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Simultaneous analysis of shikimate-derived secondary metabolites in *Lithospermum erythrorhizon* cell suspension cultures by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatography (HPLC) analysis system based on a water–acetonitrile gradient program was established for simultaneous quantification of shikimate-derived secondary metabolites in cultured cells of *Lithospermum erythrorhizon*. The cells cultured in pigment production medium (M-9) are capable of producing five highly hydrophilic compounds such as *p*-hydroxybenzoic acid-*O*-glucoside and lithospermic acid B, as well as eleven lipophilic compounds including echinofuran B and acetylshikonin. In addition to the wide polarities of those compounds, many of them are unstable under light, dryness, oxygen and heating. Thus, a new extraction procedure for all these compounds was also established by use of ultrasonication under ice-water chilling with MeOH as the solvent. This procedure was applied to the quantitative analyses of these compounds in cell cultures and hairy root cultures of *Lithospermum*, and in the intact plants as well.

Keywords: *Lithospermum erythrorhizon*; *p*-Hydroxybenzoic acid-*O*-glucoside; Shikonin; Caffeic acid; Echinofuran

1. Introduction

Shikonin derivatives, red naphthoquinone pigments found in the root of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae), show antimicrobial [1], anti-inflammatory [2], wound-healing [2], and anti-tumor activities [3], and have been used as a crude drug in Japan and China. Cell cultures of *L. erythrorhizon*, which was originally established by Tabata et al. [4], produce shikonin derivatives in a large quantity (4 g/l medium) when cells are cultured in pigment production medium M-9 [5]. This culture system has been utilized not only for the industrial production of shikonin, but also as a model system of secondary metabolism in plant cells in basic research. Shikonin is biosynthesized from two key precursors, *p*-hydroxybenzoic acid (PHB) derived from shikimate/phenylpropanoid pathway and geranyl pyrophosphate from mevalonate pathway [6–9]. In Linsmaier–Skoog's (LS) liquid medium [10] used for subculture of *Lithospermum* cells, shikonin production fails and a large amount of PHB-*O*-

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glucoside (PHBOG) is accumulated in the vacuoles [11,12]. This compound is, therefore, presumed to be a storage form of shikonin precursor.

Besides shikonin derivatives and PHBOG, *L. erythrorhizon* cell cultures also produced two other groups of shikimate-derived secondary metabolites, furobenzoquinone derivatives and caffeic acid oligomers. Echinofurans B, C, dihydroechinofuran, deoxyshikonofuran, and shikonofuran E, which are either benzoquinone or hydroquinone derivatives, have been isolated from *L. erythrorhizon* cell cultures as unusual metabolites [13–15]. Caffeic acid oligomers such as rosmarinic acid, biosynthesized through phenylpropanoid pathway, have been also isolated from shikonin non-producing *Lithospermum* cells [16]. The production of rosmarinic acid in this cell culture was reported to be stimulated by yeast extract [17], methyl jasmonate [18], and blue light [19]. Recently we have found two caffeic acid tetramers, lithospermic acid B and (+)-rabdosiin, in *Lithospermum* cell cultures, and the production of the former was highly stimulated in M-9 medium, to reach almost the same amount as shikonin derivatives [20].

L. erythrorhizon cells produce more than ten variable compounds which can be classified into four groups; shikonin derivatives, furobenzoquinone derivatives, caffeic acid oligomers and PHBOG (Fig. 1). To clarify the metabolic linkage and the regula-

