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Supported liquid extraction in the quantitation of plasma enterolignans using isotope dilution GC/MS with application to flaxseed consumption in healthy adults

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ABSTRACT

Dietary interventions involving foods that are enriched in lignans, such as flaxseed, are drawing attention due to their beneficial protective effects in various diseases and human conditions. Accurate quantitation of key lignan metabolites such as enterodiol (END) and enterolactone (ENL) is necessary in order to identify factors that may influence overall bioavailability. Here we describe the validation of a novel supported liquid extraction (SLE) method for isolation of plasma enterolignans, END and ENL, using $^{2}H_{6}$-labeled isotopes with gas chromatography–mass spectrometry in micro selected ion storage (GC/MS-μSIS) mode. Following enzymatic hydrolysis and SLE extraction with 70:30 diethyl ether:ethyl acetate, enterolignans were rapidly separated within 8 min. SLE in combination with GC/MS-μSIS gave high recoveries of 96.4% and 96.0% for END and ENL. Intra-assay precision ranged from 2.5 to 5.9% for both compounds whereas the inter-assay precision was 2.6–6.9%. SLE was also directly compared to liquid liquid extraction (LLE). Both techniques offered high precision and accuracy, however, SLE consistently enabled successful analyte extractions and derivatizations, unlike LLE, which had an ~4% failure rate. SLE was also tested in a study where dietary milled flaxseed supplementation (30 g/day for 1 month) and enterolignan bioavailability was examined in a healthy, human population ($n = 10$). Plasma total enterolignan levels significantly increased ($P = 0.002$) at 4 weeks relative to baseline. Average concentrations for END and ENL were 209 nM and 304 nM, respectively.

1. Introduction

Epidemiological studies have shown the beneficial effects that diets rich in whole grains [1], seeds (flaxseed and sesame) [2–4], fruits and vegetables [5] and beverages (teas and wine) [6] have on lowering cardiac risk factors. These foods are very high in plant lignans which have protective effects against various cancers and cardiovascular diseases [7,8]. Flaxseed or linseed (Linum usitatissimum) is not only one of the richest sources of the cardioprotective $n-3$ fatty acid, alpha-linolenic acid (ALA), but also of polyphenolic plant lignans. Secoisolariciresinol diglucoside (SDG) is the most prevalent lignan found in flaxseed followed by matairesinol, lariciresinol and pinoresinol [9]. In nature, lignans exist primarily as glycosides [10] and upon consumption are metabolized by microflora in the intestines to form the enterolignans enterodiol (END) and/or enterolactone (ENL) (Fig. 1). They are then absorbed by the gut and repackaged in the liver as β-glucuronide or sulfate conjugates, which then circulate within the bloodstream or are eliminated in urine or feces [11]. Information on inter-individual differences in END and ENL production have been reported in several studies in humans [11–13] and are suggested to be influenced by the nature of the host bacteria in the intestinal tract [14]. In terms of the beneficial properties of enterolignans it has been suggested that they may behave as antioxidants in vivo

Abbreviations: ALA, alpha-linolenic acid; BSTFA, N,O-bis(trimethylsilyl)-trifluoroacetamide; END, enterodiol; ENL, enterolactone; GC/MS-μSIS, gas chromatography/mass spectrometry-micro selected ion storage; HPLC–CEAD, high performance liquid chromatography–coulometric electrode array detection; LLE, liquid liquid extraction; LOD, limit of detection; LOQ, limit of quantitation; SDG, secoisolariciresinol diglucoside; SIM, single-ion monitoring; SLE, supported liquid extraction; SPE, solid phase extraction; TMS, trimethylsilylated; TR-FIA, time resolved fluoroimmunoassays.

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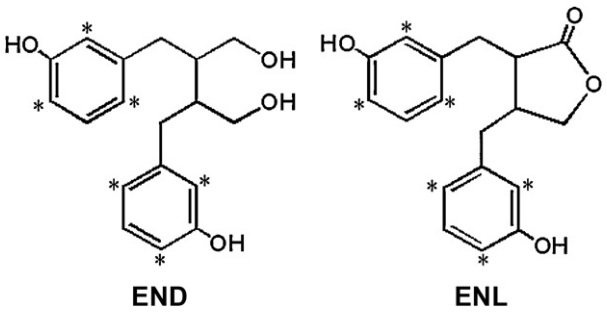

Fig. 1. Chemical structures of END, $^{2}H_6$-END, ENL and $^{2}H_6$-ENL. * Refers to the location of the deuterium ions in [2,2',4,4',6,6'-$^{2}H_6$] enterodiol (596 g/mol) and [2,2',4,4',6,6'-$^{2}H_6$] enterolactone (448 g/mol).

as these properties have already been demonstrated in vitro [15].

Traditional methods of extracting enterolignans from plasma involve lengthy liquid liquid extraction (LLE) [16] or solid phase extraction (SPE) steps [17]. Supported liquid extraction (SLE) is a relatively new technology that offers advantages of high recoveries, removal of the matrix effect, reduced sample preparation times, loss of emulsions and reduced sample volumes. SLE and LLE are comparable in analyte recoveries yet SLE offers improvements due to matrix cleanup qualities [18]. SLE is conceptually analogous to traditional LLE, however, it uses an inert support material of modified diatomaceous earth, with a high surface area for extraction interface and a modified flow-through technology [19]. Unlike SPE, SLE does not require solvent preconditioning and is directly loaded with aqueous sample to which water absorbs to the column bed, thus effectively removing it from the equation. Analytes are eluted from both SPE and SLE columns using solvents, however, the initial loading conditions, as predetermined by the stationary phase, differentiate the flow-through technology of the two techniques. Current applications involving SLE related to plasma bioanalysis include matrix purification [20], circulating drugs [20–23], steroid hormones [19], vitamin D [24] and phospholipid removal [25].

