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Discrimination between steam processed and unprocessed Tubers of *Gastrodia elata* Blume by HPLC

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Abstract: In this study, to evaluate the effectiveness and safety of oral therapy using Gastrodiae Rhizoma, a new HPLC-PDA analysis method was developed for the simultaneous quantitation of the three major components: (**1**) gastrodin, (**2**) gastrodigenin, and (**3**) *p*-hydroxybenzaldehyde, in steam processed and unprocessed tubers of *Gastrodia elata* Blume. The clear separation of the three components was achieved on a C18 column (250 × 4.6 mm, 5 μm) by gradient elution using water (including 0.1 % formic acid) and acetonitrile as the mobile phase. The flow rate was 1.0 mL/min, and the UV detector wavelength was set at 270 nm. The results demonstrate satisfactory linearity, recovery, precision, accuracy, stability, and robustness. The established HPLC-PDA method was applied to quantify three major compounds in 59 samples of G. *elata* Blume tubers. Finally, the steam processed and unprocessed tubers of G. *elata* Blume were successfully distinguished by pattern recognition analysis.

Key words: gastrodiae rhizoma, gastrodin, gastrodigenin, *p*-hydroxybenzaldehyde

1. Introduction

Gastrodiae Rhizoma, the steam processed tuber of *Gastrodia elata* Blume (Family: Orchidaceae) has a long history of use as a traditional herb for improving liver meridian. It is distributed in various East Asian countries, such as China, India, Korea, and Japan.[1] In recent years, a wide variety of practical applications for Gastrodiae Rhizoma have been found in many countries, in its capacity as a health supplement.[2] Modern pharmacology has demonstrated that Gastrodiae Rhizoma not only has a protective effect on the liver, but also has various other medicinal benefits such as for anti-convulsion, anti-anxiety, anti-tumor, anti-inflammation, anti-oxidation, anti-aging, etc.[3-6] Currently, more than 80 compounds from Gastrodiae Rhizoma have been isolated and identified, including phenolics, alkaloids, polysaccharides, steroids, flavonoids, and amino acids.[1]

In the pharmacopoeia of China, the content regulation of Gastrodiae Rhizoma has been prescribed as containing more than 0.25 % of both ingredients

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with gastrodin and *p*-hydroxybenzaldehyde.[7] However, the prescribed ingredient for Gastrodiae Rhizoma has not been stipulated in the Korean Pharmacopeia and Japanese Pharmacopeia.[8-9]

In previous studies, high performance liquid chromatography (HPLC) analytical methods have been developed for Gastrodiae Rhizoma. Bi *et al.* quantified *β*-sitosterol by HPLC from 22 different origins, but *β*-sitosterol was not a uniquely marked compound in Gastrodiae Rhizoma.[10] Li *et al.* established an HPLC method for the simultaneous determination at 220 and 254 nm of six bioactive constituents (gastrodin, *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, adenosine, parishin A, and 4,4'-dihydroxydibenzyl ether) of the steam unprocessed tuber of *Gastridia elata* extract.[11] However, this method was too complex (gradient elution of three-steps in the mobile phase) and has the disadvantage of a long detection time (80 min). Wong *et al.* analyzed gastrodin, parishin B, parishin C, and parishin in Gastrodiae Rhizoma by direct ionization mass spectrometry to distinguish between the wild and cultivated types.[12] Zhang et al. employed the HPLC method to identify the un-fumigated and sulfur-fumigated Gastrodiae Rhizoma.[13] However, little research has been carried out on distinguishing between steam processed and unprocessed tubers of *Gastrodia elata* Blume. In a study on the discrimination of the steam processed and unprocessed tubers of *Gastrodia elata*, Kwon *et al*. applied a metabolomics approach using LC-QTOF-MS, and suggested six metabolites that were key components for identification: gastrodin, mono-substituted parishin, di-substituted parishin, parishin B, parishin, and *S*-(4-hydroxybenzyl)glutathione.[14] However, researchers have overlooked the content relationship between gastrodin and gastrodigenin. In addition, other studies were analyzed and quantitated for the constituents of marker or isolated compounds from steam unprocessed[15,16] or steam unprocessed[17] *G. elata* Blume.

