Supercritical fluid extraction of lipids from linseed with on-line evaporative light scattering detection.

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Abstract

Supercritical fluid extraction (SFE) is a green alternative method of extraction for neutral lipids in seeds compared to conventional methods utilizing organic solvents. In this work a novel method where SFE is hyphenated with an evaporative light scattering detector is presented. The method was subsequently applied to determine lipid content in crushed linseed. The new method enables rapid quantification of extracted lipids as well as be ability to continuously monitor the extraction rate in real-time, thus being able to determine the time point of completed extraction.

Both the detector and the method was validated. The results show that any of several tested oils can be used to calibrate the detection method for the determination of lipids extraction from linseed. The overall method repeatability and intermediate precision was 2.6% relative standard deviations. The extracted amount was significantly less than that obtained using the standard method of Soxhlet with petroleum ether, 26.0 ± 0.4% (95% CI, n = 9) compared to 32.3 ± 1.3% (95% CI, n = 3) of extracted amounts.

It was found that channeling effects were present, and by either performing sequential repeated extractions with decompression in-between or by using a relatively large vessel a more complete extraction could be obtained. Interestingly, a substantially higher extracted amount (approximately 50%) was obtained compared to both a single extraction by SFE and the Soxhlet method. Therefore, it is recommended that an additional extraction including a rapid decompression in-between should be included in the validation of a method using supercritical fluid extraction, in order to either rule out channeling effects or to acquire a full recovery.

Abbreviations

BPR, back pressure regulator; ELSD, evaporative light scattering detector; $scCO_2$, supercritical carbon dioxide; SFE, supercritical fluid extraction

1. Introduction

Linseeds contain plentiful of omega-3 fatty acids, linoleic acid as well as the phytoestrogen lignan. A diet that includes linseeds has proven to decrease both total and low-density lipoprotein cholesterol [1]. Besides having beneficial health effects linseeds oil also have some industrial applications in e.g. resin, plastic, varnish and paint. The fibers can be used in high quality products within the paper and textile industry [2]. The seeds contain a large fraction of lipids ranging from approximately 35% to 43% in dry weight, of which polyunsaturated fatty acids exists to the largest extent followed by monounsaturated and lastly saturated fatty acids. Linseeds contain quite little polar lipids such as phospholipids which are in the mg g^{-1} range [3].

Standard methods are usually based on the original the Folch method, the simplified version of the previously mentioned namely the Bligh & Dyer method or Soxhlet extraction. Numerous alternatives to these methods are available in the literature [4]. It has however been noted that the Folch method and the Bligh & Dyer method are not suitable for efficient extraction of neutral lipids but rather designed for the retrieval of somewhat more polar lipids [5].

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A common factor for all of these conventional methods is the use of toxic and environmentally burdensome organic solvents like chloroform or hexane.

In relation to traditional methods utilizing organic solvents, supercritical fluid extraction (SFE) offers an alternative for extracting lipids or lipophilic compounds which is more environmentally benign. Carbon dioxide is the usual choice for performing extraction and merits benefits such as being non-toxic, non-flammable and cheap. SFE also provides convenient recovery of the extracted compounds as the CO_2 is removed by depressurization. Supercritical fluids have desirable properties in the context of extraction due to densities close to those of liquids and higher diffusivity as well as low viscosity similar to those of gases. Therefore, supercritical carbon dioxide ($scCO_2$) readily penetrates the sample matrix and solubilizes non-polar compounds. Thus SFE can offer quicker and more efficient extraction processes than traditional techniques based on organic solvents for extraction of lipophilic compounds. However, mineral salts, proteins, polysaccharides and sugars remain insoluble in $scCO_2$ [6, 7].

Previous work already exist with the aim to optimize the extraction of lipids in linseed. Ivanov and Čolović [8] optimized the operating conditions for extraction of lipids from linseed using SFE. The conclusions were that a minor addition of ethanol mixed with the sample and the highest possible pressure of 62 MPa generated the highest recovery of lipids. The effects of temperature was not evaluated. A thorough discussion on extractability of lipids and comparison of SFE with conventional methods applied to other matrices can be found elsewhere [7]. Furthermore, the use of SFE for extraction of lipids from oilseeds also exist as a standard method (AOCS Am 3-96).

SFE is usually operated in continuous mode, also known as dynamic extraction, although batch extraction and combinations of these two are utilized as well. The continuous extraction process can be generalized and thus be regarded as a flow through a randomly packed column, also known as a fixed bed. A common problem using non-supercritical fluids is however that the flow profile is rarely uniform and the bed is not homogenous in a randomly packed column [9]. In packed beds and fluidized beds (sludge) channeling effects often occur due to agglomeration of particles. This is often the case when the material has a high initial moisture content and thus forms a cake during the extraction. Agitation is commonly applied to hinder the formation of agglomerates and thus increasing fluidization [10].

Channeling effects have been observed in SFE [11, 12]. However it has been relatively unexplored, although it has been noted that a general trend exist within the literature to add dispersants such as glass beads with the motivation to hinder channeling effects.

In order to study extraction kinetics and other related extraction phenomena such as channeling effects, continuous data describing the extraction process from start to the end is needed. On-line detection offers benefits in regards to sample handling by both minimizing work and additional steps which could inflate the overall measurement uncertainty. It also allows for a real-time evaluation of when the extraction can be determined as completed and the ability to study the extraction rate of the process. The continuous data could also allow for studying other aspects of the extraction such as the kinetics, potential channeling effects and the effects of compression and decompression of the sample matrix.

There are numerous of applications in which SFE has been coupled to mainly chromatographic instruments including liquid chromatography, gas chromatography and supercritical fluid chromatography but also capillary electrophoresis [13]. Such setups are only able to have a sampling rate equivalent to the total analysis time of the

subsequent separation method and therefore might not provide sufficient resolution in order to detect sudden changes during the extraction.

