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Quantitative Analysis of Anti-inflammatory and Radical Scavenging Triterpenoid Esters in Evening Primrose Oil

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Cold pressed, nonraffinated evening primrose oil (EPO) was recently found to contain lipophilic triterpenoidal esters with radical scavenging and anti-inflammatory properties. A simple and robust method for the quantitative analysis of these 3-*O*-*trans*-caffeoyl derivatives of betulinic, morolic, and oleanolic acid was developed and validated. Separation was achieved by normal phase chromatography on a Diol column and with hexane/ethyl acetate (50:50) as eluent. The analytes could be determined directly in the oil matrix, without need of a previous removal of the triglycerides. Normal phase LC ESI-MS with a makeup flow of polar modifier was used for checking the identity and purity of analyte peaks. Samples from 22 commercially available EPOs were analyzed. The average caffeoyl ester contents were 58 mg/100 g in cold pressed oils and 4.7 mg/100 g in partially raffinated oils. In fully raffinated EPO samples, the concentration was below the limit of detection. The influence of extraction temperature on the content of caffeoyl esters in nonraffinated EPO was investigated with seeds of *Oenothera biennis* and *Oenothera lamarckiana*, respectively. With *O. lamarckiana*, the concentration of caffeoyl esters in the oil increased with rising pressure and temperature, whereas no such dependency was found with *O. biennis*. Microscopic analysis revealed some differences in the histology of the seed testa, which may explain in part the differing behaviors in the extraction experiments. There was a difference between *O. biennis* and *O. lamarckiana* oils with respect to the relative amounts of the three esters. The temperature of the extraction process had no effect on the ratio of the compounds.

KEYWORDS: Evening primrose oil; *Oenothera biennis*; *Oenothera lamarckiana*; Onagraceae; caffeoyl ester; triterpenoid; HPLC; radical scavenger; anti-inflammatory; normal-phase LC ESI-MS

INTRODUCTION

Evening primrose oil (EPO) is a dietary supplement that is used for its high concentration of polyunsaturated fatty acids, in particular in γ-linolenic acid (18:3*n*−6). Beneficial effects have been reported in various ailments (*1*). In the course of our investigations on natural products with anti-inflammatory (*2*, *3*) and antioxidant activities (*4*), we carried out a phytochemical investigation on the non-triglyceride (NTG) fraction of cold-pressed EPO. Three caffeoyl derivatives of betulinic, morolic, and oleanolic acid (**1**−**3**, **Figure 1**) with radical scavenging activity were isolated and characterized (*5*). The compounds showed potent in vitro inhibitory effects on cyclooxygenases 1 and 2 and on human leucocytic elastase, key enzymes of inflammatory processes (*6*, *7*). It thus appeared that these lipophilic caffeoyl derivatives could be of importance for the quality of EPO. Their radical scavenging effect serves for a natural stabilization of the polyunsaturated fatty acids in EPO, and the anti-inflammatory activity may possibly contribute to some of the beneficial properties reported for the oil.

A preliminary survey of some oils carried out in the course of the isolation of caffeoyl esters **1**−**3** already suggested that their concentrations in EPO were highly variable and apparently depended on the production process (*5*). To further substantiate these qualitative observations and the putative health-promoting role of these esters, a quantitative assay was needed. We here describe the development and validation of an HPLC assay for the direct and simultaneous determination of compounds **1**−**3** in EPO samples without prepurification from the oil matrix. We then carried out a systematic survey on the concentration of these compounds in commercial oil samples. We also describe the results of a study on the influence of the extraction temperature on the ester content in oils obtained from *Oenothera biennis* and *Oenothera lamarckiana*.

MATERIALS AND METHODS

Chemicals and Reference Compounds. HPLC grade water was obtained from a Sersdest SD 2800 purification system (Seral, Ransbach-Baumbach, Germany). Chemicals and solvents were of analytical grade,

