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Short communication

Determination of oregonin in *Alnus* plants and biological samples by capillary electrophoresis

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Abstract

Oregonin, existing primarily in the *Alnus* plants, displayed anti-inflammatory and antioxidative activities. The capillary zone electrophoresis (CZE) method was developed in this study to quantitatively determine oregonin content in the *Alnus* plants for the first time. Various parameters, including buffer concentration, pH and applied voltage, were evaluated for their optimum analytical conditions. The optimized buffer was composed of 30 mM sodium tetraborate at pH 8.0. The separation voltage was set at 30 kV and the UV detection wavelength was set at 220 nm. Oregonin could be determined within 6 min under such optimized conditions. Relative standard deviation (R.S.D.) of the run-to-run repeatability and intermediate precision of the retention time of oregonin was within 1.36%. Run-to-run repeatability and intermediate precision of the peak area ratios of oregonin to internal standard, theophylline, were both within 1.55% R.S.D. The presented method was applied to analyze oregonin in leaves of *Alnus formosana*, seeds of various *Alnus* plants as well as biological samples. The stability of oregonin in biological system was indicated in this study. It demonstrates the potential of this developed method in natural product research.

Keywords: Oregonin; *Alnus* plants; Capillary zone electrophoresis; Biological samples

1. Introduction

Anti-inflammatory drugs are one of the highest consumption pharmaceutical products worldwide. Inflammatory conditions are characterized by the activation of the transcription factor nuclear factor kappa B (NF-κB), resulting in the expression of NF-κB-regulated, inflammation-related genes, such as inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) [1]. A lot of effort has been made on the development of anti-inflammatory agents which interfere with the transcriptional induction or inhibition of the enzymatic activities of COX-2 and iNOS [2]. Oregonin, existing primarily in *Alnus* plants [3–6], is a diarylheptanoid glycoside containing 3-carbonyl and 5-xylosyloxy groups. It displayed potent inhibitory activities against PKCα [7], COX-2 [8], and NO synthesis [9,10]. In addition, the presence of oregonin could inhibit stimulation of murine macrophages by LPS, iNOS mRNA expression, and NO synthesis [11]. Various studies demonstrated that oregonin has the potential to be developed as an anti-inflammatory agent.

The *Alnus* genus comprises about 30 species of trees and shrubs found in the Northern Hemisphere [5]. In view of the large number of *Alnus* species, efficient analytical methods are required to facilitate qualitative and quantitative studies of oregonin in these plants. The HPLC method has been used to screen oregonin in several *Alnus* plants [6]. However, validation and quantitation were not included in the report. Recently, capillary electrophoresis (CE) has gained its status as a powerful separation technique in natural product researches due to the strength of high efficiency, short analysis time and economic of sample and reagent consumption. The aim of this study is to develop an efficient and reliable CE method to analyze oregonin in *Alnus* plants and to facilitate the pharmacokinetic study of oregonin. We used various *Alnus* plants extract and biological samples to demonstrate the practicability of the method developed in this study. To the best of our knowledge, this is the first study to analyze oregonin with CE.

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2. Experimental

2.1. Chemicals and materials

The seeds of *Alnus glutinosa*, *A. cordata*, *A. hirsute*, *A. viridis* and *A. incana* were purchased from B&T World Seeds sarl, Pauguignan, 34210 Olonzac, France. Sodium tetraborate was purchased from Merck (Darmstadt, Germany). Sodium hydroxide was purchased from Fluka (Buchs, Switzerland). All the reagents used were of analytical and chromatographic grades.

2.2. Stock and standard preparations

Oregonin stock solution (1000 μg/ml) was prepared by dissolving 10 mg oregonin in 10 ml MeOH. Stock solution of theophylline, the internal standard, was prepared in the same way. Adequate amounts of oregonin and theophylline stock solutions were mixed at different ratios to prepare standard solutions for respective experiments.

2.3. Apparatus

The experiments of capillary electrophoresis were carried out with Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) equipped with photodiode array detector. Separations were performed using a 59 cm (49 cm effective length) × 50 μm ID fused-silica capillary from Polymicro Technologies (Phoenix, AZ, USA). The electropherograms were recorded using an EZChrom (Scientific Software, San Ramon, CA, USA) chromatographic data system.