Although Gastrodiae Rhizoma has been used as a traditional herbal medicine for millennia, occasional cases of *G. elata* poisoning have occurred in clinics when administered orally or by injection. Such poisoning could be due to the toxicity of *G. elata.* Traditionally, herbal medicines have been generally administered after processing via methods such as steaming, pickling, and parching to reduce the toxicity.[18-20] It is considered that the toxicity of Gastrodiae Rhizoma can also be reduced by a steaming process.[14] However, in traditional herbal markets, two types of *G. elata* tuber (steam processed and unprocessed) are available. Therefore, identification and discrimination between steam processed and unprocessed tubers of the *G. elata* Blume is necessary. In this study, we firstly developed an analytical method using HPLC/PDA based on representative marker compounds of gastrodin (**1**), gastrodigenin (**2**), and *p*-hydroxybenzaldehyde (**3**) only during 40 min to discriminate between the steam processed and unprocessed tubers of *G. elata* Blume. Our developed method is advantage of economic values for popularity and reduction experimental costs. To the best of our knowledge, no reports have been presented on the systematic discrimination between steam processed and unprocessed tubers of *G. elata* Blume.

2. Experimental

2.1. Materials

Three standards [gastrodin (**1**), gastrodigenin (**2**), and *p*-hydroxybenzaldehyde (**3**)] were provided by Eun Kyoung Seo, Ewha Womans University, Seoul, Korea. Purity of standards was estimated to be higher than 95 % based on HPLC analysis. Internal

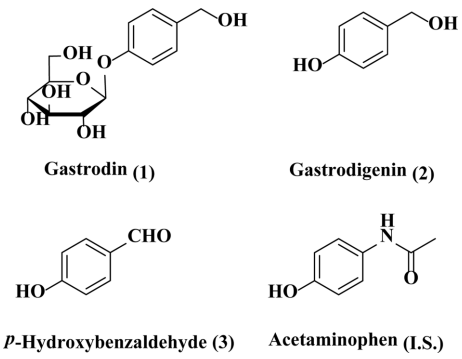

Fig. 1. Structures of standards and an internal standard.

standard (I.S.), acetaminophen, was purchased from Sigma Chemicals (St. Louis, MO, USA). All of the standard structures are shown in *Fig.* 1. Acetonitrile was purchased from Merck K GaA (Darmstadt, Germany). All other chemicals used were analytical grade unless otherwise noted. Distilled water was prepared using the Milli-Q purification system (Millipore, Bedford, MA, USA). This study adopted the following 59 samples correspondng to fifty steam processed (SGE01–SGE50) and nine unprocessed (UGE01–UGE09) tubers of *Gastodia elata* Blume.

2.2. Sample preparation

The powdered sample was sieved through 50 mesh sieve. About 0.5 g of the powder, accurately weighed, was added to 50 mL of 70 % methanol containing an internal standard (**I.S.**; 10 μg/mL acetaminophen), and then the sample was sonicated at 60W, 40 Hz for 60 minutes. The solution was filtered through a 0.45 μm membrane filter and the filtrate was used as the test solution. This extraction method was used after the optimization of the extracted condition.

2.3. HPLC/UV conditions

The HPLC equipment was a LC-20AD pump, SPD-20A diode array detector, SIL-20A auto-sampler injector, DGU-20A5 solvent degasser, and CTO-20A column oven (Shimadzu, Japan). SunFireTM C18 (250 mm × 4.6 mm, 4 μm), Luna C18 (250 mm × 4.6 mm, 5 μm), and Shiseido C18 (250 mm × 4.6 mm, 5 μm) columns were tested with the same stationary phase; 0.1 % formic acid in water (**A**) and acetonitrile (**B**) was used as the mobile phase [(0 – 25 min, 2 – 10 % (**B**); 25 – 30 min, 10 – 15% (**B**); 30 – 40 min, 15 % (**B**)]. The mobile phase was filtered under vacuum through a 0.45 μm membrane filter and degassed prior to use. The analysis was carried out at a flow rate of 1.0 mL/min with UV detection absorbance at 270 nm. The chromatograms were processed using LC solution Lite software (Shimadzu, Japan).

