Identification and LC–MS–MS Determination of Acteoside, the Main Antioxidant Compound of *Euphrasia Rostkoviana*, Using the Isolated Target Analyte as External Standard

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Abstract

The main compound of the traditionally known herbal eye-remedy, *Euphrasia rostkoviana* Hayne (eyebright) is isolated in high purity, identified and quantified in multiple reaction monitoring (MRM) mode with external calibration method using the isolated compound as standard. For structure elucidation high-performance liquid chromatography (HPLC)-electrospray ionization source (ESI)-tandem mass spectrometry, HPLC-ESI-time-of-flight, and nuclear magnetic resonance spectroscopy are used. The main compound is identified as acteoside, a phenyletanoid glycoside, exhibiting strong antioxidant activity. Purity of the isolated compound is over 97%, hence it is amenable to be used as standard for quantitation. Acteoside content is 2.56 ± 0.19 g / 100 g dry plant sample. According to the method performance test results the concentration-response plot is linear (r^2 = 0.997), precision and accuracy are all within acceptable range (highest RSD: 6.27%). SPE and method recovery are good, 98.6% (RSD 1.56%, n = 3) and 91.0% (RSD 2.1%, n = 3), respectively. Determination is carried out with a reliable, fast, characterized method, using a self produced standard.

Introduction

Eyebright (*Euphrasia rostkoviana* Hayne, Scrophulariaceae) has long been used in phytomedicine for the treatment of various eye problems such as cataracts, conjunctivitis, red-, inflamed-, irritated-, and sore-eyes (1,2). Although eyebright has been a part of the traditional folk medicine for centuries, the literature of its constituents and their way of acting is limited. A recent multinational prospective cohort trial gave support for the anti-conjunctivitis effect of eyebright eye-drops (3), which enhances the need for experimental data in respect of reinvestigation of phytochemical composition. The principal compounds in the aerial part are iridoids, phenolic acids, phenylpropane-, and flavonoid-glycosides. Previous qualitative analyses included differential spectrophotometry, planar chromatography, high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) spectroscopy and in one work mass spectrometry (4–11). Quantitative data on any active compound, in extent of the traditional use of *Euphrasia,* has not been published yet.

Because oxidative stress is a key feature of inflammation process and phenolic compounds are potent antioxidant agents, a polyamide column chromatographic fractionation of the total extract was performed to study the free-radical scavenging behaviour of the methanolic fractions (12). Methanolic fractions 8–9 were identically dominated by the main compound of the herb, which raised interest in question of identity and quantity.

The objective of the present study was to identify and quantify the isolated leading compound of *Euphrasia rostkoviana* Hayne. Quantitation was carried out after a solid phase extraction (SPE) cleaning procedure by an LC tandem mass spectrometry (MS–MS) external calibration method. The performance of the analytical method was thoroughly tested. Previously published works on the quantitation of acteoside used several analytical methods; Wu et al. applied amperometric detection, Tu et al. determined acteoside in SIM mode using the m/z 642 $[M + NH_4]^+$ adduct, and Fernandez et al. and Ma et al. used an HPLC-UV method for quantitation and Shuya et al. worked with electrophoresis (13–17). The MS–MS method used is a fast and specific method achieving quantitation without commercial standard, with the help of self provided standard by isolation.

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Experimental

Chemicals and plant material

Reagent-grade methanol and acetic acid were supplied by Reanal (Budapest, Hungary). HPLC super gradient grade methanol (G Chromasolve) was from Sigma-Aldrich (Budapest, Hungary). For all steps of sample preparation, except extraction and column chromatography, HPLC super gradient grade methanol was used. Water was prepared with a Millipore Direct Q5 water purification equipment (Millipore, Bedford, MA). MN polyamide SC-9 was purchased from ICN Biomedicals GmbH (Eschwege, Germany). All aqueous solvents for LC–MS were filtered through 0.45-µm membranes (Millipore, Bedford, MA) and degassed in an ultrasonic bath before use.

Euphrasia rostkoviana Hayne plant samples were collected in Mures county, Romania, in the stage of blooming (July 2006). Plant samples were authenticated in the Dept. of Pharmacognosy, Semmelweis University, Budapest, where the voucher specimen is deposited (E.r.008).

Extraction, isolation and SPE

Ten grams air-dried, freshly powdered (meshsize 1 mm) plant material was extracted with methanol in a Soxhlet apparatus according to the instructions of the Ph. Eur. 5.8. After filtrating the extract methanol was evaporated under reduced pressure with a rotary evaporator at 50°C.

