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Identification and Quantification of Steroidal Saponins in *Polygonatum* Species by HPLC/ESI/MS

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An HPLC/ESI/MS method has been developed to identify and quantify the spirostanol glycosides in the rhizomes of five *Polygonatum* species. The relative distribution of two steroidal saponins in each extract was established using the selective ion monitoring (SIM) mode *via* an electrospray ionization (ESI) source. It was found that there were significant differences in the amount of spirostanol glycosides among the *Polygonatum* species. The results showed that this method could be used to identify the steroidal saponins in the extracts and differentiate *Polygonatum* species with high sensitivity and reproducibility in a short time. Fragmentation patterns of the two reference compounds were also discussed with the electrospray ionization multi-stage tandem mass spectroscopy (ESI-MS^n).

Key words: HPLC/ESI/MS, *Polygonatum* species, Spirostanol glycosides, SIM mode, ESI-MS^n

INTRODUCTION

The rhizomes of *Polygonatum* species of the family Liliaceae are important herbal drugs in traditional Asian medical practices (Anonymous, 1978). These species are widely distributed in Mt. Himalaya, China, Japan, and Korea. Twenty-six species and three varieties are known worldwide (Liu *et al.*, 1993b) and seven species are found to grow in Korea (Park and Lee, 2000). Meanwhile, the heat-processed crude drugs of *Polygonatum* species are sold in Korean markets without any standardization or clarification of their origins. The botanical origins of these species are often confusing because the rhizomes of these plants are morphologically similar. Pharmacognostical studies on the *Polygonatum* species have been previously reported to differentiate *Polygonatum* species according to anatomical characteristics (Liu *et al.*, 1993a, 1993b). There are also preceding reports on classification of these species by random amplified polymorphic DNAs analysis (Jang and Kim, 1998) and quantification of primary metabolites in these species (Jang *et al.*, 2002). However, these studies involve a time consuming and tedious procedure, and did not focus on the amount of biologically active components.

Therefore, a simple HPLC-ESI-MS technique was developed to differentiate these species. This approach relies on the detection of steroidal saponins because our preliminary phytochemical study showed that two spirostanol glycosides (PO-2 and PO-3) are major saponin components of *Polygonatum* species and one of them is known to be bioactive (Kato and Miura, 1993).

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MATERIALS AND METHODS

Plant materials

P. odoratum (Mill.) Druce was collected in Seoul and *P. odoratum* var. *thunbergii* Hara in Kyeongju, *P. humile* Fisch. ex Maxim. in Dangjin, *P involucratum* (Franch & Sav.) Maxim. in Taegu, *P. sibiricum* Redouté in Hongseong and Pusan provinces of Korea in the spring of 2002. These plants were identified by Prof. J.H. Park in the College of Pharmacy, Pusan National University, Korea.

Solvents and reagents

All the solvents used in this experiment were HPLC-grade. Acetonitrile (MeCN), water and acetic acid were purchased from Mallinckrodt (U.S.A.), and methanol (MeOH) from Fischer (U.S.A.). A membrane filter (MF3-

13 PTFE, diameter-13 mm, pore size-0.50 μm, Advantec, CA, USA) was used to filterate each sample.

Standards and samples

Pure spirostanol glycosides (PO-2 and PO-3) were isolated from the rhizomes of *P. sibiricum* in our laboratory and used as standard compounds. In a 10 mL-volumetric flask, the standard compounds (approx. 1 mg) were accurately weighed and dissolved in HPLC grade methanol to make a stock solution. Working calibration solutions were prepared in a range from 9.77 to 5000 ng/mL by successive two-fold serial dilution of the stock solution with methanol.

The rhizomes of *Polygonatum* species were sliced into small pieces and lyophilized. Finely pulverized powder was weighed (1.0 mg), and transferred to a 50 mL test tube. 25 mL of methanol was added, and the mixture was stirred gently and ultrasonicated twice at 37°C for 90 min. The supernatants (20 mL) were then separated after filtration with filer papers (Whatman No. 40) and evaporated *in vacuo* followed by adding 3 mL of methanol. The sample solutions were filtered through a membrane filter prior to analysis.

