Characterization of the Fluorescent Spectra and Intensities of Various Lignans: Application to HPLC Analysis with Fluorescent Detection†

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There is considerable interest in dietary lignans since they have been shown to have antioxidant, estrogenic and lipid-lowering activity in humans. In this study, the fluorescent excitation and emission spectra of seven lignans were characterized and their relative fluorescent intensities compared. The lignans were found to have similar excitation (286.6 ± 2.5 nm, X ± SD) and emission (320.1 ± 6.4 nm) maxima; however, their fluorescence intensities on a molar basis decreased in the following order: asarinin, sesamin, sesamolin, seco-isolariciresinol, seco-isolariciresinol diglucoside and matairesinol. Enterolactone, a mammalian lignan conversion product, and sesamol, an antioxidant found in sesame oil, also exhibited significant fluorescence excitation and emission intensities. A high-performance liquid chromatographic method using photodiode array (PDA) and fluorescent detection was developed for the analysis of the individual lignans. Analysis was performed on a reversed phase C-18 column with methanol–water (70:30, v/v) as the mobile phase. With fluorescent detection, the limits of quantitation (LOQ) was 0.1 ng or 2.82 nmol for sesamin and asarinin, 2.70 nmol for sesamolin, 2.76 nmol for seco-isolariciresinol, 1.45 nmol for seco-isolariciresinol diglucoside, 2.79 nmol for matairesinol and 0.5 ng or 1.67 nmol for enterolactone. With PDA detection, the LOQ was a 1000-fold less sensitive than with fluorescent detection.

Introduction

Phytoestrogens are a group of plant-derived substances that have structural and physiological properties similar to that of the steroidal estrogens. Lignans are the major phytoestrogens; however, the isoflavone, and coumestane class of compounds also have phytoestrogenic activity (1, 2). These compounds have gained considerable attention in the past decade not only because of their phytoestrogenic activity (1, 2), but also because of their antioxidant (3–5) and lipid-lowering properties (6–10). Sesame seed lignans, such as sesamin and sesamolin, have been shown to be strong inhibitors of desaturase enzymes which are involved in polyunsaturated fatty acid biosynthesis in humans (6). In addition, many studies have shown that both sesame seed and flax seed lignans, or their oils, lower cholesterol concentrations, decrease atherosclerosis and reduce cardiovascular disease in humans and animals (7–10). Lignans have also been shown to reduce the growth of human breast tumors in athymic mice (11).

Lignans occur in many plants, however, they occur in highest concentrations in several oil seed (3, 4, 7–10, 12–16) and in various types of cereals (2). Of the food crops, sesame seed (4, 12–15) and flaxseed (7, 8, 16) have been shown to be one of the richest sources of lignans. The major lignans in sesame seed and sesame oil (Sesamum indicum L.) are sesamin, sesamolin, pinorresinol and sesaminol glucosides (4, 12), whereas the major lignans in flaxseed are seco-isolariciresinol and seco-isolariciresinol diglucoside (7, 8, 16). Coumestrol and genistein are also major coumestan and isoflavone components of flaxseed (7, 8, 16). The mammalian lignans, enterolactone and enterodiol, are also of interest since they are metabolites of lignans, isoflavones and coumestanes and have been shown to be responsible for the phytoestrogenic properties of these classes of compounds (17, 18). In humans, enterolactone and enterodiol are produced primarily from the dietary plant lignans seco-isolariciresinol, seco-isolariciresinol diglucoside and matairesinol (18).

There have been few studies on the fluorescent properties of the lignans and no studies comparing the excitation and emission spectra and the relative fluorescent intensities of the individual lignans. Several of the sesame lignans have been analyzed by TLC (13), high-performance liquid chromatographic (HPLC) (12, 14, 15, 19), GC–MS (12) and LC–MS-MS (20). Two HPLC methods with fluorescent detection have been developed for the analysis of sesamin, asarinin and sesamolin (12, 14) and one for the analysis of seco-isolariciresinol and seco-isolariciresinol diglucoside (19).

In this report, the fluorescent spectra of some of the major lignans found in sesame seed, flaxseed and soybean seed were determined and characterized. In addition, the fluorescent excitation and emission intensities of the individual lignans, as well as those of enterolactone and sesamol, were compared on a molar basis. An HPLC method for the fluorescent analysis of the individual lignans has been developed and validated. The HPLC method could be applicable for the analysis of lignans in a variety of food products, plant material and dietary supplements as well as for the isolation and purification of the individual lignans for pharmacokinetic and biological activity testing.

Experimental

Reagents and chemicals

Sesamin, sesamol and enterolactone were obtained from Sigma Life Sciences, St Louis, MO, USA; asarinin was obtained from Aldrich Chemical Co., Milwaukee, WI, USA, and sesamolin,
sec-o-isolariciresinol, sec-o-isolariciresinol diglucoside and matairesinol were all obtained from ChromaDex, Irvine, CA, USA. Methanol, HPLC grade, and ethyl alcohol were obtained from Fisher Scientific, Fair Lawn, NJ, USA and Pharmaco Products, Inc., Brookfield, CT, USA, respectively.

