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Process Development of *Lappula squarrosa* Oil Refinement: Monitoring of Pyrrolizidine Alkaloids in Boraginaceae Seed Oils

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Abstract Boraginaceous plants are characterized by high levels of polyunsaturated fatty acids and show a high ratio of ω-3/ω-6 fatty acids. In addition, *Lappula squarrosa* (Boraginaceae) shows high levels of stearidonic acid content (ω-3; 18:4; 6,9,12,15-octadecatetraenoic acid) showing interesting medical and health promoting properties. On the other hand Boraginaceous plants contain genotoxic and carcinogenic pyrrolizidine alkaloids (PA). An HPLC–ESI–MS/MS sum parameter method was developed to monitor the total sum of 1,2-unsaturated PA in seed oil. The method was used to monitor different steps in oil refinement using lab model experiments and pilot scale refinement of *L. squarrosa* seed oil. A limit of detection and a limit of quantification (LOQ) of 0.02 μg retronecine equivalents (RE)/kg and 0.05 μg RE/kg were achieved, respectively. Multiple washing steps at pH 2.2 can significantly reduce the PA content to 0.07 % of the start value. In addition, combining washing with neutralization, bleaching and deodorization can reduce the PA content of *L. squarrosa* oil below the LOQ (0.05 μg RE/kg). The newly established method was further used to analyze the PA content of commercially available Boraginaceous seed oils (*Echium* spp., *Borago officinalis*). Three out of ten products were tested as PA positive (PA content ranging from not detected to 0.6 μg RE/kg product).

Keywords Pyrrolizidine alkaloids · *Lappula squarrosa* · Oil refinement · Boraginaceous seed oils · HPLC–ESI–MS/MS · Food supplements · *Borago officinalis* · *Echium* spp.

Abbreviations

$BMDL_{10}$	Benchmark dose lower confidence limit is the 95 % lower confidence limit of the benchmark dose associated with a 10 % response
ND	Not detected

Introduction

The polyunsaturated fatty acids (PUFA) α-linolenic acid (ALA; ω-3; 18:3; 9,12,15-octadecatrienoic acid) and linoleic acid (LA; ω-6; 18:2; 9,12-octadecadienoic acid) are known to be essential for humans. Starting from ALA (ω-3) or LA (ω-6) humans/animals convert these two precursors into the long chain PUFA sharing the same set of enzymes (desaturases and elongases). This results in two series (ω-3/ω-6) of long chain PUFA like eicosapentaenoic acid (EPA; ω-3; 20:5; 5,8,11,14,17-eicosapentaenoic acid), docosapentaenoic acid (DPA; ω-3; 22:5; 7,10,13,16,19-docosapentaenoic acid), docosahexaenoic acid (DHA; ω-3; 22:6; 4,7,10,13,16,19-docosahexaenoic acid) or arachidonic acid (AA; ω-6; 20:4; 5,8,11,14-eicosatetraenoic acid), respectively [1]. While long chain PUFA play an essential role as structural components of cell membranes the C_{20}-PUFA, EPA (ω-3) and AA (ω-6) can be transformed into eicosanoids including prostaglandins, prostacyclins, thromboxanes and leukotrienes, which show a multitude of health and disease modulating properties [2,

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3]. Plant oils do not contain those long chain ω-3/ω-6 PUFA and vary considerably in the content of saturated/unsaturated fatty acids as well as in the ratio of ω-3/ω-6 fatty acids. While the European Food Safety Authority (EFSA) does not recommend a specific ratio of ω-3/ω-6 fatty acids in its opinion on dietary reference values [1], some national authorities like the Deutsche Gesellschaft für Ernährung suggest a ω-3/ω-6 of 1:5 or higher as beneficial [4]. Based on Food Consumption Studies in Germany (years 1994–1998) the actual ω-3/ω-6 ratio is instead 1:7.2–8.6 in western diets [4].

Seed oils from Boraginaceous plants hold a special position concerning their fatty acid composition. They are characterized by high levels of PUFA and at the same time a high ratio ω-3/ω-6 compared to the commonly used edible oils like olive, canola or palm oil (low in PUFA) or safflower, sunflower or soybean oils (rich in PUFA but high in ω-6 LA) [5]. In addition, these Boraginaceous plants show high levels of stearidonic acid (SDA; ω-3; 18:4; 6,9,12,15-octadecatetraenoic acid). SDA is only found in few higher plant oils and has drawn some interests due to its medicinal and health promoting properties [6–9].

In humans the conversion of ALA, catalyzed by the delta-6 desaturase, is limited. Oils rich in SDA can be more efficiently converted into longer chain PUFA like EPA, DPA, or DHA [9]. Because of this, SDA rich plant seed oils are considered as valuable sources to bypass recommended EPA/DHA (ω-3) dietary intakes without further increasing overexploitation of the aquatic environment. Although transgenic varieties of soybean were developed that produce up to 30 % SDA the negative perception of transgenic products may limit their economic potential [9, 10]. Hence, natural occurring SDA-rich plants are a promising and sustainable source for the production of SDA-rich oils.

So far, approval has been given to refined *Echium plantagineum* seed oil as novel food ingredient under Regulation (EC) No. 258/97 of the European Parliament and of the Council. The SDA content of this oil is specified as "not <10 %" [11]. Typically, *E. plantagineum* seed oil shows a SDA content range of 9.6–15.5 % [12, 13].

On the other hand, Boraginaceous plants are known to contain toxic 1,2-unsaturated pyrrolizidine alkaloids (PA) [14]. PA are toxic secondary plant compounds which are constitutively expressed as a chemical defense strategy in some plant families [14]. Recently, the EFSA has published a scientific opinion on PA in food and feed [15]. Based on a 2-year carcinogenicity study of lasiocarpine in rats, it was concluded that the PA exposure from food should be as low as possible [15]. Because of the genotoxic and carcinogenic nature of 1,2-unsaturated PA the approval of refined *E. plantagineum* seed oil includes the specification of <4 µg PA/kg oil which represents the detection limit of the TLC method used in this application.