HPLC analysis

A Hewlett-Packard 1100 series HPLC system equipped with an autosampler, a column oven, a binary pump and a degasser (Hewlett-Packard, Waldbronn, Germany) was used. A 5 μL volume of standard or sample solutions was directly injected on a Zorbax XDB C_8 column (4.6 × 150 mm, 5 μm, Agilent) using a gradient acetonitrile-water solvent system containing 0.01% acetic acid at 37°C. The flow rate was 0.3 mL/min and the solvent gradient conditions are shown in Table I. The Chemstation software (Hewlett-Packard, Avondale, CA, U.S.A.) was used to operate this HPLC system.

ESI-mass spectrometry

All ESI-MS and ESI-MS^n spectra were acquired using a Finnigan MAT LCQ ion-trap mass spectrometer (San Jose, CA, U.S.A.) equipped with a Finnigan electrospray source and capable of analyzing ions up to *m/z* 2000. Mass spectrometer conditions were optimized in order to achieve maximum sensitivity. The source voltage was set to +36.5 V and the capillary temperature to 300°C. The other conditions were as follows: capillary voltage, +36.5 V; inter-octapole lens voltage, 10 V; sheath gas flow, 80 arbitrary units; auxiliary gas flow, 20 arbitrary units. Nitrogen (>99.999%) and He (>99.999%) were used as sheath and damping gas, respectively.

Table I. Solvent gradient conditions for HPLC-MS

final time (min)	flow rate (mL/min)	water (0.01% acetic acid)	MeCN (0.01% acetic acid)
0	0.3	60	40
15	0.3	50	50
30	0.3	40	60
45	0.3	15	85
60	0.3	15	85

The sodium cationized molecular ions were isolated with an isolation width of 2 *m/z* units and fragmented using a collision energy of 50% for MS^2 experiments and 45% for MS^3, 35% for MS^4 and MS^5 experiments. The MS data were acquired in Selective ion monitoring (SIM) mode to quantify steroidal saponins.

The mass scale was calibrated in the positive-ion mode using a solution consisting of caffeine, the tetra-peptide MRFA, and Ultramark 1621 (Sigma, St. Louis, MO, U.S.A.) solution. The Xcalibur software (Finnigan MAT) was used for the operation.

RESULTS AND DISCUSSION

Saponins exhibit a variety of biological activities and are widely used in foods, medicines, and cosmetics (Waller and Yamasaki, 1996a, 1996b). Several steroidal saponins have been isolated from *Polygonatum* species (Ono *et al.*, 1988; Son and Do, 1990) and among them, a steroidal saponin, PO-2, is known to have a hypoglycemic activity in normal and diabetic mice (Kato and Miura, 1994). In fact, the processed rhizomes of *Polygonatum* species are used for diabetes in folk medicine. Accordingly, it is worthwhile to classify the *Polygonatum* species by the contents of steroidal saponins. However, it is difficult to detect and quantify saponins because saponins can be detected at a short wavelength (200-210 nm) with an UV detector and are present at low levels (He, 2000). Actually, in this study the LOD (limit of detection) was 1250 ng/mL (*S/N*, 3) for the two standard compounds at 204 nm with an UV detector. This LOD was increased to 1 mg/mL when they were co-injected with samples owing to matrix effect. Therefore, the characterization and quantification of spirostanol glycosides in the extract of *Polygonatum* species were accomplished with a quadrupole ion trap mass spectrometer that allows high sensitivity, rapid analysis time and low levels of sample consumption (Cui *et al.*, 2000).

