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Enzymatic Degradation of Echinacoside and Cynarine in *Echinacea angustifolia* Root Preparations

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Abstract

Echinacea preparations are among the bestselling herbal medicinal products in North America and Europe. Echinacoside and cynarine are the major polar constituents in the roots of *Echinacea angustifolia* DC and therefore are often used as marker compounds for the standardization of corresponding *Echinacea* preparations. This study demonstrates that echinacoside and cynarine are highly susceptible to enzymatic degradation and oxidation in hydroalcoholic solutions during the extraction process. This makes their use as analytical marker compounds still possible, although care must be taken to avoid error due to this enzymatic degradation. Batch-to-batch consistency and standardization of preparations from *E. angustifolia* roots require in particular a control of this enzymatic activity. The polyphenol oxidase (PPO), which is responsible for the oxidative degradation of echinacoside and cynarine, was isolated from the roots of *E. angustifolia*. An Electrospray Ionization-Liquid Chromotography-Mass Spectometry (ESI-LC-MS) method was used to monitor this effect and to determine the kinetics of the degradation.

Keywords: Cynarine, echinacea, echinacoside, enzymatic degradation, LC-MS analysis, polyphenol oxidase.

Introduction

Echinacea angustifolia DC G. Asteraceae is one of the three species of *Echinacea* that are commercially available for use as supportive therapy of colds and chronic infections of the respiratory tract and lower urinary tract. These preparations have been shown to have positive effects on various immunological parameters (Bauer et al., 1998). Caffeic acid derivatives (CADs), polysaccharides, and alkamides are thought to be among the compounds responsible for this complex mode of action (Bauer et al. 1999). Echinacoside **1** and cynarine **2** (Fig. 1) are the major polar constituents in the roots of *E. angustifolia* (Fig. 2) and are frequently used for the standardization of corresponding *Echinacea* preparations. Also, in the current draft of the USP/NF monograph of *E. angustifolia* roots, echinacoside and cynarine are the compounds which have to be determined in an assay for total phenolics. Immunologic effects of *Echinacea* extracts have been reported in a number of studies. An ethanolic extract from the roots of *E. angustifolia* in a concentration of 10^{-3}% enhanced phagocytosis of yeast particles by human polymorphonuclear neutrophils (PMN) *in vitro* by 17%. No activity was observed below a concentration of 10^{-5}%, and the constituents responsible for this stimulation were not determined. Also, *in vivo*, oral administration of the ethanolic extract enhanced phagocytosis of carbon particles by a factor of 1.7 (Bauer et al., 1988a, 1989). Echinacoside also provided dose-dependent protection against free radical–induced degradation of type III collagen by a reactive oxygen scavenging effect, leading the authors to suggest a protective activity of *Echinacea* polyphenols against photodamage of the skin. Similarly, echinacoside and other phenylpropanoid glycosides were able to protect against oxidative hemolysis *in vitro* (Li et al., 1993; Facino et al., 1995).

Because a commercially prepared extract was void of echinacoside, although the root material contained it, we suspected degradation of echinacoside during the extraction

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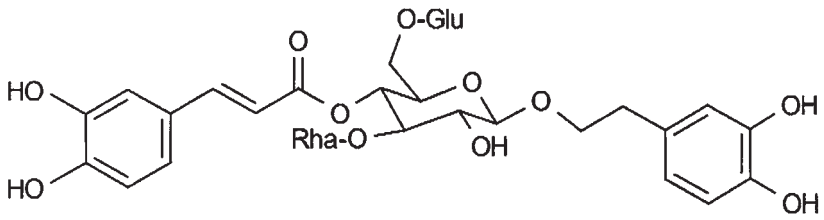
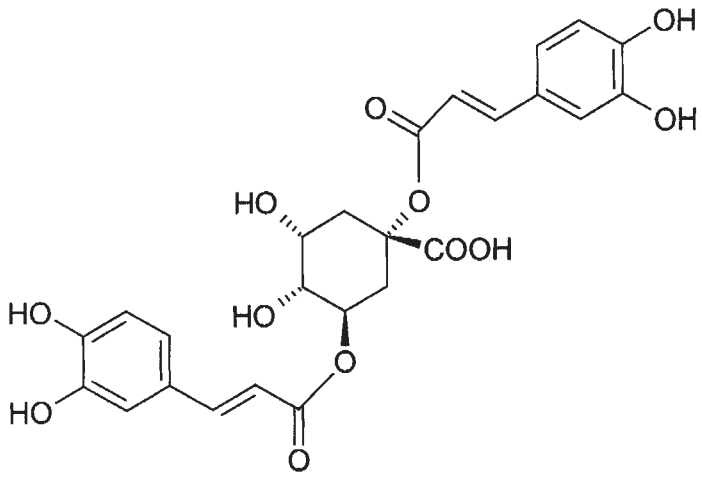


1 Echinacoside



2 Cynarine

Figure 1. Structures of compounds **1** and **2**.

process. We have now investigated and report the oxidative degradation of echinacoside and cynarine during drug processing by the action of a polyphenol oxidase present in the roots of *E. angustifolia.*

Materials and Methods

Plant material and extraction

Freshly harvested roots from 2-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 Pharmacognosy, Karl Franzens University, Graz. A voucher specimen is deposited at this Institute of Pharmacognosy.