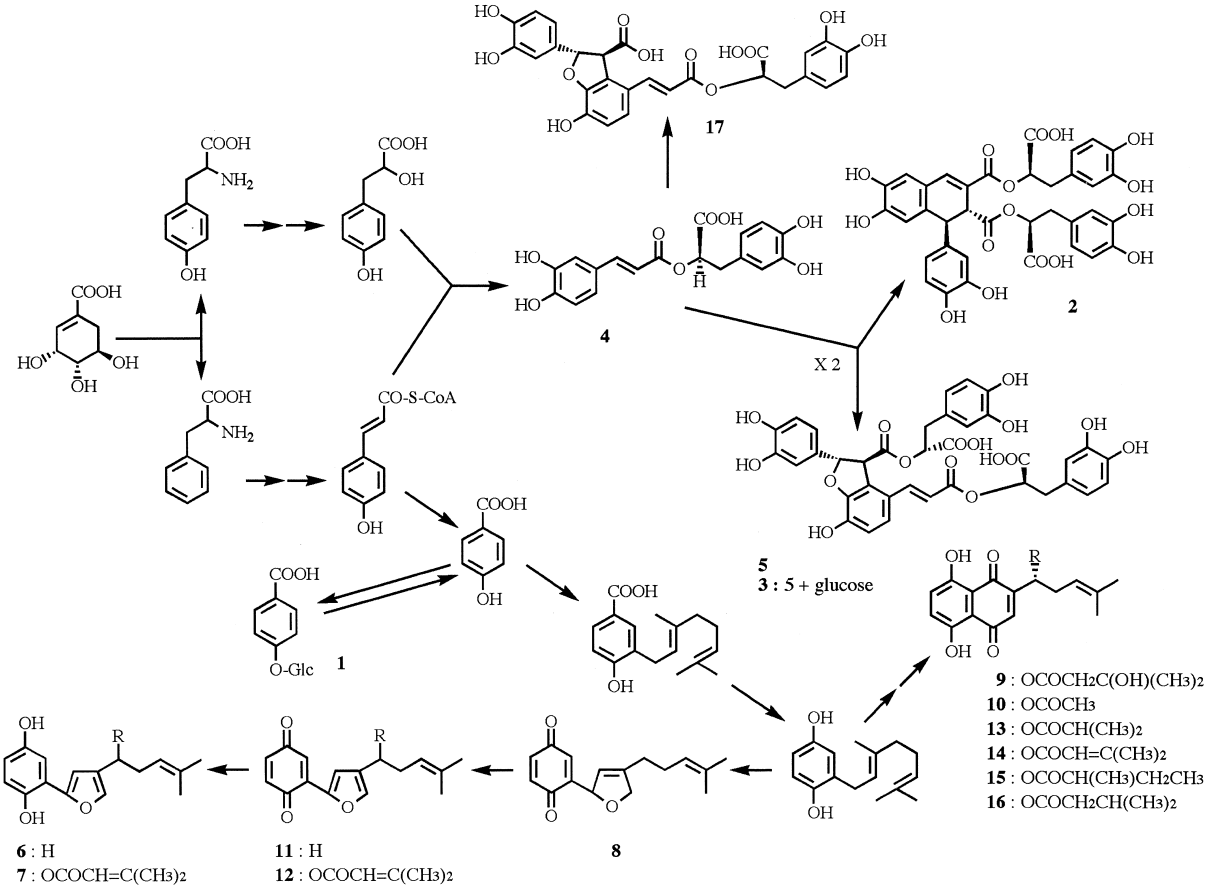

Fig. 1. Hypothetical biosynthetic pathway of shikimate-derived compounds in *Lithospermum erythrorhizon* cultured cells. Compounds; 1: *p*-hydroxybenzoic acid *O*-glucoside, 2: (+)-rabdosiin, 3: glucoside of lithospermic acid B, 4: rosmarinic acid, 5: lithospermic acid B, 6: deoxyshikonofuran, 7: shikonofuran E, 8: dihydroechinofuran, 9: β-hydroxyisovalerylshikonin, 10: acetylshikonin, 11: echinofuran B, 12: echinofuran C, 13: isobutylshikonin, 14: β,β-dimethylacrylshikonin, 15: isovalerylshikonin, 16: α-methyl-*n*-butylshikonin, 17: lithospermic acid.

tory mechanism in *Lithospermum* cell cultures, it has been necessary to analyze the dynamic accumulation patterns of all these compounds. However, because of the extreme wide polarities and instabilities of them, there has been no report concerning the simultaneous analysis of all those compounds. In this paper, we report a new method for determining the quantities of these compounds in *L. erythrorhizon* cultured cells with HPLC system at once. We also demonstrate the quantitative analyses in the hairy root cultures and the intact plants of *L. erythrorhizon* as an application of this method.

2. Experimental

2.1. Materials and culture methods

Cell suspension cultures of *L. erythrorhizon* strain M18TOM derived from strain M18 [21] have been subcultured at intervals of two weeks in a 300 ml Erlenmeyer flask containing 100 ml of LS liquid medium [10], supplemented with 1 μ*M* 3-indoleacetic acid and 10 μ*M* kinetin, on a rotary shaker (100 rpm) at 23°C in the dark. Cell strains CO and WM18 are incapable of producing shikonin even in M-9 medium. For the experiments, 30 ml media either of LS or M-9 in 100 ml Erlenmeyer flasks were used, in which 1.5 g or 1.0 g of fr. cells were inoculated respectively, and they were agitated on a rotary shaker for three weeks. The hairy root cultures were established with *Agrobacterium rhizogenes* (strain 15834) as mentioned in Ref. [22,23]. Hairy roots used in the experiment were cultured in 100 ml Erlenmeyer flasks containing 30 ml Murashige–Skoog's (MS) medium [24] on a rotary shaker (80 rpm) at 25°C in the dark, and harvested by filtration through Miracloth (Calbio Chem.) three weeks after inoculation (inoculum size: 0.5 g). An intact plant of *L. erythrorhizon* grown in the Kyoto Herbal Garden, Takeda Chemical Industries was harvested in September 1997.

2.2. Standards and solvents

As standard specimens, β-hydroxyisovalerylshikonin, acetylshikonin, isobutylshikonin, β,β-dimethylacrylshikonin [21], dihydroechinofuran [15], echinofurans B and C [13], rosmarinic acid [16], (+)-rabdosiin and lithospermic acid B [20] were isolated from strain M-18TOM cell cultures in M-9 medium. Since isovalerylshikonin and α-methyl-*n*-butylshikonin could not be separated [21,26], a mixture of these two compounds was used as the standard for the identification. Compound 3 was also isolated from strain M-18TOM cell cultures in M-9 medium in the same manner as described previously for lithospermic acid B [20]. Its structure was inferred to be a monoglucoside of lithospermic acid B by FAB–MS and ^{1}H-NMR analyses. To determine the binding site of glucose moiety to this phenolic compound as well as its absolute configuration, a large-scale isolation is now in progress. Lithospermic acid was isolated from hairy root cultures of *L. erythrorhizon* [20]. Deoxyshikonofuran and shikonofuran E were synthesized from echinofurans B and C, respectively, by reduction of them with sodium borohydride (Wako Pure Chemical Industries, Osaka, Japan) in methanol [25], followed by purification with prep. TLC [14]. PHBOG was a generous gift from Prof. Tanaka of Faculty of Pharmaceutical Sciences, Kyoto University. β-Naphthol (extra pure grade) was purchased from Wako Pure Chemical Industries. Extra pure grade acetonitrile and acetic acid (Wako Pure Chemical Industries) filtered with PTFE membrane filter T050A47A (Advantec Toyo, Tokyo, Japan) were used for HPLC solvents. Other solvents were of extra pure grade (Wako Pure Chemical Industries).

2.3. Apparatus and chromatographic conditions

Shimadzu HPLC system (Shimadzu, Kyoto, Japan) was used: pump, LC-9A; detector, SPD-6AV; column oven, CTO-6A; system controller, SIL-6B; auto injector, SIL-6B. Photodiode array detector SPD-M6A was also used for the identification of the compounds. Data analysis was performed using Chromatopac C-R4A (Shimadzu). A 5-μm Asahipak Hikarisil-C_{18} (250×4.6 mm I.D., Showa Denko, Tokyo, Japan) was used. One percent acetic acid containing acetonitrile–water system was used as basic solvent. Flow rate was 1 ml/min, column temperature was kept at 40°C. In the experiment for stability of the MeOH extracts against heating, the following linear gradient condition was used: 0–60

min, 40–100% acetonitrile; 60–75 min, 100% acetonitrile. For the routine analyses the following six linear gradient steps were programmed: 0–5 min, 5% acetonitrile; 5–6 min, 5–20% acetonitrile; 6–26 min, 20–30% acetonitrile; 26–27 min, 30–40% acetonitrile; 27–67 min, 40–60% acetonitrile; 67–82 min, 60–90% acetonitrile.

