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

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Lipophilic triterpenoidal esters with radical scavenging and cyclooxygenase inhibitory properties were recently found in cold-pressed, nonraffinated evening primrose oil (EPO). A quantitative assay for the analysis of 3-*O*-*trans*-caffeoyl derivatives of betulinic, morolic, and oleanolic acid in evening primrose seeds was developed and validated. Extraction efficiency >99% was achieved by means of pressurized liquid extraction with two extraction cycles and 80% (v/v) ethanol at 120 °C. Analysis of esters was by normal-phase high-performance liquid chromatography on a Diol column and hexane/ethyl acetate (containing 0.1% formic acid) (65:35) as the eluent. The analytes were determined without further prepurification. Seeds from defined cultures of *Oenothera biennis*, *Oenothera lamarckiana*, and *Oenothera ammophila*, grown under identical conditions, were analyzed. The cultures originated from seeds from eight collections in the wild and from selections from five cultivars. The content of total triterpenoidal esters in seeds varied between 1.34 and 2.78 mg/g. Three types of qualitative patterns were observed for the triterpenoidal esters. The influence of different harvest times and plant treatments was studied with the cultivar Anothera. Variations between 1.5 and 2.3 mg/g were found.

KEYWORDS: Evening primrose oil; *Oenothera biennis*; *Oenothera lamarckiana*; *Oenothera ammophila*; Onagraceae; caffeoyl ester; triterpenoid; pressurized liquid extraction; accelerated solvent extraction; HPLC; radical scavenger; anti-inflammatory

INTRODUCTION

Evening primrose oil (EPO) has been used for more than 30 years as a dietary supplement because of its high concentration of polyunsaturated fatty acids, in particular in γ-linolenic acid (18:3n-6) (*1*). Beneficial effects have been reported in various ailments, such as rheumatic and arthritic conditions, atopic dermatitis, premenstrual and postmenopausal syndrome, and diabetic neuropathy (*2*−*14*), and mechanisms of action of polyunsaturated fatty acids have been studied in animal models (*15*, *16*) and in humans (*17*, *18*). As part of our ongoing investigations on natural products with anti-inflammatory (*19*−*22*) and radical scavenging properties (*23*), we recently discovered three caffeoyl derivatives of betulinic, morolic, and oleanolic acid (**1**−**3**; **Figure 1**) in cold-pressed, nonraffinated EPO (*24*). The triterpenoidal esters strongly inhibited cyclooxygenases 1 and 2 (*25*) and human leucocytic elastase in vitro (*26*). Given that these lipophilic caffeoyl derivatives could possibly contribute to some of the beneficial properties reported for EPO, we carried out a quantitative survey of commercially available oils (*27*). Significant amounts of **1**−**3** were found only in cold-pressed oils. In raffinated oils or EPOs obtained by CO_2 extraction, the esters were present in traces or even below the limit of detection.

We were able to show that the triterpenoidal esters are localized in the endosperm of the seeds (*27*). However, their content in the seeds has not been investigated up to now. In an extension of our survey of triterpenoidal esters in oil samples, we here report on the development of a quantitative assay for the analysis of seeds. We also report data on the content in seeds of selected cultivars and samples originating from wild collections and on possible effects of harvest time and preharvest treatments.

MATERIALS AND METHODS

Chemicals and Reference Compounds. Analytical grade dichloromethane was purchased from Riedel de Haën (Germany), and high-performance liquid chromatography (HPLC) grade ethanol, *n*-hexane, methanol, and ethyl acetate were obtained from Scharlau Chemie (Germany). Calcinated and purified kieselgur was from Riedel de Haën.