Although hyphenating SFE directly with on-line measuring has been considerably investigated, focus has mainly been given on IR and FT-IR. Amador-Hernández and Luque de Castro [14] have reviewed prior works using on-line detection for SFE, including also UV-Vis and prototypes such as NMR and MS. Primarily, continuous monitoring using above mentioned setups has been applied to extraction of plant matrices [15, 16] and detection of lipids in water or soil [17, 18] to mention a few examples.

Detection of compounds without a chromophore can at times be difficult because of the low response using the usual UV-Vis detectors that are popular in e.g. chromatography. These compounds include carbohydrates, lipids, polymers and petroleum products. The evaporative light scattering detector (ELSD) offers an almost universal detection of most compounds that are less volatile than the mobile phase.

In the ELSD three steps take place: nebulization of the solution to an aerosol, desolvation of the solvent by heating to form free particles and measurement of the particle density by light scattering in a gas flow cell. The nebulization is usually facilitated by an auxiliary nitrogen flow. Typical limits of detection are about 1 ng or corresponding to 10 mg L^{-1} in samples using packed capillary columns in chromatography. The detector response is described by the relationship expressed in Eq. (1),

$$A = a \times m^b \tag{1}$$

where A is the detector response (peak area in chromatography), m is the analyte mass, a and b are coefficients dependent on the operating conditions [19]. Even though the response is non-linear, by limiting the range it will yet maintain an appearance of linearity.

There are no examples in the literature where ELSD has been coupled to a SFE system. However, Lesellier et al. [20] and Lecoeur, Simon [21] studied the hyphenation of supercritical fluid chromatography with ELSD including the effects of various operating conditions such as mobile phase velocity, fraction of co-solvent and nebulization temperature. Heating at the position of post-depressurization is often needed to avoid freezing and in addition a make-up flow of solvents may be needed in order to avoid precipitation due to the sudden decrease in solubility at atmospheric pressure. In summary the detector response is affected by mobile phase composition, temperature and flow rate. By comparison the detector is easily compatible with the use of supercritical fluids whereas when using other types of detectors the high pressure and the large volumes of gas needs to addressed. However, since the ELSD is positioned after depressurization and the gaseous CO_2 actually facilitates the nebulization process.

The aim of this paper is two-fold. Firstly, to hyphenate SFE with on-line ELSD for rapid determination of extracted lipids from linseed. The second purpose was to utilize the developed method to study the extraction process continuously, thus to identify and study phenomena such as channeling.

To our best of knowledge this is the first time that ELSD has been coupled to SFE for on-line detection. The instrumentation was validated in terms of limits of detection, range of linearity, precision and trueness by comparing it to the conventional method of Soxhlet extraction for extracting lipids. The newly proposed environmentally friendly

method offers quick quantification of lipids in sample matrices such as seeds. It also offers a convenient approach for studying extraction kinetics using a relatively cheap and basic detector. Therefore it enables a more precise approach of determining when an exhaustive extraction has been performed. Furthermore, the instrumentation was used to study practical aspects of the extraction such as extraction vessel dimensions, the use of dispersants to minimize channeling effects, packing strategies, effects of rapid decompression and repeated extractions.

2. Materials and methods

2.1. Chemicals

Ethanol (99.7%, Solveco, Rosenberg, Sweden) was used as make-up flow in the SFE. Ultrapure CO_2 was provided by Air Products (Amsterdam, Netherlands). Petroleum ether (60-80 °C, BDH, Poole, England) was used for the Soxhlet extraction. Crushed linseeds were bought in the local grocery store and used as they were, except in a few experiments, in which additional grinding was achieved using a Retsch mixer mill MM400 (Retsch, Haan, Germany) at 10 Hz for 15 s. The glass beads and sand used as dispersant were from Marienfeld (Lauda-Königshofen, Germany).

2.2. Instrumental setup

The system consisted of two ISCO 260D syringe-pumps (Teledyne Isco, Thousand Oaks, CA) used for pumping liquid CO_2, a Waters 515 HPLC-pump used for pumping ethanol, a HP 5890 gas-chromatography oven (Hewlett-Packard, Wilmington, DE) functioning as oven, a Tescom 26-1700 series back pressure regulator (BPR) (Tescom Europe, Selmsdorf, Germany) and an Eltherm ELTC/3 thermoregulator (Eltherm Elektrowärmetechnik GmbH, Burbach, Germany) was used for heating the lining between the BPR and the ELSD model Sedex 55 (Sedere, Alfortville, France). A Rheodyne injection valve with a 0.65 mL loop was also implemented into the system. The liquid CO_2 pump was cooled by a Julabo F12 cooling system (Julabo, Vista, CA).

The whole setup is illustrated in **Fig. 1**. The second CO_2 pump offers a feature of maintaining constant flow rate to the ELSD which is flow sensitive, even though the extraction flow rate is changed. This allows for studying the extraction at various flow rates without recalibrating the detector. The make-up flow of ethanol is used to avoid precipitation after the decompression of the CO_2 at the BPR.

2.3. Extraction conditions for the validated method

Both a detector validation of the proposed setup described in the previous section 2.2 and a complete method validation were performed. Extractions of lipids from crushed linseeds were performed by initially placing approximately 0.6 g of weighted linseeds in the extraction vessel (10 mm x 30 mm). An empty column with 0.5 µm metal filters on each side from Applied Porous Technologies (Onsala, Sweden) was used as an extraction vessel. The void volume was filled up with 3 mm Ø glass beads without packing the material and the extraction vessel was mounted so that the glass beads were at the outlet. The extraction time was 90 min. The extraction flow rate measured and set for each pump was 1.5 ml min^{-1} of liquid CO_2 and the make-up flow rate of liquid CO_2 was 0.5 ml min^{-1}, the make-up flow rate of ethanol was 2 mL min^{-1}. The extraction pressure was 30 MPa and the extraction temperature was 80 ºC, thus a density of 746 kg m^{-3}.