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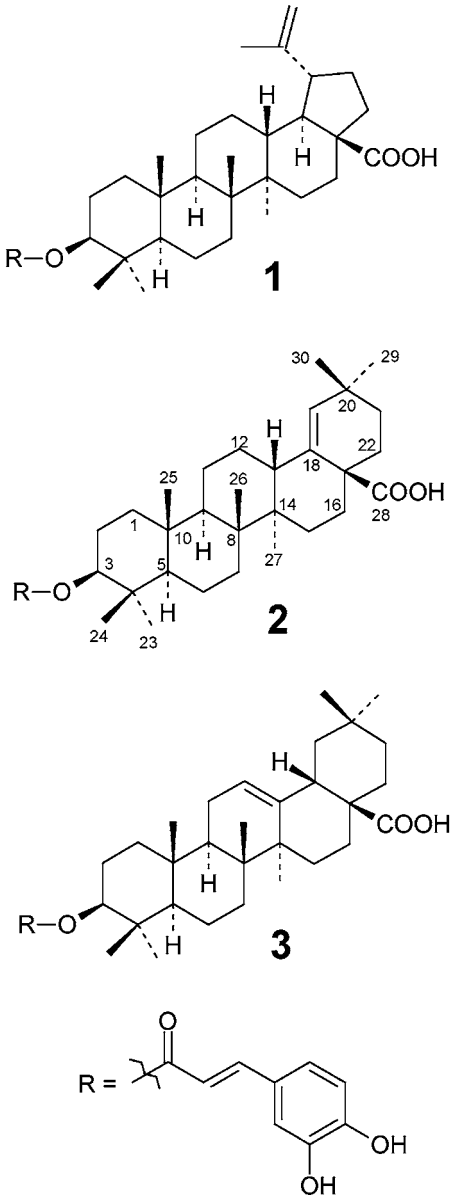

Figure 1. Chemical structures of caffeoyl esters of betulinic (**1**), morolic acid (**2**), and oleanolic acid (**3**).

unless indicated otherwise. HPLC grade acetonitrile, ethyl acetate, and hexane and a hexane isomer mixture (95%) were obtained from Roth (Karlsruhe, Germany). Technical grade ethyl acetate was purchased from Brenntag (Mühlheim/Ruhr, Germany) and was distilled prior to use.

Esters **1**−**3** were isolated from cold-pressed EPO and characterized as reported (*5*). The purity of reference compounds, as determined by reversed-phase (RP) HPLC, was ≥95%.

Evening Primrose Oils. Commercially available EPOs were from the following German suppliers: Andor Pharma (Regensburg, Germany), Bi-Ro (Sommerschenburg, Germany), Gustav Hees (Stuttgart, Germany), Henry Lamotte (Bremen, Germany), Nature (Tettau, Germany), Ölmühle Solling (Brevern, Germany), PG-Naturpharma (Edling, Germany), Primavera Life (Sulzberg, Germany), Roche Vitamine (Grenzach-Wyhlen, Germany), and Syntapharm (Mühlheim/Ruhr, Germany). A raffinated EPO (lot R0568-6A) from Paninkret (Pinneberg, Germany) served as ester-free matrix for assay development and for preparation of reference solutions.

The *O. biennis* seeds were processed by BiRo-GbR (Sommerschenburg, Germany) and the *O. lamarckiana* seeds by Kroppenstedter Ölmühle (Kroppenstedt, Germany). The seed material was provided by Pharmaplant (Artern, Germany).

Solid-phase extraction (SPE) experiments were carried out with Discovery DSC-Diol (500 mg) cartridges (Supelco, Taufkirchen, Germany). Cartridges were conditioned with hexane/ethyl acetate (90:10; 3 mL), followed by the sample introduction [100 μL of EPO in 900 μL of hexane/ethyl acetate (90:10)]. Triglycerides were removed with hexane/ethyl acetate of various proportions, followed by elution of caffeoyl esters with methanol (1−2 mL).

HPLC analysis was performed with an Agilent 1100 system consisting of solvent degasser, binary high-pressure mixing pump, autosampler, column oven, and diode array detector. HPLC separations were carried out on a LiChrosorb Diol column (5 μm; 4.0 × 250 mm i.d.) with hexane/ethyl acetate (50:50) as mobile phase. Flow rate was 1.0 mL/min, column temperature was set at 25 °C, and injection volume was 20 μL. Chromatograms were recorded at 330 nm. Photodiode array (PAD) spectra were measured from 200 to 500 nm.

For peak identity and purity checks, the HPLC was connected to a PE Sciex API 165 quadrupole mass spectrometer equipped with a Turbo Ionspray interface (Applied Biosystems, Langen, Germany). The flow rate of the HPLC pump was lowered to 0.15 mL/min. Via a T-connection, acetonitrile (containing 2% acetic acid) was added postcolumn at a flow rate of 0.15 mL/min, and the combined effluents were directed to the interface without split. MS spectra were recorded as described previously (*5*).

