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HPLC Separation of Isoquinoline Alkaloids for Quality Control of *Corydalis* species

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A simple and rapid analytical method was developed for the determination of eight isoquinoline alkaloids in *Corydalis* species. Eight isoquinoline alkaloids, including 2 aporphine alkaloids (isocorydine and glaucine) and 6 protoberberine alkaloids (coptisine, palmatine, berberine, canadine, corydaline, and tetrahydrocoptisine) were used as chemical markers which have a various biological activity and determined for quality control of *Corydalis* (*C.*) species (*C. ternata*, *C. yanhusuo*, and *C. decumbens*). To evaluate the quality of these herbal medicines, LC chromatographic separation of alkaloids were preferentially investigated on reversed-phase C18 column with pH variation and composition of mobile phase. In addition, the separation of these alkaloids in herbal extracts was found to be significantly affected on mobile phase composition using gradient elution. Especially for *C. yanhusuo* extract, berberine was seriously interfered with other alkaloid extracted from sample matrix when mobile phase composition was not optimized. As results, these compounds were successfully separated within 28 min using 10 mM ammonium acetate containing 0.2% triethylamine (adjusted at pH 5.0) as a mobile phase with gradient elution. On the basis of optimized HPLC conditions, 23 different *Corydalis* species samples were analyzed for the determination of alkaloid levels. In addition, principal component analysis (PCA) combined with the chromatographic data could be successfully classified the different geographic origin samples.

Key Words : *Corydalis* species, Alkaloids, Elution patterns, HPLC-UV

Introduction

Corydalis tuber is regarded as rich source of pharmaceutically important isoquinoline alkaloids. Several species of the genus *Corydalis* (Papaveraceae) have been used for centuries in traditional Asian medicine as *Rhizoma Corydalis* (*C.*), including *C. amabilis*, *C. ambigua*, *C. decumbens*, *C. ternata* and *C. yanhusuo*.[1,2] Their major active constituents of *C*. species consist of a number of isoquinoline alkaloids including protoberberine alkaloids (berberine, palmatine, coptisine, *etc*.), and aporphine alkaloids (typical isocorydine and glaucine).[1,3,4] These isoquinoline alkaloids frequently observed in the plants were reported to show significant cytotoxicities and therapeutic effects on allevitating pain and promoting blood circulation.[5-7] Hence, isoquinoline alkaloids were selected as markers for quality control of *C.* species. However, the determination of isoquinoline alkaloids is not easy task because of their structural diversity and common occurrence in herbal medicines.

In order to determine alkaloids in herbal medicines, reversed-phase high-performance liquid chromatography (RP-HPLC) has been popularly used, because of its high-separation power allowing simultaneous determination of a variety of protoberberine alkaloids and derivatives. Furthermore, most of these alkaloids contain strong UV-Visible chromophores, providing highly sensitive spectrophotometric detection.[8-10] Ion-pair HPLC method has been also applied for the determination of various alkaloids in herbal medicines due to the ionic character of protoberberine alkaloids and their derivatives in acidic medium.[11,12] The significant advantages of this method have been reported that peak tailing can be reduced and retention of cationic species can be decreased, due to silanol-masking effects by addition of salts.[13,14] Recently, two-dimensional RP-HPLC method has been successfully applied for the separation of alkaloids in *C. yanhusuo* species.[15] From the chromatographic view point, pH value of mobile phase was the most important factor in the HPLC separation of basic alkaloid compounds. According to pH of mobile phase, elution patterns and retention behavior of alkaloids could greatly change due to their diverse physicochemical properties.[16] In the analysis of alkaloids of herbal medicine, several interferences such as organic acids, phenolic compounds, and other basic or acidic compounds with different pK_a values can be also influenced by the pH of the solution.[16,17] Thus, the analysis of isoquinoline alkaloids in herbal extract should be systematically approached for the quality control of *Corydalis* tuber samples. Especially, the principal component analysis (PCA) and hierarchical cluster analysis (HCA) combined with HPLC chromatographic data provided on important information for the differentiation of herbal origins.[18,19]

In this paper, a simple and rapid HPLC-DAD method for the separation of eight isoquinoline alkaloids in *Corydalis*

