Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 32 No. 3, 96-104, 2019
https://doi.org/10.5806/AST.2019.32.3.96

Establishment and validation of an analytical method for quality control of health functional foods derived from *Agastache rugosa*

Keunbae Park[1], Dasom Jung[1], Yan Jin[1], Jin Hak Kim[2], Jeong Ho Geum[3], and Jeongmi Lee[1,★]

[1]*School of Pharmacy, Sungkyunkwan University, Gyeonggi 161419, Korea*
[2]*COSMAX NS Inc., Seoul 06132, Korea*
[3]*COSMAX NBT Inc., Seoul 06132, Korea*

(Received April 4, 2019; Revised May 28, 2019; Accepted May 31, 2019)

Abstract *Agastache rugosa*, known as Korean mint, is a medicinal plant with many beneficial health effects. In this study, a simple and reliable HPLC-UV method was proposed for the quantification of rosmarinic acid (RA) in the aqueous extracts of *A. rugosa*. RA was selected as a quantification marker due to its easiness in procurement and analysis. The developed method involved chromatographic separation on a C_{18} column (250 × 4.6 mm, 5 μm) at room temperature. The mobile phase consisted of water and acetonitrile both containing 2 % acetic acid and was run at a flow rate of 1 mL min^{-1}. The method was validated for specificity, linearity, precision, and accuracy. It was specific to RA and linear in the range of 50–300 μg mL^{-1} (r^2 = 0.9994). Intra-day, inter-day, and inter-analyst precisions were ≤ 0.91 % RSD, ≤ 1.40 % RSD, and 1.94 % RSD, respectively. Accuracy was 93.3–95.9 % (≤ 1.21 % RSD). The method could be applied to three batches of bulk samples and three batches of lab scale samples, which were found to be 0.64 (± 0.04) mg g^{-1} and 0.48 (± 0.02) mg g^{-1} for the dried raw materials of *A. rugosa*. The results show that the proposed method can be used as a readily applicable method for QC of health functional foods containing the aqueous extracts of *A. rugosa*.

Key words: *Agastache rugosa*, rosmarinic acid, health functional food, quality control

1. Introduction

Agastache rugosa is a mint belonging to the family of Lamiaceae.[1] Called by many names such as Korean mint, purple giant hyssop, Indiana mint, etc.,[2] *A. rugosa* has been used as traditional Chinese medicine (TCM) to cure orexia, cholera, and other diseases in South Korea and China.[3] A variety of pharmaceutical effects of *A. rugosa* have been reported including antifungal,[4,5] antimicrobial,[6] antitumor,[7,8] anti-human immunodeficiency virus intergrase,[9] anticoagluant,[10] antioxidant,[11] and anti-atherogenic[12] effects.

Numerous compounds have been isolated and identified in *A. rugosa*, and many of them have biological activities. For example, essential oils of *A. rugosa* contain methyl eugenol, eugenol, estragole, etc.[13]; methyl eugenol has antibacterial and antibiofilm activities,[14] and eugenol has antioxidant and antibacterial

★ Corresponding author
Phone : +82-(0)31-290-7784 Fax : +82-(0)31-292-8800
E-mail : jlee0610@skku.edu

activities.[15,16] Many phenolic compounds including caffeic acid, rosmarinic acid, calycosin, apigenin, tilianin, and acacetin also have been reported in *A. rugosa*.[11,17] A phenolic acid, rosmarinic acid is known to have many beneficial bioactivities including anti-Alzheimer,[18] antiviral,[19] anti-inflammatory,[19] and anticancer[20] activities. Vasorelaxation[21] and antihypertensive[22] activities have been reported for tilianin, a flavonoid glycoside.

Traditional usage of *A. rugosa* for versatile indications and its various pharmaceutical effects suggest that consumption of *A. rugosa* and its related products will increase. Indeed, *A. rugosa* has been not only consumed as tea, food material, and TCM, but also has started to be recognized as a resource for health functional food in Korea. An appropriate assay is essential for ensuring the quality of not only the raw materials but also processed materials derived from natural resources.[23] Their quality control (QC) is usually achieved using a valid analytical method based on the quantification of one or more marker compounds.[24] In the Korean Herbal Pharmacopoeia (KHP), an HPLC-UV method to determine the tilianin content has been suggested as the quantification method for the aerial part of *A. rugosa*.