Two steroidal saponins, PO-2 and PO-3, were selected as standard compounds. A saponin PO-2 displayed a quasimolecular ion peak at *m/z* 1071 $[M+Na]^+$ and the MS/MS spectrum of the $[M+Na]^+$ ion showed the peaks at *m/z* 939, 777, 615 due to the successive loss of one pentose unit (132 Da) and two hexose units (2 × 162 Da) (Fig. 1). In addition, it exhibited the peaks at *m/z* 659 [Xyl-2Glc-Gal+Na]$^+$ corresponding to a lycotetraose moiety

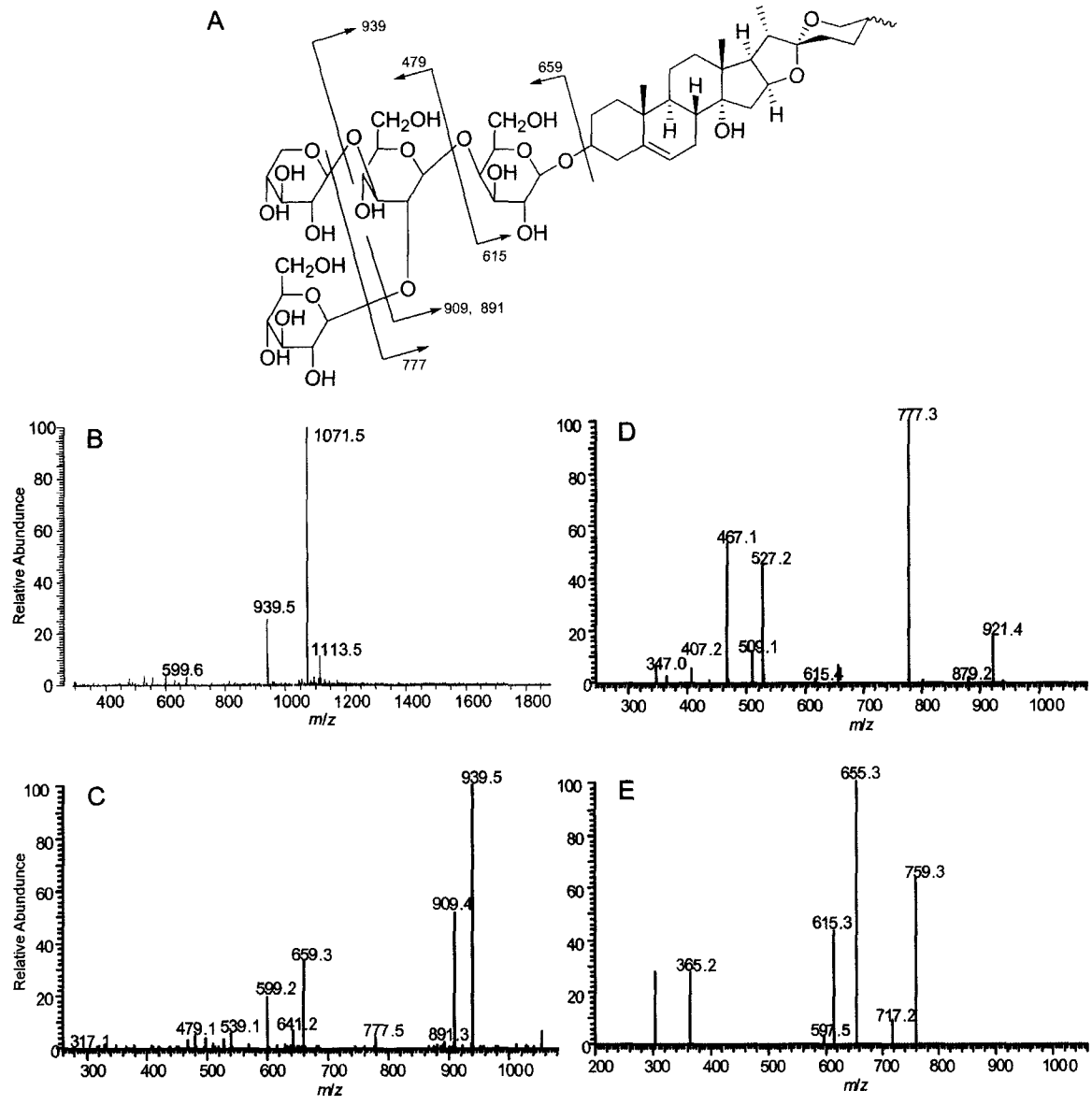

Fig. 1. (A) Structure and principal fragmentations of PO-2. (B) ESI MS spectrum of the sodium cationized PO-2. (C) MS/MS spectrum of the molecular ion at *m/z* 1071. (D) MS^3 spectrum of the ion at *m/z* 939 from MS^2 of the sodium cationized PO-2. (E) MS^4 spectrum of the ion at *m/z* 777 from MS^3 of the ion at *m/z* 939 from MS^2 of the sodium cationized PO-2.