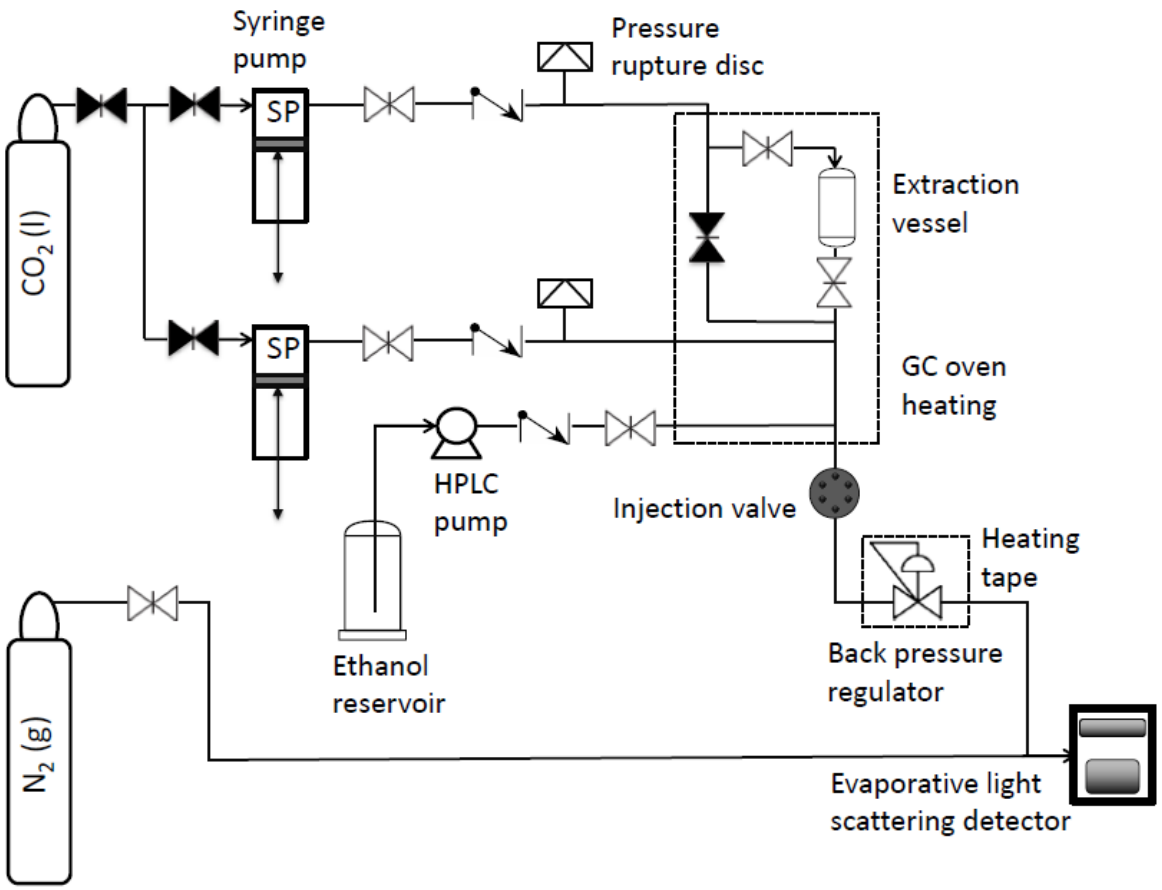

Fig. 1. Schematic of the SFE-ELSD instrumental setup.

The post-depressurization heating was set to 90 °C in order to avoid freezing, the nitrogen feed to the ELSD was 0.3 MPa of N_2, the drift tube temperature of the ELSD was 95°C and the gain factor was 5.

Initially the valves before and after the extraction vessel were closed, while the valve allowing the flow to by-pass the extraction vessel was opened. All of the components were initialized, the pumps were started and for the first run of the day a warm-up time of 30 minutes took place. The baseline of the detector was set to zero and an uptake of the signal was started. Subsequently the flow was redirected by closing the valve used for by-passing the extraction vessel and the opening the valve before the extraction vessel. When the pressure was equally high to the rest of the system the valve positioned after the extraction vessel was opened, ensuring that no ethanol was pushed into the extraction vessel.

2.4. Calibration

The calibration curve was constructed by diluted sunflower oil in heptane ranging from 1.5 to 20 mg L^{-1}, which corresponded to 1 to 12 mg of injected oil. A blank was also injected to ensure that no response was given however was not included in the calibration curve.

2.5. Reference method

The determination of lipid content in the linseed was also performed using Soxhlet with petroleum ether according to the official AOAC Method 945.16. Approximately 3 g of linseed and 80 mL of solvent was used. The extraction time was 8 h and the extract was dried using a steady flow of nitrogen and slight heating of 40 °C for two hours.

2.6. Data analysis

Analog data acquisition from the ELSD was recorded and handled using Clarity Lite v. 3.0.7 (DataApex Ltd, Prague, Czech Republic). All data processing was carried out using MATLAB R2013a including the statistical toolbox (MathWorks Inc., Natick, MA, USA). The extractograms were obtained by setting the time of initiated extraction to time zero and then integrating the raw data using the trapezoidal method. The amount extracted over time was then calculated using the previously acquired calibration curve. The extracted amount is specified as percentage (%) based on the mass fraction throughout this paper, meaning that 100% would be the equivalent of the whole sample mass being extracted.

3. Results and discussion

The purpose was to develop a method to determine total lipid content in linseeds as well as to study the SFE process continuously. Therefore, SFE was coupled with an ELSD due to the possibility to detect lipids. Thereafter extractions of lipids from linseeds were performed and the system was further developed. In this work the term lipid is used to categorize any type of organic compound, including fats, oils, waxes and sterols, which are insoluble in water but soluble in nonpolar organic solvent, and are present in the linseed. The key criteria was to obtain an equipment capable of using various extraction pressure, temperature and flow rate although yet maintaining the same response factor of the detector. Thereby the extraction conditions could be altered without the necessity to perform yet another calibration.

Various conditions including pressure (15-30 MPa), temperature (40-80 °C) and flow rates (0.5-1.5 mL min^{-1}) were tested based on a three level full factorial design, of which some selected extractions are presented (**Fig. 2**). It was found that the highest tested pressure (30 MPa), temperature (80 °C) and flow rate (1.5 mL min^{-1}) had the highest extraction rate, and were thus chosen as the extraction conditions throughout this work.

