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Article

Quantification of chlorogenic acid, rosmarinic acid, and caffeic acid contents in selected Thai medicinal plants using RP-HPLC-DAD

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The chlorogenic acid, rosmarinic acid, and caffeic acid contents in 100 selected plants were determined using reversed phase high performance liquid chromatography equipped with diode array detector. The optimum condition was 0.2% phosphoric acid in water (solvent A) and methanol (solvent B) as the mobile phase, which was set at 45% B for 20 minutes at a flow rate of 1.2 mL/min. The column temperature was maintained at 30 °C and the detection wavelength was 325 nm. Among 100 selected plants, 39.64% contained all 3 compounds, 40.54% contained 2 compounds, 14.41% contained only 1 compound, and 5.41% could not detect any of the 3 compounds. The highest contents of chlorogenic acid, rosmarinic acid, and caffeic acid were found in *Lonicera japonica* flowering buds, *Melissa officinalis* leaves, and *Coffea canephora* seeds at the concentration of 9.900 ± 0.004, 19.908 ± 0.171, and 1.233 ± 0.003 g/100 g of dried plant, respectively.

Keywords: Chlorogenic acid. Rosmarinic acid. Caffeic acid. RP-HPLC-DAD. Medicinal plant.

INTRODUCTION

Phenolic compounds or polyphenols, the secondary metabolites of plant, are one of the most abundant and extensively distributed groups of substances in the plant kingdom which appear in all plant organs. However, the polyphenolic profile of plants differs between varieties of the same species. For decades, polyphenols have interested many researchers for their antioxidant, antioxidative stress activities, and great abundance in food. The varieties of natural polyphenols range from simple molecules (such as phenolic acids) to highly polymerized compounds (such as tannins). Polyphenols occur primarily in a conjugated form with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom also exist (Bravo, 1998; Manach *et al*., 2004). Hydroxycinnamic acid, one of two major groups of phenolic acids, is usually found in plants. The hydroxycinnamic acid derivatives consist of a large group of simple phenolic acids, and are bountiful in fruits, seeds of fruits, vegetables, and cereals. In addition, they have been arranged into structural and functional constituents of plant cell walls and also as bioactive ingredients of diets. The derivatives of hydroxycinnamic acids are synthesized through the shikimate pathway in which phenylalanine and tyrosine are used as starting precursor molecules. The main hydroxycinnamic acid derivatives are ferulic acid, caffeic acid, *p*-coumaric acid, chlorogenic acid, sinapic acid, and rosmarinic acid (Lafay, Gil-Izquierdo, 2008; Manach *et al*., 2004; Teixeira *et al.*, 2013). Caffeic acid (Figure 1A) is one of the most common phenolic acids that biosynthesise by hydroxylation of *p*-coumaric acid and is more broadly present in several food sources such as berries, coffee drinks, and dietary supplements (Magnani *et al*., 2014). Chlorogenic acid (Figure 1B) is an ester form of caffeic acid and quinic acid, which is widely distributed in the human diet with plants, fruits, and vegetables especially in coffee, apples, and pears (Upadhyay, Mohan Rao, 2013). Rosmarinic acid (Figure 1C), an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid, is commonly found in species of the boraginaceae, lamiaceae, and in some ferns and hornworts (Petersen, Simmonds, 2003). High performance liquid chromatography (HPLC) is a primary method for the separation and analysis of chemical compounds in many fields such as agriculture, cosmetics, pharmaceutical industries, environments, and

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food. It is commonly used for qualitative and quantitative analyses of chemicals in herbal extracts. The identification of compounds depends on the retention time and light spectral characteristics of each chromatographic peak (Zeng *et al*., 2011).

The aim of this study was to establish a RP-HPLC-DAD condition for analysis and provide the approximate quantification of chlorogenic acid, rosmarinic acid, and caffeic acid in 100 selected Thai medicinal plants.

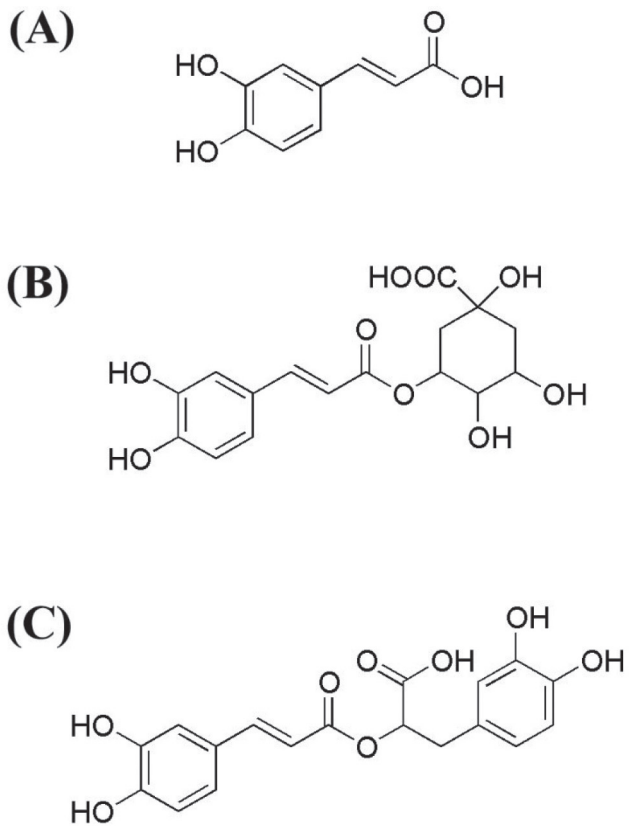

FIGURE 1 - Structures of caffeic acid (A), chlorogenic acid (B) and rosmarinic acid (C).

MATERIAL AND METHODS

Chemicals and material

Standard chlorogenic acid (CAS no. 327-97-9, purity ≥ 95%), rosmarinic acid (CAS no. 20283-92-5, purity 96%) and caffeic acid (CAS no. 331-39-5, purity ≥ 98%) were purchased from Sigma-Aldrich, USA. Methanol was of HPLC grade (RCI Labscan, Bangkok, Thailand). Ethanol, petroleum ether, and ortho-phosphoric acid were of analytical grade (RCI Labscan, Bangkok, Thailand). Ultra-pure water was prepared by an ultra-pure water system (NW20VF, Heal Force, China). The filters were 46 mm × 0.45 µm nylon membrane filters (National Scientific, Tennessee, USA) and 13 mm × 0.45 µm PTFE membrane syringe filters (ANPEL Laboratory Technology, Shanghai, China).