2.4. Electrophoretic conditions

CZE-UV experiments were performed on a 59 cm (49 cm effective length) × 50 μm i.d. fused-silica capillary. The new fused-silica capillaries were conditioned by flushing with 1.0 M NaOH for 10 min, 0.2 M NaOH for 10 min, water for 5 min and running solutions for 10 min in sequence. At the beginning of each experiment, the capillary was washed with 0.2 M NaOH for 3 min, followed by running buffer for 5 min. The background electrolyte (BGE) solution was 30 mM sodium tetraborate and the pH value was adjusted to 8.0 by titrating with 1 M HCl. Operation conditions were set as follows: voltage, 30 kV; injection time, 5.0 s (hydrodynamic, 50 mbar); detection wavelength, 220 nm; temperature, 25 °C.

All the solutions were filtered through 0.45-μm filters (Millipore, Bedford, MA, USA) prior to use.

2.5. Isolation and identification of oregonin from leaves of *Alnus formosana* [4]

Dry ground powdered material (4.1 kg) of leaves of the *A. formosana* Burk (Betulaceae) (collected in Nantou County, Taiwan) was extracted with methanol. The methanolic extract was divided into fractions soluble in hexane, chloroform, *n*-butanol (293 g) and water by liquid–liquid partitioning. Part of *n*-BuOH soluble fraction (10 g) was fractionated via a Sephadex LH-20 column (MeOH) to give an oregonin-rich fraction (2.10 g), which was repeatedly purified via centrifugal partition chromatography (CPC, model CPC-LLN, Sanki Engineering Ltd., Kyoto, Japan). The organic layer of $CHCl_3$–MeOH–H_2O (2:2:1) or $CHCl_3$–MeOH–H_2O–i-PrOH (10:10:5:1) was used as the mobile phase initially to remove the less polar constituents. The polar ingredients were then separated using the aqueous layer as the mobile phase. Through this procedure, oregonin (165 mg), which was TLC, ^{1}H NMR essentially pure and showed identical physical data to those reports was obtained [4,12].

2.6. Drug administration and sample collection

Eight hundred milligrams of oregonin-rich fraction from the leaves of *A. formosana* described above was mixed with 200 mg acacia and suspended in water. The suspension was administered at a dose of 53.3 mg/kg to a female Lanyu pig with a weight of 15 kg though an intragastric tube. Urine sample was collected at 24 h post-administration and the dialysate sample was collected at 2 h post-administration for 30 min. Both samples were stored at temperatures below −20 °C until analysis.

2.7. Sample preparation

2.7.1. Seeds extract

Ground seeds of *A. glutinosa*, *A. cordata*, *A. hirsute*, *A. viridis* and *A. incana* (each 10 g) were extracted successively with *n*-hexane, dichloromethane and methanol (200 ml each) using a soxhlet apparatus. Portion of the methanol extract obtained after evaporation was diluted with MeOH for CZE analysis.

2.7.2. Leaves of *A. formosana*

Portion of the *n*-BuOH soluble fraction of the MeOH extract from the leaves of *A. formosana* described above was dissolved in MeOH for CZE analysis.

2.7.3. Serum sample

Blood sample was collected from healthy volunteer and then centrifuged at 3000 rpm for 10 min to obtain serum sample. Oregonin was spiked to serum sample. 1.5 ml ACN was added to 1.0 ml serum sample and vortex-mixed briefly. The resulting mixture was centrifuged at 13,000 rpm for 10 min to precipitate serum proteins. A portion of the supernatant was subjected to CZE analysis.

2.7.4. Pig urine and dialysate

Pig urine and dialysate described above was separately passed through Amberlite XAD-II column. Two liters of water was used as washing solution to remove electrolyte, and 1 l of MeOH was used as eluting solution. Portion of the methanol eluate obtained after evaporation was dissolved in MeOH for CZE analysis.

2.8. Calculations

The peak efficiency was represented by the theoretical plate number (N) which was calculated based on the following

equation

$$N = 16\left(\frac{t_m}{t_w}\right)^2$$

where t_m is the peak migration time and t_w is the baseline bandwidth.

