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Simultaneous Determination of Eight Compounds in *Lysimachia christinae* by HPLC-DAD

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Abstract – *Lysimachia christinae* Hance was commonly used in Oriental medicine for treating the hepatitis virus, cholecystitis and cholagogic efficiency. According to the previous study, it possesses high antioxidant and anti-inflammatory activity. Simultaneous determination analytical method of isolated eight compounds, cynaroside (**1**), 2-(3,4-dimethoxyphenyl) ethyl O-α-L-arabinopyranosyl-(1→2)-O-[6-deoxy-α-L-mannopyranosyl-(1→3)] β-D-glucopyranoside (**2**), stearylester ricinoleic acid (**3**), (*E*)-4-(3,4-dimethoxyphenyl) but-3-en-1-yl palmitate (**4**), 2-hydroxy-24-methoxy-4-tetracosenoic acid (**5**), 2-hydroxy-24-propoxy-4-tetracosenoic acid (**6**), β-sitosterol (**7**), and androst-16-ene-3,6-diol (**8**) were established by using HPLC-DAD. This HPLC analysis was detected on a Dionex C18 column (5 μm, 120 Å, 4.6 mm × 150 mm) at 25°C. The mobile phase consisted of 0.1% trifluoroacetic acid and acetonitrile at a flow rate of 1 mL/min. Validation of the method was assessed by linearity, precision and accuracy test. Calibration curve was good at $r^2 > 0.9998$. Limits of detection (LOD) ranged from 0.19 to 8.18 g/ml and Limits of quantification (LOQ) ranged from 0.19 to 24.80 g/ml. The relative standard deviations (RSD) values of precision test, intra- and inter- day, were less than 0.99% and 1.0%. The accuracy test results ranged from 98.81% to 106.49% and RSD values were less than 0.95%. These results showed that the HPLC-DAD method was very reliable and accurate for the quantity analysis of eight compounds in *L. christinae* extract for quality control.
Keywords – *Lysimachia christinae* Hance, cynaroside, HPLC-DAD, quality control

Introduction

In the 21 centuries, the aging population goes on growing rapidly and due to a prolonged average life span, neurodegenerative disorders become the serious problem. Neurodegeneration evokes the continuous and irreversible neuronal cell death and this brings about the malfunction of cognitive and motor ability.[1] Alzheimer's disease and Parkinson's disease are the representative diseases that caused by neurodegenerative disorder.[2] Various medicines that composed by chemical compounds exist, but their medicinal effects are not that satisfactory and their side effects become the serious drawbacks. Therefore, a vast number of studies for screening the novel natural product which has outstanding neuroprotective effect are performed.[3-5]

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Herbal medicines have been one of the sources for preventing and treating various diseases worldwide since ancient times.[6] It is already known that many herbs prevent oxidation and act as anti-inflammatory agent.[7] Herbal medicines also have relatively few side effects. In recent years, as interest in health has been increasing, studies on natural products have been continuing and development of health functional foods of quasi-drug using natural products has been actively carried out. However, the quality of these herbal plants may vary depending on the origin, cultivation method, collection timing and processing method.[8] Due to the need for more systematic and efficient medicinal herb management, individual analysis methods for substances using high performance liquid chromatography (HPLC) have recently been established. However, natural products have a variety of compounds, and when they are used as a drug, their effects are complexly expressed.[9] Currently most herbal medicines use individual methods, resulting in high-economic and time loss. A more efficient quality control can be achieved by using simultaneous analysis of

the multiple components.[10]

Lysimachia christinae Hance is widely distributed in temperate climates and usually found in China.[11,12] *L. christinae* was commonly used as Chinese medicines treating for hepatitis virus, cholecystitis and cholagogic efficiency.[13] Recent studies have scientifically proved hepatoprotective, anticholecystitis, dieresis and anti-hyperlipidemic activity of *L. christinae* extract.[14-16] It contains various triterpenoids, glucopyranosides and flavonoids.[17] Based on these chemical compositions, it also showed remarkable antioxidant and anti-inflammatory activity.[18,19]

In this study, the simultaneous analysis of *L. christinae* was established by using HPLC-DAD method. We also verified the established method validation and confirmed the availability of this analysis method to *L. christinae* extract for quality control.