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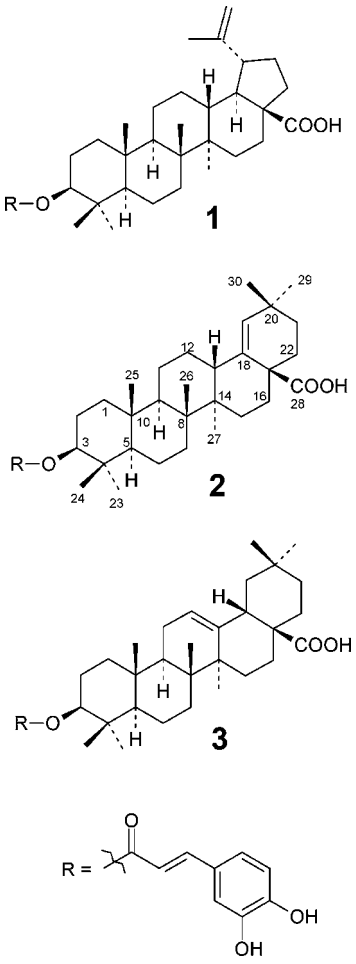

Figure 1. Chemical structures of caffeoyl esters of betulinic (**1**), morolic acid (**2**), and oleanolic acid (**3**).

Compounds **1**−**3** were isolated from cold-pressed EPO according to a procedure previously reported (*24*), with minor modifications: An enriched fraction containing triterpenoidal esters was obtained from the oil as a crude mixture by flash chromatography on a 40 mm × 150 mm i.d., 40−63 μm Chromabond silicagel cartridge (Büchi, Flawil, Switzerland) with a step gradient of ethyl acetate in *n*-hexane (0:1 → 1:0). Final purification of **1**−**3** was performed by semipreparative HPLC on a 10 mm × 250 mm i.d., 10 μm, LiChrosorb Diol column (Merck) and a 44:56 mixture of ethyl acetate (containing 0.01% formic acid) and *n*-hexane. The flow rate was 5 mL/min. Detection was at 340 nm. The purity of **1**, used as a reference compound for the quantification of **1**−**3**, was determined by HPLC to be 96.8%.

Plant Material and EPO. Cold-pressed EPO and the corresponding seed material from an *Oenothera biennis* cultivar (EP-1) were obtained from BiRo-GbR (Sommerschenburg, Germany). Further seeds samples were from selections made from three other *O. biennis* cultivars (EP-2 to EP-4), from an *Oenothera ammophila* cultivar (EP-5), and from *O. biennis* Anothera. All of the plants were grown on experimental plots at Pharmaplant GmbH (Artern, Germany).

Additional samples were from plants grown from seeds collected at different locations across Germany. These wild collections were carried out at Basdorf, Dornbusch, Rostock, Berlin, Hamburger, Greifswald, Densow, and Soltau. The seed sample from *Oenothera lamarckiana* was also from Pharmaplant. These plants were all grown on experimental plots at Pharmaplant under conditions that were identical to those for the selections from cultivars. Further details on agricultural and harvest conditions are given in the Supporting Information.

The influence of harvest time and preharvest treatment was investigated with seeds of the cultivar Anothera grown in 2004 on an experimental field of the University of Giessen at Gross-Gerau. Harvest times were on September 30 (early), October 21 (intermediate), and November 1 (late). Desiccation treatment of plants to terminate photosynthesis of plant and accelerate ripening of seeds was with a herbicide (deiquat) or by flame with a propane burner. Further details are given in the Supporting Information.

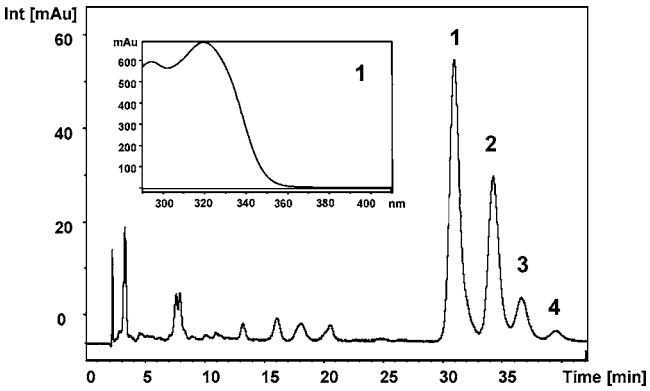

Figure 2. HPLC chromatogram of a seed extract of *O. biennis* sample EP-1. Peaks 1–3 correspond to known esters **1**–**3**, and peak 4 corresponds to an unkown isomer.