Detection and quantitation of human plasma enterolignans have been accomplished primarily using liquid chromatography–mass spectrometry (LC/MS) [26] or gas chromatography–mass spectrometry (GC/MS) [11,27–32] with more recent procedures including more tandem MS techniques [12,13,16,17,33–36]. Other methods such as time resolved fluoroimmunoassays (TR-FIA) [37–39] and liquid chromatography with coulometric electrode array detection (HPLC–CEAD) [40] have been employed, however, they lack specificity compared to techniques using mass spectrometry. To the best of our knowledge, SLE has never been applied to the extraction of plasma enterolignans and has only been used once as a sample preparatory tool in conjunction with GC/MS [41].

Compared to GC/MS operated in full scan mode, micro selected ion storage (μSIS) offers improved ion sensitivity and selection. Full scan analyses offer qualitative information yet suffer in their limits of detection (LOD) due to interfering background noise. μSIS is a similar technique to single-ion monitoring (SIM) where narrow selection windows are used to extract ions of interest at specified time points. Incorporating this technique with isotopically labeled compounds as internal standards enables accurate analyte quantitation by removing any errors that may have arisen during sample preparation and/or GC/MS analysis.

This manuscript details the validation of an SLE method to extract END and ENL from human plasma, followed by silylation and quantitation using GC/MS-μSIS. A direct comparison of SLE to LLE will demonstrate the preserved analytical integrity of this new method, with the benefit of increased sample throughput. Application toward plasma enterolignans at baseline and 4-weeks post daily ground flaxseed consumption (30 g/day) in a healthy adult population will also be discussed.

2. Materials and methods

2.1. Chemicals, materials and reagents

HPLC grade (≥95%) standards of enterodiol and enterolactone were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Hexadeuterated internal standards of $^{2}H_6$-enterodiol ($^{2}H_6$-END) and $^{2}H_6$-enterolactone ($^{2}H_6$-ENL) were purchased from Dr. Wähälä (University of Helsinki, Finland). Aqueous sodium acetate buffers (0.5 M and 0.1 M; pH 5.0) and 2600 units of β-glucuronidase-sulfatase from *Helix Pomatia* (EC 3.2.1.31) (G1512) (made up in 0.5 M sodium acetate buffer) were prepared fresh daily (Sigma Aldrich).

Pyridine and ethyl acetate were both Chromasolv Plus grade for HPLC (≥99.9%), diethyl ether and methanol were Chromasolv grade for HPLC (≥99.9%) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (derivatization grade) were all purchased from Sigma Aldrich. Buffers were made using doubly distilled water collected at 18 MΩ-cm from a Barnstead E-Pure system (Dubuque, Iowa, USA). Rabbit plasma from male New Zealand white rabbits (2.8 ± 0.1 kg, Southern, Rose Rabbitry) was used for preliminary solvent optimization experiments. Human plasma (HPEK2-0500) for preparation of standards, blanks and spikes for all of the validation studies had K_2-EDTA as the anticoagulant and was purchased from Cedarlane Laboratories Limited (Burlington, Ontario, Canada). Human plasma from healthy adults consuming a daily dosage of flaxseed is described in Section 2.2. All plasma samples were frozen at −80 °C and thawed at room temperature prior to using.

Isolute SLE+ 1 mL supported liquid extraction columns were purchased from Biotage (Charlotte, North Carolina, USA). All glass vials and vial inserts were silanized and screw caps were PTFE-lined. These were all purchased from VWR International (Mississauga, Ontario, Canada).

2.2. Human plasma samples from flaxseed study

This study was approved by the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee. Healthy human volunteers were between 18 and 49 years of age, were equally male and female and were not taking cholesterol lowering or hypertension drugs, anti-histamines or nutritional supplements. Plasma was obtained at baseline and 4 weeks following daily dietary supplementation with a muffin containing 30 g of ground flaxseed. Muffins were baked by the Canadian International Grains Institute in Winnipeg, Manitoba and stored for a maximum of one month at −20 °C. Two types of muffin flavors were prepared: banana chocolate chip and orange cranberry. Formulation details and a flavor profile are available for the orange cranberry muffin [42]. Individuals were asked to continue with their same dietary pattern with no restrictions placed on consumption of lignan containing foods, other than those containing flaxseed. Baseline plasma enterolignan levels would be indicative of typical lignan consuming patterns prior to dietary intervention. Plasma was collected into EDTA containing tubes, spun at 1800 × g at 4 °C for 5 min and then aliquoted into cryovials and stored at −80 °C until analysis. Throughout the 4 week study, participants were allowed to withdraw at any time without consequence.

2.3. Preparation of standards and blank

Pure standards of END, ENL and their $^{2}H_6$-isotopically labeled internal standards (Fig. 1) were accurately weighed and dissolved

in methanol then stored at −20 °C until needed. Blanks and standards were prepared by first adding 13.8 μL of a 17.3 μM cocktail of internal standards to each silylated glass tube. The final concentration of internal standard at the time of injection was 1000 nM. Eight standards were then made, equally distributed throughout the concentration range (33–1000 nM) by the addition of aliquots from either a 170 or 1000 nM END and ENL stock standard solution. Methanol was removed under a gentle stream of N_2 (g) and then 300 μL of control human plasma was added. Sample blanks contained only the internal standard and plasma. Calibration standards were run in duplicate at the start and end of each sample set with the combined averages of each of these injections enabling the final construction of the calibration curves. Final sample concentrations were calculated using the slope of the line equation ($y = mx + b$) where y represented the ratio of enterolignan area to its corresponding $^2H_6$-internal standard area and x was the concentration being solved for.