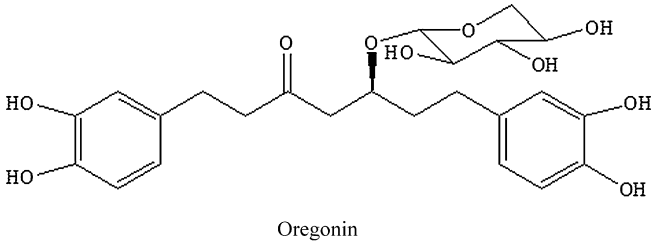
3. Results and discussion

3.1. Method development

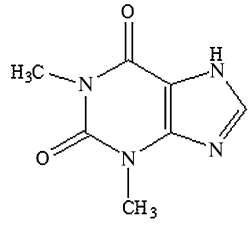
Oregonin was first analyzed by micellar electrokinetic chromatography (MEKC) mode. High percentage of organic solvents in sample matrix in MEKC system will result in low plate number or peak splitting [13–15]. Water was therefore selected as sample matrix to enhance peak efficiency. However, oregonin was unstable in aqueous solution and it degraded in sample matrix within 1 day. CZE method was therefore employed.

3.1.1. Effect of pH

Chemical structure of oregonin is shown in Fig. 1. Oregonin is unstable in acid and strong alkaline conditions. The pH values in the range of 7.5–9.5 of the solutions were chosen for better compound stability and greater buffer capacity, and the running buffer was 30 mM sodium tetraborate. The results were shown in Fig. 2. Data for plots of migration time and theoretical plate number average values were obtained by 3 repetitions. When pH was higher than 9.0, the degree of ionization increased, thus the peak efficiency increased as a result of reduced analyte adsorption. The migration time also increased when pH was higher than 9.0 due to the ionization of the phenolic groups. It was found that pH 9.0 provided good peak efficiency with the shortest analysis time. However, oregonin will co-migrate with other components in the *Alnus* plants analyzed in this study. pH 8.0 provided best selectivity with acceptable peak efficiency and short analysis time, and was considered as optimum separation pH.



Oregonin



Theophylline

Fig. 1. Structures of oregonin and theophylline (I.S.).

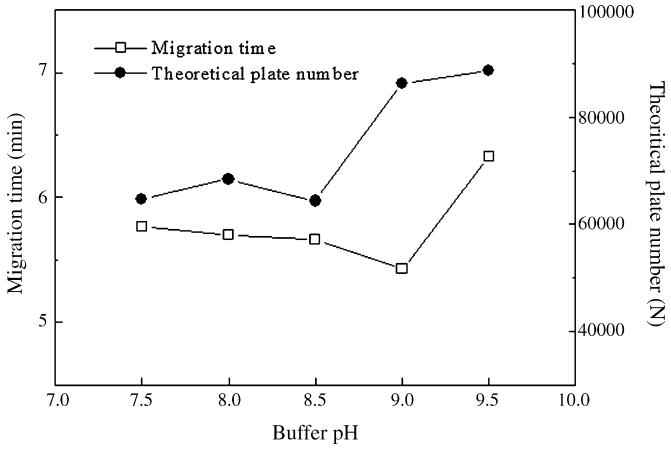


Fig. 2. Effect of buffer pH on migration time and theoretical plate number of oregonin. Separation conditions: fused-silica capillary, 59 cm × 50 μm i.d., 49 cm detection length; 30 mM sodium tetraborate; temperature, 25 °C; injection, 50 mbar, 5.0 s. The pH values are as indicated in the figure; applied voltage, 30 kV.

3.1.2. Effect of buffer concentration

Sodium tetraborate at concentrations between 20 and 40 mM with pH 8.0 was tested in this study, and the results were shown in Fig. 3. Peak efficiency was found to reach the maximum at 30 mM. Migration time prolonged with the increase of ionic strength. Concerning both separation efficiency and analysis time, 30 mM sodium tetraborate was chosen as optimum concentration. The highest applicable voltage (30 kV) of the apparatus was used to reduce the analysis time. Under the optimum separation conditions, the analysis was completed within 6 min as exhibited in Fig. 4.