Isolation of the main phenolic compound was carried out by column chromatography. An aqueous suspension of MN polyamide SC-9 was prepared and used as stationary phase filled in a homemade glass column. Before introducing the sample, the suspension was allowed to settle (final dimensions of the bed: 390 mm × 30 mm). Then, 2.4 g methanolic extract was dissolved in 4 mL methanol, adsorbed on a reasonable amount of (4–5 g) polyamide, dried until the solvent evaporated and layered on the top of the settled stationary phase. The column was eluted successively with water (1000 mL) and aqueous-methanol (100 mL 50% methanol, 100 mL 70% methanol, and 800 mL 100% methanol). Fractions of 10 mL were collected and those with similar fingerprints were combined and used for further studies. This paper deals only with methanolic fractions 8–9, containing solely the main phenolic compound of *Euphrasia*.

In order to separate the analyte(s) of interest from chlorophyll and other probable matrix constituents, all samples were subjected to an SPE procedure, as follows. The Supelclean LC-18 SPE tubes (500 mg/3 mL, Supelco, Bellefonte, PA) were conditioned with 2 × 2.5 mL methanol and with 2 × 2.5 mL 2.5% acetic acid. The dry sample extracts were redissolved in a mixture of HPLC super gradient grade methanol and 2.5% acetic acid in water (ratio 1:5) and added to the tubes. SPE tubes were eluted in three steps, with 3 mL of 25%, 70%, and 100% methanol.

LC–MS conditions

HPLC separation

For the chromatographic separation an Agilent 1100 HPLC system [G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector (DAD)] was used (Agilent Technologies, Waldbronn, Germany). Samples were separated on a Supelco ODS Hypersil (Supelco, Bellefonte, PA) (150 mm × 4.6 mm, 5 µm, pore size: 120 Å) column equipped with a guard-column of the same stationary phase, maintained at 25°C. Eluent A was acetic acid (2.5%, v/v) and eluent B was methanol. The following gradient program was applied at a flow rate of 1 mL/min; 0 min: 75:25 (A:B, v/v), 25 min: 48:52 (A–B, v/v), 30 min: 10:90 (A–B, v/v), 33 min: 75:25 (A–B, v/v). UV-spectra were recorded between 200 and 380 nm. Chromatograms were acquired at 260 and 340 nm. Injection volume was 15 µL.

MS conditions

Triple quadrupole. Scan and CID mass spectral analyses were performed with an Agilent 6410B triple quadrupole equipped with an electrospray ionization source (ESI) (Agilent Technologies, Palo Alto, CA). ESI conditions were as follows: temperature: 350°C, nebulizer pressure: 45 psi N_2, drying gas flow: 9 L/min N_2, fragmentor voltage: 130 V, capillary voltage: 4000 V. Electron multiplier voltage was 1810 V. High purity nitrogen was used as collision gas. Full mass scan spectra were recorded in negative ion mode over an *m/z* range of 50–700 dalton (1.88 cycle/s). Before quantitation both fragmentor voltage (from 70 to 140 V, with steps of 10 V) and collision energy (from 5 to 50 V, with steps of 5 V) were optimized by parameter ramping. Optimal setting for collision energy was 35 V, and for fragmentor voltage, 130 V. Quantitation was achieved in multiple reaction monitoring (MRM) mode. A postcolumn splitter (60%–40%, waste–MS) was employed for the reduction of the flowrate. The Masshunter B.01.03 software was used for data acquisition and qualitative analysis.

Time-of-flight. Accurate mass and elemental analysis was achieved by an Agilent 6210 time-of-flight (TOF) MS (Agilent Technologies, Palo Alto, CA) operating with a dual-nebulizer ESI source in the negative ion mode. ESI conditions were the same as described previously. Two reference masses (*m/z* 112.985587 and *m/z* 1033.988109) were used to recalibrate the mass axis during analysis. Full scan mass spectra were acquired over a range of *m/z* 100–1500. The Masshunter B.01.03 software (Agilent) was used for data acquisition and qualitative analysis. For confirmation the measured mass of the deprotonated molecular ion was compared with the calculated theoretical value.

Method performance characterization

Calibration plot, precision, and quality control samples

The standard solutions for calibration were prepared by use of the isolated component. Purity and identity of the isolate was thoroughly checked by means of NMR spectroscopy HPLC–DAD–MS–MS and LC-TOF before using it as a standard compound. The isolate was dried with a rotary evaporator at 50°C in an analiticaly pre-weighed flask, which was weighed after that, thus amount of the isolate was known.