2.4. Quantification of standards

Known amounts of standards were dissolved in 10 ml methanol containing 1 mg internal standard (β-naphthol), analyzed by HPLC with monitoring at 254 nm, and calibration curves were set up by plotting the peak area against the respective concentration of each standard with comparing the peak area of the internal standard. For the estimation of isovalerylshikonin and α-methyl-*n*-butylshikonin which could not be obtained pure, however, the calibration curve of acetylshikonin was used. In the assay system established in this paper, the sensitivity of each standard was as follows; PHBOG, 2.75: (+)-rabdosiin, 7.32: compound 3, 12.69: lithospermic acid, 6.32: rosmarinic acid, 5.16: lithospermic acid B, 7.52: deoxyshikonofuran, 3.06: shikonofuran E, 3.83: dihydroechinofuran, 1.64: β-hydroxyisovalerylshikonin, 2.90: acetylshikonin, 4.47: echinofuran B, 1.15: echinofuran C, 2.07: isobutylshikonin, 5.80: β,β-dimethylacrylshikonin, 2.90 (μg/10 ml).

2.5. Sample preparation

In the experiments for determination of HPLC condition, cells cultured for three weeks in M-9 medium were harvested to separate cells from medium by the filtration, and 0.5 g of cells were homogenized with 5 ml methanol by a Teflon glass homogenizer for 5 min in an ice-water bath. The homogenates were centrifuged at 12 000 *g* for 5 min to remove cell debris, and the supernatant was brought up to 10 ml with methanol and 20 μl were injected into the HPLC system.

To examine the stability of each component in MeOH extracts against heating, three g of freshly harvested cells were extracted with 30 ml methanol in the same manner as above. The methanolic extract was divided into six portions (5 ml each) and each portion was refluxed for 0, 20, 40, 60, 120 and 180 min, brought up to 10 ml with methanol, respectively, and 20 μl of each sample were analyzed in triplicate by HPLC.

The examination for establishing the extraction method was carried out using the following extracts. A 0.5 g sample of freshly harvested cells cultured for three weeks in M-9 medium was homogenized with 8 ml methanol by a Teflon glass homogenizer for 5 min in an ice-water bath. The homogenate was centrifuged at 12 000 *g* for 5 min, and the supernatant was brought up to 10 ml with methanol. A 1 mg/ml solution of β-naphthol in *n*-butanol was added to the supernatant as an internal standard and 20 μl were analyzed as a control. As samples to be tested, fresh cells (0.5 g) and dried cells obtained after lyophylization of 0.5 g of fresh cells for two days or after drying in an oven of 50°C for three days were extracted with 8 ml methanol by ultrasonication for 90 min under ice-water chilling using an ultrasonic bath UT-205 (Sharp, Osaka, Japan) or by reflux for 3 h, respectively. After the extraction, each extract was centrifuged (12 000 *g*, 5 min), and the supernatant was adjusted to 10 ml. A 1 mg/ml solution of β-naphthol in *n*-butanol was added to the supernatant and 20 μl of each sample were analyzed in triplicate.

Extraction efficiencies of the compounds from the cells by the ultrasonication method were examined by the further extraction of the cell debris with methanol. In the second extracts, only trace amounts of PHBOG and caffeic acid oligomers were detectable (lower than 5% of the first extracts) and shikonin derivatives and furobenzoquinone derivatives could not be detected.

Recovery of the internal standard from the analyte was estimated by the comparison of the area of the β-naphthol in each sample with that obtained from the analysis of 1 mg β-naphthol dissolved in 11 ml methanol.

Stability of each component in cold methanol extracts was examined after keeping the ultrasonicated extracts under the different conditions given in Table 4 for three days. The internal standard was added to each sample at the end of the preservation.

For routine analyses, 0.5–1 g of freshly harvested cells were extracted with 10 ml of MeOH for 90 min under iced water chilling and sonication. Intact plant

was separated into several tissues shown in Table 6, chopped, and 0.5–1 g of each tissue was extracted with 10 ml methanol, in the same manner. After the ultrasonication, a 1 mg/ml solution of β-naphthol in *n*-butanol was added to the homogenate. After mixing, cell debris were removed by centrifugation (12 000 *g*, 5 min) and the supernatant was subjected to reversed-phase HPLC. Compounds secreted in the cultured medium were extracted with 15 ml *n*-butanol containing 1 mg β-naphthol as an internal standard, and the *n*-BuOH extract was centrifuged (12 000 *g*, 5 min) and analyzed.

All extracts were stored at −30°C prior to HPLC analysis.

3. Results and discussion

3.1. Establishment of extraction method

Table 1 shows several procedures for quantitative analyses of secondary metabolites in *L. erythrorhizon* cultured cells reported so far, but there is no convenient method for determining all the components in *Lithospermum* cells simultaneously. From the previous studies were observed the following points; furobenzoquinone derivatives were extremely unstable under dryness. The red color of shikonin in the extracts gradually changed into dark blue to black on the process of the extraction with MeOH at room temperature from fresh plant material and the more drastic condition of the hot extraction accelerated the color change. However, in order to establish a new analytical procedure, we chose fresh cells as the starting material and MeOH as the solvent because of the extraction of the more polar constituents from the cultured cells. Since no detailed experiments on the effect of hot MeOH on components of the extract was made, the influence of the reflux period on components of the MeOH extracts of shikonin producing cells was monitored with HPLC (Fig. 2).