3.1. Instrumental development

Initially, the SFE-ELSD instrumentation was set up in order to acquire a functioning system. As illustrated in **Fig. 1**, one of the features is the additional CO_2 pump which is used to create a make-up flow added after the extraction vessel. This proposed setup enables constant flow rates at the inlet of the detector even though the extraction flow rate is altered between runs by also adjusting the make-up flow of CO_2. This is particularly important since the response of the ELSD is affected by the flow rate, and thus a recalibration of the detector would be needed otherwise.

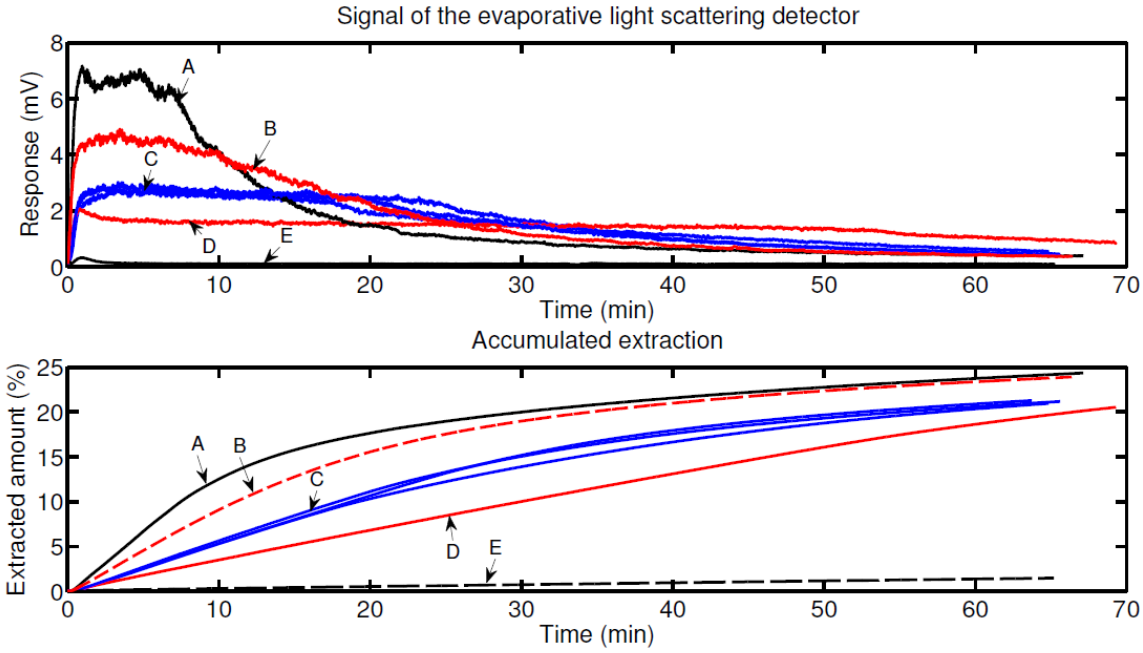

Fig. 2. Extraction using the SFE-ELSD system at various conditions. The upper figure shows the detector signal and the lower figure is the integrated response giving the extractogram. The black line (A) shows extraction at T=80 °C, P=30 MPa, ρ=746 kg m^{-3} and Q=1.5 mL min^{-1}, the red dashed line (B) shows extraction at T=40 °C, P=30 MPa, ρ=929 kg m^{-3} and Q=0.5 mL min^{-1}, the blue lines (C) show replicated extractions at T=60 °C, P=22.5 MPa, ρ=740 kg m^{-3} and Q=1 mL min^{-1}, the red line (D) shows extraction at T=60 °C, P=22.5 MPa, ρ=740 kg m^{-3} and Q=0.5 mL min^{-1} and the black dashed line (E) shows extraction at T=60 °C, P=15 MPa, ρ=563 kg m^{-3}, Q=1.5 mL min^{-1}.

Another feature of the setup is the make-up flow of ethanol, which was required in order to transport the otherwise precipitated compounds after CO_2 expansion to the ELSD. Approximately equal volumetric flow rate of total liquid CO_2 and ethanol was required to ensure efficient mass transfer to the detector. Spikes were otherwise observed in the response of the ELSD, due to formation of extracted mass in the linings which then was released in larger portions when the make-up flow rate was too low. It was also observed that the detector response factor was very sensitive towards temperature variations in the mixture that reached the detector. Thus about 30 minutes of equilibration time was needed for all the lining to reach a steady-state in temperature, otherwise a higher detector response factor was obtained. This initial warm-up of the instrumentation was only needed before the first run of the day. A practical benefit of the equipment is that any significant leakage would affect the signal by an increased noise level. Such leakage could occur inside of the heating oven and pass by otherwise unnoticed although affecting the actual extraction flow rate.

In conclusion, by using two make-up flows of CO_2 and ethanol, and by applying a sufficient initial equilibration time, a well-functioning and robust SFE-ELSD instrumentation was obtained.

3.2. Detector validation

The instrumentation was first validated by evaluating the performance of the detector. This was performed by injecting oil standards in order to construct a calibration curve as well as by performing extractions of lipids from linseed and comparing the extracted amounts determined by a gravimetric method and by using the ELSD.

The influence of oil choice on the response and thus also the calibration curve for the prediction of unknown samples was evaluated. Oil from corn, rapeseed and sunflower seed was diluted with heptane and introduced to the system in various amounts ranging from 0.6 to 20 g L^{-1}. Each injection was performed in triplicate. The injected standards appeared very similar to those obtained in chromatography, namely Gaussian shaped peaks with more tailing along with increased concentrations (**Fig. 3**). Range of linearity was evaluated by plotting the response factor as a function of the logarithm of the amount of injected oil. The linearity of the calibration curve depends on the detector response (mV), and it was determined to be between 0.3 and 11 mV which corresponded to about 0.5 mg and 13 mg of injected oil, respectively. Beyond approximately 11.8 mV the detector became saturated and was thus the highest possible signal obtained from the detector, and below 0.3 mV the variation of the response factor was large. Within the range of linearity (~10^2 mV) a good linear fit of the calibration curves was obtained (R^2>0.98).

