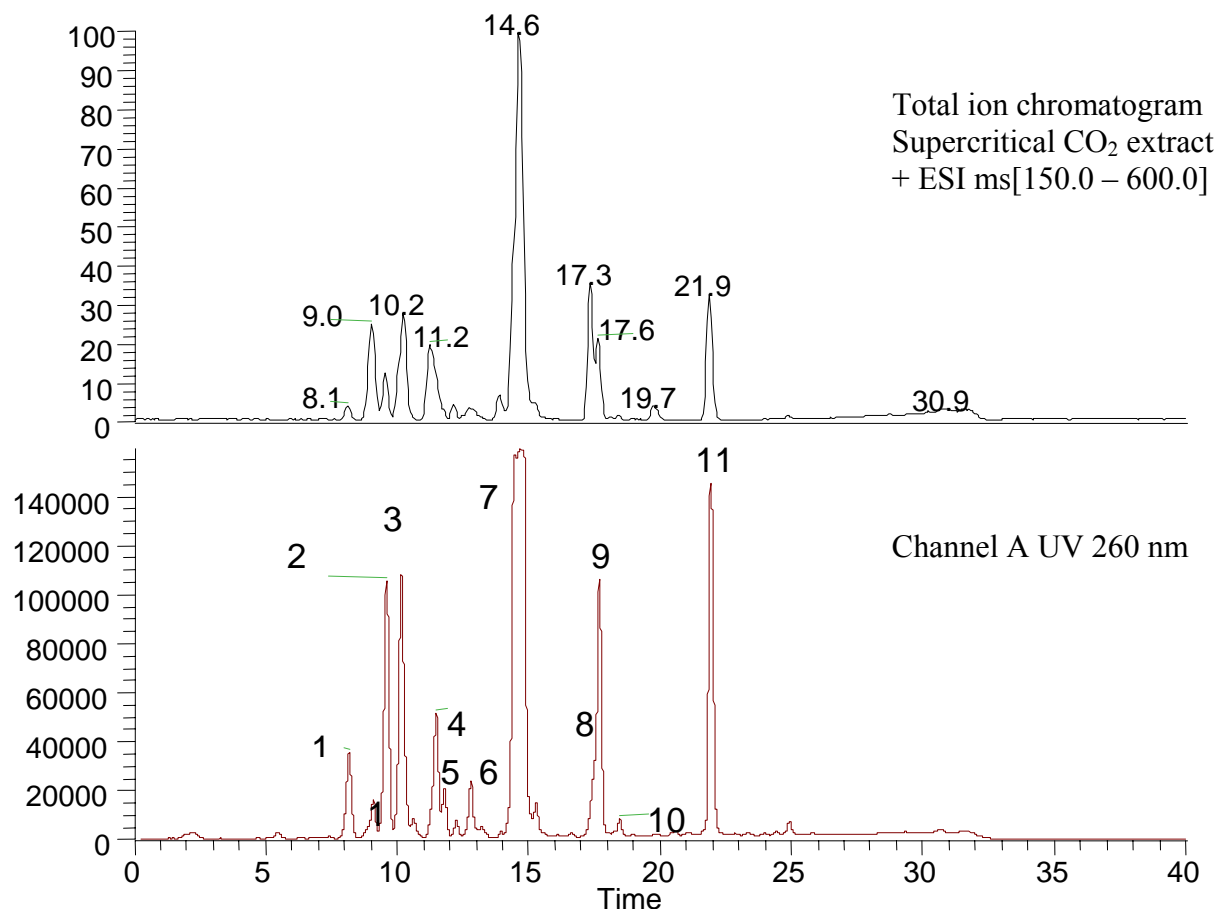
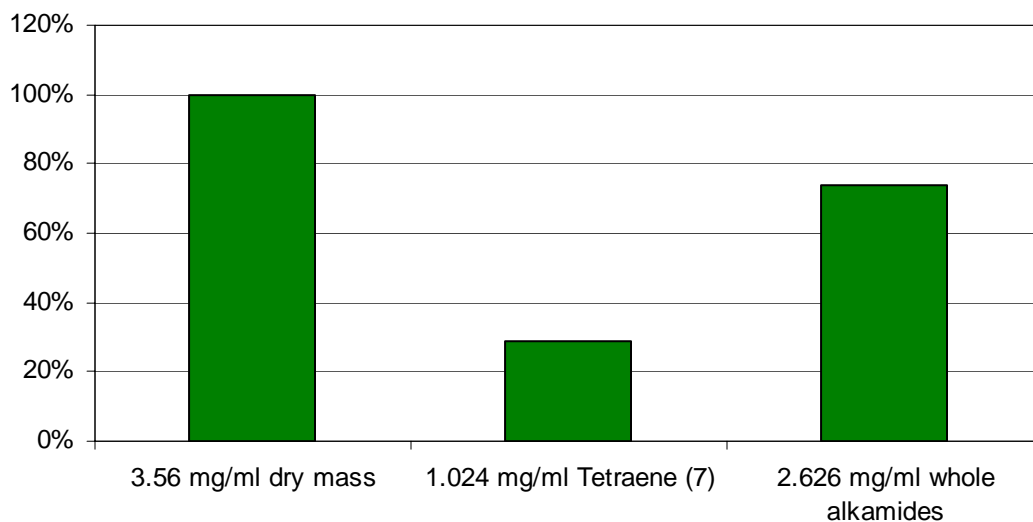