Shikonin derivatives were first converted into less polar unknown compounds within 40 min of reflux, and were then decomposed to form much less polar ones, which showed very broad peaks (Fig. 2). After

Table 1
Procedures previously reported for quantitative analyses of secondary metabolites in *L. erythrorhizon* cultured cells

Compound	Material	Extraction method		Determination	Reference
		Solvent	Condition		
Shikonins	Dried cells	$CHCl_3$	Room temp.	A_{620} of 2.5% KOHaq	[21]
	Fresh cells or medium	*n*-Amyl alcohol	Room temp.	HPLC at 520 nm $CH_3CN:H_2O:AcOH:(Et)_3N=700:300:3:3$	[26]
	Freeze-dried cells	MeOH	Room temp.	HPLC at 520 nm $CH_3CN:H_2O:AcOH=60:30:1$	[19]
Furobenzoquinones	Fresh cells or medium	*n*-Amyl alcohol	Room temp.	HPLC at 254 nm $CH_3CN:H_2O:AcOH:(Et)_3N=700:300:3:3$	[27]
p-Hydroxybenzoic acid *O*-glucoside	Fresh cells	MeOH	Room temp.	HPLC at 254 nm $H_2O:AcOH=79:1$	[13]
Rosmarinic acid	Fresh cells	MeOH	Room temp. or reflux	HPLC at 254 nm $MeOH:H_2O:AcOH=30:20:1$	[16]
	Freeze-dried cells	MeOH	Room temp.	HPLC at 333 nm $MeOH/H_2O/H_3PO_4$ gradient system	[19]

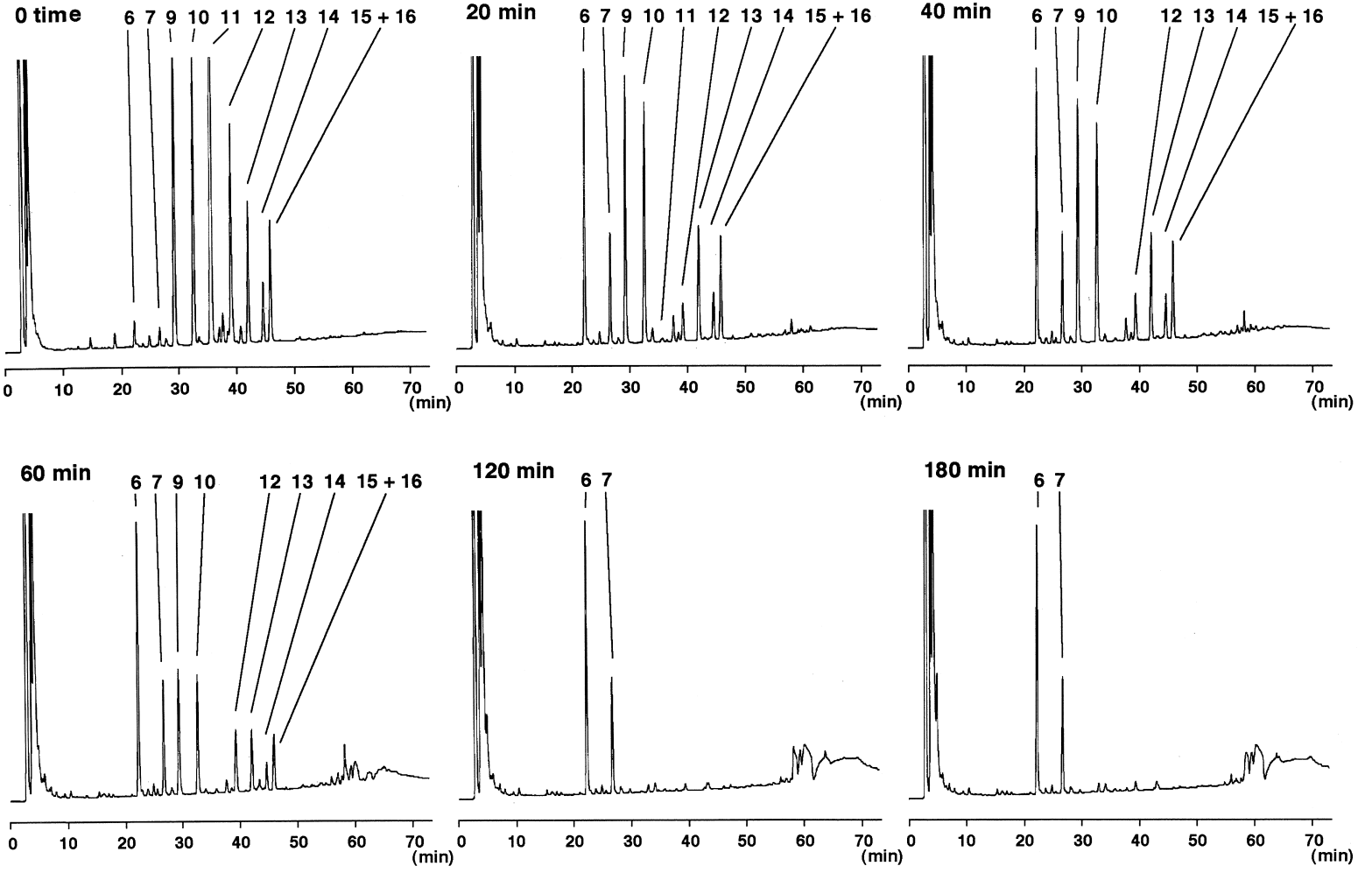

Fig. 2. Stability of MeOH extracts of shikonin-producing *L. erythrorhizon* cells against the heating. The chilled MeOH homogenates of shikonin producing cells were refluxed for three h. Time indicated in each panel is the reflux time (min), and 0 time is the MeOH extract kept on ice after extraction for 90 min.

2 h of reflux, almost all shikonin derivatives disappeared. Since a pure specimen of shikonin dissolved in MeOH was not decomposed by reflux at least in 3 h, the decomposition of shikonin derivatives in the crude extracts may be due to impurities such as metal ions and amino acids co-extracted from the fresh cells (Yazaki, unpublished data). However, no peak corresponding to the decomposed compounds was detected in the extract with chilled MeOH (Fig. 2, 0 time), indicating that the conversion was critically dependent on temperature, and therefore MeOH can be used for extraction if the procedure was done below 4°C. Benzoquinone derivatives, echinofurans B and C, were converted by reflux into the corresponding hydroquinone derivatives, deoxyshikonofuran and shikonofuran E, respectively, which were identified by photodiode array and direct comparison with standard specimens.

3.2. Establishment of HPLC condition

In HPLC analyses using an ODS column and 1% AcOH-containing acetonitrile–water linear gradient condition, PHBOG, caffeic acid oligomers, and the others were eluted with water, with 20–30% acetonitrile, and with 50–90% acetonitrile, respectively. Based on these data, the optimal gradient condition given in Section 2 was established. In Fig. 3, two representative chromatograms of the cold MeOH extracts of *Lithospermum* cells cultured either in M-9 (Fig. 3, upper) or in LS (Fig. 3, lower) medium were shown. Dihydroechinofuran which was transiently biosynthesized and secreted into the culture medium [15] was detected only in a trace amount after three weeks of culture.