In this study, we revisited the KHP method above and assessed its suitability as the analytical method for QC of health functional foods derived from *A. rugosa*. Then, a new, reliable, and readily applicable method was established and validated according to the Association of Official Analytical Chemists (AOAC) guidelines.

2. Experimental

2.1. Chemicals, reagents, and samples

Rosmarinic acid (RA; reagent grade, > 96 %) and acetic acid (AA; ACS reagent grade, > 99.7 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (FA; LC-MS grade, > 99 %) was purchased from Thermo scientific (Waltham, Massachusetts, USA). Tilianin (reagent grade, >95 %) and acacetin (reagent grade, > 95 %) were purchased from Biopurify Phytochemicals Ltd. (Chengdu, China). HPLC-grade acetonitrile (ACN), water, and methanol (MeOH) were purchased from Honeywell Burdick & Jackson (Ulsan, Korea).

Dried raw materials of the aerial part of *A. rugosa*, which were available in local market in Korea, were provided by Nutribiothech Co. Ltd. (Seoul, Korea). Their aqueous extracts (AEs) made in bulk scale were also provided by Nutribiothech Co. Ltd. The bulk AEs were produced through a hydrothermal extraction process as follows: 100 kg of dried raw materials were extracted in 1500 kg of purified water at 95 °C for 4 h, followed by filtration and freeze-drying. The AEs in lab scale were prepared under the same conditions using 2 g of dried raw materials in 30 mL of water.

2.2. Analytical instruments and the operation conditions

The HPLC analysis was performed using a Waters HPLC system (Millipore, MA, USA) equipped with a separations module (Model No. 2695) and a photodiode array (PDA) detector (Model No. 996). Chromatographic separation was achieved on a Phenomenex Gemini C_{18} column (250 × 4.6 mm, 5 μm) at room temperature. The detector wavelength was 330 nm, and the sample injection volume was 10 μL. The mobile phase consisted of solvent A (2 % AA in water) and solvent B (2 % AA in ACN) and was run at a flow rate of 1.0 mL min^{-1}. The gradient elution conditions were: 5% B for 0–1 min, 5–19 % B for 1–15 min, 19–23.5 % B for 15–23 min, 23.5–24.5 % B for 23–33 min, 24.5–100 % B for 33–34 min, and 100 % B for 34–39 min. It was returned to the initial conditions within 1 min and re-equilibrated for 15 min prior to next injection.

A qualitative analysis of the *A. rugosa* extract was conducted using an ultra-high performance liquid chromatography-quadrupole time of flight mass spectrometry (UHPLC-QTOF/MS) system. The instrument was composed of an Acquity UPLC™ system (Waters co., Milford, MA, USA) and a Waters Acquity Xevo G2 Q-TOF system (Waters Corp., Manchester, UK). The samples were chromatographed on an Acquity UPLC BEH C_{18} column (50 × 2.1 mm,

1.7 μm) maintained at 30 °C. The mobile phase consisted of solvents A (0.1 % FA in water) and solvent B (0.1 % FA in ACN) with a flow rate of 0.2 mL min^{-1}. The elution conditions were: 10–30 % B for 0–10 min, 30–100 % B for 10–10.5 min, 100–100 % B for 10.5–12.5 min.

2.3. Standard solutions

An RA stock solution with a concentration of 1000 μg mL^{-1} was prepared in methanol. Working standard solutions were prepared by diluting the stock solution to 50, 75, 100, 150, and 300 μg mL^{-1}.

2.4. Sample preparation for the HPLC analysis

Two hundred mg of AEs were added to 9 mL of MeOH in a 10 mL volumetric flask. After extraction by ultrasonic irradiation for 30 min, the mixture solution was allowed to cool down to room temperature. MeOH was added to the mark and vortexed thoroughly. After centrifugation at 2898 *g* for 3 min, the supernatant was filtered through 0.45 μm membrane filter (Whatman, Piscataway, NJ, USA) and injected into the HPLC system.