Sample collection

A selection of 100 fresh plants was obtained by randomized collection from various places in Thailand and also purchased from local markets in Thailand based on chemotaxonomy. They were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi. All plant materials were dried at 45 °C in a hot air oven, and voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. After the removal of any foreign matter, crude drugs were grounded into coarse powders before use.

Sample extraction

Ten grams of each selected plant sample were exhaustively extracted with petroleum ether and followed by 95% ethanol using a Soxhlet apparatus. The ethanolic extract was filtered through filter-paper and evaporated to dryness under reduced pressure by a rotary evaporator. The extract yields were weighed, recorded, and stored at -20 °C to avoid the possibility of degradation of the active compounds.

Preparation of standard solutions

One milligram of each standard was dissolved in 1 mL of methanol. The solution was filtered through a 0.45 µm PTFE membrane syringe filter.

Preparation of sample solutions

Fifty milligrams of each extract were dissolved in 1 mL of methanol and diluted to appropriate concentrations for further RP-HPLC analysis. The solution was filtered through a 0.45 µm PTFE membrane syringe filter.

Chromatographic conditions

The Shimadzu HPLC LC-20A system (Shimadzu, Japan) consists of a system controller (CMB-20A), two solvent delivery units (LC-20A), an on-line degassing unit (DGU-20A3), an auto-sample (SIL-20A), a column oven (CTO-20A), and a photo-diode array detector (SPD-M20A). System control and data analysis were processed with Shimadzu LC Solution software. The

chromatographic separation was performed with an Inertsil® ODS-3 5 µm C_{18} column (4.6 X 250 mm) and coupled with a ReproSil®-Pur ODS-3 C_{18} guard column (4.0 X 10 mm). The samples were analyzed using 0.2% phosphoric acid in water, pH 1.46 (solvent A), and methanol (solvent B) as a mobile phase. The isocratic program was set at 45% B for 20 minutes at a flow rate of 1.2 mL/min. The mobile phase was filtered through 0.45 µm nylon membrane filters and degassed using an ultrasonic bath before analysis. The column temperature was maintained at 30 °C and the injection volume of standards and sample solutions was 5 µl. The wavelength was set at 325 nm for monitoring chromatographic profile. All measurement was done in triplicate.

System suitability

The retention factor, theoretical plate number, and tailing factor were evaluated for system suitability parameters. The system performance was analyzed for five replicates of standard solution.

Method validation

According to the ICH guideline (ICH, 2005), the calibration range, specificity, accuracy, repeatability, intermediate precision, limit of detection (LOD), limit of quantitation (LOQ), and robustness were validated for analytical method. The *Lonicera japonica* flowering bud ethanolic extract was found to contain all 3 compounds so it was used as a sample matrix to evaluate the validity of the analytical method.

Calibration range

The calibration range was performed by plotting peak areas obtained from RP-HPLC analysis *versus* concentrations of standard. The stock solutions of chlorogenic acid, rosmarinic acid, and caffeic acid were dissolved in methanol and diluted together to give concentrations of 16.67, 33.33, 50.00, 66.67, and 83.33 µg/mL for evaluation of the calibration range. The calibration range of these standards was fitted by linear regression. The regression equation was calculated in the form of $y = ax + b$, where y is peak area and x is concentration.

Specificity

The specificity was evaluated by a peak purity test. The peak purity index of the analyte was processed by Shimadzu LC Solution software. It was determined by comparing all the spectra within the chromatographic peak to the reference spectrum at the peak apex.

Accuracy

The accuracy of each sample was tested by recovery method. Three different levels of standard solutions (10, 25, and 50 µg/mL) were spiked into the extract. The spiked and un-spiked samples were evaluated under the same condition in triplicate, then percent recoveries were calculated by comparing the measured amount of those standards with the amount added.

Precision

The precision was determined by repeatability (intra-day) and intermediate precision (inter-day) studies. The method was performed by analyzing three level concentrations of sample solution in triplicate on the same day for repeatability and in the five different days for intermediate precision. The precision was calculated in terms of percent relative standard deviation (% RSD) of compound content.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were determined from the calibration range using the following formulae:

$$LOD = \frac{3.3 \times \sigma}{S}$$

$$LOD = \frac{10 \times \sigma}{S}$$

where: σ = the residual standard deviation of the regression line; S = the slope of the regression line

Robustness

The robustness was determined for variations in flow rates (1.195, 1.200, and 1.205 mL/min), variations in column temperature (29, 30, and 31 °C), and variations in wavelength (322, 325 and 328 nm). The robustness was calculated in terms of percent relative standard deviation (% RSD) of retention time and peak area.

RESULTS AND DISCUSSION

Optimization of chromatographic condition

The chromatographic condition optimization including mobile phase, gradient elution procedure, flow rate, column temperature, and wavelength detection were performed to provide a better separation of constituents. Numerous mobile phases and gradient programs were trialled using various proportions of different aqueous phases and organic modifiers. Formic acid, phosphoric

acid, and acetic acid were usually employed to the aqueous phase to enhance the resolution, restrain the ionization, and reduce the peak tailing of compounds (Ma *et al*., 2011). The most suitable mobile phase that showed good resolution and symmetric peak shape were obtained using two parts as Solvent A (0.2% phosphoric acid in water) and Solvent B (methanol) with an isocratic program. The column temperature was held at 30 °C for the duration of analysis to improve the retention time precision. Hydroxycinnamic acids have the maximum wavelength during 270 - 360 nm (Köseoglu, Kolak, 2017). The UV spectra of standard chlorogenic, rosmarinic, and caffeic acids were compared at varying wavelengths, and based on the data from the literatures. The optimal detection wavelength in this study was to be 325 nm (Haghi, Hatami, 2010; Shan *et al*., 2013).

Chlorogenic acid, rosmarinic acid, and caffeic acid quantification

The 100 selected plants were edible vegetables, fruits, and herbal plants in Thailand. The plant samples were exhaustively extracted with petroleum ether and followed by 95% ethanol using a Soxhlet apparatus. The percent yields of crude extracts were shown in Table I.