Table 4. Alkamides with Retention time and quantity of the main 6 compounds.

compound	retention time (min)	m/z [M+H⁺]/MS/MS	quantity/ml extract
Undeca 2 <i>E</i> / <i>Z</i> ,4 <i>Z</i> / <i>E</i> -diene-8,10-diynoic acid-isobutylamides (1)	8.10, 9.53	229 230/131	0.186 mg/ml
Undeca 2 <i>E</i> / <i>Z</i> -ene-8,10-diynoic acid-isobutylamides (2)	9.05, 10.34	231 232/133	0.437 mg/ml
Dodeca-2 <i>E</i> ,4 <i>Z</i> -diene-8,10-diynoic acid-isobutylamide (3)	10.07	243 244/145	0.475 mg/ml
Dodeca-2 <i>E</i> -ene-8,10-diynoic acid-isobutylamide (4)	11.24	245 246/147	0.411 mg/ml
Dodeca-2 <i>E</i> ,4 <i>Z</i> -diene-8,10-diynoic acid 2- methylbutylamide (5)	12.17	257 258/145	
Dodeca-2 <i>E</i> -ene-8,10-diynoic acid 2- methylbutylamide (6)	13.91	259 260/147	

Dodeca-2 <i>E</i> ,4 <i>E</i> ,8 <i>Z</i> ,10 <i>E</i> / <i>Z</i> -tetraenoic acid-isobutylamides (7)	14.63	247 248/149	1.024 mg/ml
Pentadeca-2 <i>E</i> ,9 <i>Z</i> -diene-12,14-diynoic acid isobutylamide (8)	17.35	285 286/230	
Dodeca-2 <i>E</i> ,4 <i>E</i> ,8 <i>Z</i> -trienoic acid-isobutylamide (9)	17.64	249 250/167	0.268 mg/ml
Pentadeca-2 <i>E</i> ,9 <i>Z</i> -diene-12,14-diynoic acid 2-methylbutylamide (10)	19.78	299 300/185	
Dodeca-2 <i>E</i> ,4 <i>E</i> -dienoic acid-isobutylamide (11)	21.90	251 252/179	0.253 mg/ml

Phytochemical analysis of this supercritical CO₂ extract revealed that there are 73.77 % alkamides. The content of the main constituent Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid-isobutylamide (**7**) is 1.024 mg/ml from the whole 2.626 mg/ml alkamides



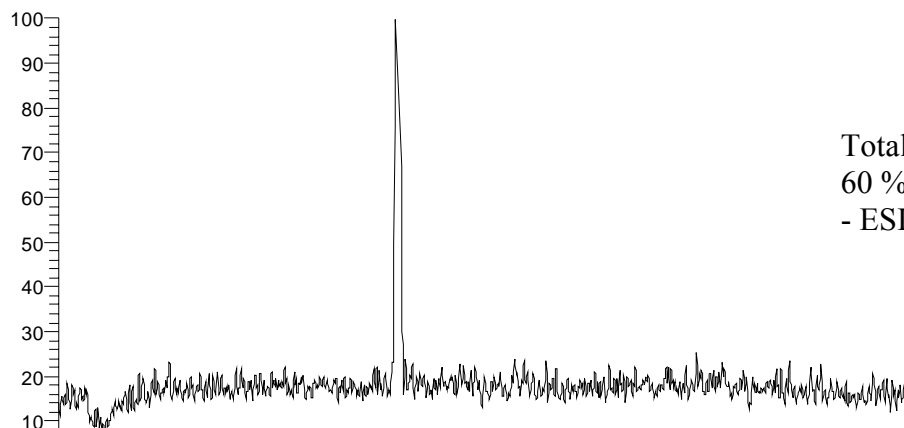
ANALYSIS OF CAFFEIC ACID DERIVATIVES: The analyses were carried out on a ZORBAX[®] 150 mm x 2.1 mm, 3.5 µm RP-18 Narrow Bore column (Agilent Technologies, Waldbronn, Germany). To protect the integrity of the analytical column, all analyses were performed with a OPTI-GUARD[®] C₁₈ 1 mm guard column (OPTIMIZE Technologies, INC). The mobile phase was (A) aqueous formic acid (0.1 %) and (B) acetonitrile. The gradient elution was modified as follows: initial 10 % B; linear gradient