Lappula is a genus of about 50 species within the Boraginaceae family commonly found in temperate Eurasian regions. *Lappula squarrosa* is an annual or biennial plant and prefers warmth, dry and sandy soils. Its natural occurrence is rare and scattered through Central Europe. The common name European stickseed relates to the fruits which are clusters of four nutlets which are coated in hooked prickles helping in the dispersal by affixing themselves to animal coats etc. [16]. The seeds are known to contain ~17–19 % SDA [17–20]. On the other hand, *L. squarrosa* is also a PA-containing plant which needs to be considered when the oil is intended for human consumption.

Here, we report the application of an HPLC–ESI–MS/MS method which was used to monitor PA content at different stages of oil refinement. The method was finally used in terms of a preventive consumer protection to optimize and implement an effective refinement procedure for *L. squarrosa* seed oil. In addition, the PA content of several Boraginaceous plant seed oils which are commercially available on the European market for cooking, food supplements, or cosmetic purposes were analyzed.

Experimental Procedures

Chemicals

All chemicals used were purchased from Roth (Karlsruhe, Germany) and Sigma-Aldrich (Seelze, Germany) and were of HPLC grade purity or redistilled before use. Lithium aluminium hydride solution AcroSeal® (1 M in THF) and pyridine AcroSeal® were acquired from Acros Organics (New Jersey, USA). For spiking experiments and method development, the certified reference material of senecionine (Sen), senecionine-*N*-oxide (SenNOx) and seneciphylline were purchased from PhytoLab (Vestenbergsgreuth, Germany).

Sun Flower Oil as a Matrix for Spiking Experiments

Common sunflower oil for cooking and frying was obtained from a local supermarket. The absence of PA and the corresponding PA-*N*-oxides (PANO) was tested and verified before it was used as a matrix in method development to optimize the refinement process.

Lappula squarrosa Plant Cultivation and Oil Production

L. squarrosa was grown under controlled organic cultivation conditions on medium to good soils located in the region "Östliches Harzvorland" (Saxony-Anhalt,

Germany). Climate conditions: annual mean temperature is 9.0 °C, annual average rainfall below 500 mm, and elevations below 100 m. Cultivation, harvest and seed cleaning was performed with standard agricultural equipment.

Seed oil production was carried out by pressing the cleaned *L. squarrosa* seeds using a Monforts Komet S 87 G/1 (IBG Monforts Oekotec GmbH & Co. KG, Mönchengladbach, Germany) at 3.0–4.5 U/min, jet 15 mm, pressing head temperature 45–60 °C using an inert atmosphere of nitrogen. The oxygen levels in the oil collection container were below 1 % during pressing. The oil content of the *L. squarrosa* seeds ranged 19.8–23.7 %, while the oil content of the residues was 4–5 %.

Oil Refinement Procedures

Washing and Deodorization Conditions to Reduce PA Content of Plant Seed Oil

First, 1.5 mg senecionine and 1.5 mg SenNOx (stock solution in methanol) were added to 13.6 kg of commercially available sunflower oil. The mixture was homogenized for 30 min at 15 °C, yielding a PA spiked starting material. Two aliquots of 500 ml were put aside to serve as a reference material for later PA analysis.

At each step in the process development, oil samples were collected and used for PA analysis.

Washing experiments were conducted at pH values of 2.2, 3.0, 4.0, 5.0, 6.0 and 7.0 using 500 g of the PA spiked material and 50 ml of the corresponding aqueous washing solution. The appropriate pH of a citric acid-phosphate buffer solution was obtained by mixing appropriate volumes of citric acid and disodium phosphate buffers of 100 and 200 mM, respectively. The final pH was adjusted using a pH-meter (WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). Washing was performed in a stirring vessel at 85 °C for 15 min. Phases were separated after 30 min settling time and the resulting oil phase as used for PA analysis.

Identical washing experiments were conducted using *L. squarrosa* seed oil.

In addition, a lab scale refinement was conducted using a modified 2-l three-neck sulfuration flask containing a draining outlet. Following an initial washing step (1,750 ml oil and 175 ml citric acid-phosphate buffer solution pH 7.0 or 2.2, respectively), the oil fraction was neutralized using appropriate amounts of aqueous 7 % NaOH based on the total acid number (TAN). Drying and bleaching were performed in a 2-l three-neck sulfuration flask at 90 °C. Drying was achieved by stirring under vacuum (30–40 mbar) for 60 min. Additional bleaching was performed by adding 1 % Tonsil Supreme 114 FF (Süd-Chemie, München, Germany). The additive was immediately removed by pressurized filtration using a cellulose acetate filter (OE 67, 0.45 μm; Whatman International Ltd, Springfield Mill, UK) using nitrogen at a pressure of 3 bar. All steps were performed under an inert atmosphere using nitrogen gas, while residual oxygen was monitored throughout the process using an electronic oxygen sensor device (GOX 100, Greisinger Electronic GmbH, Regenstauf, Germany). The oxygen levels ranged from 1.7 to 1.9 %.

Pilot Scale Refinement of Lappula squarrosa Seed Oil

Crude *L. squarrosa* seed oil was filtered using a chamber filter press (Petkus FP 250-16/10-20 (filter cloth: PPM44/025/14/660; PETKUS Technologie GmbH, Wutha-Farnroda, Germany) and 1 % of Claracel DIC (CECA, La Garenne-Colombes, France) as filtration additive. The resulting oil (one batch 50 kg) was further processed in an oil refinement mini plant (ÖHMI Engineering GmbH, Magdeburg, Deutschland) using nitrogen as the inert atmosphere throughout the whole process. The different conditions for each step are briefly summarized. One washing step at pH 2.2 (5 l each); neutralization in two steps using appropriate amounts of aqueous 7 % NaOH based on the TAN followed by five washing steps with water. These steps were conducted at 90 °C and 20 min processing time for each step. Drying and bleaching including a final filtration were conducted under a vacuum (25–30 mbar). One percent Fuller's earth was added as a filtration additive. Final deodorization was achieved using a temperature of 240 °C for 65 min at 3–5 mbar.