Pressurized Liquid Extraction (PLE). Extraction was performed on a ASE 200 extractor (Dionex, Sunnyvale, United States). The pressure was set at 120 bar, and the extraction cycle time was at 5 min. Different solvents [dichloromethane, *n*-hexane, methanol, and ethanol/water (80:20)] were tested. The temperature (50−130 °C) and number of extraction cycles (1−5) were optimized.

Prior to extraction, aliquots of seed material were cryomilled (0.75 mm) in an ultracentrifugal mill (Retsch ZM1, Haan, Germany) under continuous cooling with liquid nitrogen. For each extraction experiment, 1.00 g of ground seeds was mixed with approximately 5 g of kieselgur and packed into a 22 mL extraction cartridge. With the exception of the last step of the factorial optimization process, extracts obtained from a sample by multiple extraction steps were pooled together and evaporated to dryness. The residues were redissolved in ethyl acetate (1.0 mL). In the third step of optimization, the extracts of each extraction cycle were evaporated separately. Prior to HPLC analysis, the dry residues resulting from cycles 1, 2, and 3−5 were redissolved in 5.0, 2.0, and 1.0 mL of ethyl acetate, respectively.

HPLC Analysis. Analyses were performed on an Agilent 1100 system consisting of a degasser, a quaternary pump, and a diode array detector. A Gilson liquid handler 215 was used as an autosampler. Separation was performed on a 4.0 mm × 125 mm i.d., 5 μm, LiChrospher Diol column (Merck, Darmstadt, Germany). Samples used for the optimization of extraction conditions were separated with *n*-hexane/ethyl acetate (containing 0.1% formic acid) (56:44) as a mobile phase. The injection volume was 30 μL, and the flow rate was 1.0 mL/min. For the repeatability assessment of the extraction method and quantitative HPLC analyses of the different seed samples, *n*-hexane/ethyl acetate (containing 0.1% formic acid) (65:35) was used as the mobile phase. The injection volume was 20 μL, and the flow rate was 1.0 mL/min. Chromatograms were recorded at 330 nm, while photodiode array detector (PDA) spectra were measured from 290 to 450 nm. Data processing was carried out with HyStar 3.0 software (Bruker Daltonic, Bremen, Germany).

Calibration Curve. Compound **1** was used for the quantification of all triterpenoid esters. A stock solution (5.11 mg/mL) was prepared in ethyl acetate. Dilutions were made in ethyl acetate over a concentration range of 1.28−0.05 mg/mL. The calibration curve was as follows: $y = 1874.7x$, $R^2 = 0.9884$.

Repeatability of Extraction Method. Repeatability of the optimized extraction procedure was assessed with seeds of cultivar EP-1 (BiRo-GbR). Six samples were independently extracted and analyzed with HPLC-PDA. The standard deviation was found to be 5.5%. Repeatability of the HPLC method was determined by repeating the analysis of the same sample six times; the standard deviation was 3.0%.

RESULTS

Development of the Extraction Procedure. Seeds from the cultivar EP-1 were used for the experiments. A representative