to 22 % B in 20 min; recycle to initial conditions in 1 min and hold for 9 min. The total running time was 30 minutes. The flow rate was 300 $\mu\text{l}/\text{min}$. The detector monitored the eluent at 330 nm. The column temperature was set at 26 $^{\circ}\text{C}$. The sample injection volume was 5 μl for the 60 % ethanol and CO_2 extract and for the 20% extract 10 μl .

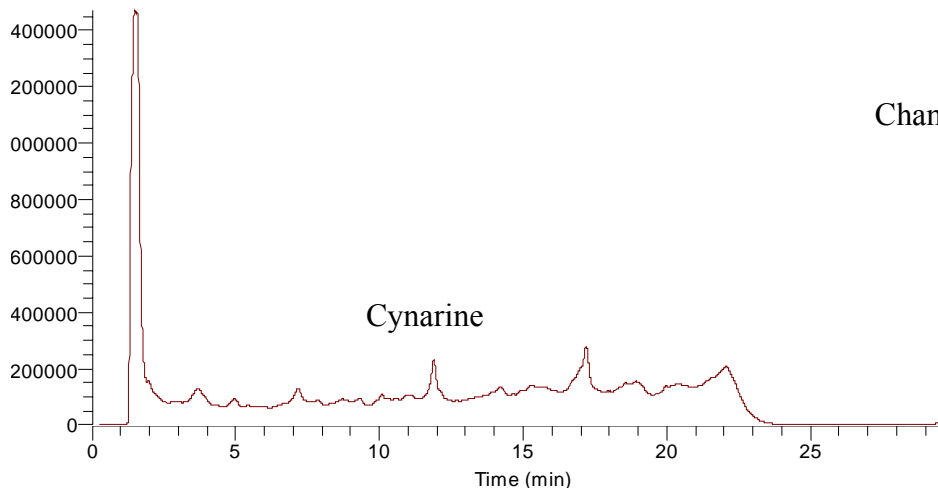
The LC was interfaced with a Finnigan LCQ Deca XP Plus ion trap mass spectrometer operating in the electrospray ionization (ESI) negative mode by XCALIBUR software. The mass spectra were generally recorded from 300 – 1500 m/z in full-scan-mode which provided the total ion current (TIC) chromatogram. The signal was optimized on the total ion current in MS mode, leading to a transfer capillary temperature of 350 $^{\circ}\text{C}$, a spray voltage of 4.50 kV a sheath gas flow of 70 units, and a Aux/Sweep Gas Flow of 15.00 units (units refer to arbitrary values set by the Excalibur software). At the same time, the selection of ions and the collision voltages were optimized using Xcalibur software.

Standards and Calibration curve: Cynarine was purchased from Sequoia Research Products Ltd. Oxford, United Kingdom. The level of Cynarine in the extract was quantified by comparison to an external standard. After calibration, a linear relationship was established for analysis of 2 to 2000 ng cynarine. The correlation of peak area and concentration was found to be highly linear for cynarine detected with ESI Selected Reaction Monitoring (SRM) m_s2 with R^2 value of 0.9992.