Commercial Boraginaceous Plant Seed Oils

Ten different Boraginaceous plant seed oils and food supplement capsules containing Boraginaceous plant seed oils were purchased from several supermarkets, pharmacies and German language internet shops. The products contained either *Echium* spp. or *Borago officinalis* seed oil and the intended use was food, food supplement and cosmetics.

Sample Preparation Procedures

Sample Preparation of PA Spiking Experiments in Sunflower Oil

Twenty grams of oil was emulsified with 18 ml 0.05 M sulfuric acid. One hundred and ten nanograms seneciphylline (11 μl of a 10 μg/ml stock solution in methanol) was added as the internal standard. The mixture was shaken for 3 min, followed by centrifugation (10 min at 12,096×*g* at 22 °C). The oil phase was extracted one more time with

5 ml 0.05 M sulfuric acid. The aqueous phases were combined and 8 ml dichloromethane was added. The mixture was centrifuged (2 min at 12,096×*g* at 4 °C) and the aqueous phase was separated (pipetted off) and extracted with 8 ml dichloromethane again. Then, 0.5 g zinc dust was added to convert the PA-*N*-oxide to the corresponding tertiary PA. This mixture was stirred for 3 h at room temperature. Afterwards, the mixture was alkalized with 4 ml NH_4OH_{conc} to a pH value of around 12 and applied on a 20-g Isolute®-column (Biotage, Eke, Belgium). After an exposure time of 20 min the PA were eluted with 2 × 40 ml dichloromethane and the organic phase was dried under a stream of nitrogen gas. After redissolving in 0.5 ml deionized water and 0.5 ml acetonitrile the sample was directly analyzed by HPLC–ESI–MS/MS.

Sample Preparation of PA Spiking Experiments in Lappula squarrosa Oil

The absence of senecionine, SenNOx and seneciphylline was confirmed prior to the method development.

A similar sample preparation was performed on *L. squarrosa* oil as described above for sunflower oil. Differences were extraction three times, first with 15 ml then two times using 7.5 ml of 0.05 M sulfuric acid.

Sample Preparation for Lab and Pilot Scale Refinement of Lappula squarrosa Oil

The sample work-up was based on the recent GC–MS sum parameter method [22, 23]. Major modifications to simplify and to increase time efficiency were introduced and are briefly described here.

Two to 20 g *L. squarrosa* oil (depending on the expected PA content of the sample) were emulsified with 6–10 ml 0.05 M sulfuric acid. 7-*O*,9-*O*-dibutyroyl-[9,9-$^2H_2$]-retronecine was added as an internal standard (30–100 ng according to the expected PA content) from a 2 µg/ml stock solution in methanol. The mixture was shaken for 3 min and the two phases were separated by centrifugation (10 min at 12,096×*g* at 22 °C). The extraction was repeated two more times with 6 ml 0.05 M sulfuric acid each. The combined aqueous phases were filtered through glass-wool onto a solid phase extraction cartridge (SCX-SPE, 6 ml/500 mg, Phenomenex, Aschaffenburg, Germany). Pre-conditioning of the SPE cartridge was performed by 6 ml methanol and 6 ml 0.05 M sulfuric acid. The loaded column was first washed with 12 ml deionized water followed by 12 ml methanol. Elution (flow: 1 ml/min) of the target compounds was accomplished with 3 × 6 ml ammoniacal methanol (6 ml NH_4OH_{cc} in 100 ml methanol). The eluate was dried under a stream of nitrogen gas and redissolved in 50 µl methanol. Six hundred microliters $LiAlH_4$ (1 M in THF) were added and the mixture was sealed, immediately vortexed and kept at 4 °C for 30 min. After that, the reaction was stopped by adding 1 ml dichloromethane and 200 µl NaOH (10 %). 0.5 g sodium sulfate was added and the mixture was briefly vortexed. The organic phase was extracted by filtrating through glass wool. The extraction was repeated three more times with 6 ml dichloromethane each time. The resulting organic phases were dried under a stream of nitrogen. For derivatization, the residue was redissolved with 0.06 g phthalic acid in 0.3 ml pyridine and kept at 85 °C for 60 min. After a cool-down-period of 2 min the reaction was stopped by adding 0.7 ml deionized water. The vortexed sample was measured by HPLC–ESI–MS/MS the same day.

Sample Preparation of Commercial Boraginaceous Plant Seed Oils

The same sample work-up was performed with commercial Boraginaceous plant seed oils and food supplement capsules as described above (see "Sample Preparation for Lab and Pilot Scale Refinement of *L. squarrosa* Oil") In the case of food supplement capsules the capsules were either cut open with a scalpel to press out the oil or they were directly mixed with 0.05 M sulfuric acid and shaken till the soft capsule burst open.

Analytical Methods

HPLC–ESI–MS/MS Method for PA Spiking Experiments in Sunflower Oil and Lappula squarrosa Oil

Recently, an HPLC–ESI–MS/MS method was developed and validated to quantitate PA in a plant leaf matrix [21]. This method and sample preparation were modified to analyze PA in a Boraginaceae oil matrix. An HPLC (Agilent 1200 Series, Waldbronn, Germany) was coupled to a 3200 QTrap mass spectrometer (Applied Biosystems MDS Sciex, Darmstadt, Germany). A Gemini-NX C18 column (150 mm × 2.0 mm, Phenomenex, Aschaffenburg, Germany) with a particle size of 3 µm and a pore size of 110 Å was used for chromatographic separation. The gradient of two solvents (A: 25 mM ammonium bicarbonate solution, B: acetonitrile) was used to separate the target compounds (flow rate 250 µl/min): 0–3 min (95 % A), 3–28 min (95–0 % A), 28–31 min (0 % A), 31–32 min (0–95 % A), 32–42 min (re-equilibration 95 % A).

