APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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A New, Sensitive Method for Lignan Metabolite Detection of Flaxseed-Fed Mice Using LC/MS/MS

Introduction

Dietary phytochemicals such as polyphenols, carotenoids, saponins and glucosinolates have been suggested to play important roles in human health and may contribute to the prevention of diseases. While the intrinsic food bioactives have been found to act as strong antioxidants and exert various activities in vitro, the actual bioactive forms in vivo are not clear. In fact, it is often the metabolites of a phytochemical that exert bioactivities. Among the various bioactive polyphenols found in plant-based foods, lignans are one of the most important subgroups. For example, plant lignans such as secoisolarisiresinol diglucoside (SDG) glycoside in flax seed are not free molecules but part of the lignan complex. These lignan glycosides are released by digestive enzymes and further hydrolyzed to produce the aglycones secoisolariciresinol (SEC) and matairesinol (MAT), which are converted by the microorganisms in the human colon to the bioactive mammalian lignans enterodiol (END) and enterolactone (ENL) (Figure 1).[1] However, due to low bioavailability, metabolites of lignans are at minute concentrations in biological fluid samples. This presents a significant challenge for food and nutritional scientists using conventional detectors coupled with liquid chromatography. LC/MS/MS technology provides an excellent alternative because of its sensitivity.[2]

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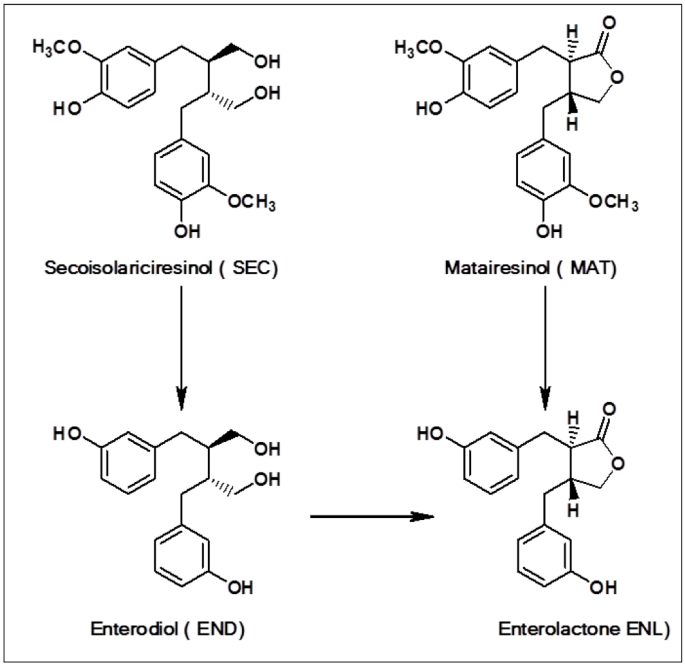

Figure 1. Flax lignans and mammalian metabolites.

Experimental

All mouse serum samples were taken from a feeding trial with a diet containing different amounts and fractions of flaxseed content. The serum (50 mL) was mixed with 100 mL of freshly prepared hydrolysis reagent (contained 2 mg/mL b-glucuronidase/sulfatase in 50 mM NaOAc buffer, pH 4) and incubated at 37 °C for 19 h. 600 mL MeOH was added to each sample (80% final MeOH concentration) after hydrolysis. The samples were vigorously vortexed and placed on a shaker for 1 h., then centrifuged at 20,000 g for 10 min. at 4 °C. The supernatant was transferred to a new microfuge tube, and the pellet was washed with 400 mL of 80% MeOH and then centrifuged. The supernatant was combined and evaporated to dryness using Speed-Vac with a solvent compatible ultra-cold trap and vacuum pump. The dried samples were re-dissolved into 50 mL of 80% MeOH by vortexing, sonication for 10 min. and centrifuged at 20,000 g for 10 min. at 4°C. The supernatant was transferred and analyzed using the QSight™ 220 Triple Quadrupole Mass Spectrometer.

To verify the accuracy of the sample preparation procedure, recovering was determined by calculating the percent recovery of known amounts of lignan standards added to serum from the mouse, which was on a basal diet. Serum was spiked with 10 mL of 5 mg/mL of lignan standard mixture (MAT, SEC, END and ENL) and hydrolyzed and extracted the same way as unspiked samples.

The LC/MS/MS analysis was performed using a QSight 220 Triple Quadrupole Mass Spectrometer. Table 1 outlines the parameter settings used during this method.

Table 1. QSight 220 instrument settings.