Powdered root material (1 g) was extracted in an ultrasonic bath (for 5–7 min at room temperature) three-times with 15 ml solvent (60% v/v ethanol in water) each. The extracts (n = 3) were combined and the volume was completed up to 50 ml to a final concentration equivalent to 0.02 g roots/ml.

LC-MS analysis

LC-MS analysis of extracts was carried out using a Thermo Finnigan Surveyor liquid chromatograph (San Jose, CA, USA) equipped with a LiChroCART 55-2, 3 µm RP-18 end-capped Purospher STAR column, using a gradient elution from acetonitrile + 0.1% formic acid/water + 0.1% formic acid 5/95 (v/v) to 25/75 (v/v) in 12 min, at a flow rate of 250 µl/min with an injection volume of 10 µl. Ultraviolet, detection with a Surveyor PDA was used at wavelength 330 nm.

The LC was interfaced with a LCQ Deca XP Plus mass detector (San Jose, CA, USA) operating in the Electrospray Ionization (ESI) negative mode. The mass spectra were generally recorded from 300 to 1500 *m/z* in full-scan mode, which provided the total ion current (TIC) chromatogram. For quantification, ESI selected reaction monitoring (SRM) was used, which is a scan mode with two stages of mass analysis and in which a particular reaction or set of reactions, such as the fragmentation of an ion or the loss of a neutral moiety, is monitored. Selected reaction monitoring allows the very rapid analysis of trace components in complex mixtures.

Standards

Echinacoside was isolated from dried *E. angustifolia* roots as previously described by Bauer et al. (1986), and cynarine was purchased from Sequoia Research Products Ltd. (Oxford, UK). For both compounds, a calibration curve was prepared. The levels of the constituents in the extract were quantified by comparison to an external standard. After calibration, a linear relationship was established for analysis of 8 to 8000 ng echinacoside and 2 to 2000 ng cynarine. The correlation of peak area and concentration was found to be highly linear, both for echinacoside and cynarine detected with ESI SRM MS/MS with R^2 values of 0.9999 and 0.9992, respectively.

Extraction of the enzyme

Polyphenol oxidase (PPO) was isolated from the roots of *E. angustifolia* and from *Solanum tuberosum* L. tubers using a technique based on ammonium sulfate precipitation of protein. Ground root material (20 g) and potato tubers, respectively, were homogenized with 35 ml of 4 °C cold sodium fluoride (NaF) in a cooled blender. After filtration, the enzyme caused a floculent white precipitate when an equal volume of saturated ammonium sulfate solution was added. The pellets were then resuspended in citrate buffer, pH 4.8. PPO was insoluble in 50% ammonium sulfate but is soluble in the citrate buffer. All steps were carried out on ice, and enzyme extract solutions yields of 16.5 ml *E. angustifolia* extract and 20 ml *S. tuberosum* extract were stored at 4 °C (Matheis et al., 1977; Dean, 1997).

Determination of enzymatic activity

For the characterization of the isolated polyphenol oxidase, the catalytic action was determined. PPO catalyzes the conversion of L-DOPA to dopachrome. A standard curve with L-3,4-dihydroxyphenylalanine (CAS. no. 59-92-7) purchased from Sigma-Aldrich (Vienna, Austria) was prepared to monitor the activity of the enzyme by analyzing the appearance of dopachrome pigment at an absorbance maximum of 475 nm. The activity is a measurement of the velocity at which substrate is converted to product (Matheis et al., 1977; Mazzafera & Robinson, 2000) (Fig. 3).