A quantitative analysis of chlorogenic acid, rosmarinic acid, and caffeic acid in selected plants was performed by RP-HPLC analysis. The standard markers to quantify in this study are chlorogenic acid, rosmarinic acid, and caffeic acid which are hydroxycinnamic acid derivatives. Hydroxycinnamic acid derivatives, a subgroup of phenylpropanoids, are synthesised by the shikimate pathway where the starter precursor molecules are phenylalanine and tyrosine. Chlorogenic acid, rosmarinic acid, and caffeic acid in extracts were identified by comparing the retention time and UV spectrum of each peak with a reference of standard compounds (Figure 2). The contents of chlorogenic acid, rosmarinic acid, and caffeic acid in the 100 selected plants were shown in Table I. The results of RP-HPLC analysis demonstrated that the distribution of these 3 phenolic compounds varied in many samples. Among 100 selected plants, 39.64% contained all 3 compounds, 40.54% contained 2 compounds, 14.41% contained only 1 compound, and 5.41% could not detect these 3 compounds. *Lonicera japonica* flowering buds were found to be the richest source of chlorogenic acid content at 9.90 g/100 g of dried crude drug, and *Melissa officinalis* leaves showed the most rosmarinic acid content at 19.91 g/100 g of dried crude drug. The most caffeic acid content was found in *Coffea canephora* seeds at 1.23 g/100 g of dried crude drug. Chlorogenic acid was found in many families and is the main active constituent in *L. japonica* flowering bud (Chaowuttikul, Palanuvej, Ruangrungsi, 2017). It is also the main phenolic compound in coffee (*Coffea* spp.) that supported this study (Ayelign, Sabally, 2013). Rosmarinic acid was mostly found in the Labiatae family, relating to a previous report of high rosmarinic acid content in plants of this family, especially in *Mentha spicata*, *Salvia officicalis,* and *Melissa officinalis* (Shekarchi *et al*., 2012).

System suitability

The retention factor, theoretical plate number, and tailing factor were found to be 4.30 ± 0.01, 2745.17 ± 158.17, and 1.027 ± 0.07, respectively (Table II). These parameters confirmed that the condition is appropriate for analysis according to the FDA criteria.

Method validation

The analytical method validation is the process that confirms precise, accurate, and reliable quantitative data. According to the ICH guideline, calibration range, specificity, accuracy, repeatability, intermediate precision, limit of detection, limit of quantitation, and robustness should be validated for analytical analysis.

Standard chlorogenic acid, rosmarinic acid, and caffeic acid at 5 concentrations were investigated for linearity by RP-HPLC method. The calibration curves of standard compounds were linear in the range of 16.67 - 83.33 µg/mL. The regression equation of chlorogenic acid, rosmarinic acid, and caffeic acid were $y = 2874.5x + 813.03$, $y = 2833.8x - 1858.3$, and $y = 5202.2x + 673.32$, respectively (Figures 3 - 5). The linearity showed good correlation ($R^2 \geq 0.999$). An analytical technique is acceptable when the correlation of method (R^2) value achieved is 0.99 or better.

The specificity was evaluated by peak purity test and confirmed that analyte chromatographic peak is not attributable with another compound. This test is based on the absorbance spectrum, which is detected by diode array detectors. If all of the individual spectra recorded during the elution of a peak are identical, even if detected at any periods of a peak, the peak is considered pure (Hansen, Pedersen-Bjergaard, Rasmussen, 2011). An identical peak resulted in a peak purity index of 100% or peak purity index of 1.0, indicating that all spectra are similar. The results showed the peak purity index of the three compounds was more than 0.999 (Figures 6-8), thus no impurity was detected in these peaks.

The accuracy was evaluated by the recovery method.

TABLE I - The contents of chlorogenic, rosmarinic and caffeic acids in plant samples

Voucher specimen Number	Scientific plant name	Plant parts used	% yield (g/100 g)	Content (g/100 g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Acanthaceae					
CCPh001	*Andrographis paniculata* (Burm.f.) Wall. ex Nees	Leaves	25.595	0.625 ± 0.001	0.338 ± 0.000	0.045 ± 0.000
	Family: Alliaceae					
CCPh002	*Allium sativum* L.	Bulbs	10.393	-	-	0.005 ± 0.000
CCPh003	*Allium cepa* L.	Bulbs	66.467	0.034 ± 0.000	-	-
	Family: Amaranthaceae					
CCPh004	*Spinacia oleracea* L.	Leaves	21.103	-	0.039 ± 0.000	0.037 ± 0.000
	Family: Anacardiaceae					
CCPh005	*Mangifera indica* L. cv. Okrong	Leaves	28.698	0.354 ± 0.003	-	1.008 ± 0.008
	Family: Apiaceae					
CCPh006	*Anethum graveolens* L.	Aerial part	28.714	7.361 ± 0.038	-	0.184 ± 0.002
CCPh007	*Apium graveolens* L.	Aerial part	36.739	0.913 ± 0.003	2.880 ± 0.018	0.088 ± 0.001
CCPh008	*Apium graveolens* L. var. *secalinum*	Aerial part	36.066	2.574 ± 0.011	2.556 ± 0.014	0.112 ± 0.000
CCPh009	*Centella asiatica* (L.) Urb.	Aerial part	31.917	0.848 ± 0.003	0.910 ± 0.003	0.086 ± 0.002
CCPh010	*Coriandrum sativum* L.	Seeds	4.770	0.028 ± 0.000	0.061 ± 0.001	0.024 ± 0.000
CCPh011	*Daucus carota* L. subsp. *sativus* (Hoffm.) Arcang.	Roots	52.063	0.326 ± 0.003	-	0.085 ± 0.000
CCPh012	*Eryngium foetidum* L.	Leaves	30.451	4.979 ± 0.006	4.302 ± 0.100	0.160 ± 0.007
CCPh013	*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill	Aerial part	31.826	0.058 ± 0.004	9.153 ± 0.602	0.048 ± 0.001
	Family: Apocynaceae					
CCPh014	*Telosma cordata* (Burm. f.) Merr.	Flowers	34.584	0.350 ± 0.000	-	0.184 ± 0.002
	Family: Asteraceae					
CCPh015	*Artemisia dracunculus* L.	Aerial part	28.885	5.253 ± 0.047	5.359 ± 0.037	0.062 ± 0.008
CCPh016	*Artemisia pallens* Wall. ex DC.	Aerial part	8.141	0.042 ± 0.000	-	0.011 ± 0.000
CCPh017	*Chromolaena odorata* (L.) R. M. King & H. Rob.	Leaves	28.239	4.137 ± 0.035	0.279 ± 0.009	0.621 ± 0.018
CCPh018	*Gnaphalium polycaulon* Pers.	Aerial part	23.409	0.699 ± 0.009	1.207 ± 0.012	0.236 ± 0.009
CCPh019	*Helianthus annuus* L.	Pericarps	2.880	0.038 ± 0.000	0.004 ± 0.000	0.003 ± 0.000
CCPh020	*Helianthus annuus* L.	Seeds	11.523	2.144 ± 0.006	0.008 ± 0.000	0.110 ± 0.000
CCPh021	*Helianthus annuus* L.	Sprouts	30.726	2.199 ± 0.005	3.117 ± 0.029	0.093 ± 0.002
CCPh022	*Lactuca sativa* L.	Leaves	24.088	1.396 ± 0.036	0.343 ± 0.001	0.258 ± 0.002
	Family: Brassicaceae					
CCPh023	*Brassica juncea* (L.) Czern.	Leaves	28.414	0.351 ± 0.002	-	0.083 ± 0.001
CCPh024	*Brassica oleracea* L. Group *Capitata*	Aerial part	38.510	0.172 ± 0.002	-	-
CCPh025	*Brassica rapa* L. Group *Pekinensis*	Aerial part	41.573	0.154 ± 0.001	-	-
CCPh026	*Raphanus sativus* L.	Roots	52.566	0.072 ± 0.002	-	0.033 ± 0.001
	Family: Caprifoliaceae					
CCPh027	*Lonicera japonica* Thunb.	Flowering bud	32.734	9.896 ± 0.004	2.543 ± 0.007	0.195 ± 0.002
	Family: Caricaceae					
CCPh028	*Carica papaya* L.	Leaves	17.759	0.049 ± 0.000	0.023 ± 0.001	0.508 ± 0.000
	Family: Convolvulaceae					
CCPh029	*Ipomoea aquatica* Forssk.	Aerial part	30.503	2.541 ± 0.014	8.303 ± 0.042	0.113 ± 0.002
	Family: Cucurbitaceae					
CCPh030	*Momordica charantia* L. (Thai varieties)	Fruits	28.359	0.042 ± 0.000	-	<LOQ