2.5. Method validation

The analytical method was validated for specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. Specificity was evaluated by comparing the retention times, PDA spectra, and mass spectra of the RA peak in the solutions of RA standard, AE sample, and AE sample spiked with RA at 100 μg mL^{-1}. Linearity was assessed based on the calibration curve, which was established by triplicate analyses of the RA standard solutions at five concentrations (50, 75, 100, 150, and 300 μg mL^{-1}). The LOD and LOQ were calculated using the formulae:

$$LOD = 3.3 \times SD_{y\text{-intercept}} / S$$
$$LOQ = 10 \times SD_{y\text{-intercept}} / S$$

Where S is the slope and $SD_{y\text{-intercept}}$ is the standard deviation of the y-intercept of the calibration curve. Precision, which was expressed as % RSD from quintuplicate experiments, was evaluated in terms of repeatability and reproducibility according to the AOAC guidelines. Repeatability indicates the intra-day precision; reproducibility was measured as inter-day and inter-analyst precisions. The intra-day and inter-day precisions were assessed using the AE samples spiked with RA at three concentration levels (0, 50, and 100 μg mL^{-1}). The inter-analyst precision was measured by two different analysts, who separately analyzed six replicates of the unspiked AE samples. Accuracy was determined using the AE samples spiked with RA (0, 50, 100, and 150 μg mL^{-1}). It was estimated as the % relative recovery, which was calculated using the following equation:

$$\% \text{ Relative recovery} = 100 \times (C_{found} - C_{unspiked}) / C_{added}$$

in which C_{found}, $C_{unspiked}$, and C_{added} were the RA concentrations of a spiked sample, an unspiked sample, and an added standard solution, respectively, measured from five replicate experiments.

2.6. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 7.00 for Windows (GraphPad Software, San Diego, CA, USA). The RA contents of the three batches of AE samples were compared using one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test.

3. Results and discussion

3.1. Selection of a quantification marker compound

Tilianin is known as one of the major compounds in *A. rugosa*[25] and was suggested as the marker compound in the HPLC-based quantification method for the aerial part of *A. rugosa* in KHP. The KHP method involves 2 h of ultrasound-assisted extraction of the raw materials in methanol prior to HPLC analysis. The resulting extracts would be rich in relatively nonpolar compounds such as flavonoids. With regard to health functional foods, they are usually manufactured using aqueous or aqueous ethanol extracts of raw materials such as AEs in this study. This implies that the KHP method might not

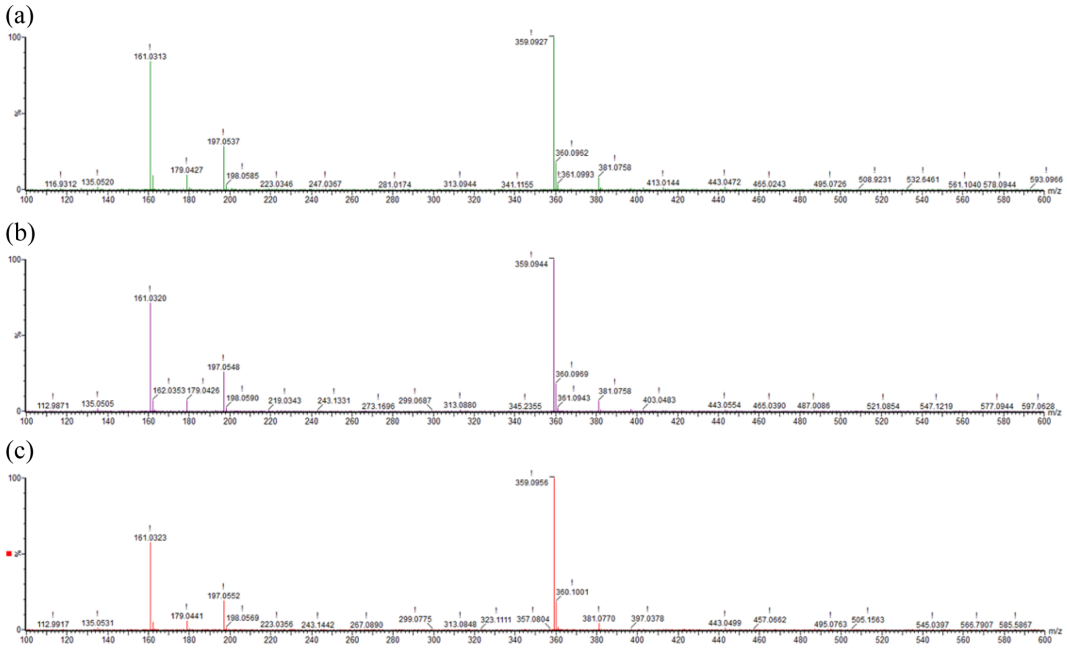

Fig. 1. UHPLC-QTOF/MS spectra of (a) RA standard in methanol (100 μg mL^{-1}), (b) non-spiked AE sample, and (c) AE sample spiked with RA at 100 μg mL^{-1}.

be well-suited to QC of health functional foods derived from *A. rugosa*. A relatively high price of tilianin with limited accessibility also suggested necessity for an analytical method with an alternative marker compound for QC of AEs.