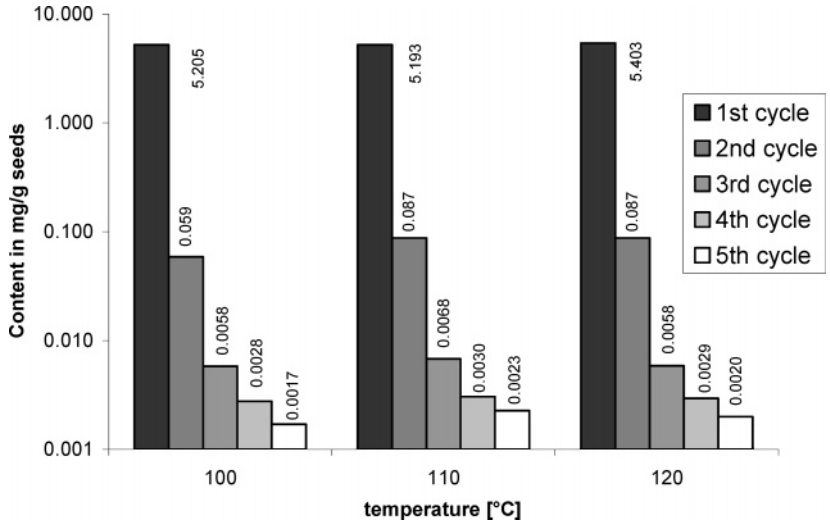

Figure 3. Final optimization and validation step of the PLE extraction procedure. Yields in TTE obtained at 100, 110, and 120 °C in five extraction cycles are shown. Each static extraction cycle was 5 min. Default settings for preheating and rinsing were used. A logarithmic scale of the *y*-axis is used for improved clarity.

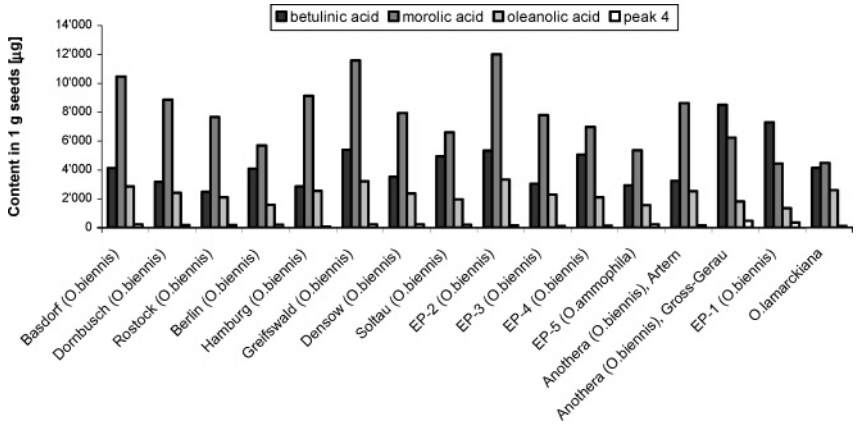

Figure 4. Content of triterpenoidal esters in seed samples from cultivars and wild collections. The individual contents of esters **1**–**3** are expressed as mg/g of seeds.

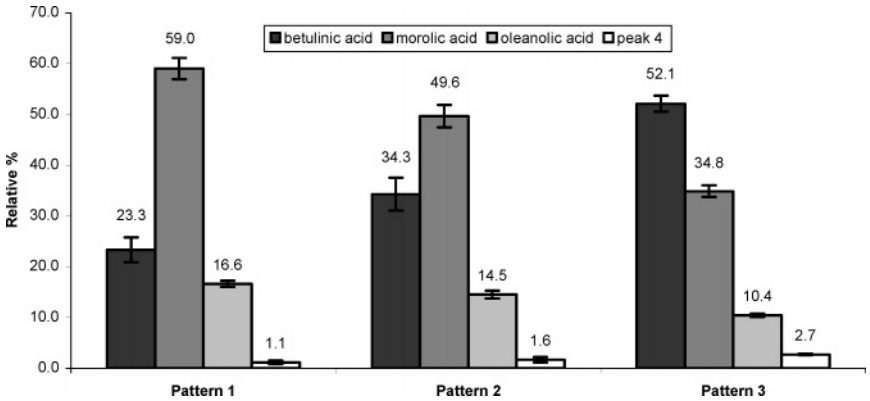

Figure 5. Patterns observed for **1**–**3** and for a minor ester (peak 4) in the analyzed seed samples.