Analytical procedures

Preparation of stock standards
Stock standards of all of the lignans were prepared in methanol at 1.0 mg/mL. Since some of the compounds were sparingly soluble in methanol, the standards were closely watched to insure that they were completely dissolved in methanol. All standards and calibrators were stored at 4 ± 3°C.

Spectrofluorometric analysis
All spectrofluorometric scans of the standards were performed on a PTI QuantaMaster Fluorescence Spectrometer (Photon Technology International, Edison, NJ, USA). The fluorescent excitation and emission spectra were made in quartz cuvettes (Hellma, USA) that had been rinsed at least three times with HPLC grade methanol and allowed to air dry between each sample.

HPLC fluorescent analysis
Chromatographic analysis was performed on an Alliance 2695 Separations Module equipped with a 2996 Photodiode Array Detector and a 474 Scanning Fluorescent Detector (all from Waters Corp, Milford MA, USA). Analysis was performed on a Waters Symmetry C-18 reversed phase column, 4.6 × 250 mm, 10 mm (WAT054215) and on a Waters Symmetry C-8 reversed phase column, 3.9 × 150 mm, 10 μm (WAT054235) using methanol–water, 80:20, v/v, as the mobile phase. The chromatographic flow rate was 0.5 mL/min and the HPLC injection volumes ranged from 5 to 20 mL. HPLC fluorescent analyses of the individual compounds were performed using the excitation and emission maxima of the individual lignans. When all of the compounds were analyzed, the excitation and emission wavelengths were 290 and 320 nm, respectively, and the gain and attenuation were set at 100 m and 320 nm, respectively, and the gain and attenuation were set at 100 and 64. PDA analyses were usually performed at 290 nm with PDA scans from 200 to 400 nm.

Results

Characterization of the ultraviolet spectra
The ultraviolet (UV) spectra of the individual lignans were characterized using the PDA detector. PDA analysis indicated that sesamin, sesamolin and asarinin have similar UV absorption maxima at 206, 237 and 287 nm. Similar UV absorption maxima were found for sec-o-isolariciresinol, sec-o-isolariciresinol diglucoside, matairesinol and enterolactone.

Characterization of fluorescence spectra
The excitation and emission spectra of one of the lignans, sesamin, are shown in Figure 1. A summary of the excitation and emission maxima and the relative excitation and emission intensities of the individual lignans are given in Table I. The individual lignans had excitation maxima that ranged from 284 to 290 nm.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Excitation Maxima, nm</th>
<th>Normalized Excitation Intensities</th>
<th>Emission Maxima, nm</th>
<th>Normalized Emission Intensities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asarinin</td>
<td>289</td>
<td>9.980 × 10^4</td>
<td>321</td>
<td>1.001 × 10^3</td>
</tr>
<tr>
<td>Sesamin</td>
<td>289</td>
<td>6.318 × 10^4</td>
<td>321</td>
<td>8.202 × 10^3</td>
</tr>
<tr>
<td>Sesamolin</td>
<td>290</td>
<td>4.197 × 10^5</td>
<td>335</td>
<td>4.157 × 10^5</td>
</tr>
<tr>
<td>Seco-isolariciresinol</td>
<td>284</td>
<td>4.393 × 10^5</td>
<td>316</td>
<td>3.872 × 10^5</td>
</tr>
<tr>
<td>Seco-isolariciresinol diglucoside</td>
<td>284</td>
<td>4.231 × 10^5</td>
<td>316</td>
<td>3.747 × 10^5</td>
</tr>
<tr>
<td>Matairesinol</td>
<td>286</td>
<td>2.207 × 10^5</td>
<td>316</td>
<td>2.113 × 10^5</td>
</tr>
<tr>
<td>Sesamolin</td>
<td>303</td>
<td>1.074 × 10^5</td>
<td>341</td>
<td>1.049 × 10^5</td>
</tr>
<tr>
<td>Enterolactone</td>
<td>281</td>
<td>9.276 × 10^4</td>
<td>306</td>
<td>9.311 × 10^4</td>
</tr>
</tbody>
</table>

All of the compounds were prepared and analyzed at a concentration of 1.0 μg/mL.

Table I

Excitation and Emission Maxima and Fluorescence Intensities of Various Lignans

HPLC analysis with fluorescent detection
Both the Waters Symmetry C-8 and C-18 reversed-phase columns were found to be suitable for the analysis of the individual lignans; however, the C-18 column and the methanol–water, 70:30, v/v, mobile phase gave better resolutions of the individual lignans than did the C-8 column and the methanol–water, 90:20, v/v, mobile phase. As a result, the C-18 column was used for the analysis of sesamin, sesamolin, asarinin, sec-o-isolariciresinol, sec-o-isolariciresinol diglucoside, matairesinol and enterolactone (Table I). Plots of the emission spectra for sesamin, asarinin, sesamolin, sec-o-isolariciresinol, sec-o-isolariciresinol diglucoside, matairesinol and enterolactone are given in Figure 2. The excitation and emission spectra of sesamol is presented in Figure 3.