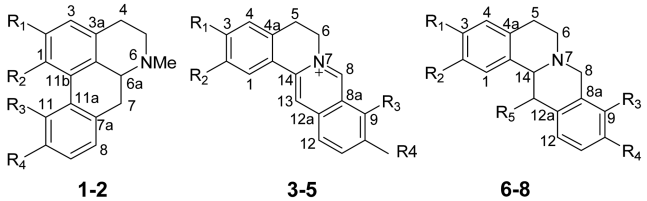
species was described. To optimize the separation conditions of alkaloids, the pH values (4.2, 5.0, 5.8 and 6.5) and composition of mobile phase were investigated on the elution patterns of isoqunoline alkaloids on reversed phase C18 column. On the basis of optimized HPLC conditions, the levels of eight alkaloids could be successfully determined for the evaluation of different *Corydalis* species.

Experiment

Materials and Reagents. All reagents were of analytical grade. Acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Isoquinoline alkaloids such as glucine, coptisine, canadine and tetrahydrocoptisine in *Corydalis* species were isolated by method outlined in previous report.[5] Their purities were determined by HPLC-DAD. Isocorydine, corydaline, and palmatine (purity > 97%) was purchased from Sigma Aldrich (Steinheim, Germany) and Aldrich (Milwaukee, WI, USA), respectively. Berberine (purity > 99%) were purchased from Wako Chemical (Osaka, Japan). Coptisine, canadine, and glaucine were purchased from both ChromaDex (Irvine, CA) and MP Biomedicals (Illkirch, France). Isopropylantipyrine used as internal standard were purchased from Tokyo Chemical Industry (Tokyo, Japan).

HPLC Conditions. Analysis was performed on an Agilent Series 1100 HPLC system consisting of a quaternary delivery system, an auto-sampler and a diode array detector (DAD). The chromatographic separation analysis was carried out on a Shiseido UG 120 C18 (250 × 4.6 mm, i.d., 5 μm) column. The mobile phases were consisted of solvent A (10 mM ammonium acetate contained 0.2% TEA at pH 5.0 adjusted by acetic acid) and solvent B (acetonitrile, ACN). Gradient elution mode was programmed as follows: 27% B for 0-10 min, 27-33% B for 10-15 min, and 33-95% B for 15-30 min. UV detection wavelength was set at 280 nm. The flow rate and injection volume were set at 1 mL/min and 20 μL, respectively.

Sample Preparation. Dried *Corydalis* tuber was pulverized and the powders screened through 30 mesh sieves. One gram powder was placed into 10 mL of 70% methanol in beaker. The sample mixture was extracted for 20 min in an ultrasonic bath at room temperature. After extraction, the sample mixture was centrifuged at 10,000 rpm for 10 min, 2 times. The supernatant was collected and filtrated through a 0.22 μm membrane filter, and 20 μL aliquots from the filtrate were then injected into the HPLC system.



Number	Compound	R_1	R_2	R_3	R_4	R_5
1	isocorydine	OCH_3	OCH_3	OH	OCH_3	-
2	glaucine	OCH_3	OCH_3	OCH_3	OCH_3	-
3	coptisine	OCH_2O		OCH_2O		-
4	palmatine	OCH_3	OCH_3	OCH_3	OCH_3	-
5	berberine	OCH_2O		OCH_3	OCH_3	-
6	canadine	OCH_2O		OCH_3	OCH_3	H
7	corydaline	OCH_3	OCH_3	OCH_3	OCH_3	CH_3
8	tetrahydocoptisine	OCH_2O		OCH_2O		H

Figure 1. Chemical structures of isoquinoline alkaloids used as marker compounds of *Corydalis* species.

Results and Discussion

Optimization of Mobile Phase pH Value. The pH value of mobile phase is the most important factor in the separation of alkaloids on reversed phase HPLC. Alkaloids are generally basic and nucleophilic compounds that have one or two nitrogen atoms with lone pair electron. Thus, retention behavior of ionizable alkaloids on RP-column is strongly dependent on the composition of mobile phase system together with the percentage of organic solvent.[16] Furthermore, retention behavior of alkaloids is closely related with their hydrophobic property on C18 column. When the alkaloids were ionized under acidic or basic medium conditions, they become less hydrophobic. In other words, alkaloids tend to easily gain proton and become ionization under acidic medium. Thus, their retentions on C18 column significantly decrease at lowered pH. On the other hands, the retention of quarternary protoberberine alkaloids such as berberine, coptisine, and palmatine was not significantly influenced at moderate acidic and neutral medium due to naturally cation form on nitrogen atom.