Standard solutions were prepared at 40, 100, 300, 500, 800, and 1500 ng/mL with 70% methanol. Range of calibration was choosed in accordance with that of previous studies (13,14). Each standard solution was prepared in triplicate and injected once. Calibration plot was constructed by plotting peak areas against corresponding concentrations. Slope, intercept, and correlation coefficient were determined by least squares linear regression analysis. Quality control samples were prepared in the

same manner at concentrations of 100, 300, and 800 ng/mL and used for the determination of the intra-day precision (low, mid, and high concentrations of the standard in three parallel runs on the same day) and inter-day precision (low, mid, and high concentrations of the standard in three parallel runs on three successive days). Retention time repeatability was checked with 6 successive runs of the isolated compound.

Accuracy, recovery test

SPE recovery. Accuracy was verified by the recovery test using fortified samples and additionally an SPE recovery test was also performed. Three parallel standard stock solutions (each 1750 µg/mL) were prepared with the mixture of 1 mL methanol and 5 mL 2.5% acetic acid in water and subjected to the SPE procedure described previously. Four hundred microliters of the stock solution was injected onto the SPE tube. The solution, collected during the SPE elution with 25% methanol, was diluted up to a volume of 10.00 mL with methanol. Five microliters of this solution was diluted up to 1 mL with 70% methanol and used for the LC–MS–MS determination. Recovery (*R*) was calculated as $R = 100\ C_{found} / C_{applied}$ (C_{found} = measured concentration in sample, $C_{applied}$ = concentration in the standard solution used).

Method recovery, fortified samples. One gram air-dried, freshly powdered plant material alone and in another set after mixed with 8 mg of the isolated compound was extracted in three parallels with methanol in a Soxhlet apparatus according to the instructions of the Ph. Eur. 5.8. After filtration methanol was evaporated under reduced pressure at 50°C. Method recovery was tested in a concentration range to match with that of acteoside in the plant sample. Forty-five milligrams of the fortified dry extract was redissolved in 1 mL of methanol and 2.5% acetic acid (1:5) solution, and 500 µL of this solution was subjected to the SPE procedure previously described. The solution, collected during the SPE elution with 25% methanol, was diluted up to a volume of 10.00 mL with methanol, and 5 µL of this solution was diluted up to 1 mL with 70% methanol and used for LC–MS–MS. Recovery (*R*) was calculated as $R = 100\ (C_{found} - C_{initial}) / C_{added}$. ($C_{found}$ = measured concentration in fortified sample, $C_{initial}$ = initial concentration in sample, C_{added} = concentration in the standard solution used).

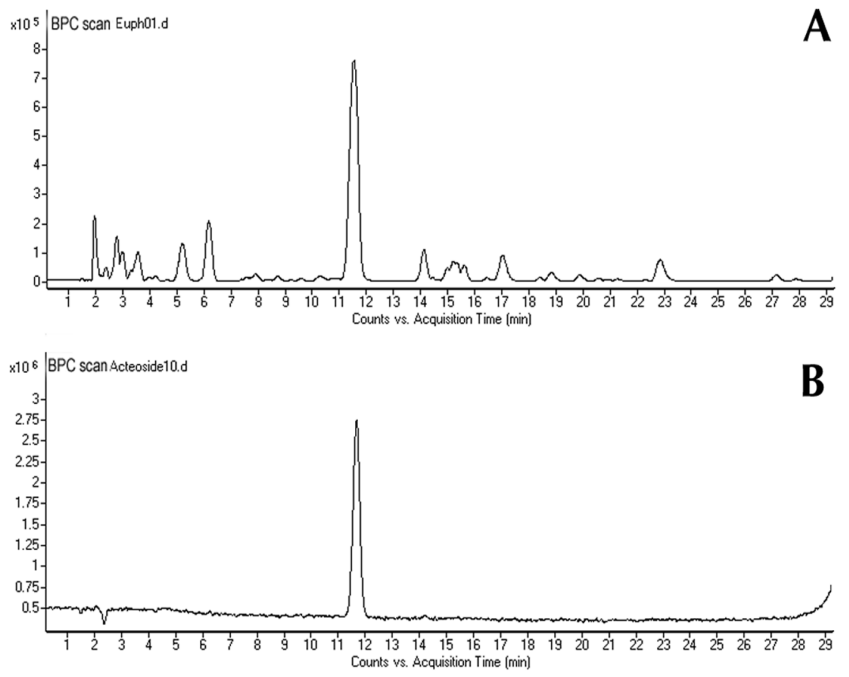

Figure 1. Base to peak chromatograms (BPC) of the methanolic extract of *Euphrasia rostkoviana* Hayne (A) and of the isolated acteoside (B).