Materials and methods

Plant materials – The dried whole plats of *L. christinae* were purchased from medicinal herbs market named Chunjigayakcho (Seoul, Korea). The voucher speci-

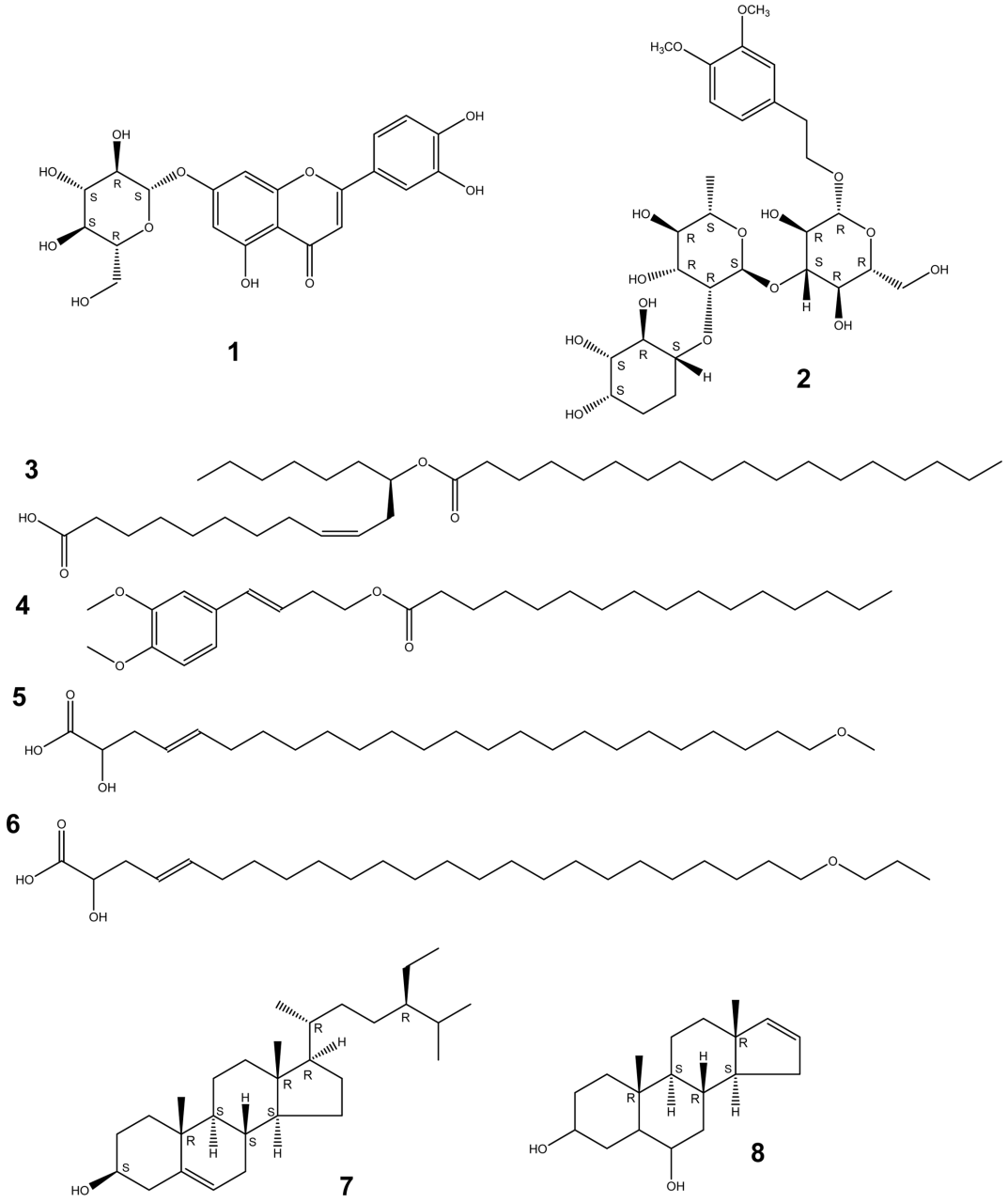

Fig. 1. Chemical structures of compounds **1** - **8** isolated from *L. christinae* extract.

men has been deposited as CH156M in the natural product laboratory, Kangwon National University (Chuncheon, Korea). The HPLC grade solvents including water, methyl alcohol and acetonitrile were purchased from J.T. Baker (U.S.A), and trifluoroacetic acid (TFA) was purchased from DAE JUNG (Seoul, Korea).