ESI Voltage (V)	5800
HSID™ Temp (°C)	250
Nebulizer Gas Setting	300
Drying Gas Setting	125
Source Temp (o°C)	250
Dwell Time (ms)	100
Pause Time (ms)	5

This method utilized an Altus™ UPLC® system. Sample injections of 5 µL were loaded onto a Brownlee™ Phenyl Hexyl (5 x 4.6 mm x 2.7 µm) guard column, using the gradient flow as shown below in Table 2 at a flow rate of 0.4 mL/min. The mobile phase consisted of solvent A (0.1% formic acid + 99.9% H_2O) and solvent B (0.1% formic acid + 99.9% MeOH).

Table 2. Optimized MRM parameters.

Compound Name	Precursor	Fragment	CCL2	CE
Secoisolariciresinol	360.7	164.8	46	32
Enterodiol	300.7	252.8	46	26
Matairesinol	356.6	82.9	40	32
Enterolactone	296.7	252.8	46	26

Table 3. LC cycle time.

Time (min.)	Solvent B %
0-10	58
12	80
13	100
16	58
23	58

Results

The conventional method for extraction and cleanup for serum samples includes enzyme hydrolysis, liquid/liquid partition, evaporation and solid phase extraction chromatography. Those procedures are often time consuming and costly. This method uses microcentrifugation with Speed-vac and proved to be a simple and valid effective protocol with good recovery rates for the compounds of interest. Table 4 shows the recovery rates for the compounds of interest, proving the method protocol to be effective.

Table 4. Recovery rate by standard addition.

Lignan	END	ENL	MAT	SEC
Before Std Addition (ng/mL)	0.7 ± 0.4	3.5 ± 2.4	0.0 ± 0.0	0.6 ± 0.6
After Std Addition (ng/mL)	549.1 ± 36.8	500.8 ± 16.8	528.1 ± 46.9	606.7 ± 22.0
Difference (ng/mL)	548.4 ± 37.1	497.3 ± 19.1	528.1 ± 46.9	606.1 ± 21.9
Expected Difference (ng/mL)	500.0	500.0	500.0	500.0
Recovery Rate %	109.7 ± 7.4	99.5 ± 3.8	105.6 ± 9.4	121.2 ± 4.4

The recovery rates for these compounds were between 99-121%, as determined by the standard addition of known amounts of serum from a mouse fed a basal diet.

All the lignans and their metabolites were well-separated and detected using the QSight 220 Triple Quadrupole Mass Spectometer. Figures 2 and 3 illustrate the results for a typical LC/MS/MS total ion chromatogram of the standards (5 ppb = 15 nM) and actual serum sample respectively. Identification of the lignans was done by congruent retention time and (-)-ESI mass spectrometry. For sensitive quantification, the MS/MS pairs were between the molecular ion $[M-H]^-$ and its most abundant daughter ion.

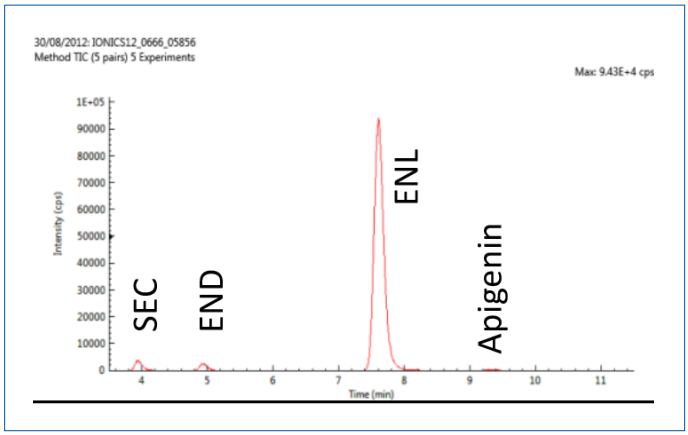

Figure 2. Example total ion chromatogram of the standards at 5 ppb = 15 nM.

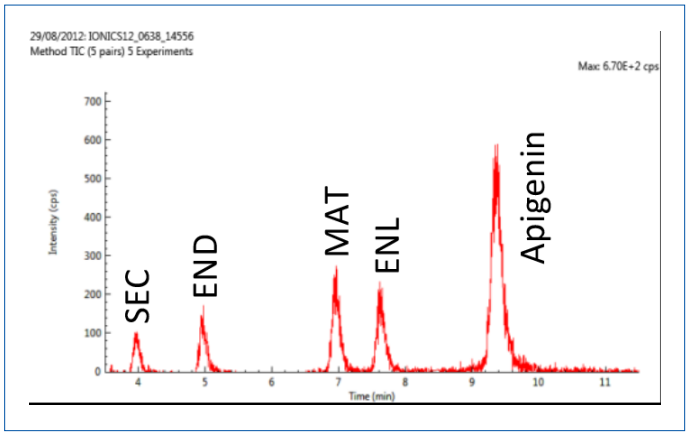

Figure 3. Example total ion chromatogram of the actual serum sample.