HPLC chromatogram of a seed extract is shown in **Figure 2**. Eighty percent aqueous ethanol as solvent gave the highest extraction yield. Temperature variations from 50 to 150 °C showed a maximum of extraction efficacy between 110 and 130 °C. The relative yields of five consecutive extraction cycles at temperatures of 100, 110, and 120 °C are shown in **Figure 3**. It appeared that >99% of triterpenoidal esters **1**−**3** was extracted with two extraction cycles of 5 min. The SD of the assay was 5.5%.

Analysis of Seed Samples. We conducted a survey of 16 seed samples. They represented selections from different cultivars and wild collections from various locations in Germany. The content in triterpenoidal esters **1**−**3** is shown in **Figure 4**. Overall, the content of total triterpenoidal esters (TTE) varied between 1.5 and 2.8 mg/g. No differences were observed between the seeds from wild collection and the selections from cultivars. The highest contents were found for *O. biennis* collected in Greifswald (wild collection) and the selection from

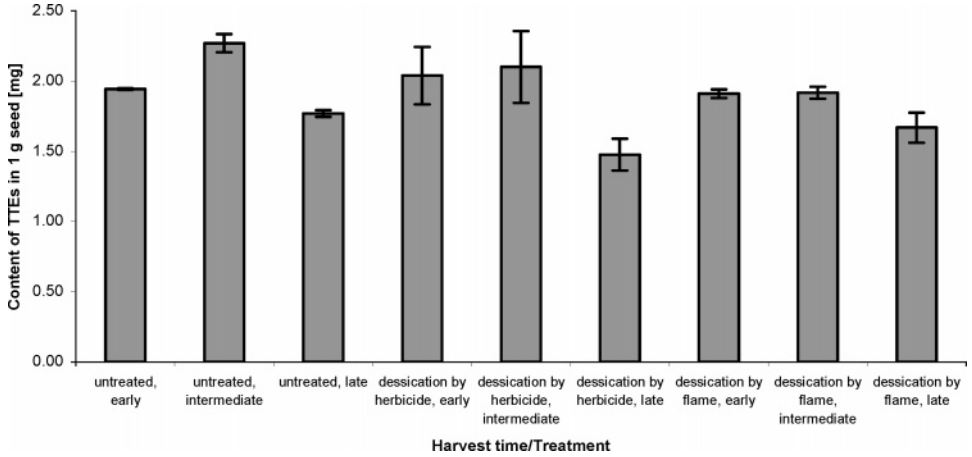

Figure 6. Influence of harvest time and preharvest treatment on content of TTEs in seeds obtained from Anothera. Harvest times were September 30, 2004 (early), October 21, 2004 (intermediate), and November 1, 2004 (late).

the *O. biennis* cultivar EP-2. The contents in seeds samples of *O. biennis* appeared higher that in seeds derived from *O. lamarckiana* and *O. ammophila* (EP-5), although only one sample of each of the latter species was analyzed. In a majority of seed samples, the caffeoyl ester of morolic acid (**2**) was dominant, whereas the betulinic acid derivative (**1**) was the major compound. In all cases, the ester of oleanolic acid (**3**) was third, and the peak of a fourth, nonidentified isomer was present in traces only in all seed samples.

According to the quantitative patterns, the samples could be grouped into three categories (**Figure 5**): Seeds in group I contained ester **2** as major constituent. These include wild collections from Basdorf, Dornbusch, Rostock, Hamburg, Greifswald, and Densow and selections from cultivars EP-2, EP-3, and Anothera (grown in Artern). In group II, compound **2** was still the dominant ester, but the difference to the content of **1** was much lower. Seeds from wild collections in Berlin and Soltau and selections from cultivars EP-4 and EP-5 were in this group. In group III, ester **1** was the major compound. Seeds from Anothera (grown in Gross-Gerau) and EP-1 were in this group. A unique pattern was observed for *O. lamarckiana*, which did not fit into any of these groups. Here, compounds **1** and **2** were almost equal, and the relative proportion of **3** was higher than in groups I−III.