Ionization was in the ESI-positive mode. Quantification of the analytes and internal standard was performed by using the MRM mode (multi reaction monitoring) with the following parameters:

Curtain gas 42 psi, ionization voltage 4,500 V, ion source temperature 450 °C, gas 1:50.0 psi, gas 2:35.0 psi, collisionally activated dissociation gas: medium, declustering potential 71.0 V, entrance potential 8.0 V, collision cell exit potential 5.0 V.

Four transitions for the analytes and three transitions for the internal standard were selected:

For the first analyte (senecionine): 336.0 amu → 94.0 amu [cell entrance potential (CEP) 19.55, collision energy (CE) 50.0], 336.0 amu → 120.0 amu (CEP 19.55, CE 35.0), 336.0 amu → 138.0 amu (CEP 19.55, CE 35.0) and 336.0 amu → 308.0 amu (CEP 19.55, CE 35.0).

For the second analyte (SenNOx): 352.0 amu → 94.0 amu (CEP 20.0, CE 60.0), 352.0 amu → 120.0 amu (CEP 20.0, CE 40.0), 352.0 amu → 136.0 amu (CEP 20.0, CE 40.0) and 352.0 amu → 324.0 amu (CEP 20.0, CE 35.0).

For the internal standard (seneciphylline): 334.0 amu → 94.0 amu (CEP 19.5, CE 50.0), 334.0 amu → 120.0 amu (CEP 19.5, CE 35.0) and 334.0 amu → 138.0 amu (CEP 19.5, CE 35.0).

Analyst 1.4.2 Software (Applied Biosystems MDS Sciex, Darmstadt, Germany) was used for data analysis and integration.

HPLC–ESI–MS/MS Method for Lab and Pilot Scale Refinement of Lappula squarrosa oil and Commercial Boraginaceous Plant Seed Oils

The same HPLC–ESI–MS/MS system was used as described above. Here, a Discovery® HS F5 column (150 mm × 2.1 mm, particle size 3 µm; Sigma Aldrich, Seelze, Germany) including a pre-column cartridge of the same material was used. The following gradient of two solvents (A: 0.1 M formic acid in water, B: acetonitrile) was applied with a flow rate of 250 µl/min: 0–3 min (95 % A), 3–13 min (95–0 % A), 13–18 min (0 % A), 18–19 min (0–95 % A), 19–29 min (re-equilibration: 95 % A).

Ionization was in the ESI-positive mode. Quantification of the analytes and internal standard was performed by using the MRM mode with the following parameters:

Curtain gas 20 psi, ionization voltage 5,500 V, ion source temperature 475 °C, gas 1:50.0 psi, gas 2:70.0 psi, collisionally activated dissociation gas: high, declustering potential 71.0 V, entrance potential 8.5 V, collision cell exit potential 4.0 V.

Four transitions for the analytes and internal standard were selected:

For the analytes (retronecine/heliotridine-diphthalate): 452.0 amu → 94.0 amu (CEP 18.0, CE 50.0), 452.0 amu → 120.0 amu (CEP 18.0, CE 36.0), 452.0 amu → 149.0 amu (CEP 18.0, CE 55.0) and 452.0 amu → 304.0 amu (CEP 24.0, CE 40.0).

For the internal standard ([9,9-$^{2}H_{2}$]-retronecine-diphthalate): 454.0 amu → 96.0 amu (CEP 18.0, CE 50.0), 454.0 amu → 122.0 amu (CEP 18.0, CE 36.0), 454.0 amu → 149.0 amu (CEP 24.0, CE 55.0) and 454.0 amu → 306.0 amu (CEP 24.0, CE 40.0).

Results and Discussion

Recently, the EFSA has published an opinion on pyrrolizidine alkaloids in food and feed [15]. A $BMDL_{10}$ for an excess cancer risk of 70 µg/kg b.w./day was calculated for induction of liver hemangiosarcomas by lasiocarpine in male rats. A margin of exposure (MOE) approach by using the $BMDL_{10}$ for excess cancer risk in male rats was chosen as the reference point for comparison. The EFSA concluded that a MOE of at least 10,000 indicated that PA doses of up to 0.007 µg/kg b.w./day are unlikely to be of concern for cancer risk [15].

So far, only the PA content of phytopharmaceuticals is regulated in several European countries, e.g. the internal use of such preparations is limited in Germany to levels of 1 µg total 1,2-unsaturated PA per day (if the period exceeds 6 weeks, then 0.1 µg PA/day) or the Netherlands 1 µg PA/kg or 1 µg PA/l [22]. In the case of the refined *E. plantagineum* oil, approved as a novel food ingredient under EU Regulations [11], this product was specified to contain <4 µg PA/kg oil, which was at the same time the limit of detection (LOD) of the TLC method applied.

Our first approach was to test the influence of the pH of the washing solution in reducing the PA content of seed oil. Since the available amounts of *L. squarrosa* seed oil are limited at the moment, we initiated our preliminary experiments using sunflower oil. This matrix was spiked with a mixture of senecionine and SenNOx (ratio 1:1, 134 µg/kg total PA) to cover for the polarity difference of both naturally occurring PA forms. As shown in Fig. 1a, there is a clear increase in PA reduction at lower pH values and the efficiency can be enhanced if the washing is repeated (Fig. 1b; shown for pH 2.2). Starting with Sen/SenNOx levels of 134 µg/kg oil, which is close to natural occurring levels in crude seed oils of Boraginaceous plants, it is obvious that washing will reduces the PA levels of such products. Furthermore, lower pH values will promote alkaloid protonation, and hence increase its polarity and water solubility. The best result was obtained at pH 2.2. Using one washing step under these conditions reduced the PA level to 1.6 %. Increasing the numbers of washings at low pH-values is an additional option to further reduce the PA levels. It was found that three washings at pH 2.2 can bring the PA level down to 0.08 % (Fig. 1b).