TABLE I - The contents of chlorogenic, rosmarinic and caffeic acids in plant samples (cont.)

Voucher specimen Number	Scientific plant name	Plant parts used	% yield (g/100 g)	Content (g/100 g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
CCPh031	*Momordica charantia* L. (Chinese varieties)	Fruits	35.728	0.085 ± 0.001	-	<LOQ
	Family: Eucommiaceae					
CCPh032	*Eucommia ulmoides* Oliv.	Stem barks	10.441	0.039 ± 0.000	-	0.019 ± 0.000
	Family: Euphorbiaceae					
CCPh033	*Euphorbia hirta* L.	Aerial part	11.150	0.234 ± 0.000	-	0.021 ± 0.000
CCPh034	*Phyllanthus emblica* L.	Fruits	28.671	0.300 ± 0.001	0.077 ± 0.000	0.029 ± 0.000
CCPh035	*Ricinus communis* L.	Leaves	22.599	0.039 ± 0.000	-	0.352 ± 0.004
	Family: Fabaceae					
CCPh036	*Pisum sativum* L.	Fruits	45.838	-	-	-
CCPh037	*Pisum sativum* L. var. *macrocarpon*	Fruits	43.713	-	-	-
CCPh038	*Sesbania grandiflora* (L.) Poir.	Flowers	42.093	0.016 ± 0.000	0.204 ± 0.007	<LOQ
CCPh039	*Sesbania grandiflora* (L.) Poir.	Stem barks	3.960	0.009 ± 0.000	-	0.006 ± 0.000
	Family: Gnetaceae					
CCPh040	*Gnetum gnemon* L. var. *tenerum* Markgr.	Leaves	22.424	0.092 ± 0.001	-	-
	Family: Labiatae					
CCPh041	*Hyptis suaveolens* (L.) Poit.	Aerial part	11.194	0.078 ± 0.001	3.961 ± 0.006	0.111 ± 0.000
CCPh042	*Leonotis nepetifolia* (L.) R. Br.	Leaves	22.287	0.171 ± 0.001	-	0.418 ± 0.010
CCPh043	*Leonurus sibiricus* L.	Aerial part	14.098	0.045 ± 0.003	-	0.012 ± 0.000
CCPh044	*Melissa officinalis* L.	Leaves	20.371	0.048 ± 0.004	19.908 ± 0.171	0.174 ± 0.006
CCPh045	*Mentha arvensis* L. var. *piperascens* Malinv.	Leaves	19.968	1.038 ± 0.002	6.809 ± 0.086	0.108 ± 0.001
CCPh046	*Mentha cordifolia* Opiz ex Fresen	Leaves	21.461	0.117 ± 0.000	7.537 ± 0.010	0.100 ± 0.001
CCPh047	*Ocimum africanum* Lour.	Leaves	15.466	0.033 ± 0.000	1.691 ± 0.002	0.092 ± 0.000
CCPh048	*Ocimum basilicum* L.	Leaves	15.756	0.243 ± 0.001	0.597 ± 0.003	0.311 ± 0.001
CCPh049	*Ocimum gratissimum* L. var. *macrophyllum* Briq.	Leaves	19.372	0.189 ± 0.002	1.756 ± 0.001	0.240 ± 0.003
CCPh050	*Ocimum tenuiflorum* L.	Leaves	17.317	0.215 ± 0.001	2.292 ± 0.003	0.112 ± 0.002
CCPh051	*Origanum majorana* L.	Leaves	24.119	-	7.954 ± 0.028	0.065 ± 0.003
CCPh052	*Origanum vulgare* L.	Leaves	31.593	0.123 ± 0.005	9.902 ± 0.091	0.410 ± 0.009
CCPh053	*Orthosiphon aristatus* (Blume) Miq.	Leaves	11.332	0.072 ± 0.002	2.101 ± 0.003	0.115 ± 0.001
CCPh054	*Perilla frutescens* (L.) Britton	Leaves	21.621	0.042 ± 0.001	13.185 ± 0.021	0.189 ± 0.001
	Family: Labiatae					
CCPh055	*Plectranthus amboinicus* (Lour.) Spreng.	Leaves	12.369	0.049 ± 0.001	0.279 ± 0.003	0.121 ± 0.001
CCPh056	*Plectranthus rotundifolius* (Poir.) Spreng.	Leaves	22.849	-	0.334 ± 0.003	0.086 ± 0.001
CCPh057	*Plectranthus rotundifolius* (Poir.) Spreng.	Tubers	6.628	-	0.669 ± 0.005	0.270 ± 0.004
CCPh058	*Plectranthus scutellarioides* (L.) R. Br.	Leaves	26.274	-	2.594 ±0.016	0.217 ± 0.001
CCPh059	*Rosmarinus officinalis* L.	Aerial part	17.989	0.124 ± 0.005	2.611 ± 0.023	0.146 ± 0.010
CCPh060	*Salvia hispanica* L.	Seeds	5.427	0.029 ± 0.000	0.576 ± 0.025	0.007 ± 0.000
CCPh061	*Salvia officinalis* L.	Aerial part	20.719	-	6.829 ± 0.070	0.166 ± 0.009
CCPh062	*Thymus citriodorus* (Pers.) Schreb.	Aerial part	19.963	0.165 ± 0.001	10.176 ± 0.417	0.128 ± 0.010
CCPh063	*Thymus vulgaris* L.	Aerial part	21.499	0.093 ± 0.005	4.349 ± 0.025	0.155 ± 0.002
	Family: Lauraceae					
CCPh064	*Persea americana* Mill.	Flesh	16.142	0.029 ± 0.000	-	-
CCPh065	*Persea americana* Mill.	Peels	15.259	0.539 ± 0.001	-	0.008 ± 0.000