2.4. Enterolignan hydrolysis

Kuijsten et al. previously demonstrated validated conditions for hydrolyzing enterolignans in plasma [16]. Briefly, all standard/sample enterolignan hydrolyses and isolations were carried out in 4 mL silanized tubes containing 1 μM of internal standards, devoid of methanol. Three hundred μL of plasma was added to each tube, followed by the addition of 300 μL of 0.1 M sodium acetate buffer, pH = 5.0. Concentrated hydrochloric acid (12 M) was used to adjust the pH. Sixty μL of freshly prepared β-glucuronidase-sulfatase from *H. Pomatia* (2600 units made up in 0.5 M sodium acetate buffer, pH 5.0) was then added to each tube. Total volume of sample (300 μL), buffer (300 μL) and enzyme (60 μL) yielded 660 μL. An additional optimization experiment was carried out using 240 μL of 0.1 M sodium acetate buffer instead of 300 μL to assess if altering the sample-to-buffer ratio from 1.2:1 to 1:1 would impact the effectiveness of the separation. Individual tubes were capped, gently vortexed (3 × 5 s) and then incubated at 37 °C for 4 h in the dark using a Model 2000 Micro Hybridization Chamber (Robbins Scientific). Enterolignan aglycones were then isolated from the matrix using either LLE or SLE.

2.5. Extraction of mammalian enterolignans using LLE

LLE was done using a slight modification of the extraction procedures described by Kuijsten et al. [16]. Following incubation, standards/samples were left at room temperature in the dark for 15 min prior to extraction. Each standard/sample was extracted twice with 1.5 mL of diethyl ether. After each 1.5 mL addition of diethyl ether, samples were vortexed 3 × 5 s each and then centrifuged at 2300 × *g* for 10 min at 10 °C. The upper diethyl ether layer was removed and added to a new 4 mL silanized tube. Both ether extracts were combined. The resulting diethyl ether was evaporated under a gentle stream of N_2 (g) at room temperature liberating the enterolignan mixture. Samples were derivatized immediately using the method described in Section 2.8.

2.6. Optimization experiments for extraction of mammalian enterolignans using SLE

Following incubation, samples were left at room temperature in the dark for 15 min prior to loading onto the SLE columns situated on a vacuum manifold (Vac Elut SPS 24 Manifold). Once total sample volume was loaded (660 μL), vacuum from the in-house system (19″Hg) was applied for 10 s, which enabled immediate entry of the sample onto the column. Following a 6 min waiting period, enterolignans were eluted by gravity into 8 mL silanized vials using a number of different test solvents including diethyl ether, ethyl acetate, dichloromethane, 1% methanol in ethyl acetate, 70:30 (v/v) diethyl ether:ethyl acetate or 50:50 (v/v) diethyl ether:ethyl acetate (4 × 1.25 mL). Once all of the organic solvent had eluted or a maximum time of 6 min elapsed, vacuum was gently pulled through the system for 1 min and then more forcefully for an additional 10 s to ensure complete sample elution. Evaporation of the solvent was accomplished using a gentle stream of N_2 (g) with the tube bases warmed to 37 °C using aluminum beads contained in a Lab-Line brand Lo-Boy Tissue Float Bath. These samples were derivatized as described in Section 2.8. Each solvent was assessed for its ability to quantitatively extract enterolignans from the SLE column. Three criteria in selecting the elution solvent required that the averaged internal standard areas for each compound have RSD values ≤15%, the calibration linearities were ≥0.98 and percent recoveries were ≥90%. Overall elution and drying times were taken into consideration as well.

2.7. Method validation

The solvent that met the above criteria was then used for all method validation experiments of which derivatization limits, carryover, sensitivity, linearity, precision and accuracy were all assessed. Successful derivatization was associated with a high linear correlation when larger concentration standards were added to the already existing calibration range. The highest concentration standard that was examined was 2300 nM. A correlation value above 0.98 indicated successful derivatization of the enterolignans and thus suitability of the derivatization method at these concentrations. To assess the reproducibility of SLE to routinely produce high analyte recoveries in a consistent manner, 5 replicates of both a medium (170 nM) and high (1000 nM) enterolignan mixture were spiked in control human plasma. Additionally, spiked plasma samples containing 250 nM and 830 nM were prepared in triplicate and calculated for their percent recoveries. Carryover was determined by running a blank sample prepared in control human plasma directly after a high concentration standard. The sensitivity of the method was calculated by determining the signal-to-noise ratio of a low concentration standard made using a standard solution that was further diluted with derivatization solvent.

2.8. Derivatization procedure

Silylation of standards/samples for GC/MS analysis was accomplished immediately following LLE and SLE using a 1:1 (v/v) pyridine:BSTFA mixture (120 μL of each reagent). Derivatization proceeded by heating at 90 °C in a Single-Wall Transite Oven (Blue M Electric Company) for 30 min and then remained at room temperature in the dark overnight. Blanks, standards and samples were analyzed within 24 h.

2.9. LLE and SLE extraction comparison

In order to determine the effectiveness of switching to SLE from LLE, it was important to compare both sample preparation techniques in tandem. A condensed calibration made up using 3 standards ranging from 170 to 1000 nM was prepared in control human plasma in triplicate and enterolignans were extracted using both techniques. The two methods were assessed for linearity across the calibration range, percent recoveries from 330 nM spikes to plasma and relative standard deviations of internal standard areas. Diethyl ether was used as the extraction solvent for LLE and 70:30 (v/v) diethyl ether:ethyl acetate was used for SLE. Cost and time analyses were calculated as well for a clinical trial involving ~300 samples.

Table 1
Target and qualifier ions of trimethylsilylated (TMS) forms of END, ENL and their hexadeuterated internal standards.