The quantities of these shikimate-derived components of the cells cultured in LS or M-9 medium are shown in Table 2. In M-9 medium, the total amounts of shikonin derivatives reached 7.6 mg/g fr. wt (ca. 6.3% of dry cell wt), among which the largest one, acetylshikonin, represented ca. 40% of the total pigment. Contrary to the induction of shikonin derivatives, the content of PHBOG in M-9 cultures decreased to one tenth of that in LS cultures. The total contents of furobenzoquinone derivatives were 2.2 mg/g fr. wt. Production of caffeic acid oligomers was observed in the cells cultured in LS and in M-9 medium as well, but the amount of lithospermic acid B in the cells cultured in M-9 medium was almost 8 times higher (12.4 mg/g fr. wt., ca. 10% dry wt) than that in LS medium. Compared with the remarkable change in the content of lithospermic acid B between LS and M-9 medium, the contents of rosmarinic acid and (+)-rabdosiin were relatively unaffected between two culture media. A new compound, a glucoside of lithospermic acid B (peak 3), was also detected in the cells cultured in M-9 medium. The binding site of the glucose moiety to the phenolic moiety is now under investigation.

Table 2
Contents of shikimate-derived compounds in *L. erythrorhizon* cell line M18TOM cultured in M-9 or in LS medium

Compound	Contents of compounds (mg/g fr. wt)	
	M-9	LS
1	0.22 (0.05)[a]	2.11 (0.02)
2	1.70 (0.28)	1.21 (0.07)
3	1.76 (0.09)	0.16 (0.05)
4	1.74 (0.29)	1.76 (0.05)
5	12.45 (0.76)	1.60 (0.08)
6	0.43 (0.06)	0.00
7	0.20 (0.01)	0.00
8	2.23 (0.12)	0.00
9	3.23 (0.08)	0.00
10	3.23 (0.08)	0.00
11	1.25 (0.12)	0.00
12	0.35 (0.05)	0.00
13	0.96 (0.05)	0.00
14	0.37 (0.02)	0.00
15+16	0.79 (0.04)	0.00
Shikonins[b]	7.58 (0.28)	0.00
Furobenzoquinones[c]	2.24 (0.15)	0.00

[a] Standard deviation.
[b] Sum of compounds 9, 10, and 13–16.
[c] Sum of compounds 6, 7, 11 and 12.

3.3. Effects of extraction procedure

Using the gradient program established above, the efficiencies of variable extraction procedures were assessed (Table 3). The fresh cells, the cells lyophilized for two days, and the cells dried for three days at 50°C were extracted with MeOH by the ultrasonication in ice-water bath for 90 min or the reflux for 3

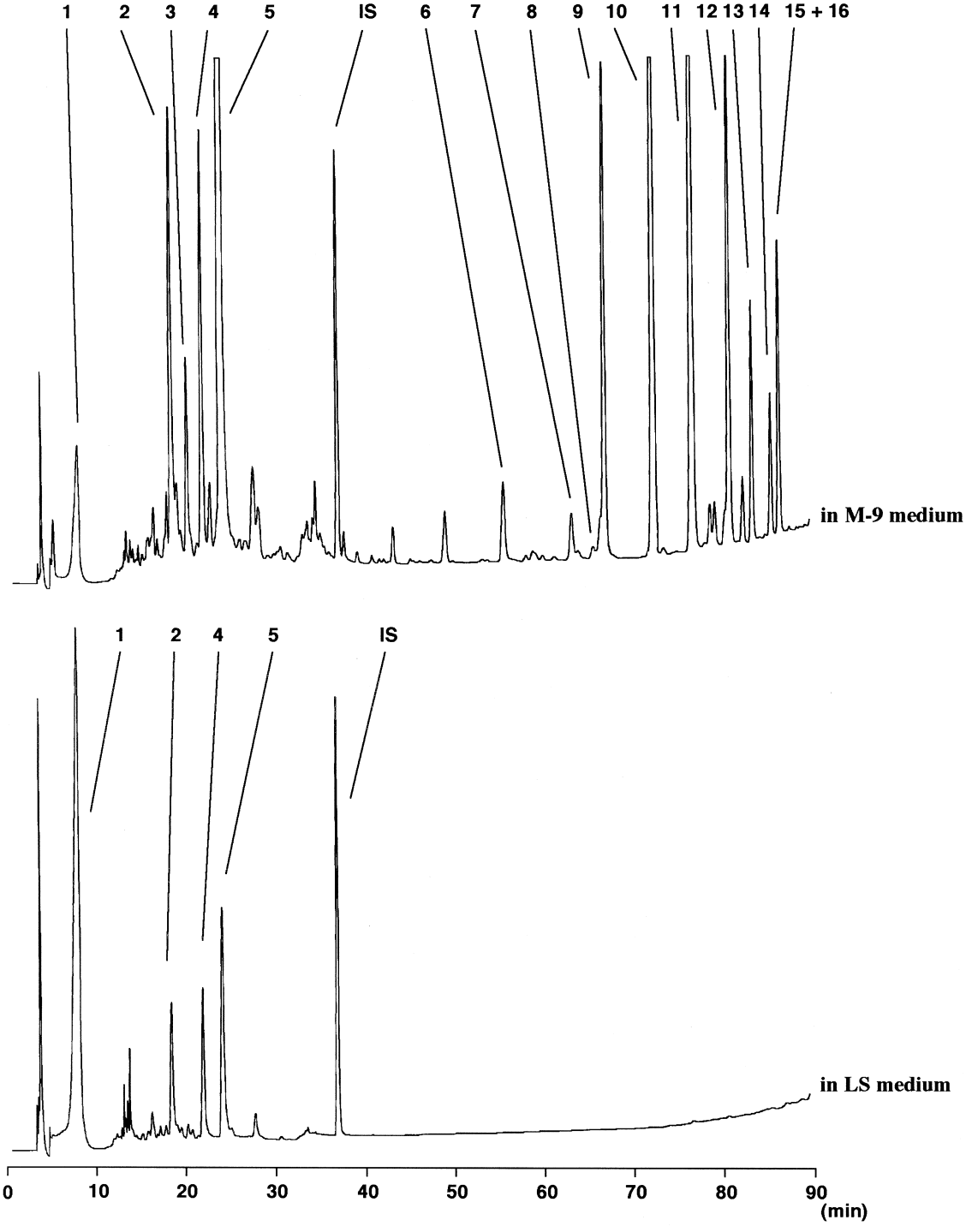

Fig. 3. HPLC chromatograms of MeOH extracts of *L. erythrorhizon* cell line M18TOM cultured in M-9 (upper) or LS (lower) medium for three weeks. IS: β-naphthol. Lithospermic acid (17 in Fig. 1), not detectable in cultured cells of *L. erythrorhizon*, but in the hairy root cultures and in the intact plants, was eluted ca. 1 min slower than rosmarinic acid.