Calibration Curves and Assay Validation. Stock solutions (0.20 mg/mL) of reference compounds **1**−**3** were prepared in ethyl acetate. Dilutions were obtained, either in ethyl acetate or in diluted matrix [ester-free EPO/ethyl acetate (1:10)], to afford a concentration range from 100 to 0.4 μg/mL. Calibration curves determined in diluted oil matrix and in ethyl acetate (values in parentheses) were as follows:

compound **1**: $y = 1.4389x$ ($y = 1.4112x$) $R^2 = 1$

compound **2**: $y = 1.4786x$ ($y = 1.3910x$) $R^2 = 0.9997$

compound **3**: $y = 0.9243x$ ($y = 0.9392x$) $R^2 = 0.9997$

Robustness testing was carried out with a LiChrosorb Diol column of a different batch and by replacing HPLC grade *n*-hexane and ethyl acetate by hexane isomer mixture and distilled technical grade ethyl acetate.

Microscopic Analysis. Fluorescence microphotographic pictures were obtained on an Axioskop 2 equipped with an Axiocam (Zeiss, Jena, Germany) and a fluorescence filter (365 nm). Seeds were embedded in epoxide resin (Uhu, Bühl, Germany) and cross sections obtained by hand-sectioning.

RESULTS

Development and Validation of an HPLC Assay. Initial experiments for a quantitative HPLC assay were based on the method previously used for the purification of esters **1**−**3** (*5*). RP chromatographic separation of the esters, however, required the removal of the lipid matrix prior to analysis. Various normal-phase supports such as silica gel and amino and diol sorbents were tested as sorbents for SPE. A combination of a diol phase with hexane/ethyl actetate mixtures for removal of triglycerides and elution of analytes with methanol appeared to be the most favorable combination. A complete removal of triglycerides was not achievable, however, and the residual lipids led to an unsatisfactory separation of analytes on the RP-8 and RP-18 columns. On the basis of the SPE experiments, a normal-phase HPLC method on a Diol column was developed. Combinations of hexane with ethyl acetate, acetone, ethanol, and ethyl methyl ketone were tested as eluents, and hexane/ethyl acetate (50:50) was found to be the most suitable mobile phase. Baseline separation of esters **1**−**3** was achieved within 15 min, and a hitherto unknown minor triterpenoidal ester (peak 4) could also be separated (**Figure 2c**). The identity and purity of peaks 1−3 in EPO samples were confirmed by electrospray ionization mass spectrometry (ESI-MS) and PDA spectra. The data for peak 4