Compound	Retention time (min)	Compound Mw[a] (g/mol)	Ion monitored	Target ion (*m*/*z*)	Qualifier ion (*m*/*z*)
Enterolignans					
END	5.42	590	[M−180]	410	180
ENL	6.36	442	[M−262]	180	442
Internal Standards					
$^{2}H_6$-END	5.40	596	[M−180]	416	183
$^{2}H_6$-ENL	6.33	448	[M−265]	183	448

[a] Molecular weights (Mw) are representative of the TMS derivatized sample.

2.10. Gas chromatography/mass spectrometry conditions

A Varian 450-Gas Chromatographic instrument connected to a 240-Mass Spectrometry detector from Agilent Technologies was used to separate and detect the trimethylsilylated (TMS) enterolignans. A splitless injection of 1 μL of standard/sample was injected using a Varian CP-8400 autosampler. The injector temperature was maintained at 240 °C throughout analyses. A Varian FactorFour capillary column (VF-5ms 30 m × 0.25 mm × 0.25 μm) from Agilent Technologies was used. The sample was carried at 1.0 mL/min using ultra-high purity helium as the carrier gas through the column which was rapidly heated from 120 to 290 °C at a rate of 50 °C/min in 3.4 min. The temperature was then held at 290 °C for 4.6 min completing the run at 8 min. The external ion trap was operated in positive EI mode (70 V) using micro-selected ion storage (μSIS) mode. Table 1 identifies the retention times as well as the target and qualifier ions that were used to identify the enterolignans and their corresponding internal standards. ENL and $^{2}H_6$-ENL (target *m*/*z*'s of 180 and 183, respectively) had an isolation window of 2 *m*/*z* whereas END and $^{2}H_6$-END (410 and 416) was 3 *m*/*z*. Trap and manifold temperatures were operated at 150 and 50 °C, respectively, with the transferline and ion source temperatures set to 250 °C. Peak areas of analytes were integrated using MS Workstation version 6.9.3 software (Agilent Technologies). Quantitation was based upon the most abundant fragment ion, with confirmation based upon the next most abundant fragment. The relative area of each standard compared to its corresponding stable isotope was used for quantitation.

2.11. Stability of derivatized enterolignans

Derivatized samples were typically analyzed within 24 h however stability of analytes beyond this was investigated further. Samples were immediately recapped and stored at −80 °C following each GC/MS analysis. Upon reinjection, samples were allowed to return to room temperature, vortexed (3 × 5 s) and reinjected at 48 and 144 h post-initial analysis.

2.12. Statistical analysis

Statistical significance was calculated with Sigma Stat software (version 2.03, SPSS) using the paired Student's *t*-test. Differences between means were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Chromatographic identification of enterolignans and internal standards

Confirmation of pure forms (without matrix effects) of TMS derivatives of END, ENL and their corresponding internal standards was first accomplished using higher concentrations (17 μM) of each compound and analyzed individually using GC/MS in full scan mode (data not shown). $^{2}H_6$-END and END eluted at 5.40 and 5.42 min, respectively whereas $^{2}H_6$-ENL and ENL had retention times of 6.33 and 6.36 min (Table 1). Application of μSIS as an ion storage mode greatly improved instrument sensitivity of enterolignans compared to full scan mode as seen in Fig. 2. This technique is used in ion trap mass spectrometers to store desired ions and reject unwanted ones thus removing interfering matrix ions and enhancing overall signal-to-noise. μSIS offered a two-fold increase in ion sensitivity relative to SIS mode. Improved limits of detection (LOD) with μSIS-MS compared to SIS-MS mode has been demonstrated previously in a MS detection comparison study involving pesticides [43]. Target ions were the most abundant fragment ions formed and were used for quantitation using μSIS analysis whereas qualifier ions were used as confirmation ions to ensure the absence of interfering compounds (Fig. 3). Mass spectral data agrees with that previously published for both labeled [44] and unlabeled compounds [45] (data not shown). No detectable analyte carryover was observed following analysis of the highest standard (1300 nM) as monitored by subsequent blank solvent injections.

Other groups using LC/MS/MS and GC/MS-SIM have noted the presence of phytoestrogens coming from the enzyme β-glucuronidase from *H. Pomatia* [34,46]. Although different sources of the enzyme were used in each of these studies, Grace [34] detected small levels of genistein and trace levels of O-desmethylangolensin within the enzyme itself and Adlercreutz et al. [46] determined that the contribution was from daidzein. In our study, small interfering shoulder peaks were observed at both retention times of END and ENL when examining the sample blank. In order to determine the source of this compound, alternate blanks were prepared in which one contained plasma alone and the other plasma and enzyme. Internal standards were not added to either blank, however buffers were present. All sample pretreatment was continued as usual with final analysis using GC/MS in both full scan and μSIS modes. No detectable differences were visible in full scan mode due to both the poor sensitivity of this technique and the low levels of this interference within the sample. In μSIS mode, the interfering peaks did not appear when plasma alone was used, but did when plasma and enzyme were combined. The most abundant *m*/*z*'s of these peaks were at 183.0 and 411.2 for END and 180.1 and 449.2 for ENL when examined in μSIS mode. This pointed to the enzyme as being the source of interference. To correct for this, sample blanks were prepared with every sample set and subtracted from the measured enterolignan values.