When analysis is performed at neutral pH or moderately basic conditions, both protonated form and free base form of alkaloid due to alkaloid's partial protonation can co-exist, resulting in irregular peak shape. It was already reported that, in alkaline medium, quarternary protoberberine alkaloids could be converted into their free bases whileas tertrahydroyprotoberberine and aporpine alkaloids could be converted into their OH group adducted forms at C-6 position.[20] Thus, the use of acidic mobile phase is generally preferable in the HPLC analysis of alkaloids.

To find an optimal pH value of the mobile phase, four pH values (pH 4.2, 5.0, 5.8 and 6.5) were examined for the retention behavior of alkaloids on C18 column. Figure 2 shows HPLC chromatograms of eight isoquinoline alkaloids under acidic conditions (pH 4.2, 5.0, 5.8 and 6.5, respectively). As can be seen in Figure 2, the retention times of tetrahydroprotoberberine and aporphine alkaloids was increased due to decreasing their polarity and hydrophilicity as pH values increases from 4.2 to 6.5. On the other hand, the retention times of quaternary protoberberine alkaloids (coptisine, palmatine and berberine) did not significantly change according to change of pH values. These phenomena can be explained that quaternary protoberberine alkaloids with charged form did not change their chemical structures at acidic conditions but tetrahydroprotoberberines and

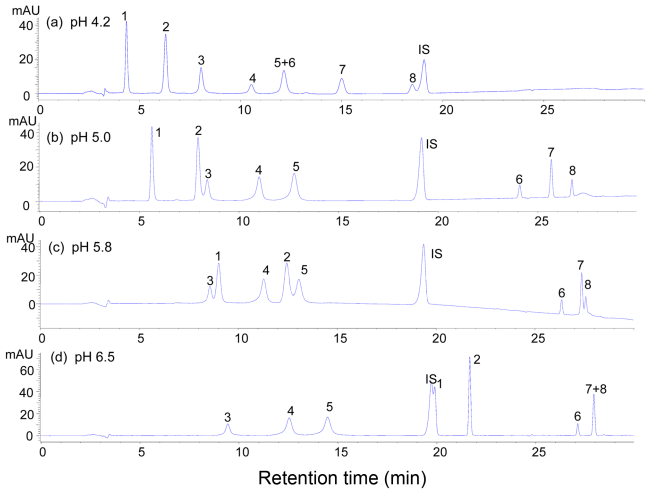


Figure 2. HPLC chromatograms of standard alkaloid mixture according to changing pH values: (a) pH 4.2, (b) pH 5.0, (c) pH 5.8, and (d) pH 6.5, Peak's identities as follow: 1. isocorydine, 2. glaucine, 3. coptisine, 4. palmatine, 5. berberine, 6. canadine, 7. corydaline, and 8. tetrahydrocoptisine, IS: isopropylantipyrine, HPLC conditions: column; Shiseido UG 120 C18 (250 × 4.6 mm, 5 μL), mobile phase; (a) 10 mM ammonium acetate in 0.2% TEA pH 5.0 adjusted by acetic acid and (b) acetonitrile, Gradient elution: 27% ACN for 0-10 min, 27-33% ACN for 10-15 min, and 33-95% ACN for 15-30 min. UV 280 nm, injection volume 20 μL, and flow rate: 1.0 mL/min.

aporphines were converted into their protonated form under acidic medium, resulting in decreasing retention time on C18 column.

As shown in Figure 2, the elution order of alkaloids at below pH 5.0 was as follow: aporphines > quaternary protoberberines > tetrahydroprotoberberines. Meanwhile, the elution order of alkaloids at above pH 6.5 was as follow: quaternary protoberberines > aporphines > tetrahydroprotoberberines. As pH of mobile phase increases, the overall elution time of aporphine alkaloids and tetrahydroprotoberberine alkaloids were delayed. The delayed elution of these alkaloids might be attributed to the decrease of their polarities. In contrast, the lowering pH of mobile phase increases the polarity of alkaloids and results in reducing their elution times on reversed phase column.[20] As shown in Figure 2, berberine and canadine were completely overlapped at pH 4.2 and corydaline and tetrahydrocoptisine were partially separated at pH 6.5. Aporphine and quaternary alkaloids were closely eluted within 8-13 min and the peak shape of aporphine alkaloids was shown broad at pH 5.8. From the consideration of peak resolution, peak shape, and run time, the separation of alkaloids by C18 column was found to be suitable at pH 5.0.