In our preliminary experiments on AE samples, RA, which was identified by UHPLC-QTOF/MS analysis (*Fig*. 1), showed high peak areas comparable to but more steadily than tilianin among various batches (data not shown). RA is one of the major compounds found in *A. rugosa*,[11] with many pharmaceutical effects.[18-20,26,27] It is easy to acquire at a relatively low price compared to tilianin. Consequently, RA was selected as a QC marker candidate for AEs of *A. rugosa*, and a quantitative analytical method was established as described below.

3.2. Optimization of the HPLC conditions

AE samples and RA standard solutions were monitored in the range of 210–400 nm by HPLC-PDA, showing that RA had strong absorbance at 328 nm. Thus, the detection wavelength was set at 330 nm. Phenomenex Gemini C_{18} columns with two different lengths (150 × 4.6 mm and 250 × 4.6 mm, 5 μm) were compared; a longer column could yield baseline separation of RA from other interferences. When the column temperature was increased from room temperature to 35 °C, the RA peak resolution decreased. Thus, room temperature (~25 °C) was selected for the column temperature. Mixtures of water and organic solvent (MeOH or ACN) with 0.5 %, 1 %, and 2 % AA concentrations were compared as the mobile phase. As a result, a gradient elution of aqueous mixture of ACN containing 2 % AA showed the best resolution of RA peak. Under the final HPLC conditions described in section 2.2, RA was eluted at 29.3 min, having no interferences in vicinity.

3.3. Method validation

The established method was validated according to the guidelines of AOAC and Korea Drug and Food Administration. The specificity was assessed by comparing the chromatograms and PDA spectra of non-spiked AE sample, spiked AE sample, and RA standard solutions (*Figs*. 2 and 3). The method was linear for RA in the range of 50–300 μg mL^{-1} (r^2 =