The mean excitation wavelength was 286.6 ± 2.5 (X ± SD). The emission maxima of the individual lignans ranged from 316 to 335 nm with a mean emission wavelength of 320.1 ± 6.4 nm. Sesamol, while not a lignan, exhibited excitation and emission maxima of 303 and 341 nm, respectively (Table I). The most intense fluorescent emission was found for asarinin and sesamin followed by sesamolin, sec-o-isolariciresinol, sec-o-isolariciresinol diglucoside, matairesinol and enterolactone (Table I). Plots of the emission spectra for sesamin, asarinin, sesamolin, sec-o-isolariciresinol, sec-o-isolariciresinol diglucoside, matairesinol and enterolactone are given in Figure 2. The excitation and emission spectra of sesamol is presented in Figure 3.
Chromatographic peak resolution was similar to that with the Waters Symmetry C-18 column.

**Limits of quantitation**

With the fluorescent detector, the limits of quantitation (LOQ) for sesamin, asarinin, sesamolin, seco-isolariciresinol, seco-isolariciresinol diglucoside and matairesinol were 0.1 ng and 0.5 ng for enterolactone. On a mole basis, the LOQ would be 2.82 nmol for sesamin and asarinin, 2.70 nmol for sesamolin, 2.76 nmol for seco-isolariciresinol, 1.45 nmol for seco-isolariciresinol diglucoside, 2.79 nmol for matairesinol and 1.67 nmol for enterolactone. With PDA detection, the LOQ of the individual lignans was 0.1 and 0.5 μg for enterolactone. On a mole basis, the LOQ would be 2.82 μmol for sesamin and asarinin, 2.70 μmol for sesamolin, 2.76 μmol for seco-isolariciresinol, 1.45 μmol for seco-isolariciresinol diglucoside, 2.79 μmol for matairesinol and 1.67 μmol for enterolactone. Standard curves of sesamin, asarinin, sesamolin, seco-isolariciresinol and seco-isolariciresinol diglucoside were found to be linear from 1.0 to 1000 ng/mL with the fluorometric detector and 100–1000 μg/mL with the PDA detector. We did not evaluate the LOQ with the Waters Acuity chromatographic system and Acuity UPLC column.

**Discussion**

The UV and fluorescent spectra of some of the major lignans were characterized and their relative fluorescent intensities compared. While the excitation and emission patterns of the various lignans were very similar, the fluorescent intensities varied 4-fold or more on a molar basis. HPLC with fluorescent detection was found to be a 1000-fold more sensitive than UV detection. This was true for sesamin, asarinin, sesamolin, seco-isolariciresinol, seco-isolariciresinol diglucoside, matairesinol and enterolactone. Even though
fluorometric analysis of the lignans was more sensitive than UV analysis, the PDA method can be useful where high sensitivity is not required. To our knowledge, there have not been any studies comparing the fluorescent spectra or the fluorescence intensities of sesamin, arsinarin, sesamolin, seco-isolariciresinol, seco-isolariciresinol diglucoside, matairesinol and entero lactone. 

Our results indicate that the individual lignans can be analyzed on C-18 columns with methanol–water, 70:30, v/v, or methanol–water, 80:20, v/v, as the mobile phase. Seco-isolariciresinol and seco-isolariciresinol diglucoside has been analyzed by HPLC previously (19). That method, however, differed from our method in that they used a chromatographic gradient for the analysis. In addition, their analysis was performed at excitation and emission wavelengths of 277 and 617 nm, respectively (19), whereas our analyses were performed at 290 and 320 nm, respectively.

The chromatographic conditions described in this report would permit the preparative isolation and purification of the individual lignans for use in biological and clinical studies. It is important that the composition of the individual lignans be quantified prior to any nutritional or biological study and that the purity of the compound or compounds is verified before use. Such quantitation of the individual lignans would greatly increase the reproducibility and validity of the nutritional and biological studies as well as provide critical information on the dose being administered. Depending on the dose used, the HPLC method with fluorescent detection may be sensitive enough to perform pharmacokinetic analysis of the individual lignans in serum or plasma. There may be a need for some modification of the HPLC method due to the matrix effect of serum or plasma; however, the sensitivity and specificity of fluorescent detection should not be affected since co-extractable interferences are less of a problem with fluorescent detection than with UV detection.

Conclusion

This study shows that lignans like sesamin, arsinarin, sesamolin, seco-isolariciresinol, seco-isolariciresinol diglucoside and matairesinol have strong fluorescent excitation and emission properties. The HPLC fluorescent method developed for analysis of the individual lignans was found to be a 1000-fold more sensitive than HPLC methods based on UV detection and, for many of the lignans, the sensitivity levels were comparable to that achieved with mass spectrometry (20). Depending on the sensitivity requirements, the HPLC and UPLC fluorescent methods could be used to analyze and quantify the concentrations of these compounds in a variety of plant material, food products and nutraceuticals. In addition, the HPLC fluorescent method could be used to verify the purity and concentration of the individual lignans prior to their use in biological and clinical studies.

References