Results

After extraction of *E. angustifolia* roots with 60% (v/v) ethanol/water and storage at 4 °C, we observed a decline in

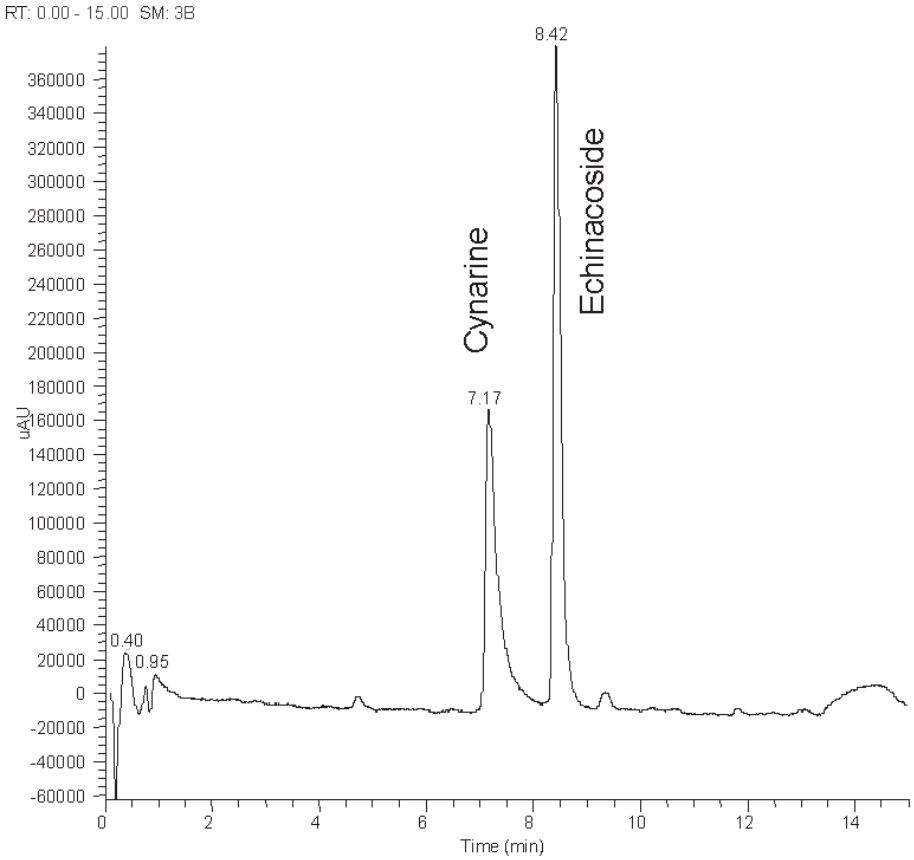


Figure 2. HPLC separation of the polar constituents of a 60% EtOH *Echinacea angustifolia* root extract. (Separation conditions see Material and Methods)

the content of echinacoside and cynarine from 0.247 mg/ml extract and 0.091 mg/ml extract, respectively, to 0 over a 16-day period. However, we did not observe any degradation product. The content of caffeic acid, one possible hydrolysis product, did not increase during incubation. The time course shown in Figure 4 could be followed easily by LC-MS analysis. We suspected that this decline was related to the enzymatic activity of a PPO, as it had been shown for cichoric acid in *Echinacea purpurea* Moench (Nüsslein et al., 2000).

We subsequently isolated a PPO from *E. angustifolia* roots. We compared the activity with that of a PPO isolated from *S. tuberosum*, as potato tubers are known to be rich in PPO. The enzymatic activity of PPO from *E. angustifolia* roots was approximately three-times lower than of that from *S. tuberosum* tubers. Expressed in units, we calculated 2.49 µmol/min against 7.12 µmol/min in the potato tubers.

Discussion

It is known that plant enzymes can become active even after harvesting and after drying the plant material. As for herbal medicinal products, plant enzymes can alter the quality of the raw materials (e.g., by influencing the content of the active principles). Although such processes have often been observed, the related biochemical reactions have not been well characterized (Ray & Hammerschmidt, 1998; Nüsslein et al., 2000).

Because we observed the lack of echinacoside in a commercially prepared large-scale extract of *E. angustifolia* roots (data not shown), although the root material had contained

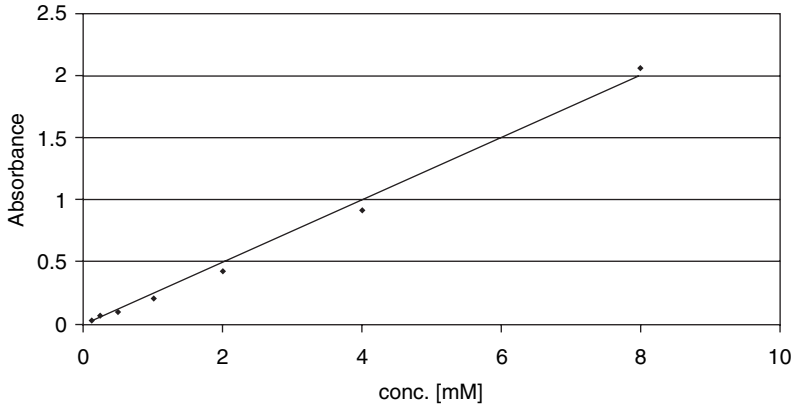


Figure 3. Dopachrome standard curve for the PPO from the roots of *E. angustifolia*; $y = 0.2495 \times R^2 = 0.9945$.

