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산지별 밤꿀에 함유된 Kynurenic Acid의 정량 분석과 분석법 검증

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Quantitative Analysis of Kynurenic Acid in Chestnut Honey from Different Regions and Method Validation

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Abstract – Chestnut honey is a sweet dark-colored honey with a distinct bitter aftertaste. It contains numerous phenolic compounds and alkaloids and is noted for its antioxidant and anti-inflammatory activities. However, it has been established that there are differences in the composition and activity of chestnut honey constituents depending on the region of origin, the sources of which warrant further research. In this study, we analyzed the kynurenic acid (KA) contents in chestnut honey produced in nine different regions in Korea, using high-performance liquid chromatography in conjunction with ultraviolet detection, and validated the analytical method developed. Use of a reverse-phase column and detection at a wavelength of 240 nm were found to be optimal for the detection of KA. Similar evaluation of an optimal method for extracting KA from chestnut honey revealed that extraction using 10% EtOH at 20 times the sample volume over a 6 h period was the most suitable for obtaining a high content of KA. Among the nine regional chestnut honeys assessed, KA content was found to be highest in the "Gongju" sample (1.14 mg/g), followed by that in the "Cheongdo" and "Damyang" samples. Validation of the KA analytical method revealed a good analyte linearity, with a correlation coefficient (r^2) of 0.9995, an accuracy of between 92.37% and 107.35%, and good precision (RSD ≤ 1.05%). Our findings in this study, based on a validated quantitative analytical method for KA, could make an important contribution to establishing a data profiling procedure for characterizing chestnut honeys produced in different regions, and may also provide basic data for the identification of functional honey.

Keywords – Chestnut honey, HPLC-UV, Kynurenic acid, Method validation, Quantitative analysis

Honey is a sweet, viscous food produced by honeybees and some other bees, which has been used for centuries in cooking and baking, and as a sweetener added to beverages.[1,2] It contains sugars, vitamins, minerals, volatiles, organic acids, and enzymes,[3] and the findings of numerous studies have indicated that honey has a range of valuable biological activities, including anticancer,[4] gastro-protective effects.[5] In particular, since ancient times, honey has been used as an antibacterial agent for the treatment of burns, wounds, and ulcers.[6,7]

Honey is generally classified according to the floral source from which nectar is obtained, and further categorized on the basis of specific processing or packaging. Polyfloral honey, for example, is produced from the nectar of multiple flower types, whereas mono/unifloral honey is derived from the nectar of a single type of flower and is typically characterized by distinct colors and flavors that reflect the differences in nectar sources.

An example of the latter type of honey is chestnut honey, which is noted for its dark color and bitter aftertaste, and as the name suggest, it tis derived from the blossoms of chest-

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nut trees. Chestnuts are deciduous tree in the family Fagaceae, with origins distributed over the four continents of Asia, Europe, America, and Africa, the four representative types of which are *Castanea crenata* S. et Z. in Korea and Japan, *Castanea mollissima* Blume in China, *Castanea sativa* Miller in Europe, and *Castanea dentata* (Marshall) Borkhausen in the USA. In Korea, chestnut is one of the various types of honey are harvested, along with acacia, jujube, citrus, honeysuckle, rapeseed, and multi-floral honey. As previously mentioned, one of the distinctive characteristics of chestnut honey is its dark color, which is taken to be indicative of polyphenol content, with darker honeys containing larger amounts of polyphenols than light-colored honeys, and having more pronounced antioxidant and antimicrobial activities.[8,9] In addition, compared with light-colored honeys, darker honeys contain large amounts of nitrate (NO_3^-), which is considered to confer protective effects against gastrointestinal ailments. In the past two decades, a number of studies have examined the anti-inflammatory,[10] and antioxidant properties of chestnut honey,[11] whereas others have assessed the effects of this honey in the treatment of burn wounds and evaluated antimelanogenic effects.[12,13]