The difference in slope of the calibration curves was determined to be insignificant ($p>0.05$) by analysis of covariance and thus the choice of oil for calibration was deemed trivial (**Fig. 4**). Due to the response being a function of the analyte mass and no difference was observed between different types of oils, any oil could be used to determine total extracted lipids from any kind of sample matrix.

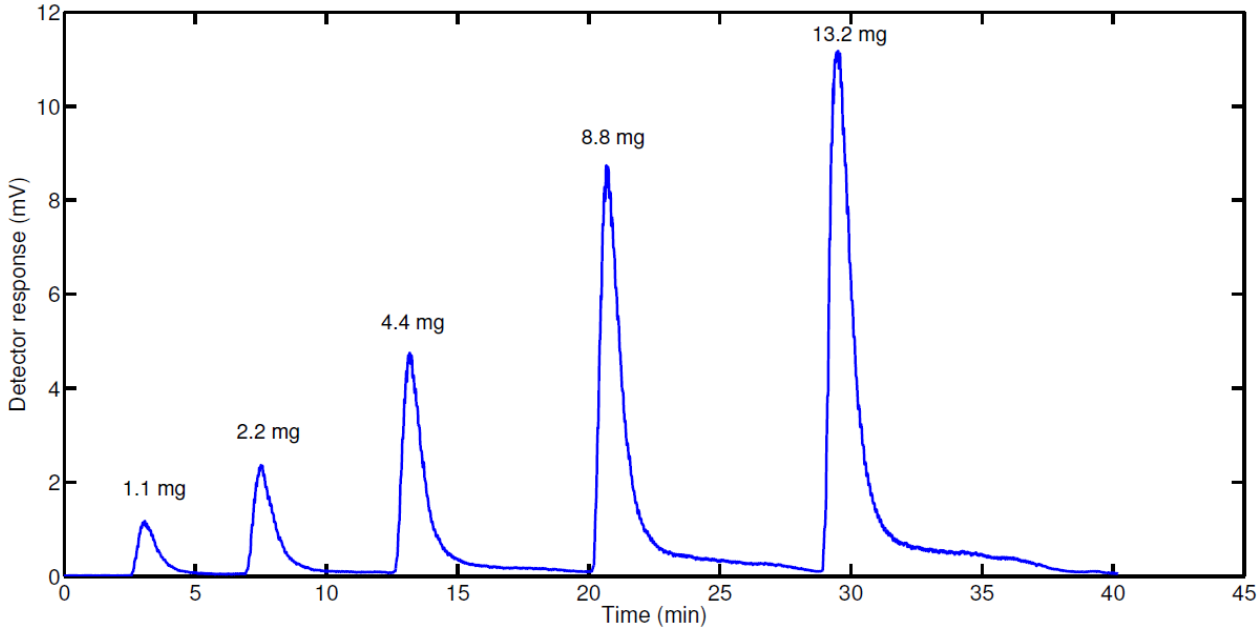

Fig. 3. Injections of sunflower oil diluted in heptane ranging from 1.1 to 13.2 mg of introduced oil.

The trueness and the precision of the detection method was evaluated by comparing gravimetric determination of the extracted mass from linseeds with the determination using the ELSD. The gravimetric determination was performed by weighting the extraction vessel filled with crushed linseeds and glass beads, before and after the extraction. Thus every extraction was determined using both methods and the runs could easily be compared in pairs. By paired t-test statistics of the total nine extractions performed three each day over three different days, the lipid content in the seeds was determined to be significantly ($p<0.001$) lower by 1.1 ± 0.4% (95% CI, n = 9) extracted amount by determination using the ELSD compared to gravimetric measurements. The determined extracted amount was 26.0 ± 0.4% (95% CI, n = 9) using the ELSD and 27.1 ± 0.7% (95% CI, n = 9) by gravimetric determination. The estimate of the measurement error in the linseed lipid determination includes variation due to the extraction, while the paired t-test only takes into account the variation between the two quantification approaches.

The precision of the detector compared to a balance can be interpreted as good considering the low confidence interval (0.4%, 95% CI) derived from the paired t-test. The trueness of the ELSD can also be interpreted as good, although providing a lower result which could possibly be explained by the water content which was determined to be 3.1 ± 0.1% (95% CI, n = 3). The small amounts of water should not give rise to any detector signal. The conclusion is that there is a small bias in the measurements using the gravimetric method.

3.3. Method validation

The SFE-ELSD method was also validated in regards to the whole method including both the extraction process and the detection method. Both the precision and the trueness were evaluated by applying the setup to crushed linseeds. In this work we have compared our proposed SFE-ELSD method with the AOAC Method 945.16, a modification of the AOCS Method Ba 3-38, both based on Soxhlet extraction. On a more general note, this is the method that is usually seen as the reference method of choice in the literature when proposing new methods for total lipid determination, for example [8, 22-23]. The other suggested reference method is based on solvent extraction using a Butt-type apparatus, much like the Soxhlet apparatus. Previous comparisons of various extraction apparatus, also including Goldfisch has shown that there is no significant difference when applied to linseed [24].

The lipid content was determined to be 32.3 ± 1.3% (95% CI, n = 3) and thus a 1.6% relative standard deviation (RSD) using the Soxhlet method. By instead using the SFE-ELSD method, only 26 ± 0.4% (95% CI, n = 9) of lipid was extracted from the linseeds. The considerably lower extracted amounts using SFE compared to Soxhlet is further discussed in section 3.4.

Both the overall repeatability and intermediate precision of the SFE-ELSD also including the extraction process were evaluated. The between-day variation was insignificant compared to the within-day variation and thus both the repeatability and the intermediate precision were determined to be 2.6% RSD, compared to the 3.8% RSD when using gravimetric analysis. The precision is seen as satisfactory compared to the repeatability of 1.6% RSD achieved by the Soxhlet method.