and at *m/z* 921 $[M+Na\text{-}Xyl\text{-}H_2O]^+$, 891 $[M+Na\text{-}Glc\text{-}H_2O]^+$, 759 $[M+Na\text{-}Xyl\text{-}Glc\text{-}H_2O]^+$ resulting from a hydroxy group in sapogenin moiety.

The other standard saponin, PO-3 not having a hydroxy group at C-14 showed the $[M+Na]^+$ ion at *m/z* 1055 in positive mode (Fig. 2). In MS^n spectrum of the $[M+Na]^+$ ion, there are several fragment ions at *m/z* 923, 761 and 599, corresponding to the successive loss of one pentose unit (132 Da) and two hexose units (2 × 162 Da) similar to PO-2. There is also a fragment ion, $[M+Na\text{-}Xyl\text{-}2Glc\text{-}Gal]^+$ at *m/z* 437, which corresponds to the aglycone moiety (Fig. 2). These fragmentation patterns by ESI-MS^n spectra gave useful information about the structure of the steroidal saponins PO-2 and PO-3.

Calibration curves were constructed for each of the reference standards in concentrations of 9.77, 19.5, 39.1, 78.1, 156, 313, 625, 1250, 2500 ng/mL in duplicates. The calibration curves showed good linearity and the correlation coefficients were found to be 0.9992 and 0.9966 for PO-2 and PO-3, respectively, over this concentration range. The limit of quantification (LOQ) was 39.1 ng/mL and the limit of detection (LOD) was 9.8 ng/mL for both PO-2 and PO-3.