3.2. Optimal solvent and buffer selection with enterolignans and isolute SLE+

Preliminary investigation into selection of appropriate solvent systems began by examining spiked rabbit plasma samples at levels within our mid-to-upper concentration range. Four separate sets of calibration standards (330, 660 and 1000 nM) were prepared and loaded onto Isolute SLE+ columns. Four solvent systems were investigated for their ability to effectively desorb all of the compounds of interest. These included diethyl ether, ethyl acetate, 1% methanol with ethyl acetate and dichloromethane. Diethyl ether was chosen due to its previously demonstrated high extraction capabilities with enterolignans when using LLE [16], the low levels

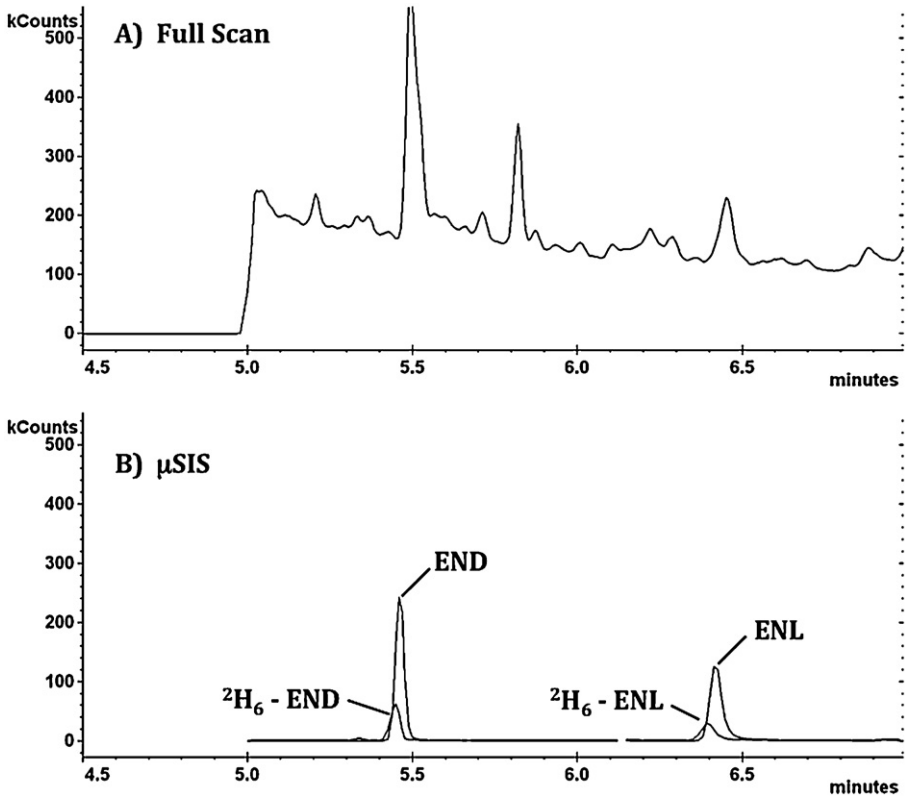

Fig. 2. Full scan (A) and μSIS (B) GC/MS chromatograms of a standard containing 2.3 μM END and ENL with 1.0 μM of both internal standards. For Fig. B the following m/z's were used: 410 for enterodiol (END), 416 for $^2H_6$-END, 180 for enterolactone (ENL) and 183 for $^2H_6$-ENL.

of interfering plasma phospholipids that are eluted using this solvent with SLE [47] and its high volatility. The remaining three solvents were recommended by the manufacturer as part of their SLE+ method development guidelines [48]. Optimal extraction conditions were initially assessed by comparing the relative standard deviations (RSD values) of the three sets of duplicate internal standards and of the linearity coefficients (R^2). Dichloromethane was immediately disqualified as it did not solubilize END or $^2H_6$-END. $^2H_6$-END and $^2H_6$-ENL both had the lowest RSD values when using 100% ethyl acetate (4.6% and 4.0%, respectively) and 1% methanol in ethyl acetate (5.3% and 3.3%). Diethyl ether gave slightly higher RSD values of 10.1% and 8.5%. Linearity coefficients across this calibration range were similar for diethyl ether and ethyl acetate for both compounds as defined by R^2 values $\geq$0.99, whereas the use of 1% methanol in ethyl acetate yielded a poor R^2 value of 0.97 for END and 1.00 for ENL. The addition of methanol was initially selected due to the known solubility of enterolignans in this solvent. However, as methanol and water are miscible, it was decided to abandon the use

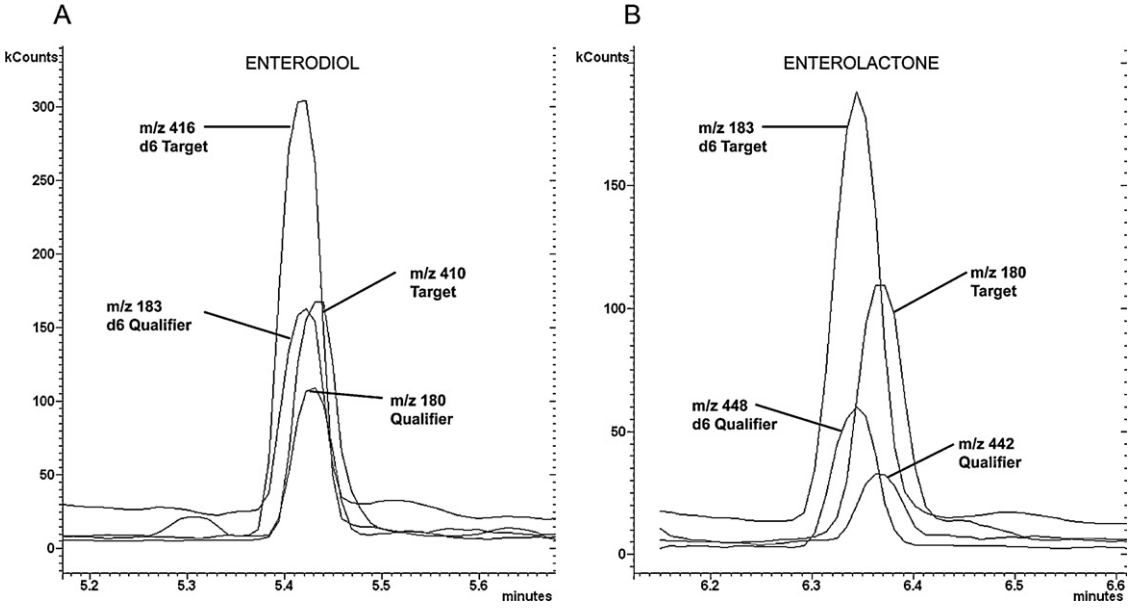

Fig. 3. GC/MS chromatographic spectra of TMS derivatives of $^2H_6$-END, $^2H_6$-ENL (1000 nM), END and ENL (270 nM) in μSIS mode.