These first model experiments were useful to establish our analytical method. It allowed us to test and optimize our sample extraction procedure. The resulting extracts

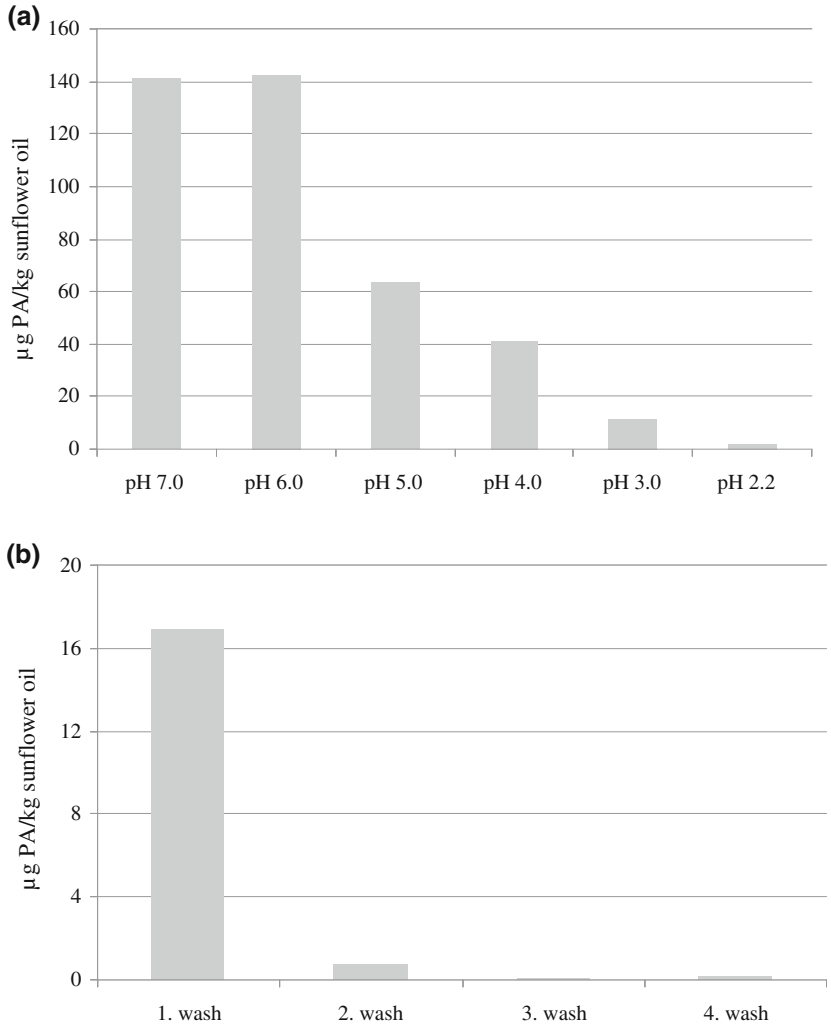

Fig. 1 Reduction of the PA content of PA-spiked sunflower oil performing washing steps at **a** different pH values or **b** multiple washings at pH 2.2

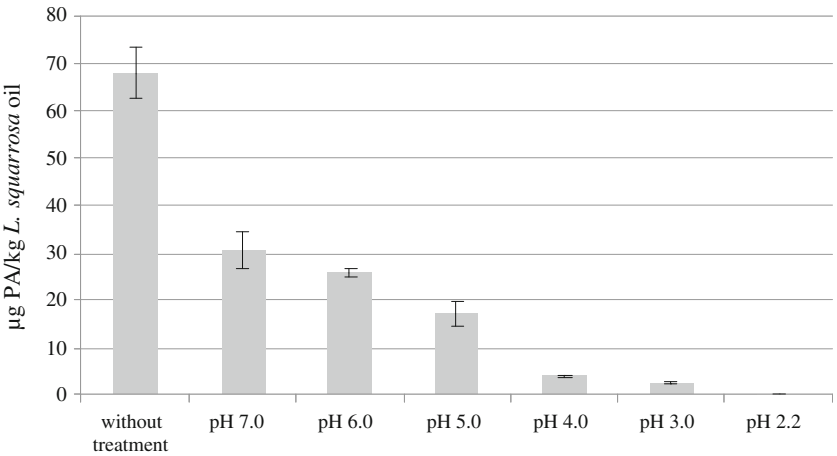

Fig. 2 Reduction of the senecionine/senecionine-*N*-oxide content of PA spiked *Lappula squarrosa* oil at different pH values ($n = 3$)

were analyzed with a selective HPLC–ESI–MS/MS method to quickly track the fate and the behavior of Sen/SenNOx under such model conditions.

Compared to sunflower oil, crude *L. squarrosa* oil has a dark greenish/brown color and intense odor representing a more complex and difficult matrix. In addition, *L. squarrosa* oil is characterized by large amounts of PUFA. Hence, it was of interest to verify the results obtained with sunflower oil to find the most efficient and gentle conditions for *L. squarrosa* refinement. *L. squarrosa* oil was spiked