As can be seen in Figure 3, the retention times of tetrahydroprotoberberine alkaloids were steeply increased until to pH 5.0 and then gradually increased from pH 5.0 to pH 6.5. Compared to the retention behavior of tetrahydroprotoberberine alkaloids, retention times of aporphine alkaloids were exponentially increased as increasing pH value from 4.2 to 6.5. However, since all quaternary protoberberine alkaloids

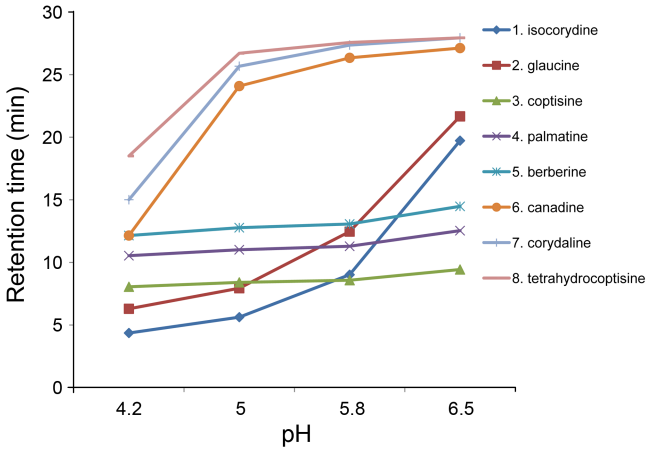


Figure 3. Profile of retention times of aporphine, quaternary protoberberine, and tetrahydroprotoberberine alkaloids according to change of pH values.

have originally cation form on nitrogen atom, their retention times did not influence by pH value of mobile phase. Generally, pK_a values on nitrogen atom are 7.10-7.16 for aporphine alkaloids (7.16 for isocorydine and 7.10 for glaucine) and about 5.43-5.81 for tetrahydroprotoberberine alkaloids (5.47 for canadine, 5.81 for corydaline, and 5.43 for tetrahydrocoptisine).[21] From these pK_a values, aporphine alkaloids with methyl group at nitrogen atom are shown to be more basic compounds than tetrahydroprotoberberine alkaloids, reflecting the relative strength of protonation. The retention times of aporphine alkaloids were shown to be more influenced than those of tetrahydroprotoberberine alkaloids according to changing pH values. It can be explained that the retention times of aporphine alkaloids were dramatically increased at above pH 5.8, resulting from increasing their hydrophobicity. The decrease of pH value from 6.5 to 4.2 can greatly affect the molar ratio of ionized/unionized forms for tetrahydroprotoberberine and aporphine alkaloids according to Henderson-Hasselbalch equation.[22] Most of aporphine and tetrahydroprotoberberine alkaloids were existed as free form above at pH 6.5, respectively. In contrast, all alkaloids investigated in this study might be presented as ionized form below at pH 4.2.

Optimization of Programming Solvent Composition by Gradient Elution. The programming solvent composition by gradient elution is one of important separation factors for complex samples, especially from herbal medicines. In this study, the mixture of 10 mM ammonium acetate solution and acetonitrile were used as mobile phase in HPLC analysis of alkaloids. It is already demonstrated that the use of ammonium acetate buffer solution can be achieved higher and narrower peaks.[15] The concentration of ammonium acetate did not greatly influence on the retention time of alkaloids at a fixed pH condition. However, change of solvent composition by gradient elution was significantly affected on both the retention times and separation of alkaloids extracted from herbal medicines. Therefore, the programming solvent composition should be also optimized to precisely determine the level of alkaloids in related herbal