TABLE I - The contents of chlorogenic, rosmarinic and caffeic acids in plant samples (cont.)

Voucher specimen Number	Scientific plant name	Plant parts used	% yield (g/100 g)	Content (g/100 g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
CCPh066	*Persea americana* Mill.	Seeds	22.143	1.381 ± 0.005	-	-
	Family: Malvaceae					
CCPh067	*Hibiscus sabdariffa* L.	Leaves	31.829	1.117 ± 0.002	-	0.351 ± 0.002
	Family: Meliaceae					
CCPh068	*Azadirachta indica* A. Juss.	Leaves	16.665	-	-	-
	Family: Moraceae					
CCPh069	*Morus alba* L.	Leaves	20.045	3.028 ± 0.005	0.050 ± 0.001	0.025 ± 0.001
	Family: Moringaceae					
CCPh070	*Moringa oleifera* Lam.	Leaves	16.509	0.525 ± 0.002	-	0.030 ± 0.000
CCPh071	*Moringa oleifera* Lam.	Seeds	8.414	-	-	0.004 ± 0.000
	Family: Myrtaceae					
CCPh072	*Psidium guajava* L.	Fruits	50.035	-	-	0.022 ± 0.000
CCPh073	*Syzygium antisepticum* (Blume) Merr. & L. M. Perry	Leaves	23.821	-	-	-
	Family: Oxalidaceae					
CCPh074	*Averrhoa carambola* L.	Fruits	63.412	0.045 ± 0.001	-	0.009 ± 0.000
	Family: Piperaceae					
CCPh075	*Piper betle* L.	Leaves	21.431	0.136 ± 0.000	-	0.041 ± 0.000
CCPh076	*Piper nigrum* L. (Black pepper)	Fruits	7.985	-	-	-
CCPh077	*Piper nigrum* L. (White pepper)	Seeds	5.619	-	-	-
	Family: Poaceae					
CCPh078	*Cymbopogon citratus* (DC.) Stapf	Rhizomes	26.886	0.132 ± 0.002	-	0.073 ± 0.003
	Family: Polygonaceae					
CCPh079	*Persicaria odorata* (Lour.) Soják	Leaves	17.391	0.289 ± 0.017	-	0.091 ± 0.000
	Family: Punicaceae					
CCPh080	*Punica granatum* L. var. *granatum*	Leaves	43.525	0.194 ± 0.009	-	0.305 ± 0.006
CCPh081	*Punica granatum* L. var. *granatum*	Peels	40.172	-	0.049 ± 0.000	0.363 ± 0.003
	Family: Rosaceae					
CCPh082	*Fragaria vesca* L.	Fruits	70.506	0.046 ± 0.001	-	-
CCPh083	*Malus domestica* Borkh.	Fruits	85.332	0.277 ± 0.001	-	-
CCPh084	*Pyrus communis* L.	Fruits	74.522	0.407 ± 0.001	-	-
	Family: Rubiaceae					
CCPh085	*Coffea arabica* L.	Seeds	8.228	5.967 ± 0.007	0.845 ± 0.002	0.380 ± 0.001
CCPh086	*Coffea canephora* Pierre ex A. Froehner	Seeds	12.262	7.843 ± 0.037	1.663 ± 0.007	1.233 ± 0.003
CCPh087	*Morinda citrifolia* L.	Fruits	23.686	0.046 ± 0.001	-	0.010 ± 0.000
CCPh088	*Morinda citrifolia* L.	Leaves	28.441	0.081 ± 0.000	-	-
	Family: Scrophulariaceae					
CCPh089	*Limnophila aromatica* (Lam.) Merr.	Aerial part	16.237	0.649 ± 0.001	-	0.058 ± 0.001
	Family: Solanaceae					
CCPh090	*Capsicum annuum* L. (Green bell pepper)	Fruits	51.863	0.148 ± 0.000	-	0.025 ± 0.000
CCPh091	*Capsicum annuum* L. (Orange bell pepper)	Fruits	55.366	0.169 ± 0.001	-	0.026 ± 0.001
CCPh092	*Capsicum annuum* L. (Red bell pepper)	Fruits	58.196	0.164 ± 0.002	-	0.085 ± 0.002
CCPh093	*Capsicum annuum* L. (Yellow bell pepper)	Fruits	58.237	0.175 ± 0.003	-	0.026 ± 0.001

TABLE I - The contents of chlorogenic, rosmarinic and caffeic acids in plant samples (cont.)