2.4. Analytical method validation

Three standards of gastrodin (**1**), gastrodigenin (**2**), and *p*-hydroxybenzaldehyde (**3**) were each accurately weighed and then dissolved with 70 % methanol containing I.S. (1.25 μg/mL acetaminophen) to produce stock standard solutions of 100 ppm. The calibration curves were generated after diluting the each stock solution with 70 % methanol containing I.S. (10 μg/mL of acetaminophen). The regression equations were calculated in the form of $y = ax + b$, where y and x correspond to peak ratio (standard area / I.S. area) and compound concentration, respectively. Recovery tests were executed by mixing a powdered sample (0.5 g) of the reference compounds at three control levels (near the LOQ, medium and higher concentrations from the calibration curves). The mixture was then extracted by sonication in 50 mL of 70 % methanol at room temperature for 60 min. The extract solution was filtered through a 0.45-μm membrane. The HPLC/PDA analysis experiments were performed in triplicate for each control level. The data from the standard solution and the extracted sample were compared. Precision and accuracy were determined by multiple analyses ($n = 5$) of quality control samples prepared at low, medium and high concentrations spanning the calibration range. Repeatability was discussed with RSD% for retention time and peak area ($n = 6$). The robustness of the method was studied by introducing changes in three different columns (i.e. Sunfire, Luna, and Shiseido), and separation temperatures (i.e. 35, 40, and 45 °C). Cross-matching test performed between two different laboratories and HPLC equipment (Waters and Shimadzu), and investigated by T-test and P-test.

2.5. Pattern recognition analysis

To evaluate the phytochemical equivalency among the 59 samples [fifty steam processed tubers of *G. elata* Blume (SGE01–SGE50) and nine steam unprocessed tubers of *G. elata* Blume (UGE01–UGE09)], pattern recognition analysis was conducted by multivariate analysis technique that is used to sort samples into groups. The similarity or dissimilarity between steamed and un-steamed samples was represented in G graph and dendrogram for ease of interpretation. In this study, we used three standards

[gastrodin (1), gastrodigenin (**2**), and *p*-hydroxybenzaldehyde (**3**)] for pattern recognition analysis using software SPSS statistics 19.0.

3. Result and Discussion

3.1. Optimization of chromatographic and extraction conditions

HPLC conditions were selected according to the requirement for obtaining a good resolution on chromatograms. In mobile phase optimization, the water and acetonitrile system was verified to be sufficient for separating the four standards, including internal standard of acetaminophen within 40 minutes (*Fig*. 2). In order to improve the accuracy of the determination, acetaminophen was elected as the internal standard, which has similar structure with three targeting compounds. Gastrodin (**1**), gastrodigenin (**2**), *p*-hydroxybenzaldehyde (**3**), and acetaminophen (**I.S.**) showed maximum UV absorptions at about 270 nm. In addition, we also tested the impact of adding 0.01 %, 0.1 %, and 1 % acid (acetic acid, formic acid, and phosphoric acid) to the mobile phase. The water including 0.1 % formic acid resulted in good resolution for all standards, as well as satisfactory peak symmetry and shape (data not shown). In optimization of extracted condition, we investigated six extraction solvents (90 % ethanol, 70 % ethanol, 50 % ethanol, 90 % methanol, 70 % methanol, and 50 % methanol), two extraction methods (ultra-sonication and reflux), and four extraction times (30, 45, 60, and 75 min). Finally, the extracted condition was chosen as 70 % methanol, sonication, and 60 min (data not shown).

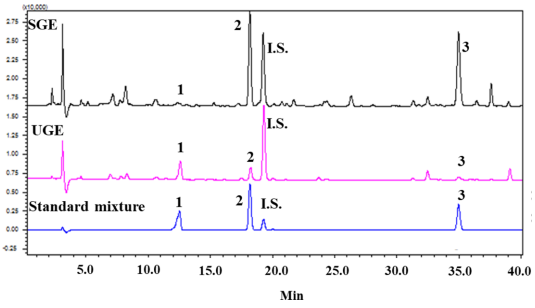

Fig. 2. The difference of HPLC chromatograms of standards mixture, SGE (**SGE01**), and UGE (**UGE03**) samples. Gastrodin (**1**), gastrodigenin (**2**), *p*-hydroxybenzaldehyde (**3**), and acetaminophen (**I.S.**) with concentrations of 10 μg/mL for samples and 1.25 μg/mL for standard mixture.

3.2. The HPLC method validation

Each coefficient of correlation (r^2) for all marker compounds was > 0.999, as determined by the least squares analysis, suggesting excellent correlation and linearity between the peak area ratio and the compound concentrations. In the results, the regression equations for gastrodin (**1**), gastrodigenin (**2**), and *p*-hydroxybenzaldehyde (**3**) was $y = 0.0125x - 0.0038$, $y = 0.0404x + 0.0019$, and $y = 0.5305x - 0.0024$, respectively. The appropriate linear-range confirmed by the content of the sample. LOD (S/N = 3) and LOQ (S/N = 10) obtained under our experimental conditions are listed in *Table* 1. The values obtained for both LOD and LOQ for these three standards were low enough that traces of these standards could be detected in either a crude extract or its preparation.

