A simple and sensitive method has been developed for determining honokiol and magnolol in fresh Magnolia obovata (M. obovata) or Magnolia officinalis (M. officinalis) has been used in traditional Japanese herbal medicine, called Magnolia Bark in the Japanese Pharmacopoeia, for the treatment of thrombotic stroke, typhoid fever, fever, and headaches.1) It has been reported that Magnolia Bark suppresses mitogen-induced proliferation of human peripheral blood lymphocytes2) and has central depressant effects.3) Honokiol and magnolol (structures shown in Fig. 1), isomers of neolignans, have been isolated from the bark of this plant and other Magnoliaceae.4) These compounds have been found to exhibit muscle relaxant activity,5) to inhibit intracellular calcium mobilization in platelets caused by collagen, even in the presence of indomethacin,6) to relax vascular smooth muscles by releasing an endothelium-derived relaxing factor and to inhibit calcium influx through voltage-gated calcium channels,7) and to have antithrombotic and antiendothelial effects.8) Recent studies indicate that honokiol has an antagonistic effect on calmodulin9) and magnolol has anti-inflammatory and analgesic effects.10) In addition, honokiol has been reported to induce calcium mobilization, and to show neurotrophic activity in rat cortical neurons.11,12) Thus the quality control of Magnolia Bark by determining honokiol and magnolol is important for pharmaceutical companies in the production of traditional Japanese herbal medicines. According to the Chinese Pharmacopoeia, the branch bark, root bark, flower buds, as well as the stem bark of M. officinalis var. biloba are also used for medicinal purposes. The distribution of honokiol and magnolol is significant for harvest of these crude herbal medicines in the optimal season, and/or for the selection of trees.

Several methods that have been reported for the determination of honokiol or magnolol are: ion-pair high-performance liquid chromatography (HPLC),13) HPLC with UV detection (HPLC-UV),14–16) and capillary zone electrophoresis (CZE).17) However, these methods lack the sensitivity and selectivity required for determining contents of honokiol and magnolol in a small part of a sample tree.

Electrochemical detection (ECD) is respected for its high sensitivity and selective determination of honokiol and magnolol, because it is both selective and sensitive for redox compounds such as phenolic compounds. Yet, no paper has been published on the determination of honokiol and magnolol by HPLC with ECD (HPLC-ECD).

In our previous reports, we successfully developed a more than 30 fold sensitive HPLC-ECD method for determining catechins21) and quercetin22) in human plasma, and ortho-phenylphenol in lemon rind23) using a microbore octadecysilica (ODS) column, when compared to a reported HPLC-ECD method that used a conventional ODS column.21–23) So, the present HPLC-ECD method using a microbore column (μHPLC-ECD) was expected to be an even more highly sensitive method. In the present study, we developed a μHPLC-ECD method for determining honokiol and magnolol, using diethylstilbestrol (Fig. 1) as an internal standard (I.S.). We applied the present μHPLC-ECD method to the determination of honokiol and magnolol contents in Magnolia Bark and analysis of the distribution of branches and leaves of Magnolia obovata.

Fig. 1. Structures of Honokiol, Magnolol and Diethylstilbestrol
leaves of *M. obovata*.

### Experimental

**Materials and Reagents** Honokiol (>98.0%), magnolol (>99.0%), and diethylstilbestrol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol (HPLC grade) and phosphoric acid (85% reagent grade) were purchased from the same source. Other reagents were of reagent grade and available from commercial sources. Magnolia Bark of the Japanese Pharmacopoeia was obtained from Uchida Wakanyaku Co., Ltd. (Tokyo, Japan).

**μHPLC-ECD System and Conditions** The μHPLC-ECD was comprised of an LC-26A vacuum degasser (BAS, Tokyo), an LC-100 pump (BAS), a 7125 injector fitted with a 5 μl injection loop (Reodyne), a Mightysil RP-18GP ODS column (150×4.6 mm i.d., 5 μm, Kanto Kagaku, Tokyo), and an LC-4000 UV detection (Hitachi). The honokiol and magnolol were also quantified by conventional HPLC according to the following conditions described in the Japanese Pharmacopoeia: the mobile phase of a mixture of acetonitrile–water–acetic acid (50:50:1, v/v/v), the flow rate of 1.4 ml/min, and the wavelength for detection of 289 nm.