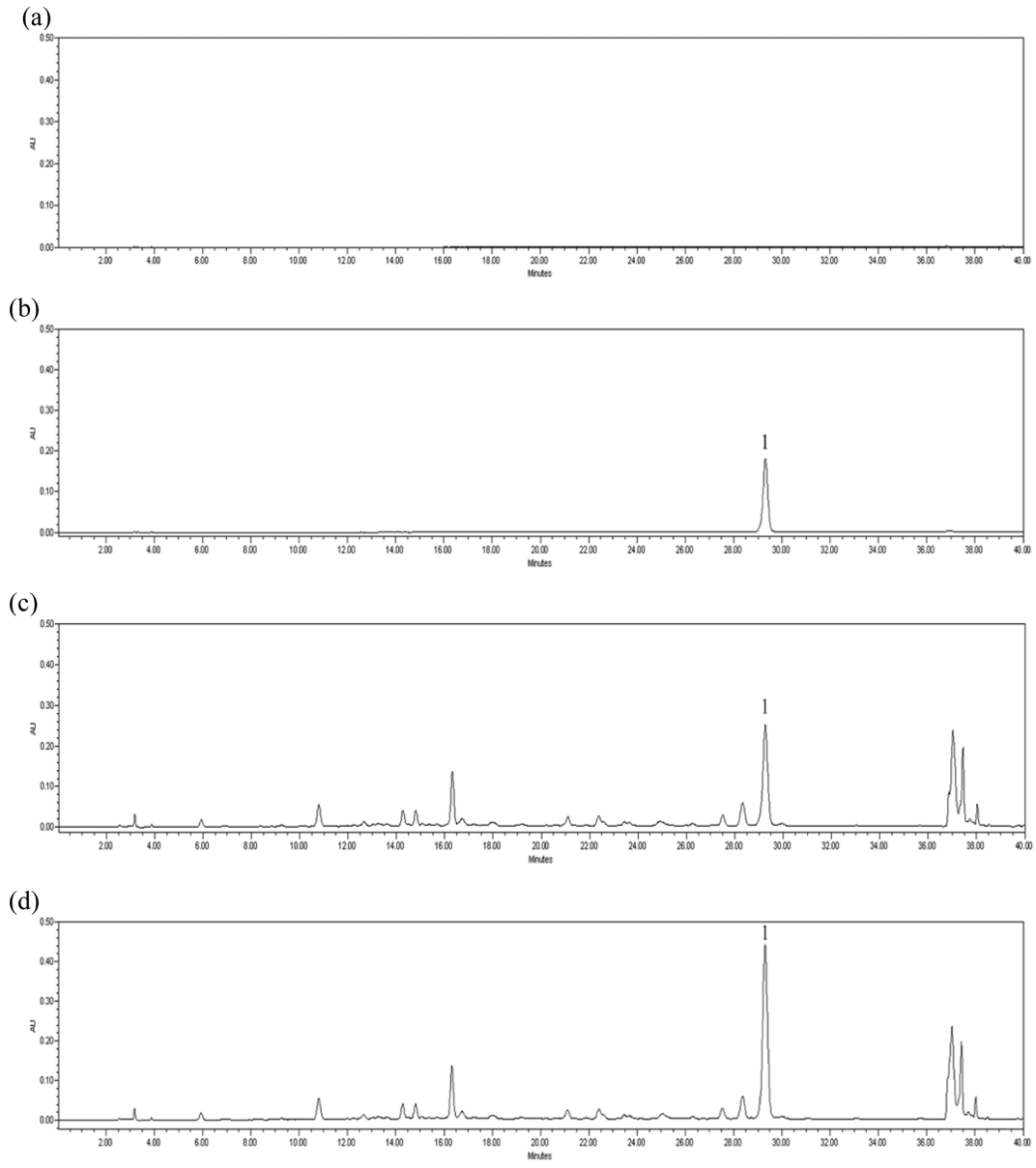

Fig. 2. HPLC-UV chromatograms of (a) methanol, (b) RA standard in methanol (100 μg mL^{-1}), (c) non-spiked AE sample, and (d) AE sample spiked with RA at 100 μg mL^{-1}. Peak ID: 1, RA.

0.9994). The calculated LOD and LOQ values were 1.93 μg mL^{-1} and 5.84 μg mL^{-1}, respectively (*Table* 1). LOD and LOQ values could be lowered with a conventional LC-MS/MS system operated at multiple reaction monitoring (MRM) mode. A rough approximation was made based on the signal-to-noise ratios using an ultra-high performance liquid chromatograph coupled to triple quadrupole mass spectrometer,[28] suggesting an LOQ that is much smaller than 100 ng mL^{-1}.

The criteria of the AOAC guidelines request that the intra-day, inter-day, and inter-analyst precisions should be below 3 %, 6 %, 6 % RSD, respectively, when the content is 0.1 %. The precisions were