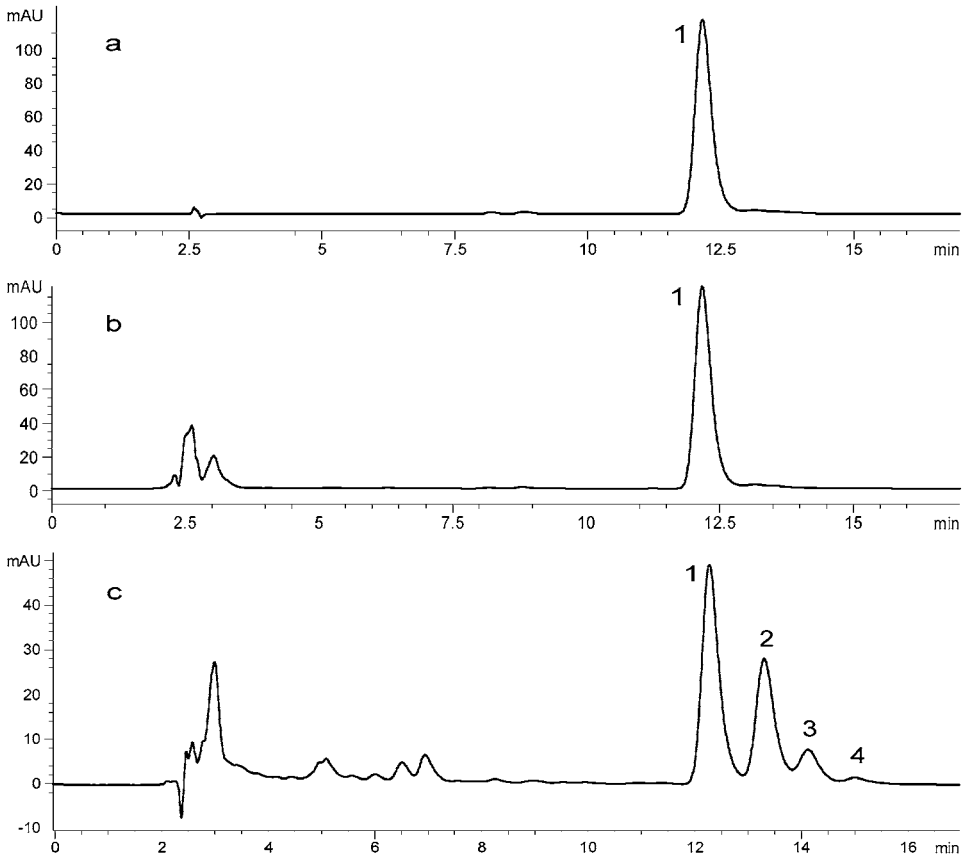

Figure 2. HPLC chromatograms of reference **1** and of cold-pressed EPO: (**a**) compound **1** dissolved in ethyl acetate; (**b**) compound **1** dissolved in EPO matrix/ethyl acetate (1:10); (**c**) representative sample of cold-pressed EPO diluted with ethyl acetate (1:10). Peaks 1–3 correspond to known esters **1**–**3**, and peak 4 corresponds to the unkown isomer mentioned under Results. Conditions: column, LiChrosorb Diol (5 μm, 250 × 4 mm i.d.); solvent, *n*-hexane/ethyl acetate (50:50); flow rate, 1 mL/min; detection, 330 nm.

were comparable to those of **1**−**3** (*5*), and the compound was, therefore, an isomer of these esters.

Injection of reference compounds in ethyl acetate or in a triglyceride matrix [raffinated EPO with an ester content below the limit of detection (LOD), diluted with ethyl acetate] showed that retention times and response factors for analytes were identical. Typical chromatograms for compound **1** are shown in panels a and b, respectively, of **Figure 2**. Specificity of the HPLC assay was assessed with the aid of ESI-MS and PDA spectra. Peaks 1−3 in EPO oil samples appeared to be homogeneous, and the spectra corresponded with reported data (*5*). Robustness of the method was tested with HPLC columns from two different batches and with solvents of differing qualities. The stability of the solutions of reference compounds was determined. Storage for 6 weeks at 4 °C led to a decrease of 0.47% as compared to the values obtained with the freshly prepared solutions. The repeatability of the assay was assessed with five samples of cold-pressed EPO from *O. biennis*. On three consecutive days, samples were weighed, freshly diluted, and analyzed in triplicate. The means of the standard deviation (SD) of the triplicate analyses were 0.15% on the first day and 0.14 and 0.33% on the second and third days, respectively. The relative SDs of all nine analyses for each oil sample were between 0.63 and 1.23%. Injection of 5 ng of ester **1** afforded a signal-to-noise (S/N) ratio of 11:1. When EPO samples were analyzed, the limit of quantification (LOQ) was at 10 ng per injection, corresponding to 0.5 mg/100 mL EPO. Calibration curves for reference compounds **1**−**3** with and without oil matrix were virtually identical and showed linearity ($R^2 = 0.9997-1$) in the range of 2 μg−8 ng on column.

Analysis of Commercial EPO Samples. We conducted a survey of 22 EPOs (A−V) of differing qualities, which were provided by various German suppliers. Among these, 16 oils (F−W) were labeled as raffinated or partially raffinated, 5 samples (A−E) were cold-pressed EPOs, and one (V) was an oil obtained by extraction with carbon dioxide. The contents in esters **1**−**3** in these oil samples are shown in **Figure 3**. In 9 of 16 raffinated oils, the analytes were either not detectable or below the LOQ (<0.5 mg/100 g of EPO). The remaining 7 samples had an average total ester content of 4.7 mg/100 g. The average ester content in cold-pressed oils was 58 mg/100 g, whereas the carbon dioxide extracted oil contained 27 mg/100 g (data not shown in **Figure 3**).