HPLC analysis – *L. christinae* solutions were analyzed by HPLC-DAD. HPLC (Dionex) was composed of an LPG 3X00 pump, an ACC-3000 auto-sampler, a DAD-3000(RS) diode array UV/VIS detector, and a column oven. Each sample was injected and isolated through a Dionex C18 column (5 μm, 120 Å, 4.6 mm × 150 mm) at 25 °C. The mobile phase consisted of 0.1% TFA water and acetonitrile. The injection volume of samples was 10 μl. The UV wavelength was set at the wavelength of 205, 254, 280 and 330 nm, respectively. And the chromatograms were acquired at the wavelength of 205 nm to show all the peaks simultaneously and effectively.

Preparation of standard solutions for HPLC analysis – Standard stock solutions were produced by dissolving the 8 isolated compounds; cynaroside (**1**), 2-(3,4-dimethoxyphenyl)ethylO-α-L-arabinopyranosyl-(1→2)-O-[6-deoxy-α-L-manno-pyranosyl-(1→3)]-β-D-glucopyranoside (**2**), stearylester ricinoleic acid (**3**), (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-yl palmitate (**4**), 2-hydroxy-24-methoxy-4-tetracosenoic acid (**5**), 2-hydroxy-24-propoxy-4-tetracosenoic acid (**6**), β-sitosterol (**7**), and androst-16-ene-3,6-diol (**8**) with 80% methanol at the concentration of 1000.00 μg/ml, 489.84 μg/ml, 502.48 μg/ml, 901.54 μg/ml, 1312.50 μg/ml, 408.68 μg/ml, 833.23 μg/ml and 972.39 μg/ml respectively (Fig. 1). 1/2, 1/4, 1/8 and 1/16 times diluted working solutions were prepared by dissolving the each standard stock solutions in 80% MeOH. Diluted samples were filtered through a 0.45 μm pore sized filter. The manufactured working solutions were used to establish calibration curve.

Preparation of *L. christinae* extract sample – Dried whole plants of *L. christinae* (100 g) were extracted in 80% methanol for 90 min with three times by ultrasonication method. The extracts were obtained in powder form through reduced pressure concentration and dissolved in HPLC grade MeOH at a concentration of 20 mg/mL. The *L. christinae* sample was filtered through 45 um membrane filter and injected into HPLC.

Validation of method

Linearity – The calibration curves were built by plotting the peak area versus concentration of each working solution. To prepare the working solution, compound **1** (62.50, 125.00, 250.00, 500.00 and 1000.00 μg/ml), compound **2** (60.77, 121.55, 243.10, 486.20 and 972.39 μg/ml), compound **3** (82.03, 164.06, 328.13, 656.25 and 1312.50 μg/ml), compound **4** (52.08, 104.16, 208.33, 416.66 and 833.32 μg/ml), compound **5** (56.35, 112.69, 225.39, 450.77 and 901.54 μg/ml), compound **6** (31.41, 62.81, 125.62, 251.24 and 502.48 μg/ml), compound **7** (25.54, 51.09, 102.27, 204.34 and 408.68 μg/ml), and compound **8** (31.18, 62.36, 124.71, 249.42 and 489.84 μg/ml) were dissolved in methanol. The linear regression equations were calculated by $y = ax \pm b$, which x and y are concentration and the peak areas of each compound respectively. The linearity was established according to the least squares treatment (r^2). Analysis at each working standard concentration was done in triplicate.

Limit of detection (LOD) and limit of quantification (LOQ) – The value of limit of detection (LOD) was determined as the lowest concentration of sample and limit of detection quantification (LOQ) was determined as the lowest concentration of compounds by injecting the diluted standard solution when the signal-to-noise ratio reached at (3.3 ~ 10).