The relative distribution of these compounds in each

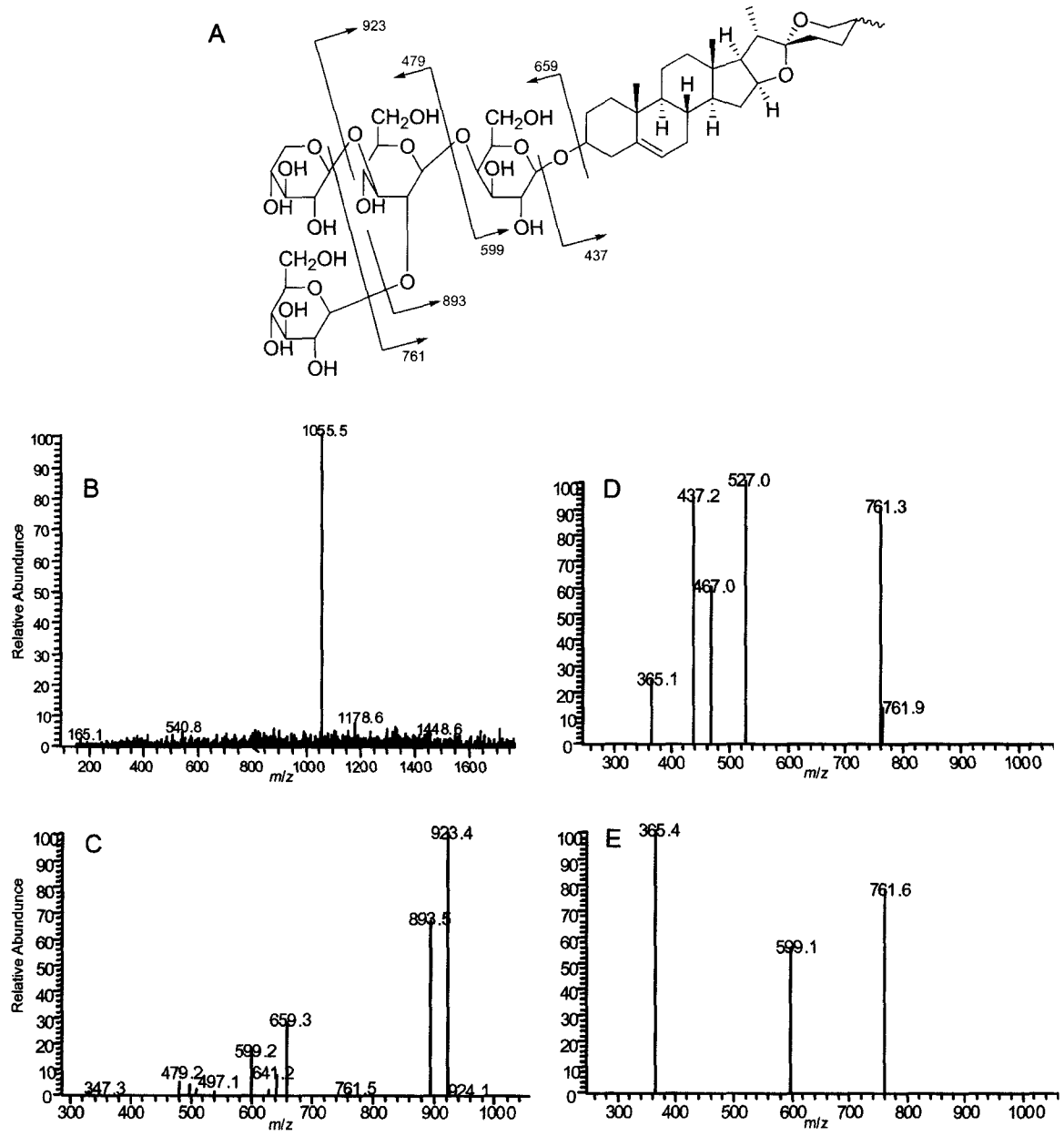

Fig. 2. (A) Structure and principal fragmentations of PO-3. (B) ESI MS spectrum of the sodium cationized PO-3. (C) MS/MS spectrum of the molecular ion at *m/z* 1055. (D) MS^3 spectrum of the ion at *m/z* 923 from MS^2 of the sodium cationized PO-3. (E) MS^4 spectrum of the ion at *m/z* 761 from MS^3 of the ion at *m/z* 923 from MS^2 of the sodium cationized PO-3.

extract of five *Polygonatum* species was established by HPLC/ESI/MS with SIM mode (Table II, Fig. 3 and Fig. 4). While the amounts of PO-2 were more than 200 μg/g in *P. odoratum* var. *thunbergii*, *P. odoratum*, *P. involucratum* and *P. falcatum*, those were less than 23 μg/g in *P. sibiricum*-I and II. For PO-3, a much greater amount of this saponin was found in the *P. odoratum*, *P. humile*, and *P. falcatum* extracts than in the other species. Similar patterns were shown in *P. sibiricum* I and II which were collected at different places.

The reproducibilities (coefficient of variance, CV) ranged from 9.8 to 20.6, 1.2 to 9.6% for PO-2 and PO-3, respectively. These results are consistent with the previous report that the hypoglycemic effect of *P. odoratum* is more potent than *P. sibiricum* (Miura and Kato, 1995).

In this study, HPLC/ESI/MS was firstly applied to quantify steroidal saponins in *Polygonatum* species. The results showed that this method could be used to identify steroidal saponins in the extracts and to differentiate *Polygonatum* species with high sensitivity and reproducibility in a short time.

Table II. Quantification of PO-2 and PO-3 of *Polygonatum* species

	PO-2		PO-3	
	mean (μg/g)[a]	CV	mean (μg/g)[b]	CV
P. odoratum var. *thunbergii*	639.5 ± 79.1	12.4	132.9 ± 7.7	5.8
P. odoratum	321.6 ± 61.4	19.1	1485.2 ± 143.1	9.6
P. humile	632.5 ± 62.2	9.8	2080.5 ± 149.8	7.2
P. involucratum	236.3 ± 30.5	12.9	221.4 ± 6.6	3.0
P. falcatum	766.7 ± 114.1	14.9	1448.9 ± 17.4	1.2
P. sibiricum - I[c]	23.1 ± 4.8	20.6	218.8 ± 12.3	5.6
P. sibiricum - II[c]	10.1 ± 1.5	14.9	40.6 ± 1.8	4.5

[a] Data are expressed as mean ± S.E. of three independent experiments.