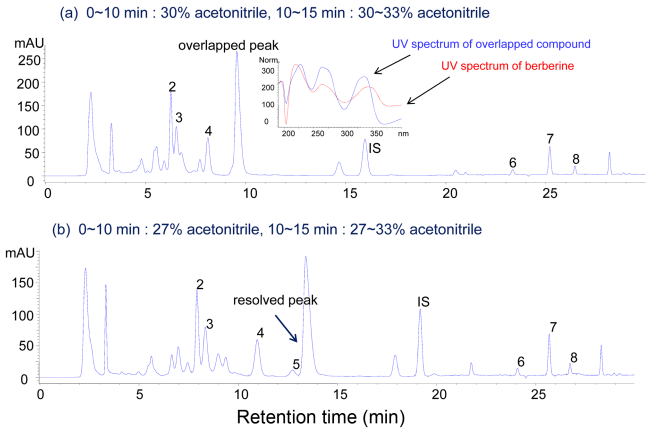


Figure 4. HPLC chromatograms of extract of *C. yanhusuo* using different programming solvent composition by gradient elution. Peak identities: 1. isocorydine, 2. glaucine, 3. coptisine 4. palmatine, 5. berberine, 6. canadine, 7. corydaline, 8. tetrahydrocoptisine, IS: isopropylantipyrine. Gradient elution: (a) 30% ACN for 0-10 min, 30-33% ACN for 10-15 min, and 33-95% ACN for 15-30 min and (b) 27% ACN for 0-10 min, 27-33% ACN for 10-15 min, and 33-95% ACN for 15-30 min.

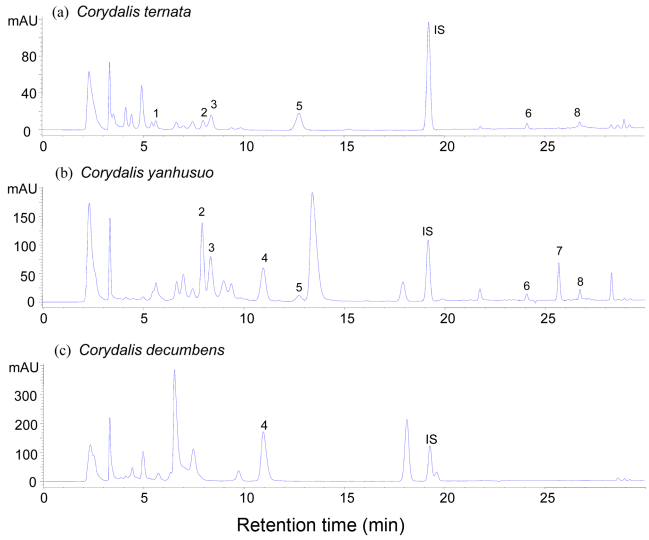


Figure 5. HPLC chromatograms of alkaloids in crude extracts of different *C.* species. Peak: 1. isocorydine, 2. glaucine, 3. coptisine, 4. palmatine, 5. berberine, 6. canadine, 7. corydaline, 8. tetrahydrocoptisine, and IS: isopropylantipyrine. HPLC conditions are the same as Figure 2.

medicines.

As can be seen in Figure 4, the overall retention time of alkaloids in *C. yanhusuo* extract was increased due to their hydrophobicity when the portion of acetonitrile of mobile phase decreases using gradient elution. However, all alkaloids in herbal extract can be shown similar chromatographic patterns because they have similar physicochemical properties. In some case, the quantity of some alkaloids could be over-estimated if alkaloids in herbal extract were not well separated from sample matrix or other alkaloids. As indicated in Figure 4(a), berberine was shown to be observed as like

Table 1. Concentration levels of marker compounds in extracts of *Corydalis* species