In addition to the aforementioned polyphenols, chestnut honey contains a diverse range of nutrients and secondary metabolites, including alkaloids, which may have potential utility as lead compounds in pharmaceuticals products.[14] Among these constituents is kynurenic acid (KA), a product of tryptophan metabolism that is noted for its neuroactive activity.[15] KA is assumed to acts as an endogenous antagonist at excitatory amino acid receptors, such those of *N*-methyl-D-aspartate (NMDA), the activation of which is known to promote neuronal damage, eventually resulting in neurodegenerative diseases, such as Alzheimer's disease. In addition, KA has been demonstrated to play a role in controlling midbrain dopaminergic activity, and thus may have potential utility as a pharmaceutical agent for the treatment of neurological disorders, such as schizophrenia.[16] Several previous studies have reported the detection of quinoline alkaloids, mainly KA, in chestnut honey,[17-19] with some finding notably large amounts of KA.[20,21] Other studies have sought to identify potential marker compounds in honey that are unique to particular unifloral types, among which, KA in chestnut honey is considered a strong candidate.[22,23] In a further study, Ronsisvalle *et al*. (2019) evaluated the antibacterial and antioxidant activities and chemical composition of two chestnut honeys produced in different geographical regions, and compared the findings with those reported in the literature for other types of honey, including Manuka honey.[24] Both chestnut honeys showed interesting activities, with neither the antioxidant nor antibacterial activities of either being inferior to those of Manuka honey. Manuka honey is also a monofloral-type honey produced from the nectar of *Leptospermum scoparium*, mainly in New Zealand.[25] It contains bioactive compounds, such as methyl syringate, leptosin, glyoxal, and methylglyoxal, which have myeloperoxidase inhibition and antibacterial activities, and is accordingly particularly noted as a functional honey.[26,27] Consequently, it can be anticipated that chestnut honey would be a promising research target for the development of a functional honey containing unique intrinsic bioactive compounds.

Notable in this regard, however, is that the composition of constituents, and thus often the biological activity, of honey is mainly dependent on its geographical or floral source of origin,[28,29] and accordingly, it may be difficult to obtain consistent bioactive activities for clinical application. Schievano *et al*. (2013) isolated and characterized 23 compounds, including several classes of terpenes, flavonoids, and organic acids, from extracts of unifloral (chestnut, acacia, linden, eucalyptus, orange, and honeydew) and polyfloral honeys.[30] Among these compounds, deoxyvasicinone, 2-quinolone, γ-LACT-3-PKA, and 4-quinolone, which are known as marker compounds or have several therapeutic effects, including antifungal and antibacterial activities, were identified in chestnut honey.[31,32] Consequently, from the perspective of potential clinical application, it is important to take into consideration the origin and variety of a particular honey, which accordingly necessitates the quantification or determination of the respective bioactive constituents. In addition, although there have previously been a few studies regarding the botanical origin of chestnut honey in other countries,[11,22,33] there has to date been little research on the chemical composition and biological activity of chestnut honey originating from different regions in Korea.

In this study, we thus analyzed the KA profiles of chestnut honey produced in nine different regions of Korea using high-performance liquid chromatography in conjunction with ultraviolet detection (HPLC-UV) and validated the performance of an analytical method for KA.

Materials and Methods

Collection of chestnut honey – Samples of chestnut honey

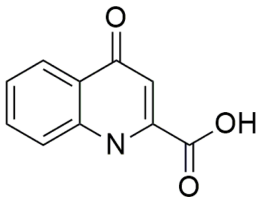


Fig. 1. The chemical structure of KA.

produced in nine different regions of Korea (Pocheon, Chuncheon, Yeongdong, Chungju, Gongju, Jinan, Damyang, Cheongdo, and Geochang) were provided by KEDEM Inc., Korea, a voucher specimen of each of which has been deposited in the herbarium of the Department of Plant Science and Technology, Chung-Ang University, Korea.