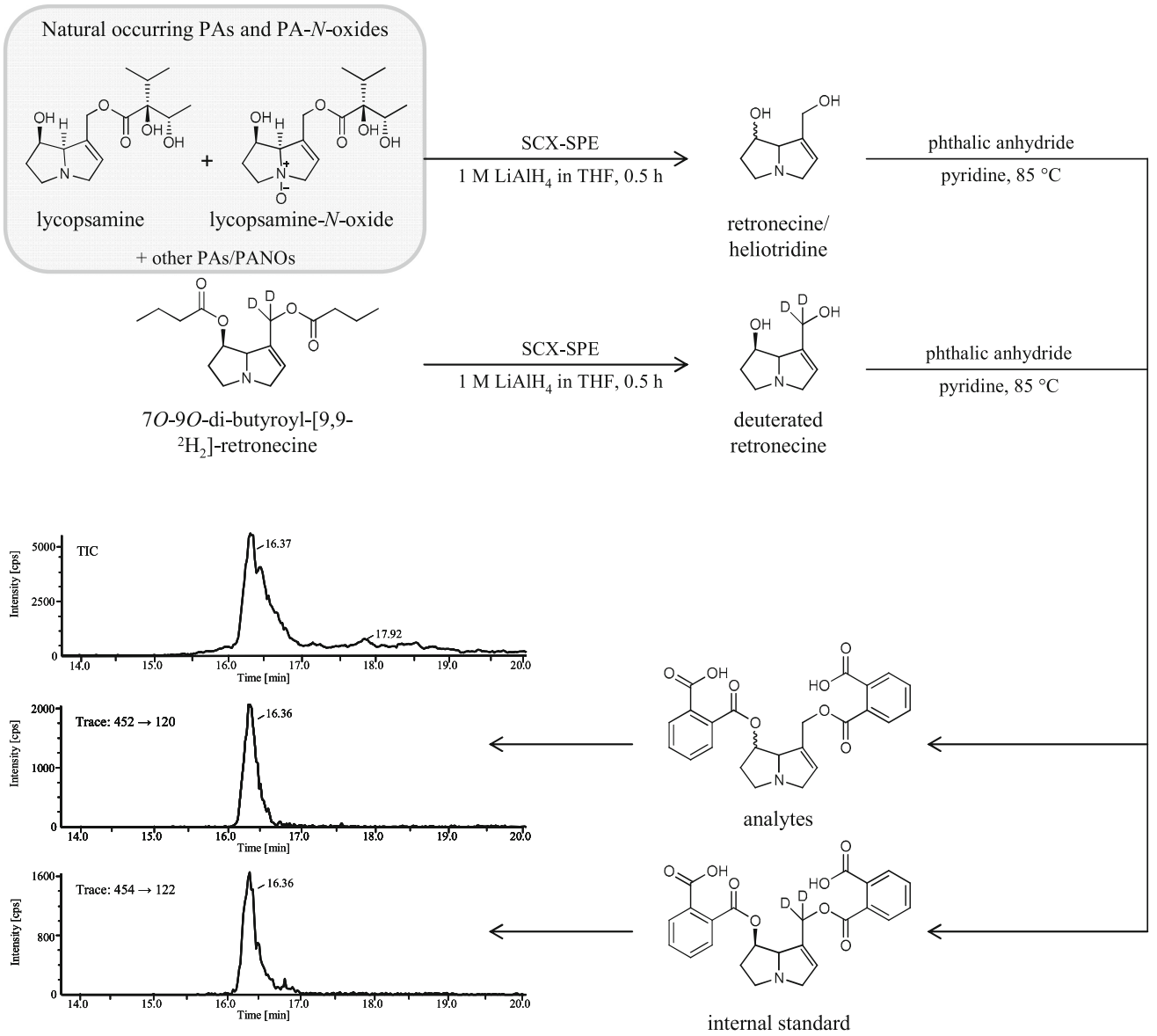

Fig. 3 Scheme of the sample preparation and HPLC–ESI–MS/MS sum parameter analysis of the total 1,2-unsaturated PA content in oil and oil seeds

with known amounts of Sen/SenNOx and seneciphylline was used as an internal standard to quantify Sen/SenNOx and to calculate recovery rates of the PA in the matrix crude *L. squarrosa* oil. All three PA are common for *Senecio* spp. (Asteraceae) but do not occur in *L. squarrosa* (Boraginaceae). As shown in Fig. 2, washing at pH 6–7 can bring down the PA level to 13–15 %, but again only washing at pH 2.2 efficiently reduced the PA content down to 0.1 % of the starting value (Fig. 2).

While the Sen/SenNOx model experiments were helpful for establishing sample preparation/PA concentration and pinpointing crucial steps in oil refinement, they lacked specificity in terms of the PA relevant for *L. squarrosa*.

So far, the PA of *L. squarrosa* are not known and standards for target analysis are not available. Hence, we decided to apply a PA sum parameter method to quantify the total PA content in seed oil at different stages of the refinement. After extraction and concentration on SCX-SPE cartridges, all PA/PANO of the sample are simultaneously reduced to the corresponding necine base diols (see Fig. 3). Those diols showed unfavorable characteristics in HPLC chromatography and ESI–MS/MS. Hence, prior to HPLC–ESI–MS/MS these diols had to be converted into the corresponding phthalic acid diesters using a phthalic anhydride/pyridine derivatization. Using this approach, we were able to detect all 1,2-unsaturated