Results

Polyamide fractionation and isolation

By aqueous and the following gradient change to methanolic elution provided separate fractions containing iridoid glycosides, and separate fractions of phenolics, respectively. The preparative isolation of the main compound was achieved successfully by polyamide column chromatography. Although the main constituent was present throughout methanolic fractions 6–10, after LC–DAD–MS–MS analysis, only fractions 8 and 9 (combined and named as fraction I) were found to contain it solely without any additional compound. The isolation process yielded 51 mg pure substance.

Identity, purity, and quantity

The succesful HPLC separation of phenolics partly depends on the proper choice of the acidic modifier of the mobile phase (18). The use of phosphoric acid seemed reasonable but it had to be declined because of MS incompatibility (19). Although trifluoroacetic acid provided well resolved, sharp peaks, it was also unadequate due to its intense ionization, predestinating a possible source of ion suppression. In view of the previously mentioned acetic acid was chosen as the acidic compound of the mobile phase. First a gradient scout was applied using acetonitril as organic modifier (20). It was soon concluded, that an isocratic run (based on the result of gradient scout) provided insufficient resolution, thus a gradient run was developed, and acetonitril was changed to methanol, to enhance ionization efficiency.

The LC method thus ensured good resolution regarding the analyte of interest (retention time = 11.7 min) (Figure 1A). Based on UV spectral data the compound was assumed to be a phenyl propane (cinnamic acid) derivative (λ_{max} = 332 nm). After acquiring scan mass spectra in negative ion mode, the molecular ion $[M - H]^-$ was detected at *m/z* 623 and neither adducts, nor characteristic isotope pattern were observed (Figure 2A). Referring to the "nitrogen rule," the odd number of the molecular ion means zero or an even number of nitrogen atom content, and the $^{13}C/^{12}C$ peak intensity ratio suggested an approximate

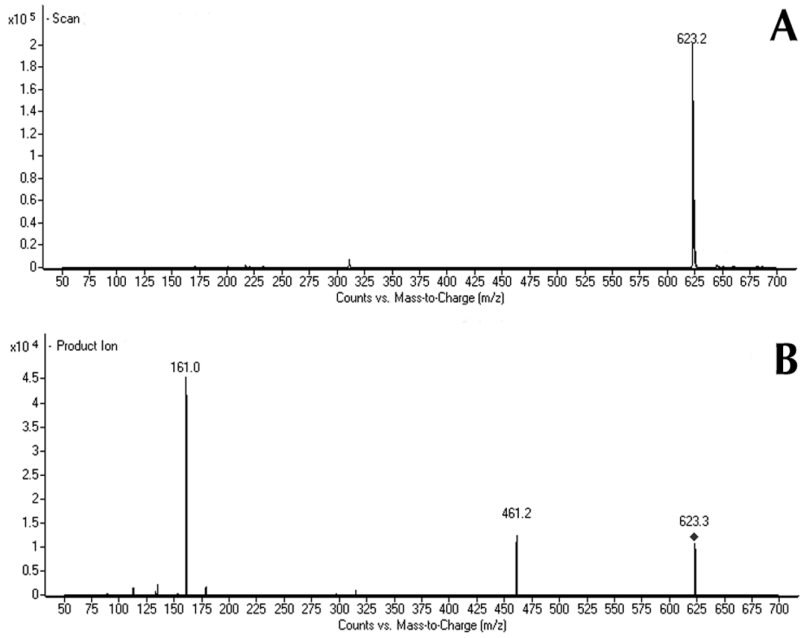

Figure 2. Scan (A) and product ion (B) mass spectra of acteoside.