Instruments, chemicals, and reagents – Chromatographic analysis was performed using an HPLC system equipped with a UV detector, pump, and an auto-sampler. A KA standard (Fig. 1) was obtained from Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea. HPLC-grade solvents (water and acetonitrile) were purchased from J. T. Baker (Avantor, Radnor, PA, USA). Trifluoroacetic acid (TFA; 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and ethanol (EtOH) for extraction was purchased from Samchun Pure Chemicals (Pyeongtaek, Korea).

Preparation of chestnut honey extracts – To establish an optimum extraction method for the validation of chestnut honey, we examined the effects of different solvent composition, solvent volume, and extraction time under reflux extraction conditions, for purpose of which, we selected "Damyang" chestnut honey. For each extraction, we used 5 g samples of honey, which were extracted using different ratios of water and EtOH (100% Water, 10% EtOH, and 30% EtOH) as solvents, the volume of which were 50 and 100 mL, which were 10 and 20 times the sample volume, respectively. Samples were extracted for either 3 or 6 h. In addition to samples of honey extracts, we also analyzed samples of unextracted and freeze-dried chestnut honey, and compared the respective KA contents. Having undergone extraction, the samples were filtered, placed in an oven (55°C) for 1 day, and freeze dried to obtain freeze-dried extracts.

Preparation of stock solutions for HPLC analysis – For quantitative analysis of KA, the experimental stock solutions were prepared by dissolving each chestnut honey sample in MeOH (100 mg/mL), sonicating for 20 min, and filtering through a 0.45-μm polyvinylidene difluoride membrane. A stock solution of KA standard (1 mg/mL) was prepared following the same procedure.

HPLC conditions – For quantitative analysis of the KA content in the nine chestnut honeys, samples were analyzed using an HPLC system equipped with a PerkinElmer Flexar QUATERNARY Pump (Waltham, MA, USA), auto-sampler, and PerkinElmer PDA LC Detector. For sample separation, we used a reverse-phase YMC Pack Pro C18 column (25 cm × 4.6 mm, 5 μm), with an injection volume of 10 μL. KA contents were determined based on UV detection at a wavelength of 240 nm. The flow rate was set at 1 mL/min, and the temperature of the column was maintained at 30°C. The mobile phase consisted of 0.2% TFA in water (A) and acetonitrile (B), and gradient elution program used was as follows: 95% A at 0 min, 82% A at 10 min, 82% A at 20 min, 40% B at 30 min, 100% B at 35 min, 100% B at 40 min, 5% B at 45 min, and 5% B at 55 min. All determinations were performed in triplicate.

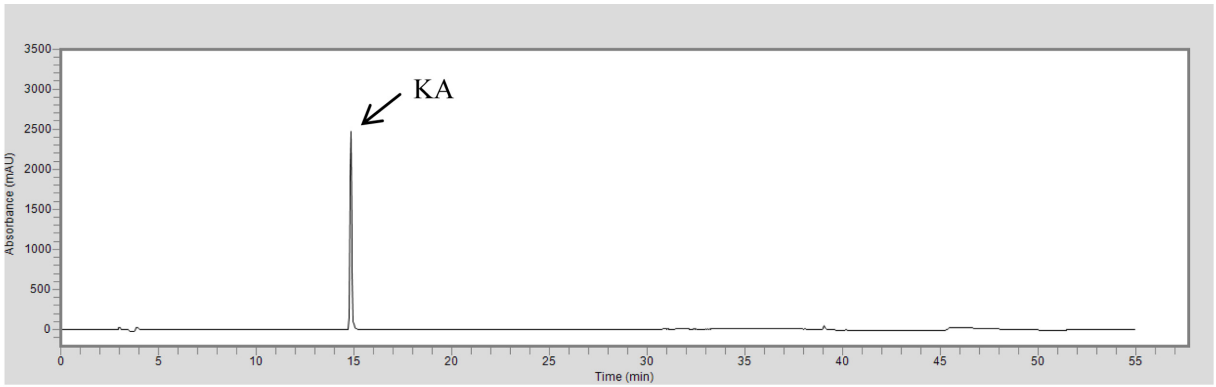
Method validation – The HPLC-UV method used to quantify KA in chestnut honey was validated in terms of its specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. Analysis was performed using an HPLC system (1290 Infinity II; Agilent Technology, Santa Clara, CA, USA) equipped with a pump, UV detector, and auto-sampler. The analytical method was validated following the guidelines provided by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH).[34] The specificity was assessed to determine potential interferences with the analyte signal. For the purposes of peak comparisons, we confirmed that the retention times and UV spectra of the standard compound and samples were identical. The linearity was confirmed using five concentrations of the standard compound (7.8125-125 μg/mL) performed in triplicate. A calibration curve was plotted using chromatogram peak areas measured at 240 nm against the known concentrations of the standard solutions. The standard curve was analyzed using linear least-squares regression, and the correlation coefficient (r^2) was used to validate the linearity. The LOD value is the lowest content of analyte that can be reliably distinguished from the base line, and the LOQ is the lowest content of analyte that can be quantified with acceptable repeatability and accuracy, the values of both of which were determined from the standard deviation of the calibration curve intercept (σ) and the slope (S). Although different methods can be used to estimate the LOQ, in general values