Repeatability and Precision – Precisions of the method were evaluated by intra-day and inter-day injections. The intra-day experiment was performed by three injections for 3 days. Both tests were examined in three different concentrations which were confirmed in calibration curves. The repeatability and precision were expressed as relative standard deviation (RSD, %).

Accuracy tested by recovery test – Accuracy of the method was evaluated by recovery study. It was conducted by adding measured amount of compound solutions to the whole *L. christinae* sample. Three different concentrations of compound **1** (125.00, 250.00 and 500.00 μg/ml), compound **2** (121.55, 243.10 and 486.20 μg/ml), compound **3** (164.06, 328.13 and 656.25 μg/ml), compound **4** (104.16, 208.33 and 416.66 μg/ml), compound **5** (112.69, 225.39 and 450.77 μg/ml), compound **6** (62.81, 125.62 and 251.24 μg/ml), compound **7** (51.09, 102.27 and 204.34 μg/ml) and compound **8** (62.36, 124.71 and 249.42 μg/ml) were added to the samples. The equation used to define the recovery percentage was (detected amount – original amount)/spiked amount × 100.

Sample analysis using established method – The efficiency of optimized simultaneous determination method was verified by analyzing the total *L. christinae* sample. According to the calibration curve of eight compounds, we calculated the amount of eight compounds from *L. christinae* extract. All compounds were detected in the chromatogram without overlap of the peak.

Statistical analysis – Whole of the experiments were replicated at least three times. Values were expressed as mean ± standard deviation (S. D) and statistical significances were decided by one-way analysis of variance (ANOVA) along with Tukey's test. Values of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were accepted to be statistically significant. Cell experiment data were expressed as relative % setting control group on 100%.