The recovery test was determined by multiple analyses ($n = 3$) at lower, medium and higher concentrations for each standards (gastrodin 12.5, 25.0, 50.0 μg/mL; gastrodigenin 6.25, 12.5, 25.0 μg/mL; *p*-hydroxybenzaldehyde 0.3125, 0.625, 1.25 μg/mL). Accurate amounts of the three standards were added into a sample of GR, which was previously quantified. The recovery for each standard was evaluated by

Table 1. Linearity, linear ranges, LOD and LOQ

Analytes	Linear range (μg/mL)	Slope	Intercept	Correlation coefficient (R^2)	LOD (μg/mL)	LOQ (μg/mL)
Gastrodin (**1**)	3.125–100	0.0125	-0.0038	0.9996	0.63	2.08
Gastrodigenin (**2**)	1.560–100	0.0404	0.0197	0.9999	0.31	1.04
p-Hydroxybenzaldehyde (**3**)	0.078–5.00	0.5305	-0.0024	0.9999	0.02	0.05

Table 2. Recovery of marker compounds through standard addition (n = 3)

Analyte	Fortified conc. (μg/mL)	Observed conc. (μg/mL)	Mean Recovery (%)	RSD (%)
1	0.000	46.51 ± 0.35	-	-
	12.500	59.39 ± 0.12	103.03	1.75
	25.000	72.27 ± 0.17	103.04	0.95
	50.000	98.15 ± 0.05	103.29	0.61
2	0.000	5.07 ± 0.06	-	-
	6.250	11.61 ± 0.09	104.56	0.73
	12.500	18.10 ± 0.02	104.18	0.29
	25.000	31.36 ± 0.22	105.14	0.87
3	0.000	0.11 ± 0.01	-	-
	0.310	0.44 ± 0.00	103.19	1.84
	0.625	0.75 ± 0.01	101.00	1.16
	1.250	1.42 ± 0.01	105.57	0.80

Table 3. Precision and accuracy of analytical results (n = 6)

Analytes	Fortified conc. (μg/mL)	Intra-day (n = 5)				Inter-day (n = 3)			
		Observed conc. (μg/mL)	SD	Accuracy (%)	Precision (RSD, %)	Observed (μg/mL)	SD	Accuracy (%)	Precision (RSD, %)
1	25.0	25.21	0.35	100.8	1.37	24.73	0.82	98.9	0.18
	100.0	106.52	0.60	106.5	0.57	106.45	0.21	105.1	0.22
	300.0	301.13	1.21	100.4	0.40	305.22	0.22	101.7	1.02
2	12.5	12.24	0.08	97.9	0.64	12.2	0.08	97.6	0.69
	50.0	53.36	0.09	106.7	0.18	53.40	0.08	106.8	0.15
	150.0	151.38	0.34	100.9	0.23	151.15	0.28	100.8	0.18
3	2.5	2.32	0.01	92.9	0.01	2.32	0.01	92.87	0.01
	10.0	10.71	0.08	107.1	0.01	106.4	0.03	106.40	0.01
	30.0	30.0	0.01	100.0	0.00	30.0	0.00	100.0	0.00

Table 4. Repeatability of analytical results (n = 6)

		Retention time	Peak area
Gastrodin (**1**)	Mean	12.39 ± 0.1	39589 ± 275.24
	RSD%	0.82	0.7
Gastrodigenin (**2**)	Mean	18.02 ± 0.10	19291 ± 217.93
	RSD%	0.54	1.13
Acetaminophen (**I.S.**)	Mean	19.07 ± 0.11	136346 ± 1326.13
	RSD%	0.58	0.97
p-Hydroxybenzaldehyde (**3**)	Mean	34.75 ± 0.07	6322 ± 242.90
	RSD%	0.21	3.84

calculating the ratio of the detected amount to the added amount. The results showed that the mean of recovery range from 101 % to 106 %, with a relative standard deviation% (RSD%) of less than 1.8 % (*Table* 2).

Intra- and inter-assay precision and accuracy were determined from the variability of multiple analyses (n = 5) of the quality control samples analyzed within

the same analytical run. The quality control samples had the intra- and inter- assay precision of below 1.4 % and 1.0 %, respectively. Furthermore, the accuracy of intra- and inter- assay exhibited between 93 % and 107 % (*Table* 3). In the repeatability test, the retention time and peak area for all of standards had below 5 % RSD levels (*Table* 4). Hence, these results indicated that this proposed method was highly reproducible.