are designated as three times that of the LOD value, with the following formulas being the most commonly used: LOD = 3.3 (σ/S) and LOQ = 10 (σ/S). The accuracy of the analytical method was evaluated by conducting recovery tests in which samples were spiked with three different concentrations of standard mixtures (mg/g), with subsequent calculation of the recovery (%) of the spiked compound in the sample. Determinations for each sample and each concentration were repeated five times. To validate method precision, samples at three different concentrations (25, 50, and 100 mg/mL) were used for intra-day analysis (repeatability), and a single concentration (50 mg/mL) for inter-day analysis (intermediate precision). Determinations for each sample and each concentration were repeated five times. Precision was expressed in terms of the percentage relative standard deviation (% RSD) of the standard on three separate days and on the same day.

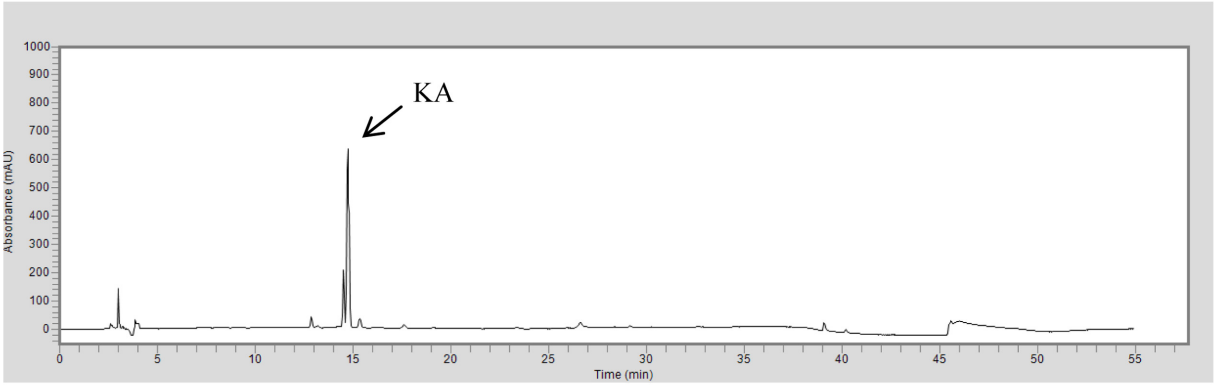
Calibration curve – The working solutions used to construct the calibration curves were prepared by serially diluting the stock solution to the desired concentrations (15.625-250 μg/mL). The linearity of the calibration curve was determined based on the correlation coefficient (r^2), and the calibration function of KA was calculated using the peak area (Y) and concentration (X, μg/mL). The values are presented as the mean value ± standard deviation (n = 3).