Voucher specimen Number	Scientific plant name	Plant parts used	% yield (g/100 g)	Content (g/100 g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
CCPh094	*Nicotiana tabacum* L.	Leaves	30.838	3.317 ± 0.001	-	0.057 ± 0.000
CCPh095	*Physalis angulata* L.	Aerial part	12.733	0.196 ± 0.002	-	0.012 ± 0.000
CCPh096	*Physalis peruviana* L.	Fruits	61.376	0.097 ± 0.001	-	<LOQ
CCPh097	*Physalis peruviana* L.	Calyx	13.857	0.474 ± 0.001	-	0.086 ± 0.001
CCPh098	*Solanum lycopersicum* L. var. *cerasiforme*	Fruits	45.374	0.225 ± 0.010	-	0.037 ± 0.000
CCPh099	*Solanum lycopersicum* L. var. *lycopersicum*	Fruits	60.356	0.280 ± 0.009	-	0.035 ± 0.000
	Family: Strychnaceae					
CCPh100	*Strychnos nux-vomica* L.	Seeds	3.228	0.389 ± 0.000	-	0.004 ± 0.000
	Family: Theaceae					
CCPh 101	*Camellia sinensis* (L.) Kuntze var. *assamica* (Mast.) Kitam.	Leaves	46.363	0.719 ± 0.003	-	0.051 ± 0.001
	Family: Thunbergiaceae					
CCPh 102	*Thunbergia laurifolia* Lindl.	Leaves	14.292	0.082 ± 0.004	11.487 ± 0.019	0.218 ± 0.001
	Family: Verbenaceae					
CCPh 103	*Clerodendrum calamitosum* L.	Leaves	20.401	0.023 ± 0.001	-	0.010 ± 0.000
CCPh 104	*Clerodendrum indicum* (L.) Kuntze	Leaves	24.400	-	-	0.011 ± 0.001
CCPh 105	*Clerodendrum quadriloculare* (Blanco) Merr.	Leaves	21.407	0.066 ± 0.001	-	-
CCPh 106	*Clerodendrum serratum* (L.) Moon	Leaves	33.686	1.804 ± 0.006	-	-
CCPh 107	*Clerodendrum thomsoniae* Balf. f.	Leaves	19.390	0.018 ± 0.002	0.903 ± 0.004	0.077 ± 0.002
CCPh 108	*Vitex agnus-castus* L.	Leaves	32.946	5.557 ± 0.068	3.083 ± 0.037	0.084 ± 0.001
CCPh 109	*Vitex negundo* L.	Leaves	28.441	1.238 ± 0.002	1.135 ± 0.005	0.054 ± 0.004
CCPh 110	*Vitex trifolia* L. subsp. *litoralis* Steenis	Leaves	28.545	0.379 ± 0.003	1.497 ± 0.008	0.167 ± 0.003
CCPh 111	*Vitex trifolia* L. subsp. *trifolia*	Leaves	31.403	2.180 ± 0.056	2.745 ± 0.098	0.177 ± 0.006

* - = cannot be detected

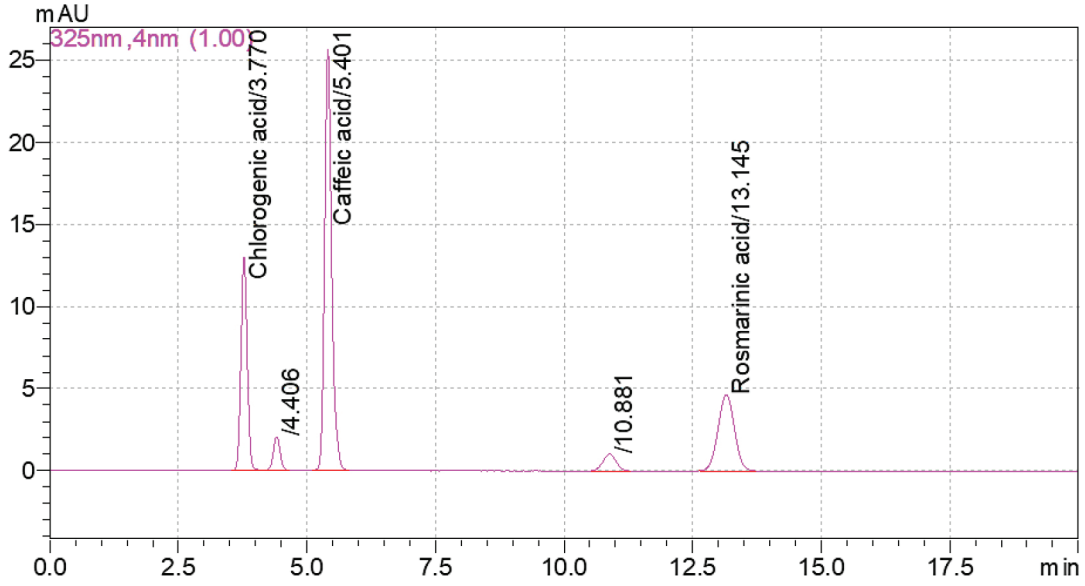

FIGURE 2 - HPLC chromatogram of standard chlorogenic acid, rosmarinic acid and caffeic acid at 325 nm by RP-HPLC-DAD.

TABLE II - System suitability of standard solution (n = 5) compared to criteria of the U.S. Food and Drug Administration (FDA, 1994)

Parameter	Result	Acceptance criteria
Retention factor (k)	4.30 ± 0.01	k > 2
Theoretical plate number (N)	2745.17 ± 158.17	N > 2000
Tailing factor (T_f)	1.027 ± 0.07	$T_f \leq 2$

Three concentrations of standard compounds were spiked into the sample. The accuracy of chlorogenic acid, rosmarinic acid, and caffeic acid quantitative analysis in *L. japonica* flowering bud ethanolic extract ranged from 103.98-108.63, 97.23-99.09, and 99.41-100.85% recoveries, respectively (Table III). The repeatability and intermediate precision were performed on samples with three different concentrations of standard compounds at the same day and five different days of experiments, respectively. The values were shown as %RSD which meant the error of the method. The repeatability and intermediate precision were shown in Table III. The acceptable range of recovery is 80 - 120% of the test concentration (ICH, 2005) and the criteria of repeatability and intermediate precision was not more than 15% RSD (U.S. Food and Drug Administration, 2001). Thus, the results indicated that this RP-HPLC analysis was accurate and precise for the quantification of the three compounds in plant samples.

LOD and LOQ analysis were calculated by the residual standard deviation of a regression line and the slope of the calibration curve. The LOD of chlorogenic acid, rosmarinic acid, and caffeic acid that is taken as the lowest concentration of analyte in a sample that could be detected was 1.64, 2.22, and 0.65 µg/mL, respectively. The LOQ of chlorogenic acid, rosmarinic acid, and caffeic acid that is taken as the lowest concentration of analyte in a sample that could be accurately quantitated was 4.97, 6.72, and 1.97 µg/mL, respectively.