60 % ethanol extract



Total ion chromatogram
60 % ethanol extract
- ESI ms[300.0 – 1500.0]



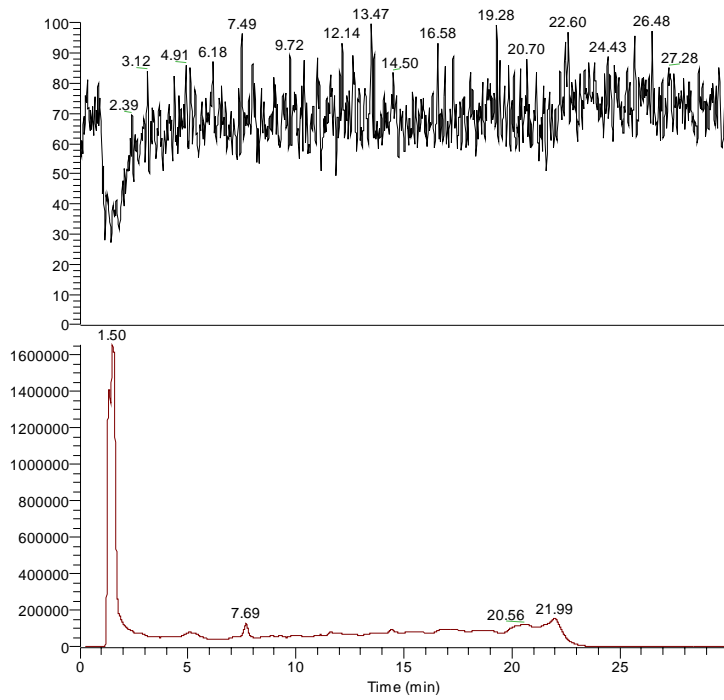
Channel A UV 330 nm

Caffeic acid derivatives with retention time and quantity

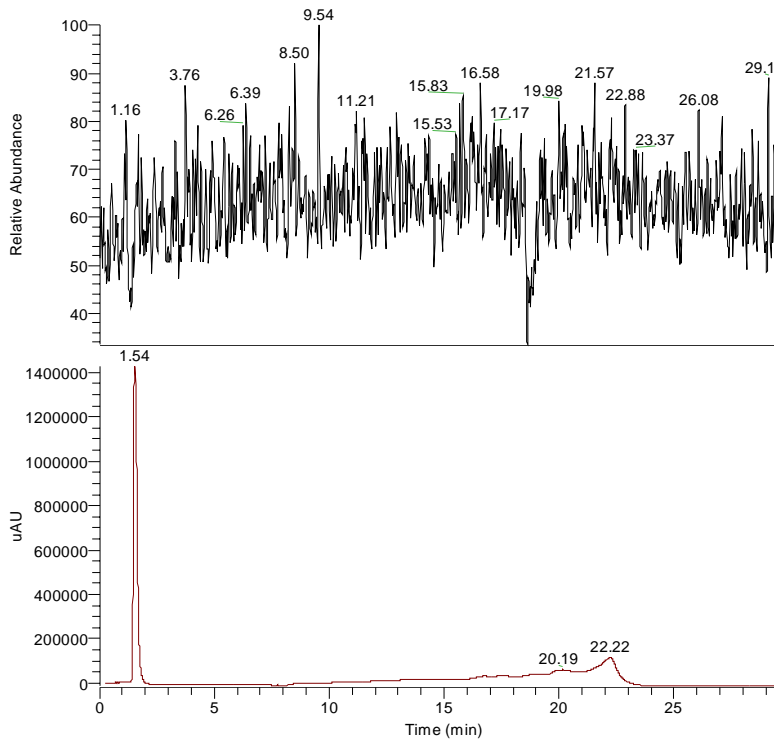
compound	retention time (min)	m/z [M+H⁺]/MS/MS	quantity/ml extract
Cynarine	11.73	516 515/353	0.155 mg/ml
Echinacoside	-	786 785/623	-

Phytochemical analysis showed the content of the caffeic acid derivatives in the 60 % extract. There are 0.155 mg/ml cynarine, but no echinacoside could be detected. As described by Woelkart et al. these CADs are highly susceptible to enzymatic degradation and oxidation in hydroalcoholic solutions during the extraction process²⁰.