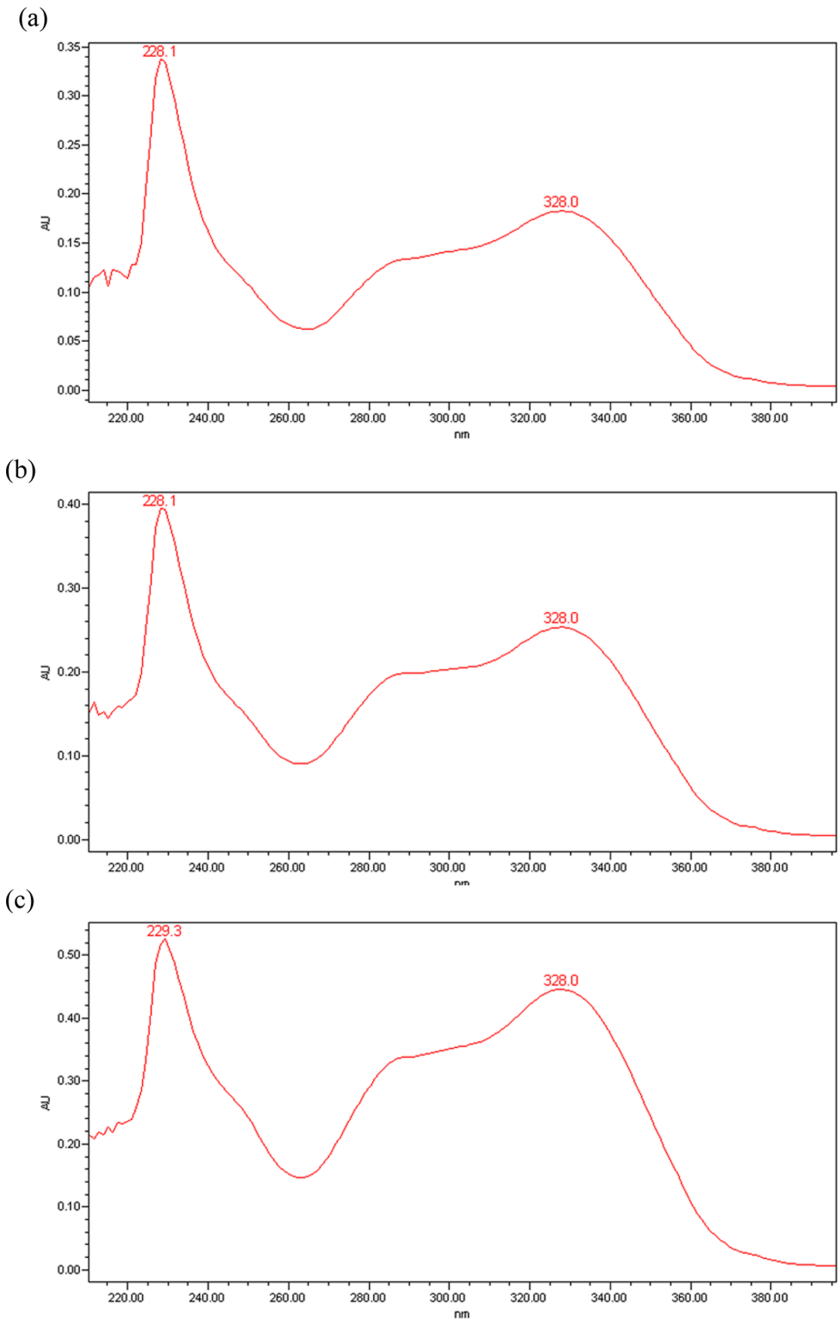

Fig. 3. HPLC-PDA spectra of (a) RA standard in methanol (100 μg mL^{-1}), (b) non-spiked AE sample, (c) AE sample spiked with RA standard at 100 μg mL^{-1}.

0.14–0.91 % RSD (intra-day), 1.13–1.40 % RSD (inter-day), and 1.94 % RSD (inter-analyst) (*Table* 2). According to the AOAC guidelines, the desirable accuracy should reside between 90 and 108 % at 0.1 % content. The accuracy values were 93.3–95.9 % with ≤ 1.21 % RSD (*Table* 3). Hence, all of the validation results met the criteria required by the guidelines, indicating that the established method is

Table 1. Linearity of the established method ($n = 3$)

Linearity range (μg mL^{-1})	Regression equation	Coefficient of determination r^2	LOD[a] (μg mL^{-1})	LOQ[b] (μg mL^{-1})
50 – 300	y = 27249x – 69183	0.9994	1.93	5.84

[a]Limit of detection.
[b]Limit of quantitation.

Table 2. Intra- and inter-day precisions of the proposed method

Spiked concentration (μg mL^{-1})	Intra-day % RSD ($n = 5$)	Inter-day % RSD ($n = 5\times3$)
0	0.91	1.13
50	0.14	1.14
100	0.51	1.40

specific, linear, precise, and accurate.