of this solvent so that water would not be brought back into the final sample eluent. Only diethyl ether and ethyl acetate were further investigated due to their immiscibility with water and their effectiveness in solubilizing enterolignans. A major drawback of using ethyl acetate was the considerably longer drying times following SLE compared to diethyl ether (70 versus 30 min). Subsequent experiments optimized the ratio of diethyl ether to ethyl acetate and incorporated moderate heat (37 °C) during solvent evaporation as a means to reduce overall extraction times.

Diethyl ether to ethyl acetate ratios of 70:30 (v/v) and 50:50 (v/v) were compared along with their parent solvents as controls in the next set of optimization experiments, now using human plasma and moderate warming to reduce evaporation times. Each of these experiments was performed in triplicate on separate days (Table 2). Linearity was high in each of the groups (>0.991). Relative standard deviations were attenuated in the 70:30 diethyl ether:ethyl acetate group with $^2H_6$-END as 7.2% and $^2H_6$-ENL as 8.7%. The other solvent systems yielded deviations ranging from 8.5 to 21.1% for both compounds. Percent recoveries were very high for each of the extraction solvents ranging from 94.8 to 104.3%. Evaporation times were much lower using 70:30 diethyl ether:ethyl acetate (30 min) relative to the 50:50 group which was 45 min. Further experiments were carried out using 70:30 diethyl ether:ethyl acetate which exhibited high recovery and linearity values, had the lowest relative standard deviations for each of the compounds of interest and had a lower evaporation time.

Manufacturer recommendations for Isolute SLE+ [49] suggest using equivalent (v/v) ratios of buffer to biological fluid so as to control sample viscosities thus promoting even flow through the column. Modeling the hydrolysis conditions established by Kuijsten et al. [16] the ratio was 1.2:1. To test a 1:1 ratio we used 240 μL of buffer. RSD and R^2 values were similar for both (data not shown). Further validation experiments were done using 300 μL of buffer.

3.3. Method validation

Using 70:30 diethyl ether:ethyl acetate for all further elutions, the accuracy and precision of the method was determined. To assess the precision of SLE as a sample preparatory tool, multiple replicates ($n = 5$) of low (170 nM) and high (1000 nM) enterolignan standards were prepared by spiking human control plasma. At low concentrations of END, levels averaged 157 ± 13 nM whereas ENL was 173 ± 13 nM yielding relative standard deviations of 5.8 and 5.9%, respectively. High concentrations averaged 1015 ± 28 nM for END and 1057 ± 46 nM for ENL with RSD values of 2.5 and 4.1% (Table 3). These values are comparable to those reported by other authors [16,34].

The inter-day variability of both medium (270 nM) and high (670 nM) END and ENL standards was acceptable. The lower concentration group had deviations below 4.3% and the higher group was less than 6.9%. It was difficult to directly compare this with literature as most studies typically monitor inter-day variability using lower concentration standards. This study reports higher concentrations than those normally monitored as these are typical levels in plasma for populations consuming foods high in lignans, such as flaxseed [36]. Kuijsten noted that enterolignan RSD values ranged from 10 to 21% across two standard concentrations, with the highest concentration being 39.2 nM [16]. These deviations are higher than ours in part because of the lower concentration they are monitoring. Adlercreutz et al. reported that in omnivorous and vegetarian women, errors ranged between 5.1 and 18.1% for END and between 1.4 and 10.7% for ENL [11]. Higher deviations were present for the lower concentrations relative to the higher ones. Smeds and Hakala reported that enterolignans prepared in plasma compared to those that are not have much higher RSD values [17] which is possibly why Grace [34] and Valentin-Blasini et al. [33] reported lower values (3.3–5.1%) as there was no matrix influence present in the preparation of their calibration curves.

To assess the percent recoveries at both lower and higher concentrations that would be typical of those observed in healthy individuals consuming flaxseed or other high lignan diets, control human plasma samples were spiked at 250 and 830 nM ($N = 3$). Percent recoveries were 96.4 ± 3.4% for END and 96.0 ± 5.2% for ENL when the spike contained 250 nM of enterolignans. Recoveries were slightly higher at the higher concentration level with END as 103.3 ± 5.1% and ENL as 114.4 ± 8.8%. These recoveries compare quite nicely with values reported by Adlercreutz et al. of 98.9% and 97.9% for END and ENL using GC/MS-SIM [11]. Lower values were reported by Nesbitt et al. (84.6 and 86.9%) [29]. Kuijsten et al. reported recoveries of 97% for END and 99% for ENL when using 100 nM spikes when extraction was done using LLE with analysis by LC/MS/MS [16]. High recoveries were also exhibited when 170 nM spikes were added to serum samples and extracted using SPE and quantitated using LC/MS/MS (END 97.0% and ENL 97.6%) [34]. Both of these studies look at lower concentration spikes typical of low to moderate lignan intake diets. However, plasma samples from individuals consuming high lignan foods like flaxseed have enterolignan levels much higher than what these groups report as "high" lignans. Therefore, spikes of 250 and 830 nM are more relevant when investigating plasma profiles of subjects consuming higher lignan containing foods like flaxseed.