The robustness was determined in order to evaluate the reliability of the established HPLC method. In robustness experiment, the column species and temperature were evaluated to optimize the chromatographic method by four parameters as theoretical plate (***N***), capacity factor (***k′***), separation factor (***α***) and resolution (***Rs***). The result showed that four analytical factors (***N***, ***k′***, ***α***, and ***Rs***) did not differ greatly, depending on the column species or temperature, and all the compounds showed a capacity for separation when the conditions were changed (*Table* 5). The stability of establishing method is also reflected in Cross-matching test. In the T-test, the *p* value for all standards between the two groups was unnoticed to the extent of over than 5 %. In addition, the correlation coefficients for all standards showed > 0.99 (*Table* 6).

Table 5. Robustness of column, and temperature

Compounds	Analytical condition		Theoretical plate (*N*)	Capacity factor (*K'*)	Separation factor (*α*)	Resolution (*Rs*)
1	Column	SunFire	93300 ± 6916	3.00 ± 0.04	1.15 ± 0.00	2.93 ± 0.13
		Luna	109163 ± 7836	2.92 ± 0.04	1.12 ± 0.00	2.75 ± 0.09
		Shiseido	170684 ± 2016	2.94 ± 0.01	1.19 ± 0.00	3.00 ± 0.10
	Temperature (°C)	35	93300 ± 6916	3.00 ± 0.04	1.15 ± 0.00	2.93 ± 0.13
		40	92090 ± 4914	2.96 ± 0.02	1.06 ± 0.00	1.28 ± 0.21
		45	60287 ± 5521	2.80 ± 0.01	1.14 ± 0.01	2.51 ± 0.11
2	Column	SunFire	268939 ± 4585	4.83 ± 0.02	1.05 ± 0.00	2.01 ± 0.01
		Luna	307419 ± 3793	4.62 ± 0.07	1.04 ± 0.00	1.71 ± 0.08
		Shiseido	105423 ± 3066	4.63 ± 0.01	1.06 ± 0.00	1.64 ± 0.01
	Temperature (°C)	35	268939 ± 4585	4.83 ± 0.02	1.05 ± 0.00	2.01 ± 0.01
		40	259852 ± 5121	4.68 ± 0.01	1.03 ± 0.00	1.25 ± 0.03
		45	203729 ± 2352	4.39 ± 0.00	1.07 ± 0.00	2.21 ± 0.04
3	Column	SunFire	493638 ± 8909	10.25 ± 0.02	1.03 ± 0.00	1.78 ± 0.04
		Luna	452856 ± 9216	9.88 ± 0.14	1.01 ± 0.00	0.89 ± 0.18
		Shiseido	485129 ± 4665	10.19 ± 0.01	1.04 ± 0.00	2.06 ± 0.12
	Temperature (°C)	35	493638 ± 8909	10.25 ± 0.02	1.03 ± 0.00	1.78 ± 0.04
		40	480140 ± 13498	10.04 ± 0.02	1.02 ± 0.00	1.68 ± 0.01
		45	312820 ± 29364	9.54 ± 0.01	1.03 ± 0.00	1.68 ± 0.12

Table 6. The cross-matching contents between two groups **A** and **B** from fifty-nine samples (fifty SGE[a], and nine UGE[b])

	Groups	Average% (*n* = 59)	Standard deviations	Correlation coefficient	Paired t-test
Gastrodin (**1**)	A	0.3947	0.2297	0.999	*p* = 0.07
	B	0.3927	0.2255		
Gastrodigenin (**2**)	A	0.1063	0.1518	0.999	*p* = 0.59
	B	0.1071	0.1608		
p-Hydroxybenzaldehyde (**3**)	A	0.0055	0.0079	0.998	*p* = 0.48
	B	0.0056	0.0082		

[a]SGE: steam processed tubers of *Gastrodia elata* Blume; [b]UGE: steam unprocessed tubers of *Gastrodia elata* Blume; Statistically significant difference ($p < 0.05$).