Sample number	Concentration (μg/g) ± relative standard deviation (n = 3)							
	isocorydine	glaucine	coptisine	palmatine	berberine	canadine	corydaline	tetrahydro coptisine
C. ternata-1	-	12.1 ± 12.3	32.6 ± 3.1	-	18.4 ± 3.9	2.9 ± 11.5	-	3.6 ± 4.8
C. ternata-2	0.8 ± 12.5	19.2 ± 1.8	18.5 ± 11.5	-	9. 3± 12.0	2.3 ± 3.8	-	4.3 ± 4.0
C. ternata-3	4.3 ± 14.7	21.8 ± 11.9	54.2 ± 3.6	4.3 ± 3.1	83.8 ± 0.5	12.6 ± 4.0	-	7.9 ± 0.8
C. ternata-4	1.9 ± 9.8	24.5 ± 7.5	33.9 ± 2.5	-	21.6 ± 0.2	6.0 ± 9.4	-	4.4 ± 1.1
C. ternata-5	-	14.1 ± 4.5	42.7 ± 2.6	-	31.6 ± 0.3	5.2 ± 12.2	-	4.9 ± 1.4
C. ternata-6	3.3 ± 2.7	11.2 ± 0.1	44.9 ± 0.9	-	15.0 ± 0.1	2.7 ± 8.3	-	0.6 ± 13.3
C. ternata-7	4.1 ± 0.7	20.1 ± 0.2	52.1 ± 0.4	-	18.5 ± 1.3	2.2 ± 10.3	-	11.2 ± 0.6
C. ternata-8	0.1 ± 5.6	11.3 ± 0.5	47.9 ± 3.1	-	29.8 ± 0.6	7.3 ± 1.1	-	-
C. yanhusuo-1	-	60.9 ± 0.3	100.6 ± 0.5	18.3 ± 0.1	1.9 ± 0.3	12.5 ± 1.3	71.5 ± 0.2	58.4 ± 0.3
C. yanhusuo-2	-	31.0 ± 0.2	72.3 ± 0.5	19.2 ± 0.2	1.5 ± 0.7	5.4 ± 4.4	33.3 ± 0.5	17.2 ± 0.1
C. yanhusuo-3	-	72.4 ± 12.6	104.7 ± 7.1	13.2 ± 9.6	-	19.9 ± 12.8	85.5 ± 9.6	66.2 ± 13.4
C. yanhusuo-4	-	47.1 ± 12.3	66.0 ± 6.0	6.5 ± 7.1	-	8.7 ± 11.2	67.3 ± 13.5	25.0 ± 14.1
C. yanhusuo-5	-	46.2 ± 8.7	70.8 ± 9.0	12.3 ± 7.2	-	6.8 ± 9.8	53.2 ± 7.5	28.5 ± 7.9
C. yanhusuo-6	-	47.3 ± 7.0	228.4 ± 7.7	56.4 ± 7.6	10.2 ± 11.9	16.0 ± 9.5	54.1 ± 6.0	36.6 ± 4.7
C. yanhusuo-7	-	64.8 ± 6.7	223.5 ± 8.8	36.6 ± 7.1	7.3 ± 13.3	10.1 ± 7.9	48.5 ± 9.8	39.3 ± 4.7
C. yanhusuo-8	-	71.4 ± 7.8	253.4 ± 9.1	42.1 ± 7.3	9.1 ± 14.6	16.6 ± 5.7	51.9 ± 9.8	30.0 ± 6.5
C. yanhusuo-9	-	49.8 ± 4.5	80.8 ± 5.9	19.3 ± 5.2	1.5 ± 15.0	15.3 ± 1.9	47.6 ± 5.8	56.5 ± 4.2
C. yanhusuo-10	-	60.7 ± 5.7	82.0 ± 7.2	10.4 ± 11.7	-	11.7 ± 8.1	78.3 ± 5.4	56.9 ± 5.3
C. yanhusuo-11	-	50.1 ± 3.7	129.4 ± 1.1	23.6 ± 0.5	2.9 ± 1.8	14.0 ± 1.6	58.6 ± 1.7	38.1 ± 1.2
C. yanhusuo-12	-	44.5 ± 2.2	78.3 ± 1.6	9.2 ± 1.0	-	13.2 ± 2.2	74.2 ± 1.8	29.0 ± 1.4
C. yanhusuo-13	-	35.7 ± 3.0	42.3 ± 1.4	11.0 ± 3.0	-	4.7 ± 4.2	34.0 ± 5.6	36.0 ± 2.6
C. yanhusuo-14	-	52.1 ± 1.3	146.8 ± 1.1	26.2 ± 0.6	5.7 ± 1.5	9.5 ± 2.1	51.3 ± 0.5	32.7 ± 0.6
C. decumbens-1	-	-	-	93.7 ± 4.4	-	-	-	-

major component although it was already known to be a minor compound of *C. yanhusuo*.[6,23] From this chromatogram, it can be deduced that berberine peak might be overlapped with other component co-extracted from *C. yanhusuo*. The overlapped compound with berberine was expected as one of alkaloids since its UV-spectrum was almost similar to that of berberine. In Figure 4(b), this overlapped peak could be successfully resolved by slightly decreasing the portion of acetonitrile in gradient elution mode, as described in Experimental section. The change of the portion of acetonitrile by gradient elution can lead to the different interaction between alkaloids and C18 column due to change of their hydrophobicity. Therefore, programming solvent composition by gradient elution should be carefully optimized to accurately determine the alkaloids in herbal extract.