Results and Discussion

Quantitative assessment of the KA content of chestnut honey produced in nine different regions was performed using HPLC-UV, with a wavelength of 240 nm being found to be optimal for KA detection. HPLC chromatograms of all chestnut honey samples showed similar patterns, with those of the standard and "Damyang" sample being shown as representative in Fig. 2. KA contents were determined by plotting chromatogram peak areas against KA concentrations of the prepared standard curve. Linear regression analysis revealed a correlation coefficient (r^2) of 0.9999. The contents of KA in honey from the nine different regions are shown in Table I, which indicates that the "Gongju" sample had the highest con-



(A)



(B)

Fig. 2. HPLC chromatograms of the KA standard (A) and chestnut honey "Damyang" (B).

Table I. Contents of KA in chestnut honey produced in nine different regions of Korea

Sample	Content (mg/g)
Pocheon	0.61 ± 0.02
Chuncheon	0.26 ± 0.00
Yeongdong	0.79 ± 0.05
Chungju	0.68 ± 0.04
Gongju	1.14 ± 0.04
Jinan	0.41 ± 0.01
Damyang	0.82 ± 0.03
Cheongdo	0.87 ± 0.05
Geochang	0.47 ± 0.02

tent (1.14 mg/g), followed by the "Cheongdo" (0.87 mg/g) and "Damyang" (0.82 mg/g) samples.

The Gongju region of Korea is well renowned for its chestnut trees, with chestnut-related specialties being heavily promoted, and studies on product development using Gongju chestnut honey are still ongoing.[35] Approximately 24% of the areas in Gongju in which chestnut is cultivated are north facing and characterized by a sandy loam soil,[36] with trees being grown on a particularly large-scale in an area referred to as "Jeongan-myeon". Given this large-scale cultivation, it can be expected that the content and quality of components, including KA, are superior to those of other regions in which chestnuts are grown on a relatively small scale. Soto *et al*. (2011) quantified the contents of KA and other tryptophan metabolites in 17 honey samples, and accordingly detected higher amounts of KA in chestnut honey (103.5-141.1 mg/kg) than in other honey samples.[37] Similarly, Turski *et al*. (2016) evaluated the content of KA in different sources, including chestnut honey, honey products, and chestnut tree parts, and accordingly demonstrated that KA contents were significantly higher in chestnut honey than in the honey derived from other sources, including pine, lavender, sunflower, ranging from 129 to 601 μg/g.[38] Comparatively, contents were relatively low in chestnut honey products (0.008-0.077 μg/g), whereas among tree parts, the content was found to be high in male flowers. In a further study, Ronsisvalle *et al*. (2019) reported qualitative and quantitative detection of the components of two chestnut honeys of different origins, among which, the KA contents of two chestnut honey samples were 0.199 and 0.046 mg/g, respectively.[24] These values are lower than those obtained in the present study, and the contents of phenolic acids were found to be higher than those of KA. These findings accordingly indicate that the content of KA in chestnut honey differs depending on botanical origin, thereby highlighting the necessity of appropriate product profiling.

Table II. Contents of KA in chestnut honey obtained using different extraction methods

Sample	Content (mg/g)
W-3H-10	0.82 ± 0.07
W-6H-10	0.88 ± 0.01
10E-3H-10	0.87 ± 0.04
10E-6H-10	0.89 ± 0.02
30E-3H-10	0.86 ± 0.01
30E-6H-10	0.85 ± 0.02
W-3H-20	0.86 ± 0.03
W-6H-20	0.84 ± 0.07
10E-3H-20	0.84 ± 0.01
10E-6H-20	0.93 ± 0.03
30E-3H-20	0.89 ± 0.03
30E-6H-20	0.90 ± 0.03
Damyang chestnut honey	0.77 ± 0.08
Freeze-dried chestnut honey	0.76 ± 0.09

W: water; 10E: 10% EtOH; 30E: 30% EtOH; 3H: 3 h; 6H: 6 h; 10: 10 times the sample volume; 20: 20 times the sample volume

Prior to method validation, we performed quantitative analysis to determine which extraction conditions yielded the highest content of KA in chestnut honey, and accordingly established that samples extracted using 10% EtOH at 20 times the sample volume for 6 h contained the highest KA contents (Table II). Thus, the "10E-6H-20" extract was selected as the sample for validating the analytical method used for determining chestnut honey KA content.