3.2. Method validation

3.2.1. Precision

Run-to-run repeatability (intraday, $n = 6$) and intermediate precision (interday, $n = 3$) of migration time and peak area ratios were tested. Theophylline of 20 μg/ml was added to the standard

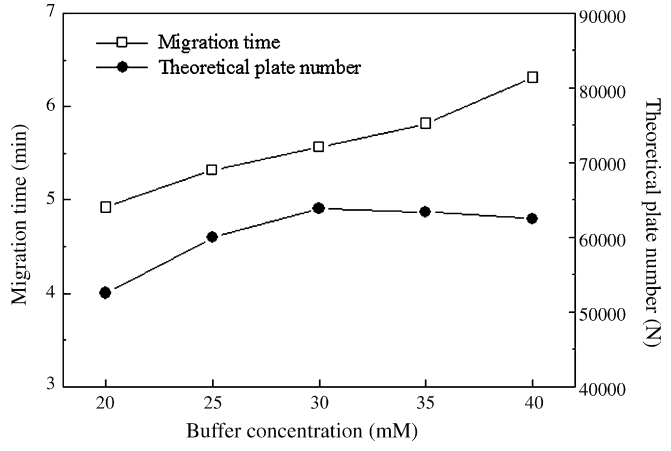


Fig. 3. Effect of buffer concentration on migration time and theoretical plate number of oregonin. Separation conditions: 20–40 mM sodium tetraborate, pH 8.0; applied voltage, 30 kV; other conditions as in Fig. 2.

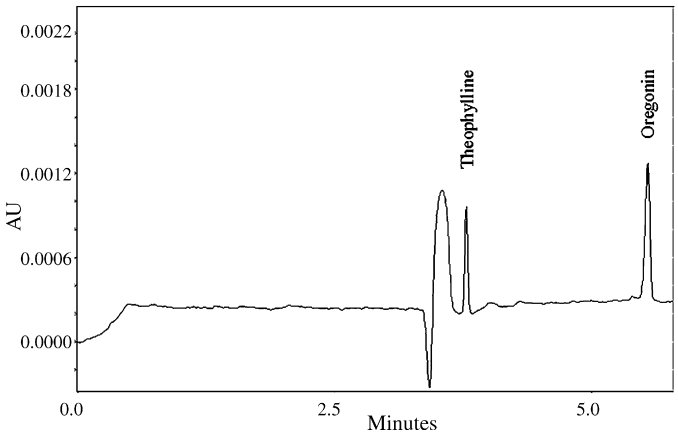


Fig. 4. Electropherogram of oregonin under optimum separation conditions. Separation conditions: fused-silica capillary, 59 cm × 50 μm i.d., 49 cm detection length; 30 mM sodium tetraborate, pH 8.0; applied voltage, 30 kV; temperature, 25 °C; injection, 50 mbar, 5.0 s.

solution of oregonin as an internal standard. Both repeatability and intermediate precision in terms of migration time were within 1.36% relative standard deviation (R.S.D.). Precision of peak area ratios was tested at 10, 50 and 100 μg/ml of oregonin (the lower, medium and higher levels of the linearity, see below). Repeatability and reproducibility of peak area ratios at 10 μg/ml level were 1.55 and 0.93% R.S.D., respectively. Precisions at 50 μg/ml level were 0.24 and 0.24% R.S.D., respectively. Precisions at 100 μg/ml level were 0.48 and 0.31% R.S.D., respectively.

3.2.2. Linearity and accuracy

Linearity of the method was tested with five standard solutions from 10 to 100 μg/ml of oregonin and measuring their responses (peak area) relative to internal standard. Each solution was tested for three times. Regression line was $y = 0.0234x - 0.0076$ with correlation coefficient (r) equal to 0.999.

Accuracy of the method was assessed via recovery. Standard solutions of 10 and 100 μg/ml of oregonin were added to the sample solutions of *A. glutinosa*. The concentrations found were calculated against the concentrations added. The recoveries were 96.7 ± 3.9% and 97.1 ± 0.3% ($n = 3$).

Table 1
Contents of oregonin in various *Alnus* species

Species (part)	Content (mg/g)	S.D.[a]
Alnus formosana (leaves)	1.983	0.087
Alnus incana (seeds)	0.131	0.006
Alnus viridis (seeds)	N.D.[b]	–
Alnus glutisona (seeds)	0.557	0.022
Alnus cordata (seeds)	N.D.[b]	–
Alnus hirsute (seeds)	0.231	0.009

[a] Standard deviation.
[b] Not detected.