Table I. ^{13}C and ^{1}H NMR Data for Acteoside in CD_3OD

Moiety	C position	δ_{13C}*	δ_{1H} (mult; J = Hz; H)
Hydroxytyrosol	1	131.47	
	2	117.11	6.687 (d; 2.0; 1H)
	3	146.13	
	4	144.68	
	5	116.30	6.666 (d; 8.1; 1H)
	6	121.25	6.557 (dd; 8.1, 2.0; 1H)
	7	36.57	2.79 (m; 2H)
	8	72.25	4.039 (m; 1H); 3.716 (m; 1H)
Caffeic acid	1′	127.67	
	2′	115.23	7.045 (d; 2.1; 1H)
	3′	146.83	
	4′	149.78	
	5′	116.51	6.770 (d; 8.2; 1H)
	6′	123.20	6.946 (dd; 8.2, 2.1; 1H)
	7′	148.00	7.584 (d; 15.9; 1H)
	8′	114.71	6.265 (d; 15.9; 1H)
	9′	168.28	
Glucose	1	104.22	4.367 (d; 7.9; 1H)
	2	76.21	3.382 (dd; 9.1, 7.9; 1H)
	3	81.63	3.806 (t; 9.1; 1H)
	4	70.60	4.910 (t; 9.5; 1H)
	5	76.05	3.527 (m; 1H)
	6	62.37	3.614 (m; 1H); 3.523 (m; 1H)
Rhamnose	1	103.02	5.180 (d; 1.3; 1H)
	2	72.35	3.908 (dd; 3.1, 1.8; 1H)
	3	72.06	3.567 (dd; 9.7, 3.5; 1H)
	4	73.80	3.283 (t; 9.5; 1H)
	5	70.41	3.558 (m; 1H)
	6	18.45	1.084 (d; 6.2; 1H)

* Chemical shifts (δ) are given in ppm relative to TMS (0.00 ppm).

carbon atom number of 30. The molar mass of 624 Da and the high carbon number indicated that the molecule is more than a simple phenyl propane derivative (e.g., sugar moieties might be attached). CID experiments resulted two high-abundant product ions at *m/z* 161 and 461, and four more product ions appearing with lower intensities at *m/z* 113, 135, 179 and 315 (Figure 2B). The loss of 162 amu $[M - H - 162]^-$ (*m/z* 623 → *m/z* 461) typically refers to a hexose sugar unit. The low-abundant fragment at *m/z* 179, resulting the ion *m/z* 161 after loss of a water molecule, may support the phenyl propane theory, if the molar weight (M_r) of caffeic acid (M_r = 180 g/mol) is considered. The loss of water may point to a phenolic hydroxyl or carboxyl group.

To gain more information about the elemental composition, ESI-TOF in conjunction with molecule formula calculation was employed. Mass accuracy of TOF analysers may change over a broad ppm range according to the ion intensity of the analyte compared to that of the lock-mass (21). Therefore sample was investigated in three different concentrations [at S/N (signal to noise ratio) = 3, at S/N = 5.7, and at S/N =12.3]. At levels of S/N = 3 and S/N =12.3 the formula calculator algorithm ranked the formula $C_{29}H_{36}O_{15}$ as first (100%) with –1.37 ppm and –0.16 ppm mass error, respectively. At S/N = 5.7 the formula $C_{24}H_{36}N_2O_{17}$ was ranked first (100%) with –2.25 ppm mass error, though $C_{29}H_{36}O_{15}$ only as fourth (72.1%), with +4.01 ppm mass error. The formula $C_{24}H_{36}N_2O_{17}$ is unlikely, if the $^{13}C/^{12}C$ intensity ratio of the molecule ion and the UV spectra is taken into consideration. All spectra were acquired at FWHM of the corresponding chromatographic peaks.

Based on the UV and MS results the molecule was tentatively identified as acteoside (Figure 3) which was confirmed by NMR analysis (Table I). After chromatographic tests purity of the acteoside isolate was proved to be 98.2 ± 0.82% (DAD) and 97.1 ± 0.79% (TIC) (Figure 1B). NMR spectroscopy served as a further tool to confirm purity. As there were only trace residual solvents observable in the NMR spectra, according to the chromatographic purity test, the purity of acteoside was over 97%. According to the comprehensive investigation regarding purity, the isolate was equal to that of commercially available phyto-

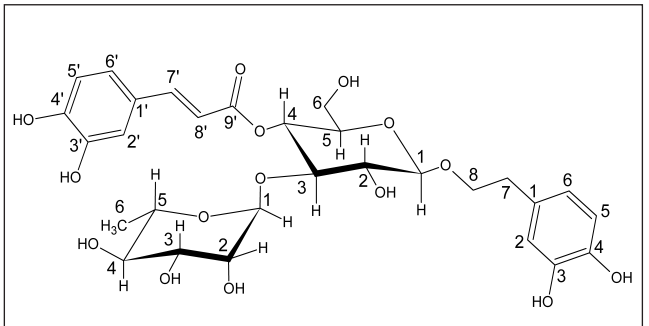

Figure 3. Structure and numbering of acteoside.

chemical reference standards, which enabled us to use it as a standard for quantitation. The result for acteoside content was 2.56 ± 0.19 g / 100 g dry plant material (RSD 7.57%, n = 3), which is prominently high, and may explain, in part, the beneficial effect of *Euphrasia* concerning inflamed eye-disorders.