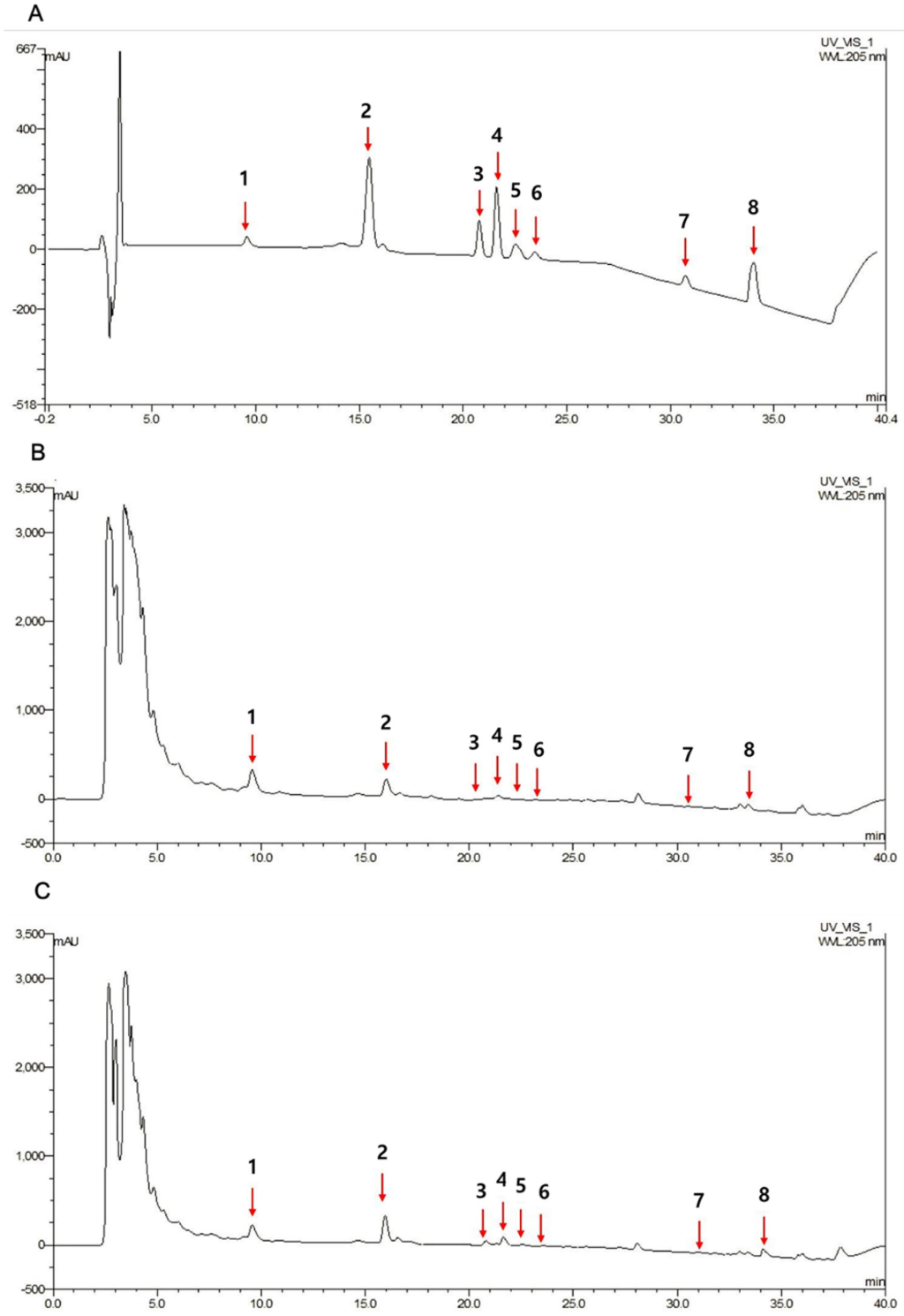

Fig. 2. The HPLC chromatogram of standard mixture (A), *L. christinae* extract (B) and co-injection of total *L. christinae* extract and standard mixture (C).

Results and discussion

The reason for optimized experiment using HPLC was to gain higher separation efficiency and peak resolution of target compounds in a shorter run-time. To achieve this goal in *L. christinae*, the effective HPLC-DAD conditions including appropriate column, mobile phase condition and the wavelength of UV spectrum were chosen through many preliminary tests. The selected column was Dionex C18 column, one of reversed phase column. The multi-step gradient solution system consisted of 0.1% TFA water (A) and methanol (B) was used as a mobile phase.

Table 1. The regression data, LOD and LOQs of eight isolated compounds analyzed by HPLC-DAD

Compound	Regression equation[a]	R^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
1	y = 0.0175x + 0.0105	0.9999	25-800	2.93	8.88
2	y = 0.2171x – 0.4777	1	25-800	1.44	4.36
3	y = 0.0643x + 0.1106	0.9997	25-800	2.40	7.29
4	y = 0.1529x + 0.6618	0.9999	25-800	5.39	16.35
5	y = 0.0665x + 0.2399	0.9999	25-800	8.18	24.80
6	y = 0.0274x – 0.1114	0.9999	25-800	0.52	1.57
7	y = 0.0467x + 0.0093	0.9998	25-800	0.06	0.19
8	y = 0.0216x + 0.0339	0.9998	25-800	1.21	3.67

[a] *y*: peak area, *x*: amount (μg)