3.2.3. Limit of detection (LOD) and limit of quantification (LOQ)

LOD of the method was defined as the concentration of oregonin being detected at S/N = 3. It was found to be 1.15 μg/ml. LOQ was tested with a concentration of oregonin at S/N = 10. Its value was found to be 3.84 μg/ml.

3.3. Application

Six *Alnus* species, *A. cordata*, *A. glutinosa*, *A. hirsute*, *A. viridis*, *A. incana*, and *A. formosana*, were examined with the developed CZE method to qualitatively and quantitatively analyze oregonin in these plants. With analyte spiking and

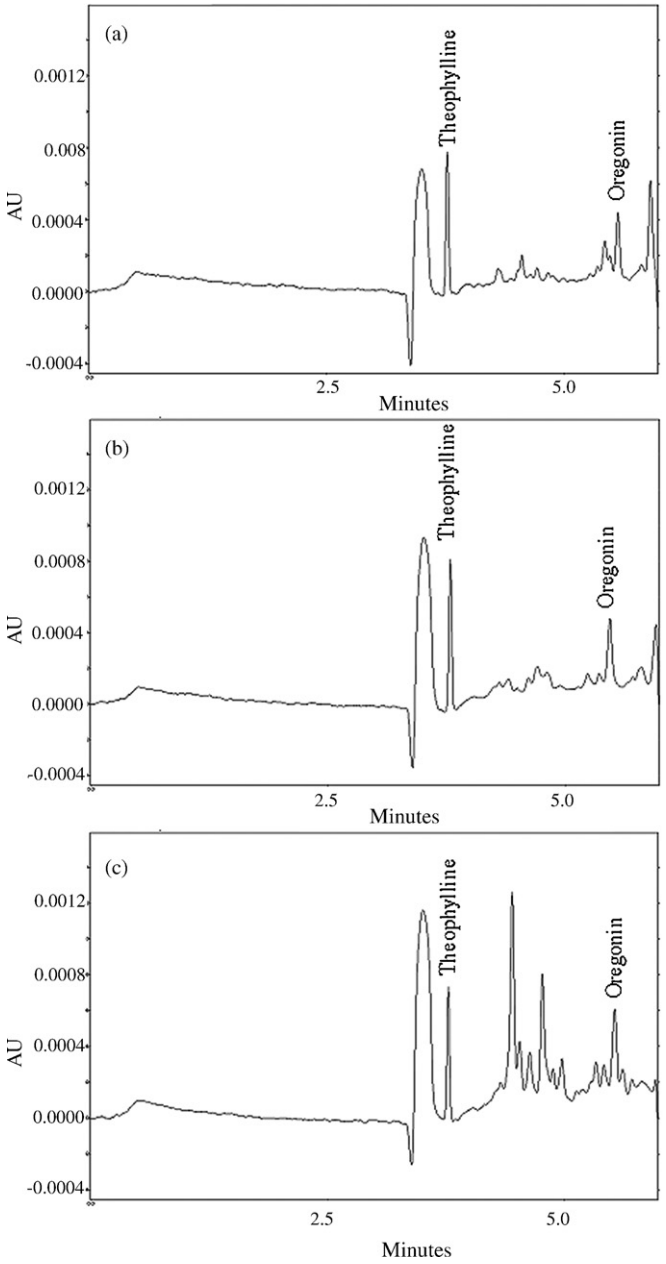


Fig. 5. Electropherograms obtained from crude extract of (a) *Alnus formosana* (leaves), (b) *A. glutisona* (seeds) and (c) *A. hirsute* (seeds). Separation conditions are as described in Fig. 4.

spectrum-matching techniques, oregonin was identified in the crude extract of four species. The similarity indexes of spectra for the comparing peaks were all greater than 0.97 (1.000 for complete overlap). The contents of oregonin in seeds or leaves of these species were shown in Table 1. Three representative electropherograms of the extracts were shown in Fig. 5. Guz et al. had used HPLC to screen oregonin in the bark of *A. glutinosa*, *A. viridis*, *A. incana* and *A. cordata* and the leaves of *A. incana* and *A. cordata*. In their study, oregonin was detected in all extracts except the leaves of *A. viridis* [6]. Same species were analyzed in this study, and both *A. glutinosa* and *A. incana* were found to contain oregonin in their seeds.