The robustness of sample and standard compounds was determined during the analysis of the RP-HPLC method when the flow rate of the mobile phase varied from 1.195-1.205 mL/min, the column temperature varied from 29-31 °C, and the wavelength varied from 322-328 nm. The results demonstrated no differences (%RSD <4) in the area of the curve and retention time as shown in Tables IV and V. However, the method validation in this study used *L. japonica* flowering bud ethanolic extract as a sample matrix which might not represent all of the plant samples. It was recommended that further quantification of chlorogenic acid, rosmarinic acid, and caffeic acid in each plant material extract as stated in this study should be verified for each sample matrix.

The RP-HPLC analysis in this study demonstrated the contents of 3 phenolic compounds in selected plants that could be useful as a chemical marker for quality control of plant material. The interesting plants with special reference to these markers could be further investigated for their biological activities involving hydroxycinnamic acid derivatives.

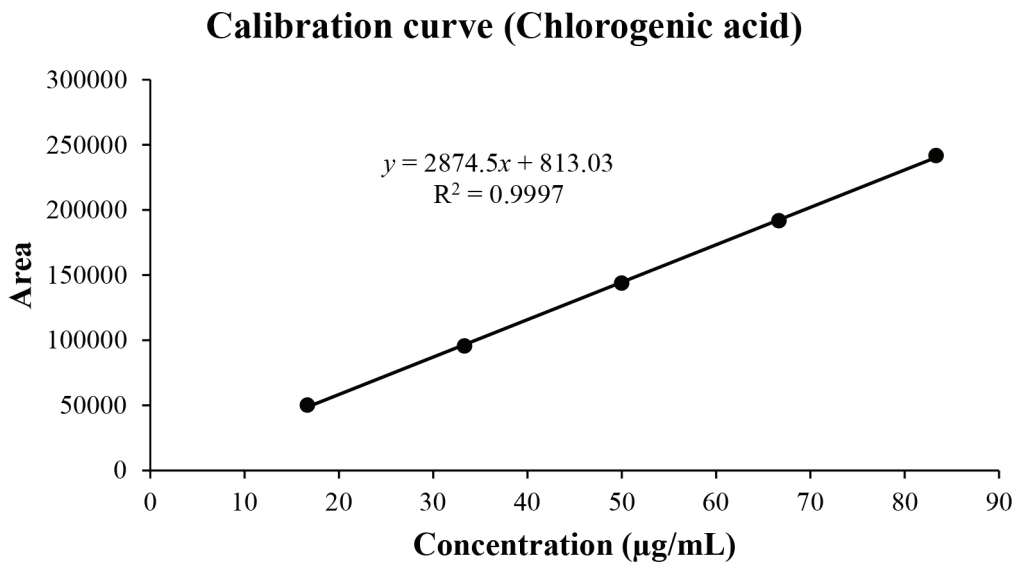

FIGURE 3 - The calibration curve of chlorogenic acid.

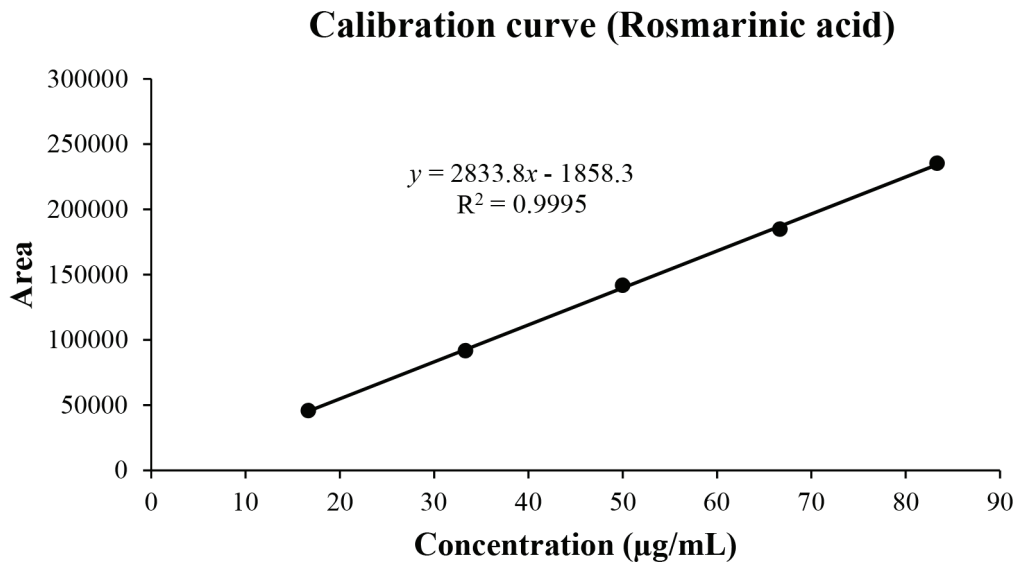

FIGURE 4 - The calibration curve of rosmarinic acid

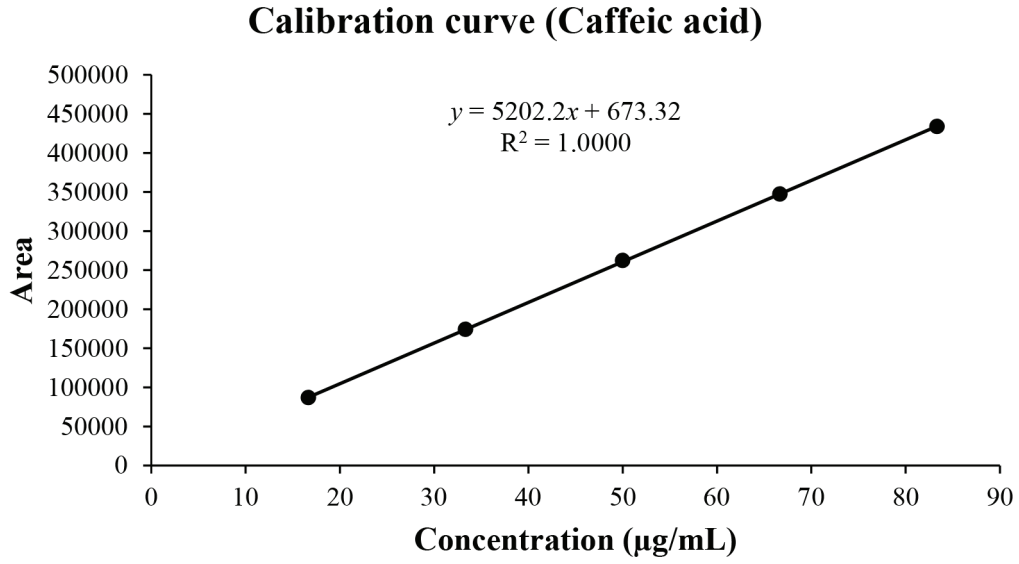

FIGURE 5 - The calibration curve of caffeic acid.