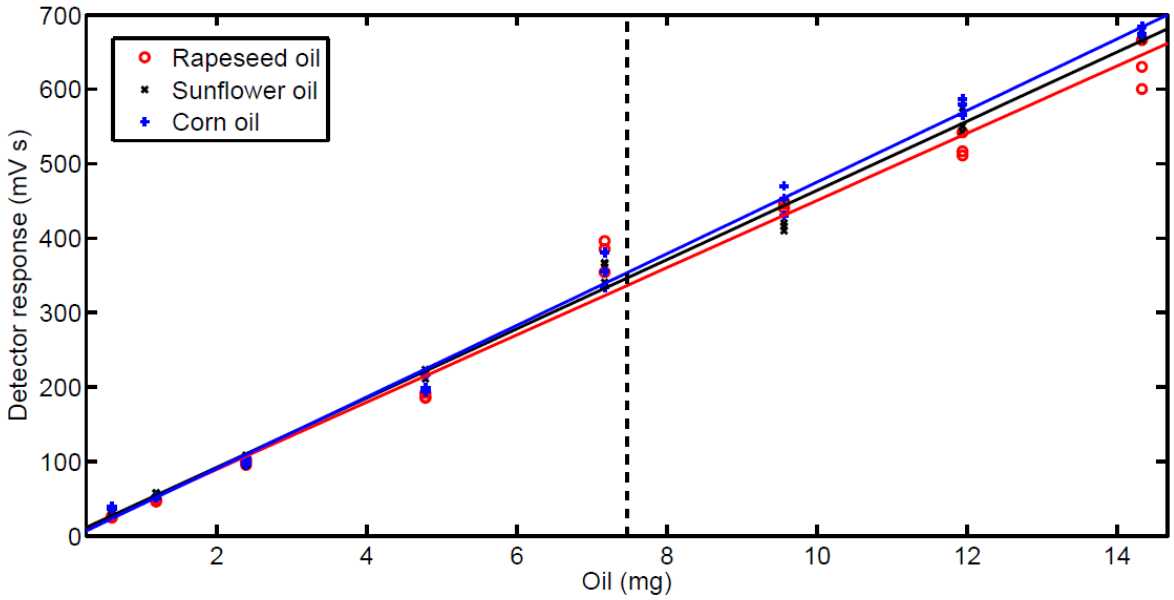

Fig. 4. Comparison of calibration curves based on oil from rapeseed, sunflower and corn diluted in heptane. The curves were not significantly different ($p>0.05$).

Limit of detection was not properly evaluated because the instrument is much more sensitive than what is required by the suggested application. The ELSD by itself has a limit of detection in the range of nanograms when injecting standards [19]. The instrument has an adjustable gain factor allowing for higher sensitivity however the range of linearity is as previously mentioned rather limited. In this application the injected amounts are in the range of milligrams. As a proof of concept an extraction was performed using 0.06 g, one tenth of the otherwise used sample amount. The gain factor and the calibration range was adjusted accordingly and the lipid content was quantified. A lower lipid content of 22% was acquired. The reason for the lower yield is probably due to the difficulties analyzing a representative sample due to the very small sample amount. Nonetheless, in regards to the detector it is possible to detect and quantify very low concentrations or from small sample amounts.

Various extraction conditions were tested by altering the temperature, pressure and the extraction flow rate however maintaining the flow rate at the detector by adjusting the make-up CO_2 flow rate (**Fig. 2**). The repeatability of the continuous extraction data was considerably good and the mean of the root mean square error was only 1.4% of extracted amount, and the highest was 2.2% of extracted amount occurring about half-way through the extraction. Variation between the extraction curves could be observed due to the different extraction conditions. This kind of data could be highly useful for studying the extraction kinetics. In these scenarios the method should not only be robust in regards to the final extracted amount, but should also be similar at any given time point throughout the extraction process. Therefore, the generated data would be suitable for calibration and determination of coefficients in mathematical modeling of SFE.

3.4. Extraction performance

As previously described in section 3.3, determination using the SFE-ELSD was 26 ± 0.4% (95% CI, n = 9) of extracted lipids compared to 32.3 ± 1.3% (95% CI, n = 3) using Soxhlet with petroleum ether (**Table 1**). The SFE-ELSD method was extracting substantially less compared to the Soxhlet method. Attempts were made to extract more by several approaches such as repeated extractions, using various extraction vessel dimensions, packing strategies and further grinding of the sample. Experiments were also carried out to investigate possible channeling effects.

It was concluded that additional extraction time would not be the solution due to very little mass being extracted after 90 minutes, and thus seemed to have converged to complete extraction (**Fig. 5**, curve A).

However, upon performing subsequent extractions of the crushed seeds it was discovered that more mass was readily extracted, almost 50%, even after almost converging to a near completed extraction during the first extraction (**Fig. 5**, curve B). In practice the vessel is depressurized and then pressurized once more when the next extraction begins. Another study also observed that a few consecutive extractions with decompression in-between improved the yield using SFE [25], however not nearly with the major impact as described in this work. The authors explain the results by appointing the increased yield to a modification or a rupture of the seeds thus increasing the availability of lipids.

The results were compared to those found in the literature with the purpose of finding a general consensus whether either method is able to extract more lipids than the other from seeds. Several other authors have also studied the comparison between conventional extractions methods using organic solvents to SFE applied to seeds in general. These studies did however not investigate the impact of repeated extractions with a sudden decompression step. Some authors acquired the same yield [26, 27], whilst others acquired less using SFE compared to the conventional method [25, 28-30], perhaps in some cases partly due to too short extraction time. However, most importantly no author has stated that SFE extracted higher amounts of lipids than using conventional organic solvents, even at pressures as high as 68 MPa which Taylor, King [27] carried out the extraction at.

Table 1. A summary of the SFE-ELSD method validation experiments. The SFE was performed at P = 30 MPa, T = 80 °C, ρ = 746 kg m^{-3} and Q = 1.5 mL min^{-1} with 0.6 g of linseeds for 90 min. Soxhlet extraction was performed using petroleum ether with 3 g of linseeds for 8 h.