SPE method

Though specificity offered by the MRM mode of the triple quadrupole is a useful tool for quantitation from complex matrices, it does not solve the problem of possibly occurring matrix effects in the ion source, like ion supression. To minimize this effect an SPE purification step was employed. The 3 mL 25% methanol eluted practically all amount of acteoside added to the SPE sorbent and no acteoside was lost during the sample addition. Chromatographic analysis showed the absence of acteoside content in the elute of the sample addition step and in the following elute step after the 25% methanol (70% methanol) as well; the S/N ratio of the peak of acteoside was below 3 in both samples. As a result, optimal elution of acteoside was achieved with high recovery and good precision, SPE recovery was 98.6% (RSD 1.56%, n = 3).

Method characterization

Selectivity and specificity

High selectivity was ensured by the MRM mode of the triple quadrupole instrument. The *m/z* 161 fragment ion (transition *m/z* 623 → *m/z* 161) was chosen as the quantifier ion, and to avoid the quantitation of any similar ion providing the same fragment, *m/z* 461 was chosen as the qualifier ion. Pure solvent (70% methanol) was injected onto the HPLC column and analyzed in order to exclude co-elution which may be a possible source of ion suppression. No interfering peak appeared at the retention time of acteoside.

Linearity, precision, and accuracy

Five calibration standard samples (40–1500 ng/mL) were analyzed in triplicate in order to determine the linearity. The assay possessed acceptable linearity (r^2 = 0.997) within the investigated range with good precision and accuracy. Linear regression equation was y = 16.232x – 69.975 (y = ax + b, where y is the peak area, a is the slope, x is the analyte concentration in ng/mL and b is the intercept). Retention time repeatability was satisfactory (RSD: 0.7%, n = 6). Intra- and inter-day precision, studied by the quality control samples, were also found to be acceptable (highest RSD 6.27%) (Table II). Accuracy was acceptable, referring to the results of the fortified method recovery test, recovery was 91.0% with good standard deviation (RSD 2.1%, n = 3).

Table II. Precision (RSD) and Accuracy of the Quantitative Method Studied with the Isolated Acteoside

Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Precision (RSD %)	Accuracy (%)
Intra-day			
100	102.1	1.51	102.1
300	297.9	0.84	99.3
800	811.2	0.87	101.4
Inter-day			
100	98.7	6.27	98.7
300	292.6	6.18	97.5
800	852.1	3.66	106.5

LOD and LOQ

According to the lowest point of the calibration curve the LOQ was 40 ng/mL for acteoside. LLOQ (S/N = 10) was 15 ng/mL, and the LOD (S/N = 3) was found to be 5 ng/mL.

Discussion and Conclusion

According to the literature search, acteoside had not been identified in *Euphrasia rostkoviana*, neither had it been quantified in the plant before. Isolation purity of acteoside was as high (≥ 97.1%) as its calibration standard application was feasible. The same result as Lei et al. and Li et al. regarding isolation purity of acteoside was achieved (22,23). The LC separation ensured acceptable resolution for almost all compounds, and good resolution for the main compound. LC–MS–MS was chosen for selectivity, sensitivity and peak purity considerations. However, acteoside is a chief constituent in *Euphrasia rostkoviana*, not needing strictly MS–MS sensitivity, the method was developed with the prospect of wider application including medicinal plant screening, biofluid analysis, quality control of raw plant materials, especially complex herbal products, where contents and interfering components may be unknown and variable. Besides, acteoside can occur as minor component in other species, and its determination might be of importance, because the molecule is of current interest, as a very recent publication of Chen et al. supports as well (24).