Table 2. Intra- and inter- day precision data of eight compounds

Compound	Concentration (μg/mL)	Intra-day			Inter-day		
		Mean (μg/mL)	RSD[a] (%)	Accuracy (%)	Mean (μg/mL)	RSD[a] (%)	Accuracy (%)
1	500.00	503.98 ± 0.12	0.02	103.37	503.24 ± 0.62	0.14	100.31
	250.00	251.71 ± 1.12	0.89	102.09	251.13 ± 0.83	0.35	102.51
	125.00	123.77 ± 3.74	0.65	99.15	123.12 ± 0.24	0.19	103.32
2	486.20	485.14 ± 0.97	0.20	100.37	483.24 ± 0.76	0.18	98.25
	243.10	244.21 ± 0.40	0.16	102.51	243.73 ± 0.51	0.56	97.83
	121.55	123.50 ± 1.21	0.98	99.84	120.92 ± 0.69	0.58	100.21
3	656.25	657.36 ± 1.22	0.31	99.21	654.13 ± 0.49	0.12	101.03
	328.13	325.95 ± 1.15	0.59	97.87	329.52 ± 0.30	0.15	97.76
	164.06	162.32 ± 0.25	0.27	94.87	164.76 ± 0.95	1.00	94.76
4	416.66	415.66 ± 0.72	0.18	102.20	414.76 ± 0.71	0.18	101.19
	208.33	206.45 ± 0.54	0.28	97.29	209.06 ± 0.69	0.35	99.53
	104.16	100.63 ± 0.65	0.67	98.07	101.52 ± 0.62	0.61	101.52
5	450.77	451.52 ± 0.39	0.10	101.32	454.39 ± 1.17	0.29	101.10
	225.39	229.72 ± 0.43	0.21	101.83	224.86 ± 0.33	0.16	101.43
	112.69	112.23 ± 1.02	0.99	102.94	114.37 ± 0.97	0.93	104.37
6	251.24	249.83 ± 0.82	0.20	102.68	251.03 ± 1.58	0.39	100.76
	125.62	126.27 ± 0.59	0.30	98.93	125.12 ± 0.27	0.13	100.06
	62.81	65.17 ± 0.71	0.71	98.86	64.09 ± 0.56	0.57	98.09
7	204.34	204.25 ± 1.53	0.38	101.99	200.66 ± 0.60	0.15	100.16
	102.27	102.63 ± 0.69	0.34	100.88	104.27 ± 0.49	0.24	102.14
	51.09	51.23 ± 0.83	0.83	99.29	52.77 ± 0.39	0.39	98.77
8	249.42	244.58 ± 0.51	0.12	102.96	248.43 ± 0.58	0.15	100.61
	124.71	122.89 ± 0.47	0.23	101.38	125.03 ± 0.91	0.45	101.52
	62.36	62.53 ± 0.78	0.77	102.03	61.92 ± 0.21	0.20	101.92

[a] Relative Standard Deviation

The most appropriate condition for the separation of isolated compounds was: 0-10 min, 10% B; 10-20 min, 10-20% B; 20-25 min, 20-30% B; and 25-30 min 30% B, at a flow rate of 0.1 mL/min. This gradient led to the separation of eight peaks within 40 min. The wavelength of DAD detector was tested at 205, 254, 280 and 330 nm and chromatograms were selected at 205 nm by UV spectrum. The column temperature had no significant influence on the separation, so room temperature was used. The peak of each compound was assured by comparing retention time and UV spectrum of their corresponding marker solution. The chromatograms of the whole *L. christinae* compounds are shown in Fig. 2.

To approve the validation of this method, the experiments for verifying linearity, detection and quantification limits, precision and accuracy, and recovery were performed.

The regression equations were established by five concentration trials of each standard in triplicate. In linear regression equation $y = ax + b$, x is concentration of the marker compositions, and y is peak area. The slope and intercept of calibration curve was determined by this equation. The high correlation coefficients ($r^2 > 0.9998$) exhibits that all calibration curves had good linearity within the test ranges as shown in Table 1. According to this linear regression, the results of limits of detection (LOD) ranged from 0.06 - 8.18 μg/mL and limits of quantification (LOQ) were in the range of 0.19 - 24.80 μg/mL, respectively. This means a minimum amount of compounds can be detected and quantified.

For evaluating the repeatability and precision of the method, within-day test (intra-day analysis, n=3) and intermediate-day test (inter-day analysis, n=3) were performed. Intra-day test was examined three times during a single day and inter-day test was examined on three different days (1, 3, 5 days). The RSD values of intra-day were 0.02% - 0.99% and inter-day were 0.12% - 1.00%, respectively (Table 2). These results showed that the method was very reproducible and precise.