[b] CV: coefficient of variance (%) = (the standard deviation value (SD) / the average value of content (μg/g)) × 100

[c] *P. sibiricum* - I and *P. sibiricum* - II were collected in Hongseong and Pusan, respectively.

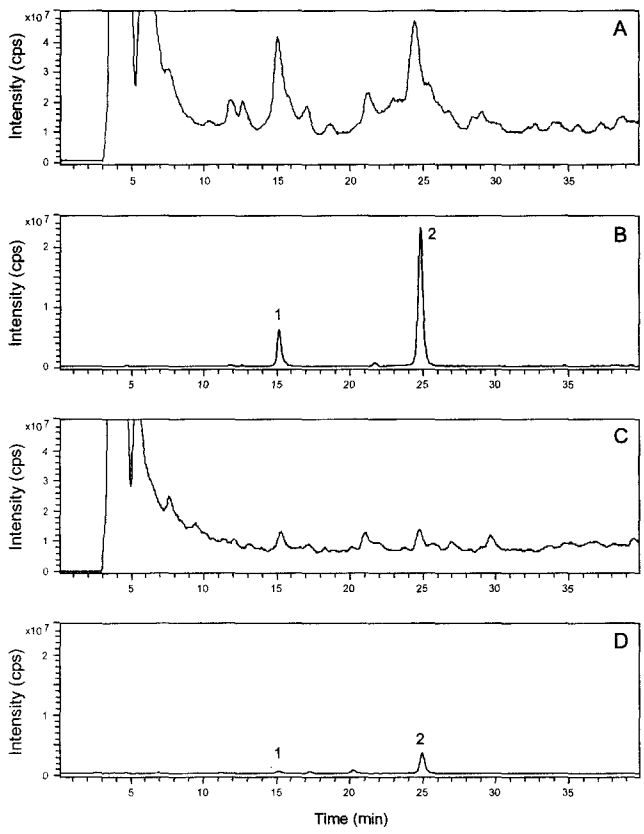

Fig. 3. Chromatograms of the methanol extracts of the rhizomes of *Polygonatum* species. (A) Total ion chromatogram of the methanol extract of the rhizome of *P. humile*. (B) Chromatogram of the extract of *P. humile* in SIM mode. (C) Total ion chromatogram of the methanol extract of the rhizome of *P. sibiricum* - I. (D) Chromatogram of the extract of *P. sibiricum* - I in SIM mode. Two segments were set as follows: the ion at *m/z* 1071 was selected for the first 20 min period and the ion at *m/z* 1055 was selected for the latter period. In this condition, the peaks 1 and 2 corresponded to PO-2 (t_R 15.1 min) and PO-3 (t_R 24.8 min), respectively.

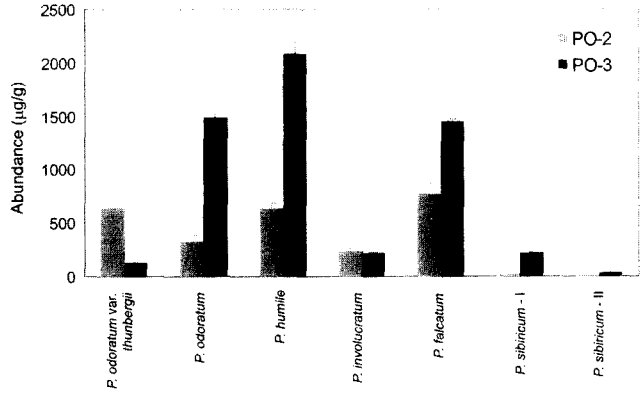

Fig. 4. Quantification of PO-2 and PO-3 of *Polygonatum* species

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