	SFE-ELSD	**SFE with gravimetric determination**	**Soxhlet**
Extracted amount (%, 95% CI)	26.0 ± 0.4 (n = 9)	27.1 ± 0.7 (n = 9)	32.3 ± 1.3 (n = 3)
Repeatability	2.6% RSD	3.8% RSD	1.6% RSD
Intermediate precision	2.6% RSD	3.8% RSD	-

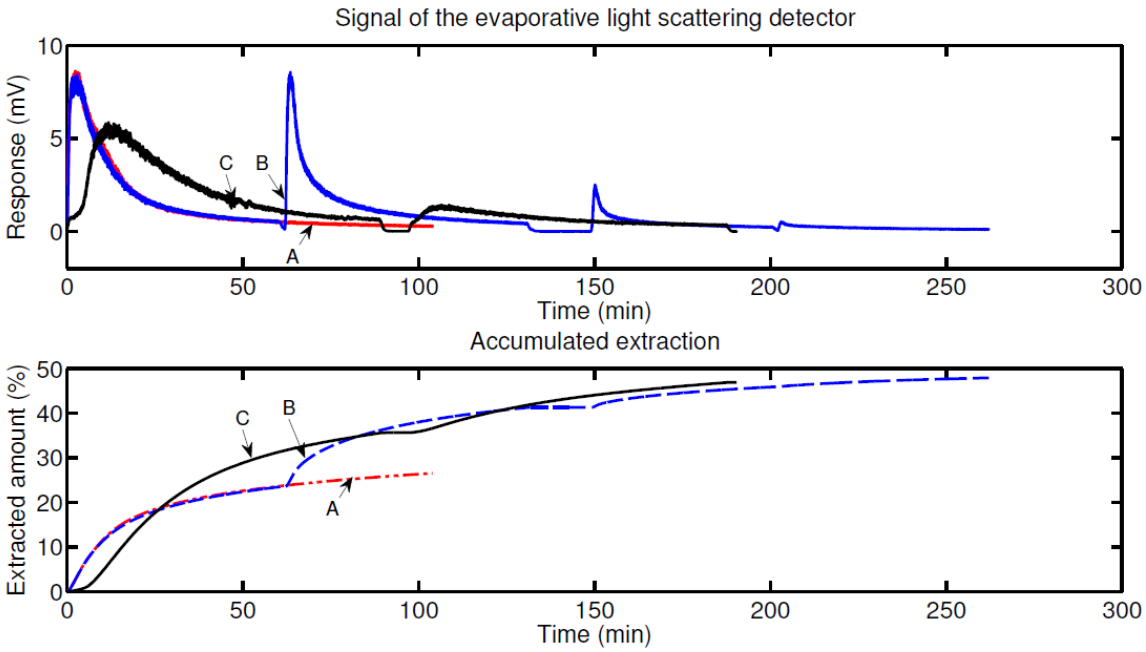

Fig. 5. Comparison of repeated extractions of linseed with depressurization in-between using a small extraction vessel (10 mm x 30 mm) and extraction using a larger vessel (17.5 mm x 125 mm). The red dot dashed line (A) shows a single extraction using the smaller vessel, the blue line (B) shows several repeated extractions with the smaller vessel and the black line (C) shows the extraction with the larger vessel. The SFE was performed at P = 30 MPa, T = 80 °C, ρ = 746 kg m^{-3} and Q = 1.5 mL min^{-1} with 0.6 g of linseed.

Another possible reason is that the incomplete extraction is caused by channeling effects. This aspect is seldom discussed in the literature associated with analytical chemistry. Several previous works of various authors have chosen to add dispersant, commonly sand or small glass beads, to the extraction vessels mixed with the sample to avoid channeling effects [29, 31]. Hence, various experiments were performed with different combinations of glass bead sizes (1-5 mm Ø), fine sand, glass wool, extraction vessel orientation and packing strategies in the same extraction vessel as described earlier (10 mm x 30 mm). Some experiments were also performed with more narrow columns (7.75 mm ID) with varying length ranging from 50 to 250 mm and thereby also altering the fraction of glass beads as the sample volume was kept constant. No effect on the extracted amounts could be observed when using different dispersants or packing strategies, besides that mixing with fine sand severely reduced the extraction efficiency (data not shown). The mixing with sand could possibly cause a packing of the sample prone to channeling effects. An additional experiment was performed where sand was added in the end of the extraction vessel, with the outlet facing vertically downwards, showed no negative effects. Thus suggesting that the sand does not function as an adsorption material for the lipids, which could otherwise be suspected.

To study the availability of lipids in the sample a few additional experiments were performed. These experiments were performed by further grinding the linseeds using a ball mill and then mixing the sample with various

types of glass beads. Although the initial extraction rate was slightly higher, the total extracted amount was not different from that of the regular crushed linseeds (data not shown).

In other areas of extraction, i.e. in the field of chemical engineering, agitation is usually applied in order to minimize unavoidable channeling affects. This approach inspired to perform experiments using a larger vessel (17.5 mm x 125 mm) with a stirring bar, and a magnetic stirrer. No glass beads were used in these experiments, mainly due to the immobilization of the stirring bar. The extraction curves are not directly comparable with the previous experiments using smaller columns, due to more void volume in the larger extraction vessel. However, the amounts of extracted lipids was much greater compared to a single extraction using the smaller extraction vessel. Actually, a single extraction using the larger vessel converged to the same extractable amounts as several repeated extractions including depressurization with the small-dimensioned extraction vessel (**Fig. 5**, curve C). Although the extraction rate was initially faster by applying stirring or using the further grinded seeds, all the yields obtained using the large vessel seemed to converge towards the same result (**Fig. 6**). Further grinding of the linseeds had a larger impact on the initial extraction rate than added stirring. Due to the large volume of the extraction vessel, a full extraction could not be carried out using only the volume of one pump and a refill was necessary. A minor increase in the extraction rate was observed initially during the second subsequent extraction. During the expansion and the refill time the compounds most likely had time to diffuse outwards in the linseeds, thus explaining the temporary increase in response.