20% ethanol extract



Supercritical CO₂ extract

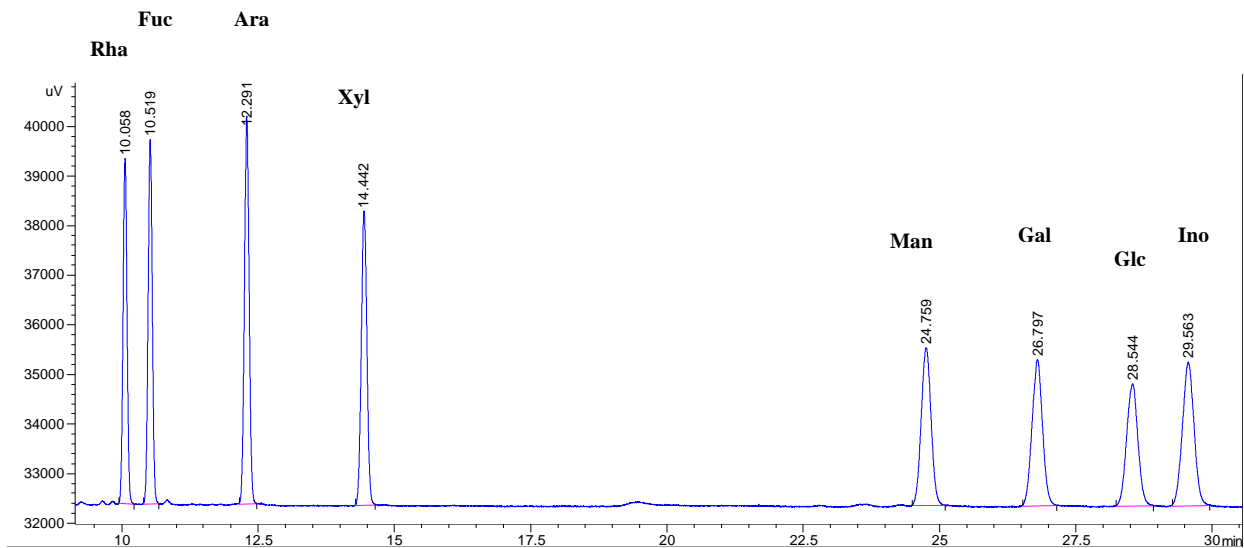


There are no caffeic acid derivatives detectable in either the 20% ethanol extract or the supercritical CO₂ extract.

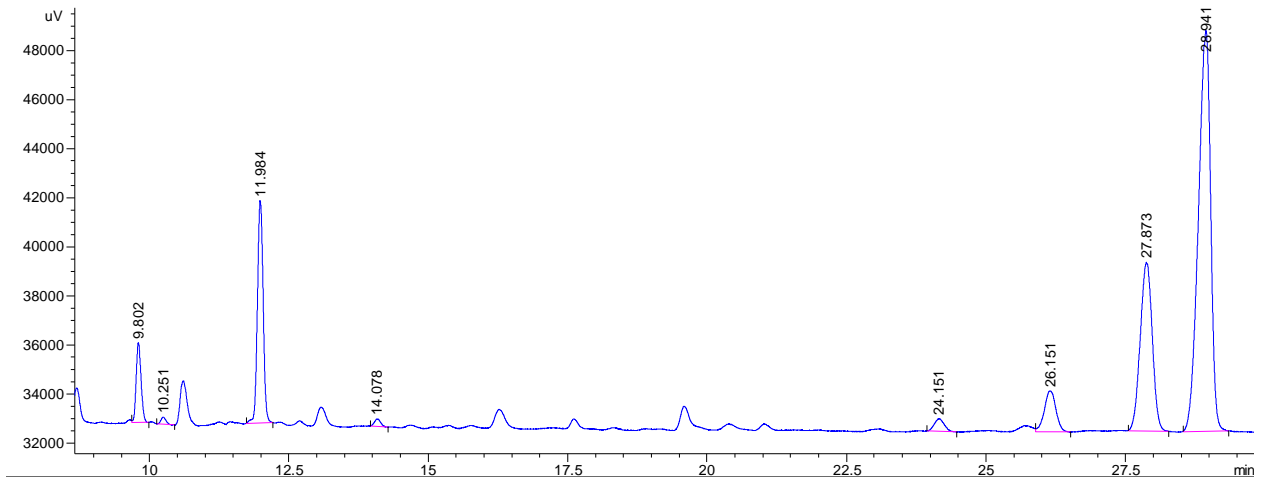
ANALYSIS OF NEUTRAL MONOSACCHARIDES: The neutral monosaccharides were analyzed by gas chromatography as described by Henry, et al.²¹.