Method Application. For the quality control of *C.* species, alkaloids were successfully extracted by ultrasonic extraction method with 70% methanol. Alkaloids in crude extracts of three different *C.* species were successfully analyzed by established HPLC conditions. Typical chromatograms of extracts of different *C.* species are shown in Figure 5. No significant interference was detected on HPLC chromatograms. The HPLC chromatographic patterns of alkaloids for *C.* species extracts showed different patterns according to their geographic origins. The quantity and number of alkaloids observed according to *C.* species origins were significantly different.

The amount of each alkaloid in *C.* species is listed in Table 1. The relative standard deviation (RSD) for marker compounds ranged from 0.1 to 15.0%. Thus, this method was successfully applicable as a quantitative analytical method for the determination of alkaloids from *Corydalis* species. Among these compounds, isocorydine with small quantity was observed only for *C. ternata* species. Interestingly, corydaline was detected only for *C. yanhusuo* species and thus can be use an important marker of *C. yanhusuo* species. The amount of berberine in *C. yanhusuo* was detected much lower than that in *C. ternata*. On the other hand, only palmatine among marker compounds was detected for *C. decumbens* (Table 2).

Principal Component Analysis. To classify *C.* species according to geographic origins, principal component analysis (PCA) was performed for chromatographic data of 23 samples obtained by established HPLC method. PCA was employed the concentrations of marker compounds observed in the extract of *C.* species. The score plots derived from PCA are shown in Figure 6. It was interesting observation that all *C.* species samples were classified into three groups, indicating clear differentiation between geographic origins. The scattered points of *C. ternata* and *C. yanuhsuo* samples were closely gathered at the upper of left and right sides, respectively, due to their chromatographic similarity within identical sample origin. The *C. decumbens* sample could be easily differentiated because of its quiet different chromatographic patterns compared with other species.

Table 2. Result of quantitative analysis of extract of *C.* species

Compound	mean concentration (μg/g)		
	C. ternata (n = 8)	*C. yanhusuo* (n = 14)	*C. decumbens* (n = 1)
isocorydine	1.8	-	-
glaucine	16.8	52.4	-
coptisine	40.9	120.0	-
palmatine	0.5	21.7	93.7
berberine	28.5	2.9	-
canadine	5.2	11.7	-
corydaline	-	57.8	-
tetrahydrocoptisine	4.6	39.3	-

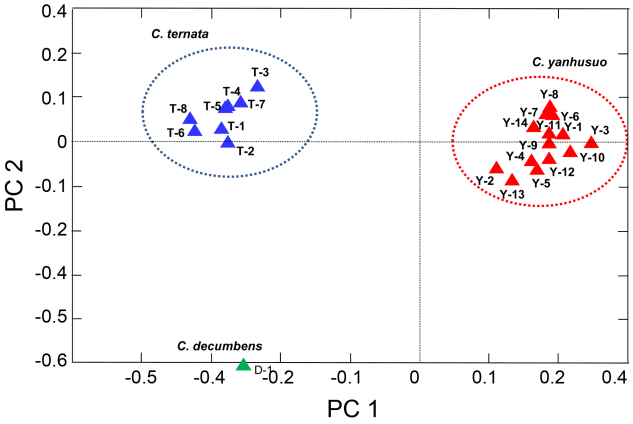


Figure 6. PCA plot of different *Corydalis* species combined with 23 chromatographic data.

Conclusion

In this study, reversed-phase HPLC elution patterns of eight isoquinoline alkaloids were investigated according to different pH values. The programming solvent composition using gradient elution was also shown to be useful method for the separation of alkaloids in complex herbal medicines. Established HPLC method is suitable for the simultaneous determination of alkaloids in three different *C.* species. In addition, PCA provided significant information on the differentiation of *C.* species with different origins. Furthermore, this developed method together with PCA will be successfully applied for the quality control of alkaloid containing herbal medicines.

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