The relative amounts of compounds **1**−**3** varied to a certain extent. In general, **1** was the most abundant, followed by **2**, whereas ester **3** usually was the least important (**Figures 3** and **4a**). When the concentrations of esters **1** and **2** relative to compound **3** were considered, the following picture emerged (**Figure 4b**): the ratio of esters **1** to **3** showed a high degree of variation, ranging from 1.6:1 to 3.5:1. The ratio of esters **2** to **3**, in contrast, was remarkably constant at approximately 2.1:1 in the cold-pressed oils. The minor peak 4 was detected in all cold-pressed EPOs.

Influence of the Extraction Conditions on Concentration of Caffeoyl Esters. EPOs obtained under defined conditions from seeds of *O. biennis* and *O. lamarckiana* were analyzed for their content in esters **1**−**3**. *O. biennis* is the economically more important species for production of EPO, but oils obtained from *O. lamarckiana* and from *Oenothera* hybrids are equally commercialized as EPO. In the case of *O. lamarckiana* seeds,

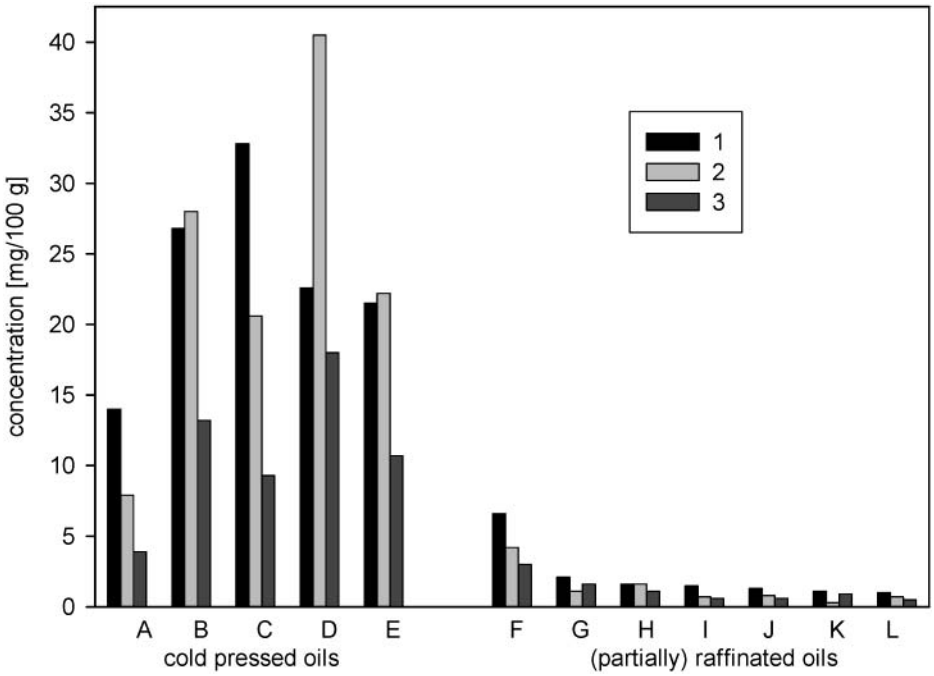

Figure 3. Concentrations of esters **1**–**3** in commercially available EPOs (samples A–L). Data of nine raffinated oils (M–U) with ester concentrations below LOQ (0.5 mg/100 g) and of the sample from carbon dioxide extracted oil (V) are not shown.