GC-chromatogram standards: Rhamnose, Fucose, Arabinose, Xylose, Mannose, Galactose, Glucose, Inositol as internal standard

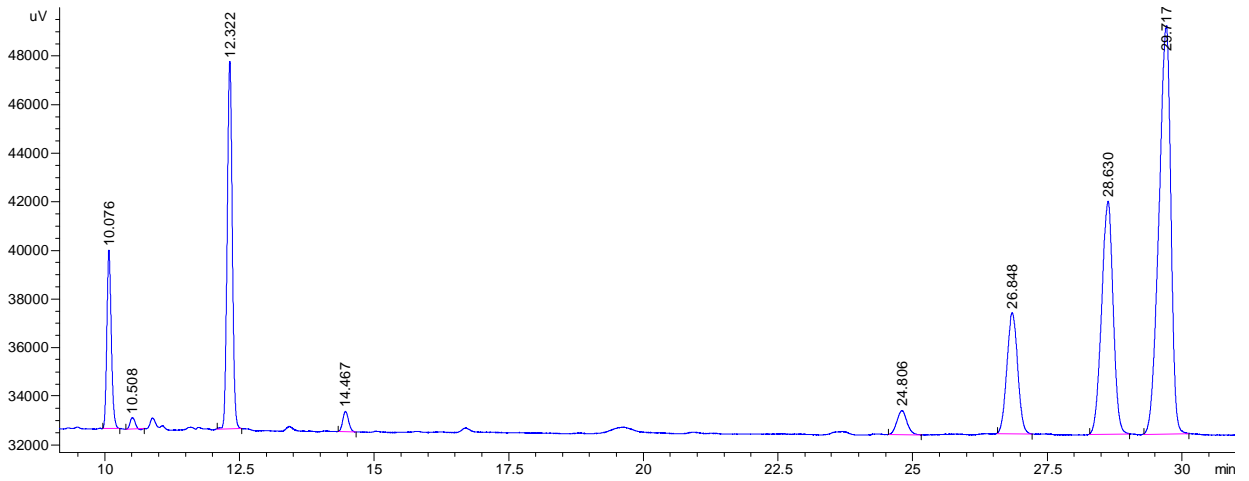
Standards



60% ethanol extract



20% ethanol extract



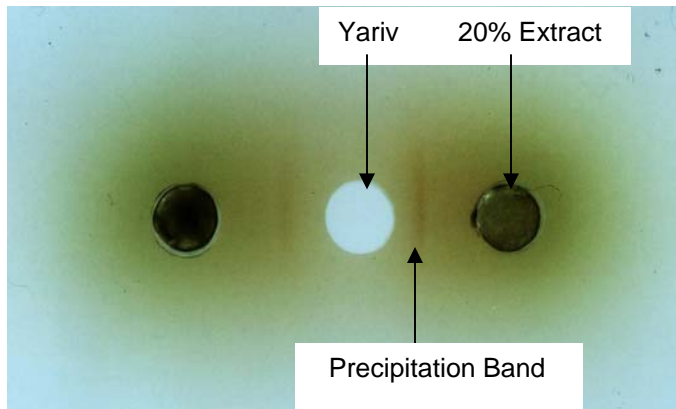
Neutral monosaccharide composition (%) by acetylation-analyses after dialysis (12- 14 kDa)

Monosaccharide	60% Ethanol Extract	20% Ethanol Extract
Rhamnose	18.3	13.3
Fucose	1.5	0.8
Arabinose	46.4	23.8
Xylose	1.8	1.6
Mannose	4.9	3.3
Galactose	18.6	18.1
Glucose	8.5	39.4

No polysaccharides were detectable in the critical CO₂ extract.

ANALYSIS OF TOTAL POLYSACCHARIDES: To determine the content of the whole polysaccharides a photometric method was used²². These analyses revealed that the 60% extract contained 48.9 % polysaccharides and the 20% extract contained 42.1% polysaccharides.

Analysis of arabinogalactan-proteins: Arabinogalactan-proteins were measured by the agar plate diffusion test with β -glucosyl Yariv's reagent. Agarose plates (1% agarose in 10 mM Tris buffer, pH 7.3, with 0.9% NaCl and 1 mM CaCl₂) were prepared. The freeze-dried, high-molecular-weight fraction (500 mg) dissolved in 50 ml of distilled water was placed in one well and Yariv's reagent in the other well. The presence of a precipitation band between the wells was evidence of the presence of arabinogalactan-protein.



By this method the 20% extract had detectable arabinogalactan-protein but the 60% extract did not.

Subsequent quantitative measurement of the arabinogalactan protein as described and performed by Classen et al.²³ revealed a content of 5.7 $\mu\text{g/ml}$ in the 20% extract and 0.675 $\mu\text{g/ml}$ in the 60% extract. The supercritical CO_2 extract had no detectable arabinogalactan-protein by either method.

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