**Sample Preparations** Japanese Pharmacopoeia Magnolia Bark: To prepare a test solution, a sample of Magnolia Bark (0.5 g) was added to 40 ml of 70% methanol, heated under a reflux condenser in a water bath for 20 min, cooled, and filtered. The above procedure was repeated with the bark residue, using 40 ml of 70% methanol to make exactly 100 ml, and this solution was used as the test solution. The test solution was then passed through a 0.45 μm membrane filter. A 20-μl volume of the test solution was injected into a conventional HPLC-UV system. Another test solution was diluted with a mixture of methanol–water–phosphoric acid (65:35:0.5, v/v/v) containing diethylstilbestrol (I.S.), and passed through a 0.45 μm membrane filter. A 5-μl volume of the test solution was injected into the μHPLC-ECD system.

### Results and Discussion

**Optimization of μHPLC-ECD Conditions** A hydrodynamic voltammogram (Fig. 2) was measured to determine the optimal detection potentials of honokiol and magnolol. Honokiol and magnolol were oxidized at potentials more than +0.6 and +0.7 V vs. Ag/AgCl, respectively. Two oxidation waves, one at +0.7—0.8 V vs. Ag/AgCl and the other at +1.1 V vs. Ag/AgCl, were observed in the hydrodynamic voltammogram. For potentials more positive than +1.1 V vs. Ag/AgCl, sensitivity was higher, but reproducibility was less, possibly due to contamination of the electrode surface by oxidation products. For highly sensitive determination without loss of selectivity and reproducibility, the value +0.8 V vs. Ag/AgCl was adopted for the present study.

An examination was made of how the ratio of water to methanol in the mobile phase influenced the separation for determinations of honokiol and magnolol. The larger the content of water, the greater was the separation of these peaks. To determine honokiol and magnolol with adequate resolution and within a short time, a mixture of methanol–water (65:35) was chosen for the most suitable mobile phase and column temperature during separation was maintained at 40°C.

Thus, the optimal HPLC conditions were: methanol–water–phosphoric acid (65:35:0.5, v/v/v); flow rate, 25 μl/min; column temperature, 40°C; and applied potential, +0.8 V vs. Ag/AgCl.

**Determination of Honokiol and Magnolol** Figure 3 shows a chromatogram of honokiol and magnolol of 0.27 ng. The detection limits (S/N=3) for honokiol and magnolol were 0.13 pg, respectively. Quantiﬁcation limits of honokiol and magnolol by the present method were compared with those of HPLC-UV.
as shown in Table 1, and the present μHPLC-ECD method was found to be more sensitive. This method is highly sensitive, because the microbore column avoids diffusing samples and slows the flow rate, thereby increasing the electrolytic efficiency of samples on the working electrode.

Honokiol and magnolol in Magnolia Bark were determined by the μHPLC-ECD method. A typical chromatogram for Magnolia Bark is shown in Fig. 4. Honokiol and magnolol contents in the Bark are listed with their recovery data in Table 2. The RSD for honokiol and magnolol were less than 0.87% (n=5). Honokiol’s and magnolol’s recoveries for spiked test solutions were more than 98.7% and RSD were less than 0.93% (n=5). By comparison of the analytical results obtained by μHPLC-ECD and conventional HPLC-UV methods (Table 2), it was noted that both results were essentially identical. By the present μHPLC-ECD method, honokiol and magnolol contents were determined to have smaller RSD than the conventional HPLC-UV method. The results demonstrate that the HPLC-ECD method is characterized by higher reproducibility than the HPLC-UV method, indicating that the present μHPLC-ECD method provides quite accurate measurements of honokiol and magnolol in Magnolia Bark.