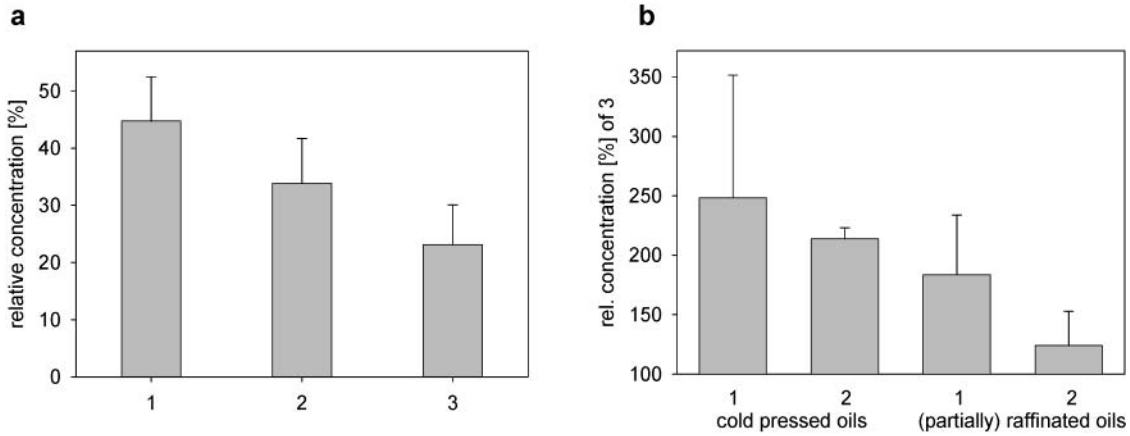

Figure 4. Pattern of caffeic acid esters in EPO samples shown in **Figure 3**: (**a**) relative concentrations (± SD) of **1**–**3** expressed as percentage of total ester content; (**b**) relative concentrations (± SD) of **1** and **2** as a percentage of **3**.

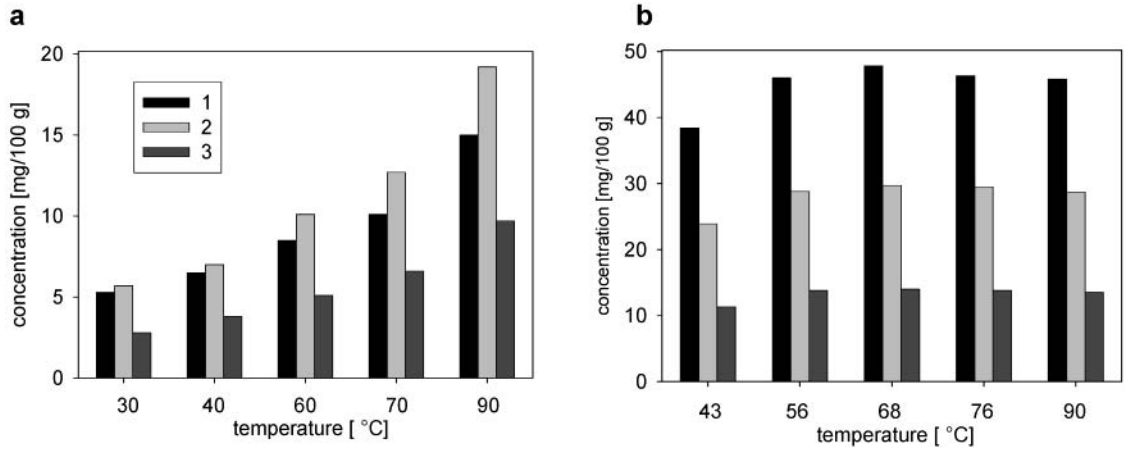

Figure 5. Effect of increasing temperature during the extraction process on the concentration of esters **1**–**3** in the oil: (**a**) *O. lamarckiana*; (**b**) *O. biennis*.

the content of total esters in the oil increased from 14 to 44 mg/100 g when the temperature was raised from 30 to 90 °C (**Figure 5a**). No temperature dependency was observed in the case of *O. biennis* seeds (**Figure 5b**). The yields remained almost constant over the entire temperature range of 43−90 °C. The total ester content, however, was significantly higher

(average of 86 mg/100 g of oil). The two *Oenothera* species also differed with respect to the relative abundance of the triterpenoidal esters **1**−**3**. Although the ratio of **1** to **3** was 1.7:1 on average for the oil samples obtained from *O. lamarckiana*, the ratio in *O. biennis* oils was approximately 3.4:1. The ratio of **2** to **3** was approximately 2:1 in the oils of both species (**Figure 5**). Peak 4 was only a trace component in *O. lamarckiana*, whereas it constituted 2.7% of the total peak area for the esters in case of the *O. biennis* oils (data not shown).

Scanning electron microscopic (SEM) and fluorescence microscopic photographs of seeds were taken in an attempt to clarify the differing behaviors in the extraction experiments. Fluorescence microscopy of cross sections revealed that the characteristic blue fluorescence of caffeic acid derivatives **1**−**3** was homogeneously distributed across the embryo (Figure 1, Supporting Information). Differences were observed, however, in the histology of the testa (Figure 2, Supporting Information). Seeds of *O. biennis* had a mesotesta of irregular thickness reaching up to several layers, whereas the mesotesta of *O. lamarckiana* was less important and of a regular thickness. SEM of the seed surface (Figure 3, Supporting Information) showed differences in surface pattern of the exotesta.