HPLC chromatograms obtained for KA and a chestnut honey sample using the validated HPLC-UV analytical method with detection at of 240 nm are presented in Fig. 3, with both showing a KA retention time of 14.1 min. Although we also detected a second smaller peak close to that of KA, this did not interfere with the validation. A comparison of standard and sample chromatograms clearly indicated identical retention times and UV spectra, respectively, thereby confirming the specificity of our developed analytical method.

For the evaluation of linearity, we constructed a calibration curve of KA using five different standard solution concentrations (7.8125-125 μg/mL, n = 3) with the regression equation and the correlation coefficient (r^2 = 0.9995) being calculated by plotting the peak areas (Y) of the prepared concentrations (X) expressed in μg/mL. This revealed a strong linear correlation between the peak area and concentration of KA, with corresponding LOD and LOQ values

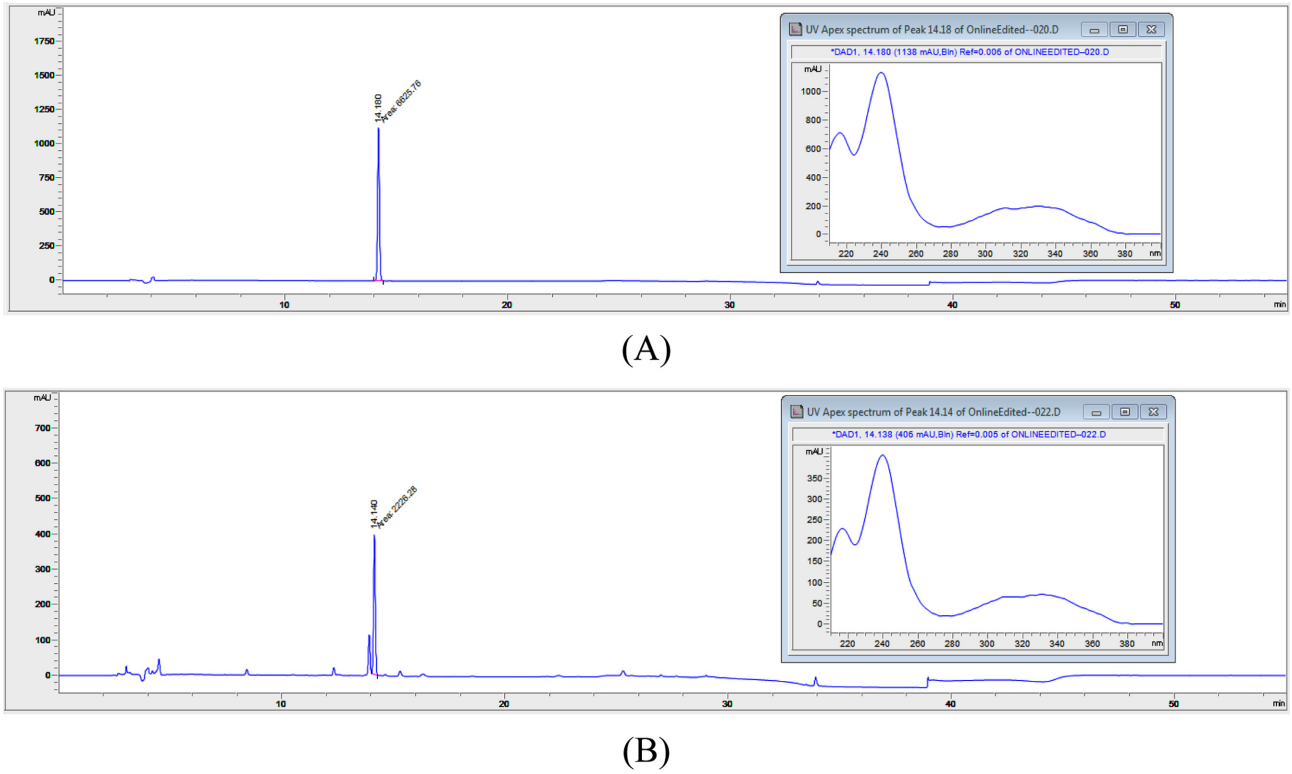


Fig. 3. HPLC chromatograms and specificity for KA (A) and chestnut honey (B).

Table III. Linearity, LOD, and LOQ values obtained for KA

Compound	t_R (min)	Range (μg/mL)	Calibration equation[a]	$r^{2\,b}$	LOD (μg/mL)	LOQ (μg/mL)
KA	14.1	7.8125-125	Y = 51.848X + 133.17	0.9995	0.711	2.156

[a]Y = peak area, X = concentration of the standard (μg/mL)
[b]r^2 = correlation coefficient for five data points in the calibration curve
t_R = retention time

Table IV. Accuracy of the validated analytical method for determining KA

Compound	Spiked Content (mg)	Recovery (%)					Average (%)	RSD (%)
		1st	2nd	3rd	4th	5th		
KA	0.0625	97.85	100.99	100.17	100.88	100.55	100.09	1.29
	0.03125	107.40	109.07	106.30	106.54	107.45	107.35	1.01
	0.015625	91.74	92.94	92.33	91.85	92.99	92.37	0.64

RSD = relative standard deviation

of 0.711 and 2.156 μg/g, respectively (Table III).