Instrument sensitivity was established using signal-to-noise (S/N) ratios involving spiked enterolignans in human plasma. The LOQ, defined as a S/N of 10:1, was 9.9 nM for END and 10.1 nM for ENL. A S/N of 3:1 defined the LOD and was 6.6 and 6.7 nM, respectively. Signal-to-noise was calculated using MS Workstation version 6.9.3 using the ratio of peak heights. Baseline noise was calculated via the software from peak to peak. As compliancy is one of the applications of this method for studying enterolignan bioavailability from flaxseed consumption, 30 nM was set as the lowest calibration standard for the remainder of the study.

3.4. Derivatization

To ensure that all enterolignans were being derivatized to completion, the linear range of the calibration was extended. Linearity was still being achieved up to 2330 nM for both END and ENL ($R^2 \geq 0.99$). Levels higher than this were not tested so no upper limits were defined. Therefore, calibration ranges within the limits of 33–2330 nM were acceptable for these two enterolignans, however a maximum of 1000 nM was suitable for our analyses.

3.5. Sample stability

Samples were typically left at room temperature overnight following the 30 min heating derivatization step at 90 °C and analyzed the next morning. However, sample stability was examined at 48 and 144 h post initial analysis after having been recapped and stored at −80 °C in-between. Comparison of internal standard areas at baseline and 48 h showed no significant changes in both $^2H_6$-END and $^2H_6$-ENL ($N = 11$). However, $^2H_6$-END was significantly attenuated at 144 h relative to baseline ($P = 0.020$) as was $^2H_6$-ENL ($P = 0.048$).

3.6. Plasma enterolignan concentrations from flaxseed consumption

Isolute SLE+ in conjunction with GC/MS-μSIS was applied to the extraction and quantitation of plasma samples from healthy adults who were on a 4 week flaxseed diet. Baseline levels of END and ENL hovered tightly around the established LOQ for each compound,

Table 2
Solvent optimization in the extraction of enterolignans from human plasma with solvent removal at 37 °C.

Compound	100% ether	70%:30% ether:EtOAc	50%:50% ether:EtOAc	100% EtOAc
RSD[a]				
$^2H_6$-END	14.0 ± 5.9%	7.2 ± 1.7%	11.0 ± 5.2%	8.5 ± 2.4%
$^2H_6$-ENL	21.1 ± 2.5%	8.7 ± 0.9%	11.5 ± 4.1%	10.6 ± 1.7%
Linearity (R^2)				
END	0.991 ± 0.007	0.998 ± 0.001	0.999 ± 0.000	0.997 ± 0.002
ENL	0.995 ± 0.001	0.998 ± 0.001	0.999 ± 0.001	0.995 ± 0.006
% Recovery[b] (%)				
END	103.2 ± 8.8	95.0 ± 6.8	99.1 ± 3.7	95.6 ± 3.4
ENL	104.3 ± 12.9	97.7 ± 9.0	94.8 ± 7.0	101.5 ± 5.5
Drying time[a] (min)	18	30	45	60

Results are represented as the mean ± S.D.
Ether refers to diethyl ether and EtOAc refers to ethyl acetate.
Results are based upon five calibration standards ranging from 100 to 1000 nM and measured as TMS derivatives using GC/MS.
[a] Based on areas of internal standards (N = 5).
[b] Calculated from an N = 3. Spike was 420 nM.

suggesting low consumption levels of lignan containing foods in young to middle-aged adults within the Winnipeg, Manitoba region. Following 4 weeks of daily consumption of muffins fortified with 30 g of ground flaxseed, total enterolignan (END + ENL) levels increased significantly in all individuals relative to baseline levels (n = 10) (P = 0.002). Final END and ENL concentrations ranged from 49 nM to a maximum of 401 nM after 4 weeks and from 35 to 1128 nM, respectively. The average value for END was 209 nM and 304 nM for ENL. Between subject variability was also very high which has been documented previously [12,36]. As all subjects exhibited increased plasma enterolignans, these compounds offer potential as reliable biomarkers of plant lignan consumption and compliancy in intervention trials involving flaxseed [13,50].

Plasma enterolignan concentrations have been reported in only a few studies where 25–30 g of flaxseed was used as the lignan source. These were short-term studies typically lasting only a maximum of 1 to 2 weeks. Saarinen et al. report average values of 731 nM for END and 755 nM for ENL following 8 days consumption of 25 g crushed flaxseed with large between subject variability [36]. Another study involving females required similar consumption patterns of 25 g of ground flaxseed daily for 8 days. Measurement every 3 h over the first 24-h period revealed a steady increase in baseline levels for both END (22.45–46.29 nM) and ENL (6.90–19.65 nM) which increased to a maximum of 3- to 4-fold after 8 days [29]. In addition, Morton et al. demonstrated that healthy postmenopausal Australian females consuming 25 g/day of flaxseed for 2 weeks had marked increases of END (582 nM) and ENL (379 nM) relative to baseline (3 nM and 17 nM, respectively) [30]. A number of factors play a role on the varying concentrations of circulating enterolignans resulting from flaxseed consumption. Some of these include the varying lignan contents within flaxseed cultivars, the type of flaxseed (milled or whole), dietary patterns, intestinal transit times, differences in gut micro flora and antibiotic usage [12,51,52].