The accuracy of the method was evaluated by carrying out the recovery test. Each sample was tested in triplicate by adding three different concentrations of solutions to the total *L. christinae* standard solution. The mean recovery was 102.03% for compound **1**, 106.49% for compound **2**, 101.57% for compound **3**, 98.81% for compound **4**,

Table 3. Recovery of the eight compounds from *L. christinae*

Compound	Spiked (μg/mL)	Found (μg/mL)	RSD (%)	Recovery (%)[a]
1	500.00	497.52 ± 0.67	0.16	102.24
	250.00	253.13 ± 0.94	0.43	103.07
	125.00	125.94 ± 0.97	0.84	100.77
2	486.20	475.98 ± 0.69	0.22	97.90
	243.10	243.79 ± 0.88	1.14	100.29
	121.55	121.28 ± 1.30	0.18	121.28
3	656.25	653.15 ± 0.69	0.15	102.19
	328.13	328.14 ± 0.88	1.10	102.64
	164.06	164.09 ± 1.30	1.61	99.89
4	412.66	412.66 ± 0.98	0.24	101.69
	208.33	206.34 ± 0.97	0.49	97.51
	104.16	107.06 ± 1.02	1.03	97.24
5	450.77	450.24 ± 0.43	0.10	100.49
	225.39	227.44 ± 0.84	0.37	102.50
	112.69	112.38 ± 0.33	0.26	104.68
6	251.24	246.66 ± 1.21	0.19	103.33
	125.62	125.14 ± 1.25	0.28	104.32
	62.81	63.99 ± 1.04	0.30	109.55
7	204.34	204.28 ± 1.40	0.25	100.11
	102.27	102.64 ± 0.68	0.19	101.56
	51.09	51.51 ± 0.85	0.33	102.47
8	249.42	247.79 ± 1.07	0.25	103.26
	124.71	124.01 ± 0.75	0.34	101.14
	62.36	63.10 ± 0.90	0.74	102.82

[a] Recovery (%) = (amount found – original amount)/spiked amount ×100 %

Table 4. Contents of eight compounds in *L. christinae* extract

Compounds	Content (μg/mg)
1	22.75 ± 1.21
2	25.81 ± 1.11
3	0.19 ± 0.02
4	0.07 ± 0.01
5	0.29 ± 0.03
6	0.10 ± 0.01
7	0.14 ± 0.02
8	1.04 ± 0.11

102.56% for compound **5**, 105.73% for compound **6**, 101.38% for compound **7** and 102.41% for compound **8** (Table 3). The accuracy of the methods was determined by the relative standard deviation (RSD). The % RSD of the average recovery was 0.48% for compound **1**, 0.51% for compound **2**, 0.95% for compound **3**, 0.59% for compound **4**, 0.24% for compound **5**, 0.26% for compound **6**, 0.26% for compound **7** and 0.44% for compound **8** (Table 3). All the analyzed compounds were in the ideal range of recovery and RSDs were also low. This data shows the high accuracy of the method. The whole results described above indicate that method was very suitable for the quantitative analysis of *L. christinae* samples.

The method described above was successfully applied to investigate the eight target compounds contest in *L. christinae*. As summarized in Table 4, the amounts of the tested compounds varied significantly. The dominant content of compound was cynaroside (**1**) and 2-(3,4-dimethoxyphenyl) ethyl O-α-L-arabinopyranosyl-(1→2)-O-[6-deoxy-α-L-manno-pyranosyl-(1→3)]-β-D-glucopyranoside (**2**) which was 22.75 mg/g and 25.81 mg/g in whole sample. Other compounds were relatively small in quantity. Total eight compounds were able to detect simultaneously by HPLC-DAD in optimized conditions.

The developed HPLC-DAD method was used for the qualitative analysis of compounds in *L. christinae*. Eight compounds were simultaneously and rapidly detected within 40 min. Also, the optimized HPLC-DAD method was selective and accurate which was verified by the validation test including linearity, limits of detection and quantification, repeatability, precision and accuracy test.

In conclusion, a new method has been applied to the separation of *L. christinae* for shorter time and higher efficiency. This allowed us to get higher peak resolution. Lineearity, detection and quantification limits, precision and accuracy, and recovery were obtained for verification of this new method. As a result, the simultaneous analysis method was found to be very reproducible and accurate. These data suggested that this qualitative and quantitative analysis method can help the quality control of *L. christinae* through the simultaneous quantification of eight compounds.

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Conflict of interest statement

The authors have declared that there are no conflicts of interest.

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