Using this method, the detection of all targeted lignans (SEC and MAT) and the two most important metabolites (END and ENL) in the serum samples of mice fed different amounts of flax seed meals, were detected. ENL and END are mammalian lignans known for their many health beneficial effects. In addition, a minute amount of apigenin, a flavonoid also found in flaxseed was detected in the serum (Figures 2 and 3).

Table 5 shows the serum concentrations of the four lignans in mice fed diets containing no or different flaxseed components, i.e. 10% whole flaxseed flour, 6% flax kernel flour or 4% flax hull powder. All these compounds were detected at the nM – µM level. There were individual differences in the ability to produce the

Table 5. Bioavailability of lignans & metabolites in healthy male mice fed different flax seed fractions (µM in serum) (selected samples).

Treatment	Sample	END µM	ENL µM	MAT µM	SEC µM
Basal diet (control)	S1	0.000	0.007	0.000	0.000
	S2	0.000	0.006	0.000	0.000
10% Flaxseed	S7	4.324	3.541	0.000	0.298
	S8	2.983	0.678	0.000	0.088
6% Flax kernel	S9	0.071	0.058	0.000	0.070
	S10	0.049	0.088	0.000	0.000
4% Flax hull	S31	3.374	1.678	0.000	0.082
	S32	2.938	0.247	0.000	0.023

metabolites, possibly due to the different microbial flora of these mice. Some produced more END than ENL, others more ENL than END. The two plant lignan aglycones SEC and MAT were of very low bioavailability, in fact, no MAT was detected in the majority of the samples. These plant lignans are mostly in the hull of the flax seed, therefore, it is highly significant to see the low concentrations of END and ENL in serum of mice fed kernel.

The concentrations of END, ENL, SEC and MAT were measured for 180 samples and they differed significantly, with END to be the most dominant metabolite ranging from 0.00452 to 100.35 µM, followed by ENL (0.00452 to 61.75 µM), SEC (0.0048 to 49.45 µM), and MAT (0.0014 to 2.76 µM). Enterodiol (END), a mammalian lignan, was the predominant metabolite in the serum whereas the plant lignan matairesinol (MAT), was found to be barely absorbed.

The standard curves around the concentrations detected in the serum samples were highly linear for all analytes, with R^2>0.993 with no weighting (Figure 4). The detection limits for all compounds were < 1 ppb, ca. 3 nM.

The calibration curves generated for SEC (360.7/164.8), END (300.7/252.8), MAT (356.6/82.9) and ENL (296.7/252.8) showed good linearity (R^2>0.993).

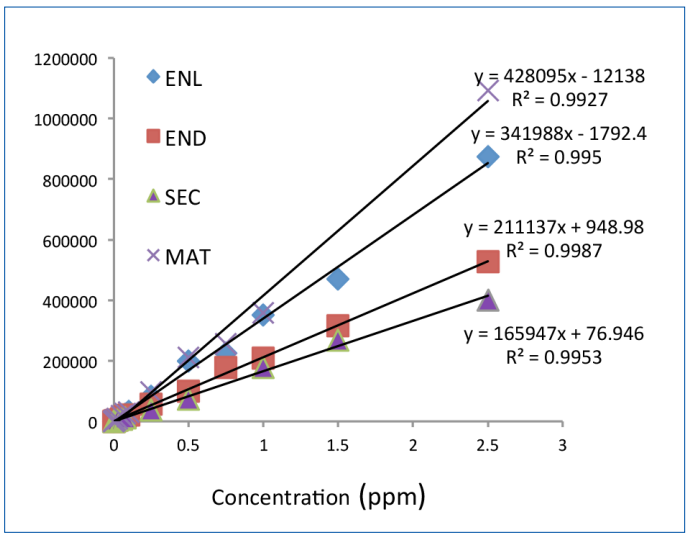

Figure 4. Standard curves of flax lignans and their metabolites.

Conclusion

Present results demonstrate that the reported LC/MS/MS method using a new highly sensitive QSight 220 triple quadrupole platform is capable of detecting <1 ppb level of dietary polyphenols and their metabolites in biological systems using only 50 µL of biological sample. This new and robust analytical method can help better understand the health benefits and risks of lignan containing foods.

References

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2. Wu, Q; Wang, M; Simon, JE. Analytical methods to determine phytoestrogenic compounds. Journal of Chromatography B, 812 (2004) 325–355

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