3.7. SLE and LLE validation comparison

Preliminary experiments in our laboratory were conducted using LLE with diethyl ether for the isolation of enterolignans from plasma. A reoccurring problem was that 1 out of every 24 samples (~4%), within at least every other sample set, never displayed internal standard or analyte peaks within the chromatogram. Repeated injections of the null sample followed by injection of a sample and standard that did previously show peaks within the same sample set verified proper injection syringe, autosampler and GC/MS function. At no time during sample preparation were there any observable differences between any of the samples within a sample set during hydrolysis, extraction or derivatization. As all samples were extracted uniformly and sequentially, it was hypothesized that water from the aqueous layer may have been transferred with the organic layer erroneously or an emulsion may have existed. It is well-known that water is a scavenger of silylating materials [53] so it was hypothesized that water may have been competing and possibly preventing this reaction from proceeding. However, additional experiments, with extra care given to not remove any of the lower aqueous layer, still resulted in this ~4% sample loss. Experiments to test whether or not water is impeding the derivation process have not been done. There are no reports to our knowledge of this problem with LLE, however, difficult emulsions associated with LLE are well documented [19,54]. Morton et al. reports using LLE with diethyl ether with final analysis of the BSTFA derivatized enterolignans using GC/MS [30]. No complications were reported. However, an additional separation step was used following LLE using a diethylaminohydroxypropyl Sephadex LH-20 short column with CO_2 bubbled methanol as the eluent. This additional separation step may have removed any residual water. An additional complication of this method was the large plasma volume of 1 mL which was required. As this sample loss problem could not be resolved without potentially adding an additional separation step like Morton used we determined that a new, single-step extraction method had to be developed that would additionally enable a smaller plasma volume to be used (300 μL instead of 1 mL). Since developing this extraction method using SLE over 400 plasma samples have been extracted with no samples lost as in the case with LLE.

Due to high sample loads that often accompany clinical investigations, it was of interest to validate our SLE method to see if

Table 3
Intra- and inter-assay precision of mid- and high-concentration enterolignans as TMS derivatives quantified using GC/MS in μSIS mode.

Enterolignan	Intra-assay precision (n = 5)		Inter-assay precision (n = 5)	
	[C] (nM)	RSD (%)	[C] (nM)	RSD (%)
END (mid)	170	5.8	270	2.6
END (high)	1000	2.5	670	6.9
ENL (mid)	170	5.9	270	4.3
ENL (high)	1000	4.1	670	3.3

Results are calculated based upon area ratios of the standard relative to its labeled isotope.

Table 4
Comparison of SLE and LLE in combination with GC/MS as an extraction technique for enterolignans from 300 μL of plasma ($N = 3$).

Compound	Linearity[a,b] (R^2)	RSD (330)[a,c] (%)	RSD (170)[a,c] (%)	Percent recovery[d] (%)	Analysis time[e] (wks)	Estimated cost[e,f] ($)
SLE					9	4500
END	0.999 ± 0.001	6.5	2.6	101.9 ± 7.5	–	–
ENL	1.000 ± 0.001	4.2	5.0	102.1 ± 7.5	–	–
LLE					12	4300
END	0.997 ± 0.004	7.4	3.0	98.2 ± 6.8	–	–
ENL	0.999 ± 0.002	5.7	7.3	100.7 ± 0.7	–	–

[a] Short calibration curves were used to construct this data (170, 330 and 1000 nM). Each standard was made in triplicate and injected twice.
[b] Values are represented as the mean ± S.D.
[c] % RSD's are based upon 330 and 170 nM standards.
[d] Percent recoveries are calculated based on 330 nM spikes. LLE spike recoveries were done in rabbit plasma.
[e] Calculations were based upon 282 clinical samples.
[f] Cost does not take into account GC/MS analysis or labor.

it could rank competitively, if not better than, LLE as a sample extraction tool. Traditional LLE methods are time consuming and labor intensive when repeated at high sample loads. Therefore, to see if there truly were advantages of switching to SLE from LLE the next series of experiments were performed in tandem using both methods (Table 4). A condensed calibration range was prepared from 170 to 1000 nM having linear correlations ≥0.99. High linearity's have previously been shown using diethyl ether as the mobile phase with both SLE (Table 2) and LLE [16]. RSD values were lower using SLE (<6.5%) than LLE (<7.4%) for each of the compounds at both mid and high concentration ranges and the percent recoveries were comparable ranging from 98.2 to 102.1%. These results indicate that switching from LLE to SLE will not result in any compromise in accuracy or precision of the separation. The next step was to calculate the time it would take to analyze ~300 clinical plasma samples and the estimated cost to complete this analysis. SLE dramatically reduced the number of weeks from 12 to 9 compared to LLE. This is a huge savings in labor expenses alone as higher throughput could be achieved. It was also interesting to calculate the overall cost of estimated consumables required to facilitate these extractions. The difference was estimated to be only $200. Therefore, SLE was chosen as the sample preparatory tool of choice for the extraction of enterolignans from plasma.

In addition to the extraction of plasma enterolignans from a number of other clinical samples within our facility, application of SLE toward extraction of urinary enterolignans is currently being investigated.

4. Conclusions

This paper outlines the successful development and validation of a method to determine END and ENL in human plasma using SLE with quantification using GC/MS-μSIS. This is the first extraction of enterolignans from plasma using SLE as well as the first time SLE has been applied as a sample preparatory technique with plasma in conjunction with GC/MS analysis. The majority of published manuscripts involving SLE use it in combination with LC/MS/MS. The advantages of using SLE over traditional LLE are faster throughput, simplified extractions, reduced costs in terms of labor and elimination of emulsions leading to zero sample loss. This technique has now been applied to the extraction of enterolignans in larger clinical trials where only minimal volumes of plasma are available. To date, there have been absolutely no samples lost due to interferences caused by emulsions in over 400 samples that have been analyzed. In addition, this technique offers an affordable and effective means to quantify END and ENL in plasma as well as providing a means to monitor compliancy in nutritional intervention trials where consumption of high lignan foods, like flaxseed, may be used. Accurate measurement of plasma enterolignans is essential for understanding their bioavailability so that associations with disease prevention can be more clearly identified.

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