3.4. Application of the proposed method to real *A. rugosa* samples

The established method was applied to various samples, *i.e*., different batches of AE samples prepared in bulk and lab scale. The three bulk samples were found to contain 6.69 (± 0.03), 5.92 (± 0.01) and 6.62 (± 0.05) mg g^{-1} for the batch #1, #2, and #3, respectively. These contents correspond to 0.67, 0.59, and 0.66 mg g^{-1}, respectively, for the dried raw materials of *A. rugosa*, because the bulk extraction yield was 10 %. Based on the ANOVA results, the RA contents in the batches #1 and #3 were not significantly different ($p > 0.05$), while those in the batches #1 and #3 and the batches #2 and #3 were significantly different ($p < 0.0001$ for both cases). When three batches of the lab scale AE samples were analyzed using the proposed method, similar chromatographic results could be acquired (*Fig.* 4), with the RA contents measured at 0.48 (± 0.02) mg g^{-1}. A relatively large difference in the sample preparation scale appeared to be responsible for the differing RA contents in the two types of samples. These results still suggest that the proposed method is applicable to various kinds of *A. rugosa* samples that contain varying levels of RA.

4. Conclusions

In this study, RA was selected as a quantification marker compound, and an HPLC-UV method was established for the aqueous extracts of *A. rugosa*. The proposed method was proven valid in terms of specificity, linearity, precision, and accuracy upon

Table 3. Accuracy of the proposed method ($n = 5$)

Original sample concentration (μg mL^{-1})	Added concentration (μg mL^{-1})	Founded concentration (μg mL^{-1})	% Recovery	% RSD
131.2	50	177.86	93.32	0.54
	100	226.35	95.15	1.21
	150	275.10	95.93	0.54

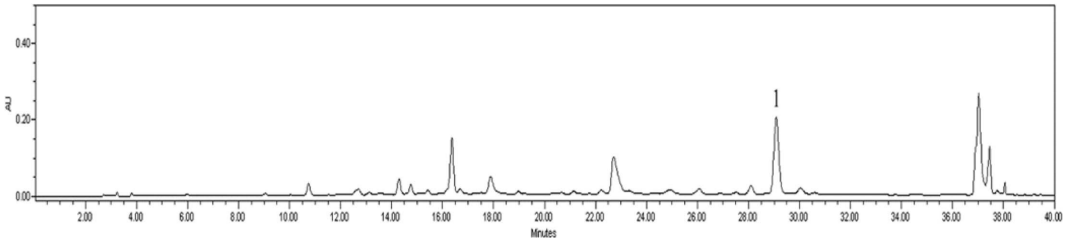

Fig. 4. An HPLC chromatogram of the AE sample prepared in lab scale. Peak ID: 1, RA.

thorough validation processes. It could be successfully applied to measure the RA contents in various types of *A. rugosa* samples prepared in bulk and lab scale. Therefore, the proposed method can be used as a readily applicable method for QC of health functional foods containing the aqueous extracts of *A. rugosa*.

Acknowledgements

This work was supported by the World Class 300 Project R&D grant (Grant No. S2435140) funded by the Korea Small and Medium Business Administration (SMBA) in 2018.

Conflicts of Interest

J. H. Kim and J. H. Geum are employed by COSMAX Inc. that might benefit from the results of the study. All other authors report no conflicts of interest relevant to this study.