3.2. Sample analysis

The HPLC/PDA method was applied to analyze fifty steam processed and nine steam unprocessed tuber samples of *G. elata* Blume. The average contents (wt%) of gastrodin (**1**), gastrodigenin (2), and *p*-hydroxybenzaldehyde (**3**) are presented in *Table* 7. Average contents in fifty steamed samples were detected as 0.45 % for gastrodin (range of 0.12 % ~ 1.27 %) and 0.05 % for gastrodigenin (range of 0.00 % ~ 0.22 %). No samples contained *p*-hydroxybenzaldehyde, except for the SGE32 (0.02 %) and SGE49 (0.01 %) samples. The average contents in the nine UGE samples were detected as 0.1 2% for gastrodin (range of 0.04 % ~ 0.21 %), 0.43 % for gastrodigenin (range of 0.26 % ~ 0.66 %), and 0.02 % for *p*-hydroxybenzaldehyde (range of 0.0 1% ~ 0.04 %). The results of the quantitation of the fifty-nine samples are shown in *Table* S1 (Supplementary). The average contents of gastrodigenin and *p*-hydroxybenzaldehyde in the un-steamed samples were (0.43 %) and (0.02 %), respectively, which were higher than those in the steamed samples, (0.05 %) and (0.00 %), respectively. In contrast, the average content of gastrodin (0.45 %) in the steam processed samples was higher than that in the unprocessed samples (0.12 %) (*Table* 7). Lv *et al.* reported that the *p*-hydroxybenzaldehyde can be converted into gastrodigenin through physical steaming.[21] While it is not difficult to understand why the Chinese Pharmacopoeia stipulated the maximum contents of gastrodin and *p*-hydrxoybenzaldehyde, in the Chinese Pharmacopoeia stipulation for Gastrodiae Rhizoma, the concentration of gastrodigenin was overlooked because, similar to gastrodin, gastrodigenin decreases with steam treatment. In the proposed biosynthetic pathway of gastrodin, the *p*-hydrxoybenzaldehyde must be converted to gastrodigenin before it becomes gastrodin.[22] These results indicate that the most appropriate quality control for Gastrodiae Rhizoma should be regulated according to the content summation of gastrodin (**1**), gastrodigenin (**2**), and *p*-hydroxybenzaldehyde (**3**).

Table 7. Average content (w/w%, n = 3) of three components in SGE, and UGE samples

	Content (w/w%)	
	SGE (n = 50)	UGE (n = 9)
Gastrodin (**1**)	0.45 ± 0.02	0.12 ± 0.01
Gastrodigenin (**2**)	0.05 ± 0.03	0.43 ± 0.03
p-Hydroxybenzaldehyde (**3**)	0.00	0.02 ± 0.01

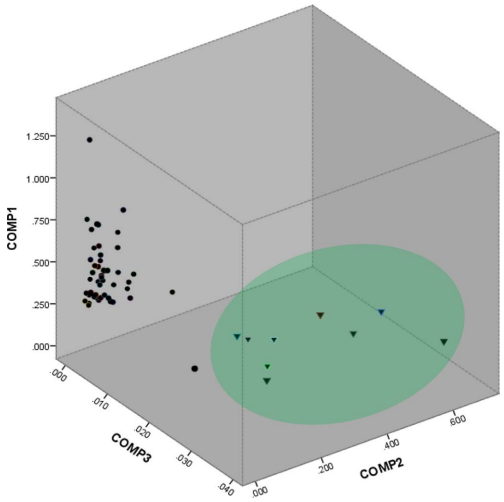

Fig. 3. The pattern analysis for fifty-nine samples [fifty SGE (**SGE01** – **SGE50**; circle), and nine UGE (**UGE01** – **UGE09**; triangle; green zone)].

3.3. Pattern recognition analysis

To evaluate the phytochemical equivalency of *G. elata*, fifty steam processed and nine steam unprocessed samples were examined by pattern recognition analysis. A 3D pattern analysis of a total of fifty-nine samples was performed using the contents of three standards [gastrodin (**1**), gastrodigenin (**2**), and *p*-hydroxybenzaldehyde (**3**)]. Based on the quantities of the three components, the results showed that the steam processed and unprocessed samples were distinguishable (*Fig.* 3). However, the *G. elata* samples were no correlation with their geographical origin, regardless of the preparation processing.

4. Conclusions

The newly established HPLC/PDA method was validated for the quantification of the bioactive components of gastrodin (**1**), gastrodigenin (2), and *p*-hydroxybenzaldehyde (**3**) from the tuber of *G.*

elata. Validation of the results indicates that this analytical method is suitable for determining the contents of the three compounds in the tuber of *G. elata*. The developed method not only affords a viable strategy for distinguishing between steam processed and unprocessed tubers of *G. elata* Blume, but also provides a reference for the assessment of the quality of Gastrodiae Rhizoma.

Conflict of Interest

The authors declare no competing financial interest.

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