Table 3
Differences of the extraction efficiencies of shikimate-derived compounds in *L. erythrorhizon* cell suspension cultures[a]

Extraction method (cells)	Contents of compounds (mg/g fr. wt)															
	1[b]	2	3	4	5	6	7	9	10	11	12	13	14	15+16	Shikonins[c]	Furobenzoquinones[d]
Homogenization	2.22	1.89	1.81	1.83	13.03	0.38	0.18	2.23	3.23	1.14	0.31	0.93	0.35	0.72	7.45	2.02
[Fresh cells]	(0.06)[e]	(0.19)	(0.10)	(0.20)	(0.90)	(0.03)	(0.02)	(0.10)	(0.12)	(0.05)	(0.03)	(0.05)	(0.02)	(0.02)	(0.27)	(0.09)
Ultrasonication	2.11	1.70	1.76	1.74	12.45	0.43	0.20	2.23	3.23	1.25	0.35	0.96	0.37	0.79	7.58	2.24
[Fresh cells]	(0.02)	(0.28)	(0.09)	(0.29)	(0.76)	(0.06)	(0.01)	(0.12)	(0.08)	(0.12)	(0.05)	(0.05)	(0.02)	(0.04)	(0.28)	(0.15)
	95.2[f]	*90.0*	*97.2*	*94.9*	*95.5*	*113.2*	*113.3*	*100.0*	*100.0*	*109.2*	*113.5*	*103.2*	*105.4*	*109.8*	*101.7*	*111.1*
Ultrasonication	1.99	0.87	1.08	1.212	7.21	0.48	0.29	2.13	3.12	1.00	0.29	0.93	0.35	0.76	7.30	2.05
[Freeze-dried]	(0.04)	(0.13)	(0.15)	(0.10)	(0.86)	(0.07)	(0.02)	(0.03)	(0.03)	(0.08)	(0.02)	(0.03)	(0.01)	(0.02)	(0.06)	(0.03)
	89.8	*45.9*	*59.9*	*66.2*	*55.3*	*126.3*	*158.3*	*95.6*	*96.7*	*87.0*	*93.2*	*100.0*	*100.0*	*106.2*	*97.9*	*101.8*
Ultrasonication	1.97	0.00	0.00	0.00	0.00	0.53	0.38	1.90	2.84	0.32	0.11	0.89	0.33	0.73	6.69	1.34
[Oven-dried]	(0.23)	(–)	(–)	(–)	(–)	(0.02)	(0.01)	(0.10)	(0.13)	(0.03)	(0.01)	(0.04)	(0.01)	(0.03)	(0.31)	(0.22)
	89.0	*0.0*	*0.0*	*0.0*	*0.0*	*138.2*	*208.9*	*85.0*	*88.0*	*28.0*	*36.0*	*94.3*	*94.3*	*102.4*	*89.7*	*66.1*
Reflux	2.08	1.57	1.63	2.30	11.01	1.69	0.63	0.00	0.00	0.15	0.08	0.00	0.00	0.00	0.00	2.55
[Fresh cells]	(0.05)	(0.03)	(0.01)	(0.46)	(0.05)	(0.06)	(0.02)	(–)	(–)	(0.02)	(0.01)	(–)	(–)	(–)	(–)	(0.06)
	94.1	*84.1*	*90.3*	*125.9*	*84.4*	*445.3*	*349.4*	*0.0*	*0.0*	*13.3*	*25.4*	*0.0*	*0.0*	*0.0*	*0.0*	*113.9*
Reflux	2.05	1.01	1.17	1.241	7.76	1.61	0.73	0.00	0.00	0.20	0.07	0.00	0.00	0.00	0.00	2.61
[Freeze-dried]	(0.18)	(0.03)	(0.02)	(0.03)	(0.13)	(0.03)	(0.01)	(–)	(–)	(0.02)	(0.01)	(–)	(–)	(–)	(–)	(0.02)
	92.5	*53.3*	*64.8*	*67.8*	*59.6*	*422.6*	*403.9*	*0.0*	*0.0*	*17.5*	*23.8*	*0.0*	*0.0*	*0.0*	*0.0*	*129.2*
Reflux	2.21	0.00	0.00	0.00	0.00	0.97	0.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.58
[Oven-dried]	(0.07)	(–)	(–)	(–)	(–)	(0.03)	(0.02)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(0.04)
	99.8	*0.0*	*0.0*	*0.0*	*0.0*	*255.5*	*338.3*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*78.3*

[a] 0.5 g fr. cells cultured in M-9 medium were extracted with 8 ml methanol.
[b] Cultured cells in LS medium.
[c] Sum of compounds 9, 10, and 13–16.
[d] Sum of compounds 6, 7, 11 and 12.
[e] Standard deviation.
[f] % of 'homogenization' method.

h, respectively. The results obtained from the homogenization of the fresh cells in chilled MeOH were employed as the control, and compared with those of other procedures. PHBOG and shikonin derivatives were relatively stable against the dryness, whereas caffeic acid oligomers were partly decomposed even by lyophilizing and were completely decomposed when cells were dried in the oven. The contents of echinofurans were also decreased by the dryness, and the increase of the corresponding reductive derivatives, shikonofurans, was observed instead. Under refluxing condition, shikonin derivatives were completely decomposed, even when dried materials were used, but PHBOG and caffeic acid oligomers were almost stable. It was noteworthy that the total amounts of shikonofurans and echinofurans were almost unchanged. This is presumably because echinofurans were nearly quantitatively converted into shikonofurans by refluxing.