DISCUSSION

A isocratic normal-phase HPLC assay for the quantitative determination of 3-*O*-*trans*-caffeoyl derivatives of betulinic, morolic, and oleanolic acid (**1**−**3**) in EPO has been developed and validated. The method proved to be simple and robust. There was no need for sample prepurification prior to analysis, because the triglyceride matrix eluted near the solvent front under the conditions used. Retention times of analyte peaks remained very stable, and we carried out up to 100 consecutive injections without need of an intermediate column wash. The selectivity of the Diol column was superior to that of RP materials tested in the assay development and enabled the baseline separation of a fourth isomeric caffeoyl ester, which was previously not detected. The advantages of normal-phase HPLC analysis of lipophilic compounds in triglyceride matrices have already been observed by Balz et al. (*8*, *9*), who successfully analyzed complex mixtures of isomeric tocopherols and related compounds on Diol columns without sample prepurification. Reports on the coupling of normal-phase HPLC with ESI-MS analysis are scarce and have mainly dealt with analysis of complex lipids (*10*, *11*). The mobile phases used in these separations, however, contained a certain proportion of water and other polar modifiers such as 2-propanol and formic acid. In our case, which required a nonaqueous, hexane/ethyl acetate mixture for separation, we found that the postcolumn addition of a polar modifier (acetonitrile with 2% of acetic acid) produced ESI spectra that were comparable to those previously recorded in RP mode (*5*). Hence, this approach may extend the ESI-MS analysis to HPLC separations requiring highly lipophilic eluents and the absence of water.

Analysis of commercially available EPO samples showed significant differences in their content in compounds **1**−**3**. Most striking was that the esters were almost quantitatively removed by the raffination processes, which are commonly used for the decoloration and desodorization of EPO. On the other hand, the esters proved to withstand temperatures of up to 90 °C during mechanical extraction of EPO. The ratio of compounds **1**−**3** remained constant over the temperature range examined, indicating that no conversion between the esters occurred. Whether the differing behaviors of *O. biennis* and *O. lamarckiana* seeds in the model extractions were due to the obvious histological differences of the testa needs further clarification by repeated experiments with various seed materials from defined cultivars. An extensive microscopic study of the seed coat anatomy in the genus *Oenothera* has been published (*12*) but, unfortunately, did not include the two species discussed here. It appears, however, that the relative abundance of caffeoyl esters **1**−**3** in the two *Oenothera* species differs substantially. Hence, there is a possibility for distinguishing the origin of EPOs on the basis of these patterns. The concentration of total esters in oil from *O. biennis* was significantly higher.

The possible implication of caffeoyl esters **1**−**3** in the clinical effects of EPO presently remains open to conjecture. Typically, oils used in intervention studies have been characterized only with respect to their content in γ-linolenic acid (*1*, *13*). Other sources of γ-linolenic acid, such as seed oils of *Ribes nigrum* (black currant) and *Borrago officinalis* (borage), reportedly were less effective at the same dosage (*14*−*17*), suggesting that additional constituents could be involved in the beneficial effects of EPO. Daily doses of a few hundred milligrams up to 5 g of γ-linolenic acid have been administered in human intervention studies (*13*). If taken as cold-pressed EPO, a daily intake of a few milligrams up to 45 mg of total caffeoyl esters would be achieved, corresponding to 0.1−1 μmol/kg of body weight. The IC_{50} values of **1**−**3** against cyclooxygenases 1 and 2 and against leukocytic elastase are in the sub-micromolar to low micromolar range (*5*). Thus, it appears plausible that these compounds could play a role in the context of usage of cold-pressed EPO. Data on the resorption, distribution, and metabolism of these compounds are, however, needed for an adequate assessment. Notwithstanding these considerations on their pharmacological relevance, the caffeoyl esters are natural antioxidants (*18*) that can efficiently protect the polyunsaturated fatty acids in nonraffinated EPO from autoxidation.

ABBREVIATIONS USED

EPO, evening primrose oil; NTG, non-triglyceride fraction; ESI, electrospray ionization; RP, rversed-phase; SPE, solid-phase extraction; PDA, photodiode array detector; LOD, limit of detection; LOQ, limit of quantification; SEM, scanning electron microscopy.

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Supporting Information Available: Fluorescence microscopic photographs and SEM of *O. biennis* and *O. lamarckiana* seeds. This information is available free of charge via the Internet at http://pubs.acs.org.

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