To evaluate the potential application of the developed CZE method in pharmacokinetic studies, 20 μg/ml of oregonin was spiked in human serum. Oregonin was well separated from sample matrix with good signal intensity. However, it was found that the peak intensity of oregonin gradually decreased and disappeared after three consecutive analysis. The stability of oregonin in human serum was greatly improved if solid phase extraction cartridge (Oasis HLB) was used to remove part of the endogenous components. The recoveries of oregonin in SPE pretreated serum at 10 μg/ml and 100 μg/ml were 93.4 ± 0.6% and 109.7 ± 4.1% ($n = 3$), respectively.

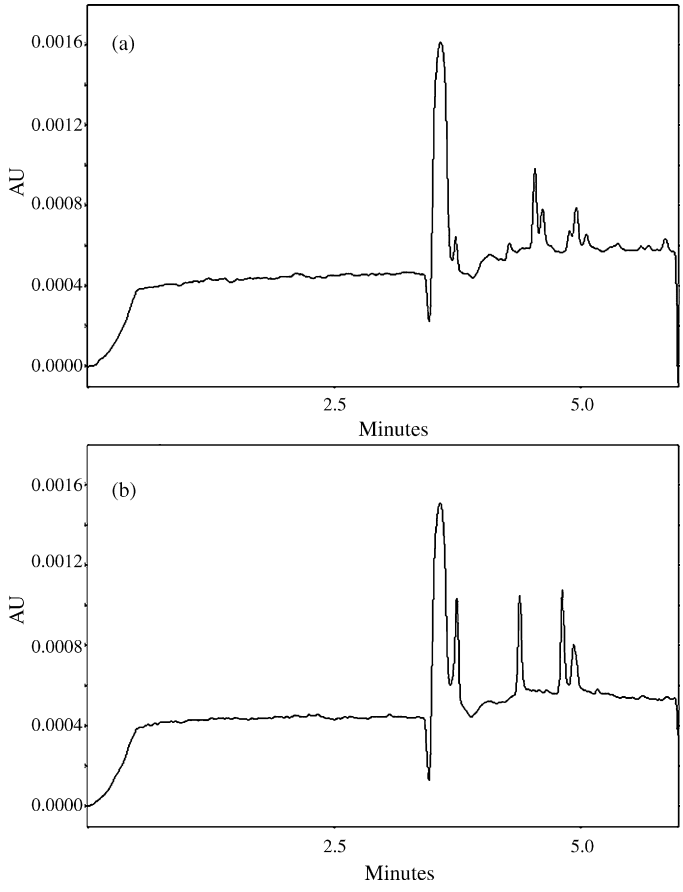


Fig. 6. Electropherograms obtained from the extracts of (a) pig dialysate and (b) pig urine after treated with oregonin suspension. Separation conditions are as described in Fig. 4.

The stability of oregonin in biological system was further evaluated in the following animal study. Oregonin enriched suspension was intragastrical administrated in pig. Extract of pig urine and dialysate were analyzed by our CZE method. The electropherograms of the extract were displayed in Fig. 6. Oregonin was not detected in the samples and considered to be degraded in biological system. The present study indicated that oregonin should be designed as a prodrug.

4. Conclusion

This study innovates an efficient CZE method to the analysis of oregonin. As stated in the beginning, NSAIDs are one of the highest consumption pharmaceutical products worldwide. Due to their gastrointestinal irritation, many efforts have been made on the development of selective COX-2 inhibitors to improve patient tolerability. However, there are only two COX-2 inhibitors – celecoxib (Celebrex®) and rofecoxib (Vioxx®) – on the market now. Oregonin has been proven to show strong anti-inflammatory activity through inhibiting the activity of the enzyme COX-2 [8]. It has the potential to be developed as a new drug. While some studies in oregonin are still ongoing, an efficient analytical method is required to study the content of oregonin in *Alnus* plants. To provide better selectivity and short analysis time, pH value and buffer concentration were systematically optimized. The applicability of the developed method was demonstrated by applying the optimum conditions to analyze various *Alnus* species and spiked human serum. It was found in human serum spiking test that oregonin should be designed as a prodrug to improve its stability in human body. This work shows that CZE is a powerful technique to analyze oregonin.

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