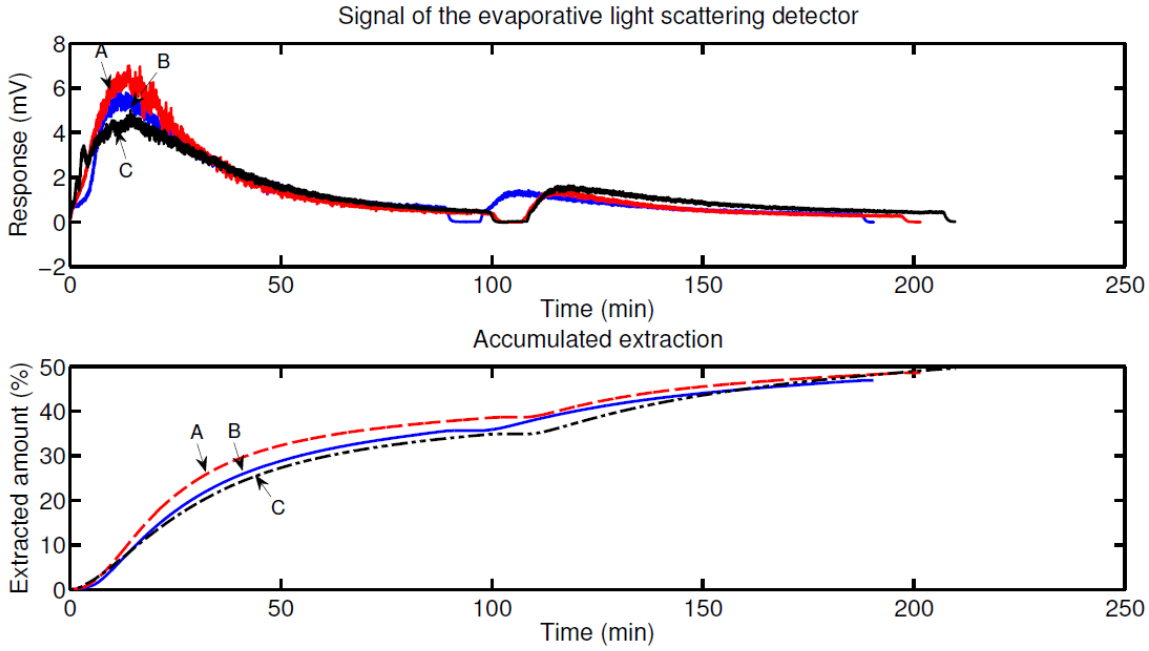

Fig. 6. Extraction performed using the larger extraction vessel (17.5 mm x 125 mm) using extra grinded linseed without stirring (A, red dashed line), regular crushed linseeds with stirring (B, blue line) and without stirring (C, black dot-dashed line). The SFE was performed at P = 30 MPa, T = 80 °C, ρ = 746 kg m^{-3} and Q = 1.5 mL min^{-1} with 0.6 g of linseed.

In addition, one extraction was performed using the smaller vessel mixed with a few 5 mm Ø glass beads although yet keeping some void volume. The extraction was carried out until the extraction was deemed completed at 90 min. At that given time, the vessel was agitated by a few swift hits with a wrench and a sudden rise in the detector response was observed (data not shown), strengthening the hypothesis that channeling causes incomplete extraction in continuous SFE.

4. Conclusions

The viability of using ELSD as an online detector hyphenated to SFE has been tested and validated. In terms of quantification of the extractable lipids the detection showed good trueness and precision. The overall method repeatability and intermediate precision was 2.6% RSD. Besides that the developed instrumentation can be used for rapid quantitative quantification of extractable lipids using SFE, it can also be used to efficiently determine the required length of an extraction to be considered complete. Spectroscopic data acquisition in-line rather than on-line is problematic at very high pressures above 40 MPa due to lack of flow cells being able to withstand these pressures. The SFE-ELSD would not be a limiting factor under those extraction conditions due to the coupling after the BPR.

The most prominent feature of the system is the rapid data acquisition of extraction data. Instead of performing several extractions and terminating at various extraction times or simply collecting fractions over time in order to study extraction kinetics, the newly developed system allows for more data with better precision. The detector provides 250 measurements per minute and only one extraction is needed whereof no fractions are collected to be used for further determination using *e.g.* a gravimetric balance. This kind of data would be ideal for the use of studying extraction kinetics, especially for development and calibration of mathematical models describing the extraction process. Also as mentioned earlier, one of the advantages is the ability to more easily determine the required extraction time. Furthermore, the initial extraction rate close to the start of the extraction between decompression and decompression could be studied more accurately. The spike in extraction rate noticed when performing agitation by brute force would otherwise not have been noticed. These things might not be as easily studied using conventional methods.

The SFE-ELSD system was utilized to study channeling effects during extraction of lipids from crushed linseeds. A single extraction using SFE acquired less extracted amounts than using Soxhlet. However, by several subsequent extractions of linseed contained in an analytical sized extraction vessel (10 mm x 30 mm), a much higher yield (approximately 50%) was obtained compared to that of both a single extraction and the Soxhlet reference method using petroleum ether, 26 ± 0.4% (95% CI, n = 9) and 32.3 ± 1.3% (95% CI, n = 3), respectively. The same result was also obtained performing only one single extraction by using a larger extraction vessel (17.5 mm x 125 mm), both with and without stirring. Further grinding of the linseeds did not improve the total extracted amount, however, it did increase the initial extraction rate.

Therefore, it is suggested that channeling plays an important role in analytical scale extraction using SFE. Even as the extraction seemed to converge towards completed during the first extraction using the smaller vessel, only approximately 55% of all extractable compounds were actually obtained.

Thus it is recommended that a decompression step and an additional subsequent extraction is performed when conducting a validation of a method including SFE. It might also be desirable to avoid a very tight packing of the

sample in order to ensure a possible change of conformation during the decompression step. For any analytical chemist this should be a crucial part of the validation work in order to ensure good trueness.

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