**Distribution of Honokiol and Magnolol in Fresh Branches and Leaves of M. obovata** Since the concentrations of honokiol and magnolol in fresh branches and leaves of M. obovata trees may often be very low, it is desirable to use a highly sensitive method for their determination. In this study, contents of honokiol and magnolol in bark, phloem, wood, leaf blades, and petioles of a fresh M. obovata were determined, and the results are listed in Table 3. The contents of honokiol and magnolol in bark of branches of M. obovata were more abundant than in the wood samples. Their contents in petioles of leaves of M. obovata were more abundant than in the leaf blades. Because the minimum amount of M. obovata necessary for determining the honokiol and magnolol contents was only 1 mg, a more detailed distribution of these contents in M. obovata can be determined by the present method. With quite a simple pretreatment, high sensitivity, and very small sample size, the present method is suitable to analyze the distribution of honokiol and magnolol in M. obovata.

**Conclusion**

Because the present μHPLC-ECD method seemed to prevent the diffusion of the injected sample compared with the regular HPLC-ECD method using a conventional column, the sensitivity by the former method would be superior to the latter. In this study, the μHPLC-ECD method has been established as a sensitive, selective, and accurate method for the determination of honokiol and magnolol with simple preparation. This method using small sample amounts was useful for the simultaneous determination of honokiol and magnolol in Magnolia Bark and the distribution analysis of a fresh tree of M. obovata. Therefore, the present method would be a

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**Table 1. Comparison of μHPLC-ECD by Several Methods for Determining Honokiol and Magnolol**

<table>
<thead>
<tr>
<th>Method</th>
<th>Column I.D. (mm)</th>
<th>Quantitation limit (pg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>μHPLC-ECD</td>
<td>1.0</td>
<td>Honokiol: 0.67, Magnolol: 0.67</td>
<td>Present method</td>
</tr>
<tr>
<td>HPLC-UV</td>
<td>4.6</td>
<td>Honokiol: 260, Magnolol: 500</td>
<td>18</td>
</tr>
<tr>
<td>HPLC-UV</td>
<td>4.6</td>
<td>Honokiol: 114000, Magnolol: 51000</td>
<td>19</td>
</tr>
<tr>
<td>CZE</td>
<td>60</td>
<td>Honokiol: 60, Magnolol: 150</td>
<td>20</td>
</tr>
</tbody>
</table>

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**Table 2. Contents of Honokiol and Magnolol in Japanese Pharmacopoeia Magnolia Bark and Recovery from Magnolia Bark Spiked with Honokiol and Magnolol Standards by μHPLC-ECD and HPLC-UV Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Content (n=5)</th>
<th>Recovery (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount (mg/g)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>μHPLC-ECD</td>
<td>Honokiol: 2.83</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>Magnolol: 10.6</td>
<td>0.58</td>
</tr>
<tr>
<td>HPLC-UV</td>
<td>Honokiol: 2.80</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>Magnolol: 10.9</td>
<td>1.72</td>
</tr>
</tbody>
</table>

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**Table 3. Distribution of Honokiol and Magnolol in Branches and Leaves, M. obovata**

<table>
<thead>
<tr>
<th>Positions</th>
<th>Honokiol (mg/g)</th>
<th>Magnolol (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branch</td>
<td>0.246</td>
<td>1.83</td>
</tr>
<tr>
<td>Phloem</td>
<td>0.155</td>
<td>1.53</td>
</tr>
<tr>
<td>Wood</td>
<td>0.024</td>
<td>0.708</td>
</tr>
<tr>
<td>Leaf</td>
<td>0.056</td>
<td>0.320</td>
</tr>
<tr>
<td>Petiole</td>
<td>0.080</td>
<td>0.875</td>
</tr>
</tbody>
</table>
good application in the quality control of Magnolia Bark and M. obovata and the harvest and processing of traditional Japanese herbal medicines, and should also be useful for further investigation of the biosynthesis and metabolism of honokiol and magnolol.

References