3.4. Stability of the secondary metabolites in MeOH extracts

The stability of these secondary metabolites extracted with cold MeOH was investigated. The MeOH extracts were kept under several conditions

for three days (Table 4). PHBOG and caffeic acid oligomers were almost stable in the MeOH solution, at 23°C both under light and dark condition except for decomposition of only small amounts of caffeic acid oligomers under the light. The temperature did not strongly affect the decomposition of furobenzoquinone derivatives, shikonofurans and echinofurans although light showed a very strong effect to decompose them. Contrary to the case of furobenzoquinone derivatives, shikonin derivatives were stable to light, but unstable around room temperature. The decomposition of shikonin derivatives was prohibited at low temperature, i.e. −25°C. Therefore, freshly prepared cell materials were extracted with chilled MeOH by the ultrasonication for 90 min, for the routine analyses, and the extracts were kept at −40°C until analysis.

3.5. Quantitative analyses of shikimate-derived components in L. erythrorhizon hairy root cultures

Table 5 shows the contents of shikimate-derived compounds in *Lithospermum* hairy root cultured in hormone-free MS or M-9 medium. In the hairy root cultures detectable amounts of both shikonin and furobenzoquinone derivatives were produced even in MS medium. The production of shikonin derivatives was strongly stimulated in M-9 medium as observed in the cultured cells. The contents of furobenzoquinone derivatives were almost unaffected or rather decreased by transferring the hairy roots into M-9 medium from MS medium. Shikonofuran E and echinofuran C were the main furobenzoquinone constituents and the ratio was higher than that in the cultured cells. Rosmarinic acid found in the cultured

Table 4
Stability of shikimate-derived compounds in the MeOH extracts of *L. erythrorhizon* cell suspension cultures[a]

Condition	Contents of compounds (mg/g fr. wt)															
	1[b]	2	3	4	5	6	7	9	10	11	12	13	14	15+16	Shikonins[c]	Furobenzoquinones[d]
0 time	2.92	1.47	1.76	0.59	9.45	0.38	0.17	1.95	1.84	4.12	1.16	0.84	0.32	0.69	5.64	5.83
	(0.03)[e]	(0.24)	(0.30)	(0.10)	(0.58)	(0.02)	(0.01)	(0.10)	(0.04)	(0.39)	(0.15)	(0.04)	(0.02)	(0.04)	(0.23)	(0.51)
23°C	2.83	1.29	1.41	0.58	8.22	0.00	0.00	0.99	0.88	0.00	0.09	0.43	0.17	0.35	2.82	0.09
under the light	(0.02)	(0.04)	(0.02)	(0.01)	(0.16)	(–)	(–)	(0.01)	(0.01)	(–)	(0.02)	(0.01)	(0.01)	(0.01)	(0.04)	(0.02)
(10 000 lx)	*97.1*[f]	*87.6*	*80.1*	*98.5*	*87.0*	*0.0*	*0.0*	*50.8*	47.9	*0.0*	*7.4*	*51.5*	*52.3*	*50.8*	*50.0*	*1.50*
23°C,	2.82	1.36	1.71	0.57	9.10	1.26	0.51	0.98	0.88	0.76	0.18	0.42	0.18	0.33	2.79	2.70
in the dark	(0.01)	(0.04)	(0.03)	(0.01)	(0.24)	(0.04)	(0.02)	(0.04)	(0.04)	(0.03)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.05)
	96.5	*92.7*	*97.0*	*96.6*	*96.2*	*334.2*	*302.4*	*50.3*	47.9	*18.4*	*15.1*	*50.1*	*54.5*	*47.6*	*49.4*	*46.4*
4°C,	2.80	1.37	1.78	0.61	9.24	1.32	0.52	1.48	1.41	0.75	0.20	0.64	0.24	0.50	4.26	2.78
in the dark	(0.03)	(0.05)	(0.23)	(0.08)	(0.16)	(0.04)	(0.02)	(0.05)	(0.06)	(0.04)	(0.01)	(0.02)	(0.01)	(0.01)	(0.13)	(0.06)
	96.0	*93.1*	*101.3*	*103.0*	*97.8*	*349.3*	*304.7*	*75.9*	76.6	*18.2*	*17.1*	*75.5*	*72.8*	*72.5*	*75.5*	*47.7*
−25°C,	2.79	1.32	1.683	0.57	8.95	0.49	0.1	1.85	1.78	3.76	1.13	0.85	0.33	0.69	5.50	5.49
in the dark	(0.01)	(0.02)	(0.08)	(0.03)	(0.12)	(0.03)	(0.03)	(0.02)	(0.05)	(0.12)	(0.02)	(0.01)	(0.01)	(0.02)	(0.06)	(0.08)
	95.7	*90.1*	*95.8*	*96.6*	*94.7*	*129.1*	*66.4*	*94.7*	97.1	*91.3*	*97.2*	*100.9*	*100.9*	*100.7*	*97.5*	*94.2*

[a] 0.5 g fr. cells cultured in M-9 medium were extracted with 10 ml MeOH under the ultrasonication in ice-water bath, and were kept for 3 days under different conditions.
[b] Cultured cells in LS medium.
[c] Sum of compounds 9, 10, and 13–16.
[d] Sum of compounds 6,7,11 and 12.
[e] Standard deviation.
[f] % of 'homogenization' method.