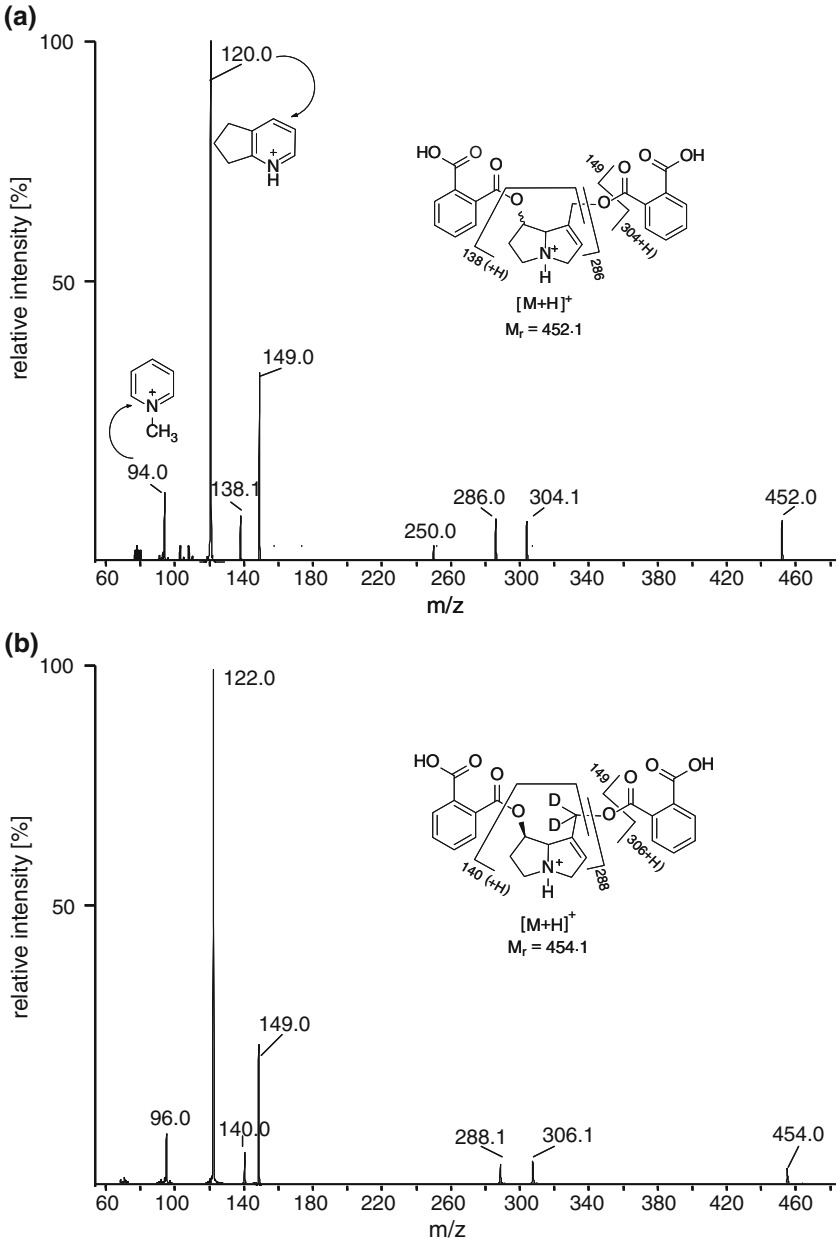

Fig. 4 Enhanced product ion (EPI)-ESI–MS/MS spectra of **a** the derivatized analytes (retronecine-diphthalate/heliotridine-diphthalate) and **b** the corresponding derivatized deuterated internal standard ([9,9-$^2H_2$]-retronecine-diphthalate) including proposed structures for fragment ions

retronecine/heliotridine-type PA in the form of a single sum parameter expressed as retronecine equivalents (RE). Accurate quantification was achieved using 7-*O*,9-*O*-dibutyroyl-[9,9-$^2H_2$]-retronecine as a stable isotope-labeled internal standard (Figs. 3, 4) [21].

The analytes (retronecine-diphthalate/heliotridine-diphthalate) co-elute together with the internal standard derivative [9,9-$^2H_2$]-retronecine-diphthalate, covering for possible matrix effects. This is especially true when larger quantities of oil are used in sample preparation to achieve low detection limits. Quantification was achieved by monitoring analyte (retronecine/heliotridine-diphthalate) specific MRM transitions and specific transitions for the internal standard, respectively. The application of this new HPLC–ESI–MS/MS method allowed the unequivocally determination of PA levels at all different stages of the refinement process, achieving a LOD and a limit of quantification (LOQ) of 0.02 and 0.05 µg RE/kg, respectively. Besides the mass spectrometric dependability (one quantifier transition, three qualifier transitions), the LOQ is 80–400 times lower than the LOD of the TLC method (LOD 4 µg/kg) applied in the novel food application of *E. plantagineum* oil or the LOD reported for a GC–MS approach (20 µg/kg) used to quantify the PA content in crude and processed Borage oil, respectively [24, 25]. This increase in sensitivity is desirable in the course of the toxicological importance of the PA and the EFSA assessment that only PA doses up to 0.007 µg/kg b.w./day are unlikely to be of concern for cancer risk [15].

Applying this sensitive sum parameter method we were able to explore the impact of additional refinement steps and the concurrent PA reductions. Figure 5 summarizes the two extremes of the experimental setup, (1) neutral conditions pH 7, being the most gentle washing step and (2) pH 2.2 representing the harshest conditions but highest efficiency in terms of PA reduction, both combined with an additional neutralization and deodorization step under laboratory conditions. At neutral pH, this combination showed a PA reduction from 33 µg RE/kg (crude *L. squarrosa* oil) down to 0.4 µg RE/kg, while the same procedure at pH 2.2 resulted in a ten times lower PA content right at the LOQ (0.04 µg RE/kg).

L. squarrosa oil is still a rare product whose market potential is still being explored. At this point it was decided that one crucial milestone for future development is food safety. Hence, a pilot scale refinement process (50 kg batch) was initiated to investigate a combination of steps which would be reasonable to produce high quality food grade *L. squarrosa* oil with the lowest possible PA content. The overall process was performed in a mini plant allowing an easy scale-up of the process to produce economic relevant amounts of *L. squarrosa* oil. The starting material was *L. squarrosa* seeds showing 16 mg RE/kg. The resulting crude *L. squarrosa* oil contained 29 µg RE/kg which was consecutively reduced to 0.9 µg RE/kg