The quantitative method provided good linearity, precision, accuracy, and low quantitation limit (Table II), which are in accordance with, or better, than previous works (13,14). The developed method meets the predetermined criteria of repeatable, low ppb level quantitation which might be necessary in the wider application. The SPE purification step may further enhance the applicability of the method. Quantitation in MRM mode, ensuring 100% selectivity, is more reliable, than the SIM ($[M+NH_4]^+$) determination of Tu et al., because adduct formation is much more sensible to changes in conditions (e.g., buffer concentration, altering water quality, and chemical memory effect) (14). It was showed here as well, that TOF mass accuracy changes according to the ion intensity of the analyte compared to that of the lock-mass (21). The NMR results pointed out some mismatch in the literature (H and C chemical shifts were inverted), as compared with previously reported data (Table I) (25,26).

Beside its antioxidant property (27,28,12) several medicinal effects of acteoside were shown, and even its production enhancement was studied in cell cultures (25,26,29–33). The previously stated facts underline the significance of the notably high acteoside content in eyebright, which may give a reasonable explanation, in part, for its anti-inflammative action concerning eye-disorders.

References

1. J.A. Duke. Eyebright. *Handbook of Medicinal Herbs*. CRC Press, Boca Raton FL, 1985, pp. 72–75.
2. G.N. Bisset. *Herbal Drugs and Phytopharmaceuticals*. CRC Press, Medpharm Scientific Publishers, Stuttgart, 1994, pp. 195–197.
3. M. Stoss, C. Michels, E. Peter, R. Beutke, and R.W. Gorter. Prospective cohort trial of *Euphrasia* single-dose eye drops in conjunctivitis *J. Altern. Comp. Med.* **6:** 499–508 (2000).
4. W. Blaschek, R. Hänsel, K. Keller, J. Reichling, H. Rimpler, and G. Schneider. *Hagers Handbuch der Pharmazeutischen Praxis*. Folgewerk, Springer, 1998, pp. 666–672.
5. I. Matlawska, M. Sikorska, and Z. Kowalewski. Flavonoid compounds in *Euphrasia* herb. *Herba Pol.* **31:** 119–123 (1985).
6. I. Matlawska, M. Sikorska, and Z. Kowalewski. Flavonoid compounds in Herba Euphrasiae. *Herba Pol.* **34:** 97–102 (1988).
7. K.J. Harkiss and P. Timmins. *Planta Med.* **23:** 342–347 (1973).
8. O. Sticher and O. Salama. Iridoidglucosides from *Euphrasia rostkoviana*. *Planta Med.* **42:** (1981) 122–123.
9. O. Sticher and O. Salama. A new lignan glucoside from *Euphrasia rostkoviana*. *Planta Med.* **42:** (1981) 123–124.
10. O. Salama and O. Sticher. Iridoidglucosides from Euphrasia rostkoviana. *Planta Med.* **47:** 90–94 (1983).
11. B. Blazics, K. Ludanyi, Sz. Szarka, and A. Kery. Investigation of Euphrasia rostkoviana Hayne Using GC–MS and LC–MS. *Chromatographia* **68:** S119–S124 (2008).
12. B. Blazics, Á. Alberti, and Á. Kéry. Antioxidant activity of different phenolic fractions separated from *Euphrasia rostkoviana* Hayne. *Act. Pharm. Hung.* **79:** 11–16 (2009)
13. Y.-T. Wu, T.-R. Tsai, L.-C. Lin, and T.-H. Tsai. Liquid chromatographic method with amperometric detection to determine acteoside in rat blood and brain microdialysates and its application to pharmacokinetic study. *J. Chromatogr., B* **853:** 281–286 (2007).
14. Y. Tu, L. Zhao, L. Zhu, G. Wang, and B. Xiang. Simultaneous Determination of Acteoside, Astragaloside IV and Icariside-I in the Traditional Chinese Medicinal Preparation Shenbao by HPLC–MS. *Chromatographia* **64:** 453–458 (2006).
15. L. Fernandez, J. Santos, A.M. Diaz-Lanza, E. Perez, and L. Villaescusa. Quantitative determination of verbascoside in *Scrophularia scorodonia* by high-performance liquid chromatography. *Pharm. Biol.* **43:** 226–229 (2005).