References

1. Y. B. Kim, J. K. Kim, M. R. Uddin, H. Xu, W. T. Park, P. A. Tuan, X. Li, E. Chung, J.-H. Lee, and S. U. Park, *PLoS One,* **8**(5), e64199 (2013).
2. H. Gong, X. Zhou, M. Zhu, X. Ma, X. Zhang, and S. Tian, *J. Essent. Oil. Bear. Pl.,* **15**(4), 534-538 (2012).
3. Y. Oh, H.-W. Lim, Y.-H. Huang, H.-S. Kwon, C. D. Jin, K. Kim, and C.-J. Lim, *J. Photochem. Photobiol. B: Biol.,* **163**, 170-176 (2016).
4. S. Shin and C. A. Kang, *Lett. Appl. Microbiol.,* **36**(2), 111-115 (2003).
5. S. Shin, *Arch. Pharmacal Res.,* **27**(3), 295-299 (2004).
6. J. Kim, *J. Plant. Biol.,* **51**(4), 276-283 (2008).
7. G. Haiyan, H. Lijuan, L. Shaoyu, Z. Chen, and M. A. Ashraf, *Saudi. J. Biol. Sci.,* **23**(4), 524-530 (2016).
8. K. J. Park, S. Yang, Y. A. Eun, S. Y. Kim, H. H. Lee, and H. Kang, *Pharm. Biol.,* **40**(3), 189-195 (2002).
9. H. K. Kim, H.-K. Lee, C.-G. Shin, and H. Huh, *Arch. Pharmacal Res.,* **22**(5), 520-523 (1999).
10. P. Cao, P. Xie, X. Wang, J. Wang, J. Wei, and W.-y. Kang, *BMC Complement. Altern. Med.,* **17**(1), 93 (2017).
11. K. T. Desta, G. S. Kim, Y. H. Kim, W. S. Lee, S. J. Lee, J. S. Jin, A. Abd El-Aty, H. C. Shin, J. H. Shim, and S. C. Shin, *Biomed. Chromatogr.,* **30**(2), 225-231 (2016).
12. J.-J. Hong, J.-H. Choi, S.-R. Oh, H.-K. Lee, J.-H. Park, K.-Y. Lee, J.-J. Kim, T.-S. Jeong, and G. T. Oh, *FEBS Lett.,* **495**(3), 142-147 (2001).
13. H. Q. Li, Q. Z. Liu, Z. L. Liu, S. S. Du, and Z. W. Deng, *Molecules,* **18**(4), 4170-4180 (2013).
14. I. A. S. V. Packiavathy, P. Agilandeswari, K. S. Musthafa, S. K. Pandian, and A. V. Ravi, *Food Res. Int.,* **45**(1), 85-92 (2012).
15. G. Ruberto and M. T. Baratta, *Food Chem.,* **69**(2), 167-174 (2000).
16. K. P. Devi, S. A. Nisha, R. Sakthivel, and S. K. Pandian, *J. Ethnopharmacol.,* **130**(1), 107-115 (2010).
17. S. Zielińska and A. Matkowski, *Phytochem. Rev.,* **13**(2), 391-416 (2014).
18. T. Hamaguchi, K. Ono, A. Murase, and M. Yamada, *Am. J. Pathol.,* **175**(6), 2557-2565 (2009).
19. V. Swarup, J. Ghosh, S. Ghosh, A. Saxena, and A. Basu, *Antimicrob. Agents Chemother.,* **51**(9), 3367 (2007).
20. J. Lee, E. Jung, Y. Kim, J. Lee, J. Park, S. Hong, C. G. Hyun, D. Park, and Y. S. Kim, *Br. J. Pharmacol.,* **148**(3), 366-375 (2006).
21. O. Hernández-Abreu, L. Durán-Gómez, R. Best-Brown, R. Villalobos-Molina, J. Rivera-Leyva, and S. Estrada-Soto, *J. Ethnopharmacol.,* **138**(2), 487-491 (2011).
22. O. Hernández-Abreu, M. Torres-Piedra, S. García-Jiménez, M. Ibarra-Barajas, R. Villalobos-Molina, S. Montes, D. Rembao, and S. Estrada-Soto, *J. Ethnopharmacol.,* **146**(1), 187-191 (2013).
23. S. Li, Q. Han, C. Qiao, J. Song, C. L. Cheng, and H. Xu, *Chin. Med.,* **3**(1), 7 (2008).
24. Y. Jin, J. Zhao, K. M. Jeong, D. E. Yoo, S. Y. Han, S.-Y. Choi, D.-H. Ko, H.-j. Kim, N.-H. Sung, and J. Lee, *Arch. Pharmacal Res.,* **40**(1), 49-56 (2017).
25. H. M. Oh, Y. J. Kang, Y. S. Lee, M. K. Park, S. H. Kim, H. J. Kim, H. G. Seo, J. H. Lee, and K. C. Chang, *J. Ethnopharmacol.,* **103**(2), 229-235 (2006).
26. N. Erkan, G. Ayranci, and E. Ayranci, *Food Chem.,* **110**(1), 76-82 (2008).
27. H. P. Bais, T. S. Walker, H. P. Schweizer, and J. A. Vivanco, *Plant. Physiol. Bioch.,* **40**(11), 983-995 (2002).
28. Y. Jin, J. Zhao, E. M. Kim, K. H. Kim, S. Kang, H. Lee, and J. Lee, *Molecules*, **24**(9). pii: E1735 (2019).

Authors' Positions

Keunbae Park : Graduate student
Dasom Jung : Graduate student
Yan Jin : Postdoctoral researcher
Jin Hak Kim : Researcher (employee) of COSMAX NS Inc
Jeong Ho Geum : Researcher (employee) of COSMAX NBT Inc.
Jeongmi Lee : Associate Professor