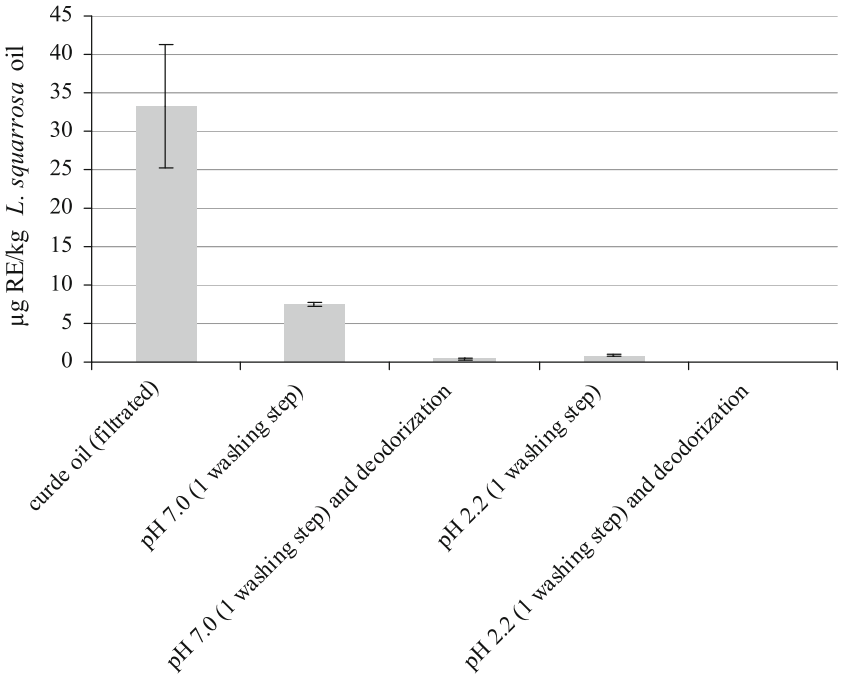

Fig. 5 Comparison of the reduction of total 1,2-unsaturated PA content of crude *Lappula squarrosa* oil using washing and deodorization at pH 7 and 2.2 ($n = 3$)

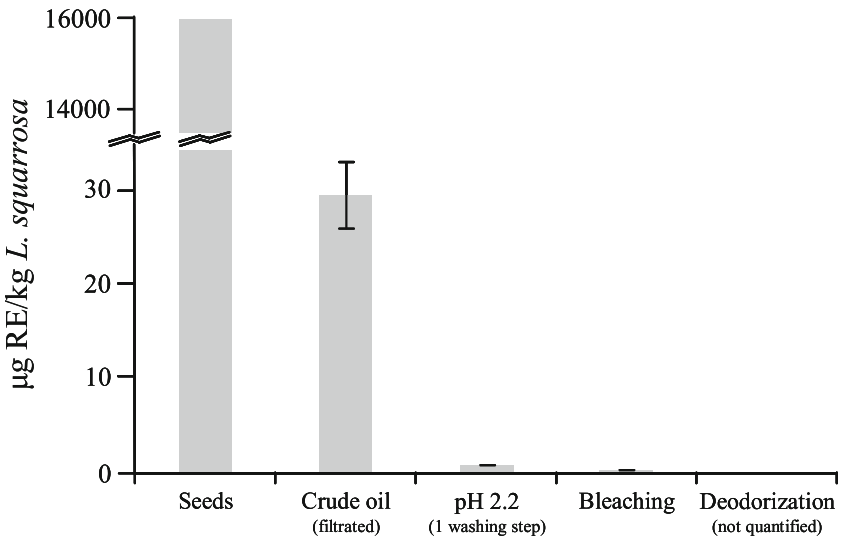

Fig. 6 Monitoring of the total 1,2-unsaturated PA content at different stages of a pilot scale refinement of crude *Lappula squarrosa* oil and the starting seed material ($n = 3$)

Table 1 Total PA content of commercially available Boraginaceous plant oil/oil products in retronecine equivalents (RE)

Sample	µg RE/kg oil
BO_1	0.08
BO_2	ND
BO_3	0.5
BO_4	ND
BO_5	ND
EO_1	ND
EO_2	ND
EO_3	ND
EOP_1	ND
EOP_2	0.6

BO, *Borago officinalis* oil; EO, *Echium* spp. oil; EOP, *Echium* spp. oil product; ND, not detected

(washing at pH 2.2) down to 0.12 µg RE/kg (after bleaching). At the next stage (deodorization) PA were below the LOQ (0.05 µg RE/kg) (Fig. 6). Using this procedure, we were able to demonstrate that PA levels of *L. squarrosa* oil can be significantly reduced to levels below any toxicological concern.

Finally, we used our established method to determine the PA content of commercially available Boraginaceous seed oil products. Overall ten different products, including pure seed oils from *Echium* spp. or *B. officinalis* and capsuled food supplements containing inter alia *Echium* spp. oil were analyzed. The analytical results are summarized in Table 1. Three out of these ten products were tested PA positive. The PA content was ranging from not detected to 0.6 µg RE/kg product (corresponding to 1.2 µg 1,2-unsaturated PA/kg using a conversion factor of 2). Ironically, one of the positive samples was labeled as "produced by using PA free *Borago* seeds" (sample BO_3; Table 1).

Assuming a daily dose of 10 g of such products would be reflected in 6 ng RE/day (assuming a conversion factor of two to calculate for the total PA content) this would be ~12 ng PA/day which most likely gives no reason for toxicological concerns. However, in terms of preventive consumer protection and other possible sources of PA intake into the food chain, our studies on the refinement of *L. squarrosa* oil and the PA negative commercial products demonstrate that the PA content can be reduced to non-detectable (LOD 0.02 µg RE/kg) using appropriate refinement conditions for such oil seeds/products.

Conclusions

The presented results pinpoint crucial steps in the refinement of *L. squarrosa* seed oil for the most effective reduction of the accompanying PA. We adopted an HPLC–ESI–MS/MS sum parameter method which enabled us to measure trace levels of PA in oil matrices. It was demonstrated that washing at low pH and deodorization are valuable tools for an efficient reduction in PA found in crude Boraginaceous seed oils. On the way to exploring the possible market potential of *L. squarrosa* oil, a pilot scale refinement was established with the aim of the best possible PA reduction for food safety reasons but without sacrificing the future options for scale up for a commercial process. The study was finalized by the analysis of commercially available Boraginaceous seed oil products and food supplements using the newly developed HPLC-ESI–MS/MS method to survey the PA content of those niche products.

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Conflict of interest The authors have no conflict of interest to report.

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