Table 5
Contents of shikimate-derived compounds in *L. erythrorhizon* hairy root cultures cultured in M-9 or in MS medium for 3 weeks[a]

Compound	Contents of compounds (mg/g fr. wt)			
	Con F-1 (MS)	Con F-1 (M-9)	Con 5 (MS)	Con 5 (M-9)
1	0.69 (0.11)[b]	0.57 (0.05)	0.70 (0.09)	0.56 (0.04)
2	1.17 (0.04)	0.25 (0.05)	0.65 (0.03)	0.42 (0.03)
17	1.60 (0.17)	3.03 (0.60)	1.76 (0.25)	4.91 (0.33)
5	0.34 (0.04)	0.87 (0.11)	0.35 (0.02)	0.87 (0.05)
7	0.61 (0.08)	0.14 (0.02)	0.91 (0.34)	0.39 (0.03)
9	0.82 (0.24)	2.00 (0.27)	0.60 (0.24)	2.08 (0.23)
10	0.61 (0.23)	5.92 (1.19)	0.90 (0.56)	5.58 (0.77)
11	0.05 (0.01)	0.07 (0.01)	0.15 (0.11)	0.22 (0.07)
12	0.15 (0.06)	0.18 (0.01)	0.17 (0.13)	0.27 (0.07)
13	0.12 (0.03)	0.61 (0.09)	0.05 (0.04)	0.48 (0.03)
14	0.09 (0.02)	0.25 (0.02)	0.03 (0.03)	0.25 (0.02)
15+16	0.29 (0.09)	1.19 (0.10)	0.20 (0.10)	1.03 (0.13)
Shikonins[c]	1.92 (0.61)	9.96 (1.56)	1.78 (0.94)	9.42 (1.18)
Furobenzoquinones[d]	0.81 (0.15)	0.40 (0.03)	1.23 (0.33)	0.87 (0.11)

[a] Compounds 3 and 6 were not detected, and only trace amount of compound 4 was detected.
[b] Standard deviation.
[c] Sum of compounds 9, 10, and 13–16.
[d] Sum of compounds 6, 7, 11 and 12.

cells was scarcely detected, whereas a considerable amount of lithospermic acid which was not detected in the cultured cells was accumulated. M-9 medium also showed a stimulatory effect on the production of lithospermic acid as well as lithospermic acid B.

3.6. Quantitative analyses of shikimate-derived components in the intact plant of L. erythrorhizon

We also investigated the distribution of shikimate-derived metabolites in the intact plant of two-year old *L. erythrorhizon* (Table 6). In the aerial parts, neither shikonin derivatives nor furobenzoquinone derivatives were detected, and rosmarinic acid was the major constituent. Both (+)-rabdosiin and lithospermic acid B were also detected, but neither PHBOG nor lithospermic acid were detectable. In the underground part, almost the same compounds as those in hairy root cultures were detected except for PHBOG. As observed in the hairy root cultures, amounts of hydroquinone derivatives were higher than those of benzoquinone derivatives. The content of rosmarinic acid was very low, but an appreciable amount of lithospermic acid was detected in the underground part as a main component, especially the content was high in the fibrous lateral roots. It was noteworthy that the contents of shikonin derivatives, furobenzoquinone derivatives, lithospermic acid and lithospermic acid B in the cork layers of the main root were 3–10 times higher than those in the cortex and the central cylinder.

4. Conclusion

In the present study, we demonstrated that the ultrasonication in the ice-water bath was most suitable to extract the compounds quantitatively without forming artifacts. Utilization of ice in the bath, which was indispensable to prevent the extract from heating, weakened the power of ultrasonication, but the prolonged treatment could compensate the extraction efficiency. After the ultrasonication for 90 min, no significant amounts of these compounds were further detected in the cell debris.

This is the first report of the quantitative analysis of all the aromatic metabolites in *L. erythrorhizon* cell cultures ranging from the water-soluble components like PHBOG to such lipophilic compounds as shikonin derivatives, in one injection to HPLC. In

Table 6
Contents of shikimate-derived compounds in *L. erythrorhizon* 2-year old plant[a,b]

Plant parts	Contents (mg/g fr. wt)														
	2	17	4	5	6	7	9	10	11	12	13	14	15+16	Shikosins[c]	Furobenzoquinones[d]
Fibrous roots	0.97	2.87	tr.[e]	0.86	0.17	0.06	0.10	0.29	-[f]	–	–	–	–	0.39	0.23
Lateral roots	0.09	2.58	tr.	2.93	0.44	0.16	0.45	2.22	0.05	0.02	0.52	0.10	0.36	3.69	0.66
Lower part of main roots (ca. 1 mm I.D.)	0.09	1.26	tr.	2.56	0.41	0.13	0.29	1.48	0.03	0.01	0.52	0.07	0.25	2.63	0.58
Middle part of main roots (ca. 5 mm I.D.)	0.05	0.81	tr.	1.52	0.47	0.14	0.24	1.42	0.04	0.01	0.51	0.07	0.24	2.51	0.66
Upper part of main roots (ca. 8 mm I.D.)	0.06	0.78	tr.	1.35	0.22	0.07	0.20	1.08	0.03	0.01	0.26	0.05	0.16	1.77	0.32
Lower leaf	1.02	–	1.26	0.08	–	–	–	–	–	–	–	–	–	–	–
Middle leaf	0.23	–	0.69	0.09	–	–	–	–	–	–	–	–	–	–	–
Upper leaf	0.79	–	1.38	0.05	–	–	–	–	–	–	–	–	–	–	–
Lower stem	0.38	–	1.97	0.94	–	–	–	–	–	–	–	–	–	–	–
Middle stem	0.16	–	0.52	0.09	–	–	–	–	–	–	–	–	–	–	–
Upper stem	0.19	–	0.50	0.01	–	–	–	–	–	–	–	–	–	–	–
Shoot apex (containing young leaf)	0.65	–	1.77	0.10	–	–	–	–	–	–	–	–	–	–	–
Cork layer of main roots	0.04	1.31	tr.	2.88	1.32	0.26	0.79	4.80	0.07	0.03	1.45	0.17	0.79	8.08	1.68
Cortex and central cylinder of main roots	0.06	0.43	tr.	0.18	0.07	–	0.04	0.07	–	–	–	–	–	0.11	0.07

[a] Compounds 1 and 3 were not detected.
[b] The material was separated into several parts and extracted with MeOH.
[c] Sum of compounds 9, 10, and 13–16.
[d] Sum of compounds 6, 7, 11 and 12.
[e] Trace amount.
[f] Not detected.

this gradient condition, only 90 min was needed for one analysis. As an application of this method, we demonstrated that most of the components were contained in the hairy root cultures as well as in the intact plant. In this study, furthermore, it was first shown that the furohydroquinone derivatives, shikonofurans, which had been presumed to be unusual metabolites in the cultured cells [13–15], were also biosynthesized and accumulated in the intact roots on a high level. The analysis system established here will be further utilized for the biochemical research works in secondary metabolism of *Lithospermum* species.

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