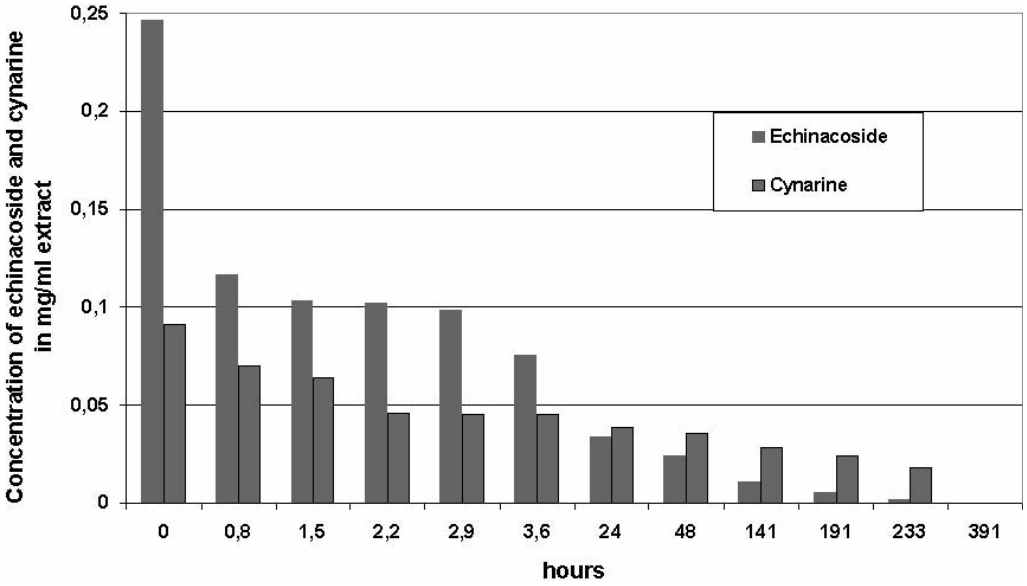


Figure 4. Content of echinacoside and cynarine in 60% EtOH *Echinacea angustifolia* root extract, during storage at 4 °C over 16 days.

echinacoside, we suspected enzymatic degradation during the preparation of *E. angustifolia* extracts. The results presented now show that the degradation of caffeic acid derivatives during the preparation of extracts from "*Echinaceae angustifoliae radix*" is an enzymatic process in which a PPO is involved. PPO (EC 1.14.18.1) is a copper-containing enzyme responsible for hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-diquinones. PPO is widely distributed in higher plants (Vaughn & Duke, 1984), but it still has no defined biological function, although many possible roles have been proposed (Spruce et al., 1987; Vaughn et al., 1988; Lax & Vaughn, 1991; Steffens et al., 1994; Hind et al., 1995; Trebst & Depka, 1995).

At present, the most likely function for PPO is its involvement in plant resistance against diseases (Bashan et al., 1985; Goy et al., 1992; Ray & Hammerschmidt, 1998) and against insect herbivory (Felton et al., 1989, 1992). In *Echinacea*, PPO activity has previously been detected in *E. purpurea* (Kreis et al., 2000).

Cichoric acid in *E. purpurea* preparations is highly susceptible to enzymatic degradation as described by Nüsslein et al. (2000) and Bergeron et al. (2002). They also found that the content of caffeic acid, one possible hydrolysis product, did not increase during incubation, although they also detected an esterase activity. Reducing agents, such as ascorbic acid or thiol reagents, added to the incubation buffer–inhibited degradation. Similar effects were seen when N_2-saturated buffers without reducing agents were used, and the soaked plant material was incubated in an N_2 atmosphere. As soon as O_2 was allowed to come into contact with the incubation mixture, degradation started and the supernatant turned brown.

These observations suggest that oxidation plays the predominant role in the degradation of caffeic acid derivatives in *Echinacea* species. The observation that there is no echinacoside and cynarine left in hydroalcoholic solutions of *E. angustifolia* extracts after 2 weeks of storage makes their use as analytical marker compounds still possible, although care must be taken to avoid error due to this enzymatic degradation. Harvest, storage, and extraction of the roots needs to be performed under controlled conditions in order to achieve a consistent content of echinacoside and cynarine. Because differences in the concentration of these compounds may be due to enzymatic postharvest degradation, determinations in plant breeding experiments have to be performed very carefully and under strict control of enzymatic activity.

In order to standardize *Echinacea* preparations and to guarantee a consistent content of caffeic acid derivatives, it is unambiguously necessary to control this enzymatic activity.

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