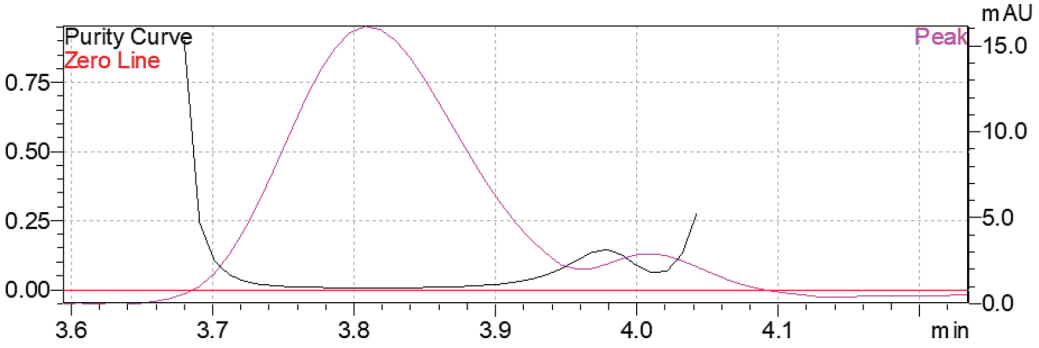

FIGURE 6 - The peak purity of chlorogenic acid in *L. japonica* flowering bud ethanolic extract (Peak purity index: 1.000000).

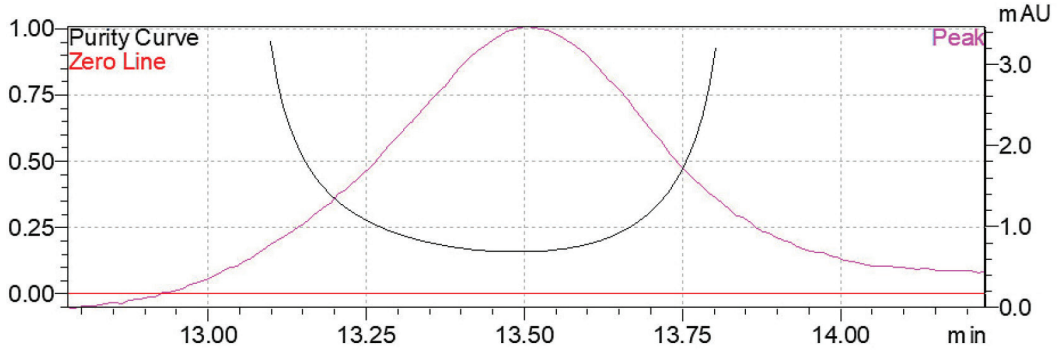

FIGURE 7 - The peak purity of rosmarinic acid in *L. japonica* flowering bud ethanolic extract (Peak purity index: 0.999952).

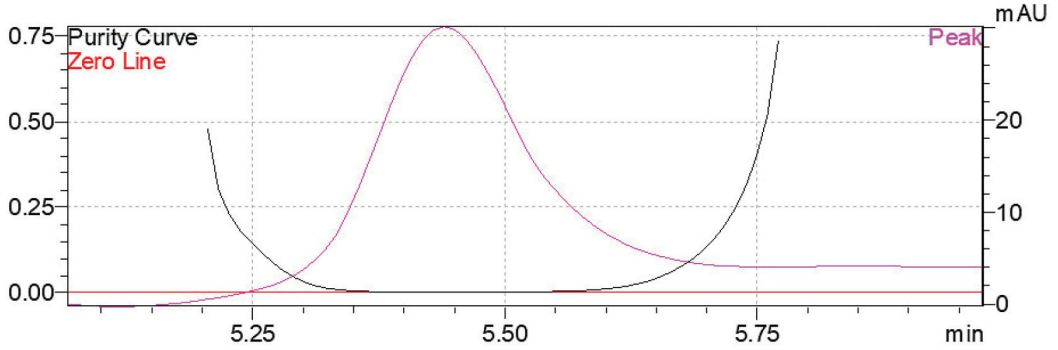

FIGURE 8 - The peak purity of caffeic acid in *L. japonica* flowering bud ethanolic extract (Peak purity index: 0.999999).

TABLE III - Accuracy and precision of chlorogenic acid, rosmarinic acid and caffeic acid in *L. japonica* flowering bud

Compounds	Spike concentration (µg/mL)	% recovery (n = 3)	%RSD	
			Repeatability precision (n = 3)	Intermediate precision (n = 5)
Chlorogenic acid	10	108.632	0.130	0.989
	25	103.976	0.077	0.699
	50	107.396	0.054	1.770
Rosmarinic acid	10	97.230	0.259	1.522
	25	99.089	0.234	1.039
	50	98.328	0.135	1.415
Caffeic acid	10	100.447	0.169	6.468
	25	99.407	0.046	5.795
	50	100.851	0.074	3.119

TABLE IV - Robustness of chlorogenic acid, rosmarinic acid and caffeic acid quantitation in *L. japonica* flowering bud

Compounds	% RSD of sample					
	Flow rate		Temperature		Wavelength	
	Rt	Area	Rt	Area	Rt	Area
Chlorogenic acid	0.31	0.50	0.79	0.78	0.06	1.14
Rosmarinic acid	0.19	0.66	2.63	0.89	0.02	1.33
Caffeic acid	0.27	0.95	1.11	3.14	0.07	2.08

* Rt = Retention time

TABLE V - Robustness of standard chlorogenic acid, rosmarinic acid and caffeic acid quantitation

Compounds	% RSD of standard compounds					
	Flow rate		Temperature		Wavelength	
	Rt	Area	Rt	Area	Rt	Area
Chlorogenic acid	0.24	0.33	0.78	0.14	0.14	1.13
Rosmarinic acid	0.11	1.38	2.57	1.10	0.03	2.41
Caffeic acid	0.23	0.29	1.20	0.25	0.09	0.87

* Rt = Retention time

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