16. Z. Ma, Z. Yang, P. Li, and C. Li. Simultaneous determination of eight phenylethanoid glycosides in different species of the *Genus cistanche* by high performance liquid chromatography. *J. Liq. Chromatogr. Relat. Tech.* **31:** 2838–2850 (2008).
17. C. Shuya, C. Xingguo, and H. Zhide. Identification and determination of ecdysone and phenylpropanoid glucoside and flavonoids in *Lamium maculatum* by capillary zone electrophoresis. *Biomed. Chrom.* **17:** 477–482 (2003).
18. D.S. Constantine. Extraction, separation, and detection methods for phenolic acids and flavonoids. *J. Sep. Sci.* **30:** 3268–3295 (2007).
19. V. Vukics, T. B. Hevesi, Á. Fukász, and Á. Kéry. Impact of flavonoid composition of medicinal plants: difficulties in selecting an LC method. *Chromatographia* **SS63:** 125–129 (2006).
20. J.W. Dolan. The perfect method, part 7: the gradient shortcut. *LC/GC. Eurpoe* **21:** 138–140 (2008).
21. V.V. Mihaleva, O. Vorst. C. Maliepaard, H.A. Verhoeven, R.C.H. de Vos, R.D. Hall, and R.C.H.J. van Ham. *Metabolomics* **4:** 171–182 (2008).
22. L. Li, R. Tsao, Z. Liu, S. Liu, R. Yang, J.C. Young, H. Zhu, Z. Deng, M. Xie, and Z. Fu. Isolation and purification of acteoside and isoacteoside from Plantago psyllium L. by high-speed counter-current chromatography. *J. Chromatogr. A* **1063:** 161–169 (2005).
23. L. Lei, F. Yang, T. Zhang, P. Tu, L. Wu, and Y. Ito. Preparative isolation and purification of acteoside and 2′-acetyl acteoside from Cistanches salsa (C.A. Mey.) G. Beck by high-speed counter-current chromatography. *J. Chromatgr., A* **912:** 181–185 (2001).
24. C.-H. Chen, T.-Y. Song,Y.-C. Liang, and M.-L. Hu. Acteoside and 6-O-Acetylacteoside downregulate cell adhesion molecules induced by IL-1β through inhibition of ERK and JNK in human vascular endothelial cells. *J. Agric. Food Chem.* **57:** 8852–8859 (2009). **DOI:** 10.1021/jf9028333
25. K.-W. Lee, H.J. Kim, Y. S. Lee, H.-J. Park, J.-W. Choi, J. Ha, and K.-T. Lee. Acteoside inhibits human promyelocytic HL-60 leukemia cell proliferation via inducing cell cycle arrest at G0/G1 phase and differentiation into monocyte. *Carcinogenesis* **28:** 1928–1936 (2007).
26. X. Pu, Z. Song, Y. Li, P. Tu, and H. Li. Acteoside from *Cistanche salsa* inhibits apoptosis by 1-methyl-4-phenylpyridinium ion in cellular granule neurons. *Planta Med.* **69:** 65–66 (2003).
27. M. Ono, E. Oda, T. Tanaka, Y. Iida, T. Yamasaki, C. Masuoka, T. Ikeda, and T.J. Nohara. DPPH radical-scavenging effect on some constituents from the aerial parts of Lippia triphylla. *J. Nat. Med.* **62:** 101–106 (2008).
28. W.F. Chiou, L.C. Lin, and C.F. Chen. Acteoside protects endothelial cells against free radical-induced oxidative stress. *J. Pharm. Pharmacol.* **56:** 743–748 (2004).
29. W. Pan, S. Jiang, P. Luo, J. Wu, and P. Gao. Isolation, purification and structure identification of antioxidant compound from the roots of *Incarvillea younghusbandii* Sprague and its life span prolonging effect in *Drosophila melanogaster*. *Nat. Prod. Res.* **228:** 719–725 (2008).
30. V. Kostyuk, A. Potapovich, T. Suhan, C. De Luca, G. Pressi, R. Dal Toso, and L. Korkina. Plant polyphenols against UV-C-induced cellular death. *Planta Med.* **74:** 509–514 (2008).
31. Z. Papoutsi , E. Kassi , S. Mitakou, N. Aligiannis, A. Tsiapara, G.P. Chrousos , and P. Moutsatsou. Acteoside and martynoside exhibit estrogenic/antiestrogenic properties. *J. Steroid. Biochem.* **98:** 63–71 (2006).
32. Q. Xiong, K. Hase, Y. Tezuka, T. Tani, T. Namba, and S. Kadota. Hepatoprotective activity of phenylethanoids from Cistanche deserticola. *Planta Med.* **64:** 120–125 (1998).
33. W.-H. Chen, C.-M. Xu, J.-L. Zeng, B. Zhao, X.-D. Wang, and Y.-C. Wang. Improvement of echinacoside and acteoside production by two-stage elicitation in cell suspension culture of Cistanche deserticola. *World J. Microbiol. Biotechnol.* **23:** 1451–1458 (2007).

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