We determined the extraction efficiency for TTE in the obtention of EPO. For that purpose, seeds of EP-1 were submitted to mechanical pressing at 50 °C. While the content of TTE in seeds was 0.18%, the concentration in the oil was only 0.05%.

Influence of Harvest Time and Preharvest Treatment. Plants of the cultivar Anothera were grown on experimental plots under identical conditions. Seeds samples were collected with different harvest times and preharvest treatments. The contents of TTE varied between 1.5 and 2.3 mg/g. Preharvest treatment to accelerate and synchronize seed maturation had no influence on the content of TTE when compared to seeds from nontreated plants (**Figure 6**). A trend was observed concerning the harvest time. Seeds obtained from a late harvest always had the lowest content of TTE regardless of the preharvest treatment.

DISCUSSION

A PLE extraction process for the quantitative analysis of anti-inflammatory and radical scavenging triterpenoidal esters in evening primrose seeds has been developed and applied to a study of seed material from cultivars and wild collections. Interestingly, optimal extraction was at a rather high temperature of 120 °C. Usually, temperatures around 70 °C (*28*−*32*) are used in PLE procedures. The experiments carried out in the assay development, however, clearly showed that such high temperature can be suitable even for structurally complex natural products with chemically labile moieties such as an ester linkage and an *o*-diphenol. The extraction was highly effective, as >99% of yield was obtained with two extraction cycles of 5 min. The extracts could be analyzed by isocratic normal-phase HPLC on a Diol column without any prepurification. To avoid possible peak interferences with the compounds of interest, the mobile phase composition was slightly modified as compared to that previously used for analysis of the oil samples (*27*).

The survey of TTE in seeds showed only moderate differences in the content across the 16 samples analyzed here and no distinction between seeds originating from wild collection or from cultivars. Given that the seeds were produced on experimental plots under identical conditions, the influence of exogenous factors on the TTE content could be excluded. A plausible explanation is that the selection of *Oenothera* cultivars has not been directed toward this particular trait, which remains, therefore, at the level of a wild plant. The qualitative pattern in all samples was found to be identical, but distinct quantitative differences were observed, which allowed the samples to be grouped into three categories. Besides the three previously known esters **1**−**3**, we consistently observed a fourth, isobaric, minor ester, which awaits full structural characterization.

A comparison of TTE in seeds and in nonraffinated EPO obtained by mechanical pressing showed that the concentration in the oil was substantially lower (28%). Visibly, the extraction process was not very efficient for these compounds. The extraction experiments for optimizing the PLE protocol showed that these moderately polar compounds were poorly extracted with solvents of low to intermediate polarity such as hexane and ethyl acetate and were readily extractable only with polar solvents such as ethanol. An explanation for this behavior may be in the *o*-diphenol structure of the caffeoyl moiety, which is prone to hydrogen bond interactions with polar biopolymers of the cell wall. Such interactions can only be effectively disrupted by polar solvents (*33*, *34*).

There is increasing evidence that nontriglyceridic compounds with antioxidant properties are involved in the beneficial effects observed in epidemiological and intervention studies with some vegetable oils (*35*, *36*). We had previously estimated that the amounts of TTE ingested could be such as to be relevant for a

pharmacological activity, if cold-pressed EPO was consumed in amounts used in the published intervention studies (*27*). Given that only a portion of TTE is extracted by mechanical pressing, improved processes could substantially increase their concentration in the final product. Also, the possibility of breeding for *Oenothera* cultivars with a high yield in TTEs has not yet been explored.

ABBREVIATIONS USED

EPO, evening primrose oil; PLE, pressurized liquid extraction; TTE, total triterpenoidal esters; PDA, photodiode array detector.

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Supporting Information Available: Information on agricultural and harvest conditions of the samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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