These results indicate the minimum concentration of KA that can be determined and quantified using the validated analytical HPLC method. In terms of accuracy, KA recovery rates ranged from 92.37% to 107.35%. Values for the average recovery rate and RSD of KA at each spiked concentration are shown in Table IV. The values thus determined were confirmed to be comparable to the true values, thereby indicating good recovery. Collectively, these finding thus indicate that the analytical method developed in the present study can be used as a method for the accurate analysis of KA in honey samples.

Furthermore, we evaluated analytical precision by performing repeated intra- and inter-day measurements of samples at three different concentrations and determining RSD values. The coefficient of variance for the intra- and inter-

Table V. Intra- and inter-day precision of the validated analytical method for determining KA

Compound	Intra-day (n = 5)			Inter-day (n = 5)		
	Concentration (mg/mL)	Measured content (mg/g)	RSD (%)	Concentration (mg/mL)	Measured content (mg/g)	RSD (%)
KA	25	0.858	0.54	50	0.830	0.57
	50	0.828	1.05		0.828	0.63
	100	0.830	0.19		0.832	0.41

RSD = relative standard deviation

day precision values for KA were found to range from 0.19% to 1.05%, and from 0.41% to 0.63%, respectively (Table V). The fact that these RSD values were lower than 2%, indicates the reliability of the analytical method for quantifying KA. Similarly, Kim *et al*. (2021) have previously established a chromatographical method for the quality control of chestnut honey.[39] Their validated UPLC method for KA in chestnut honey simply entailed dissolving chestnut honey in MeOH, with detection at a wavelength of 240 nm. Using this method, they detected the highest KA content of approximately 0.589 mg/g in a honey sample derived from Ansan.

Conclusion

In this study, we conducted quantitative analyses of the KA contents of chestnut honey produced in nine different regions of Korea, using HPLC in conjunction with UV detection, and validated the developed analytical method. We accordingly identified regional differences in the KA contents of chestnut honey, with the highest levels being detected in a sample of honey produced in Gongju, followed by those in honeys from Cheongdo and Damyang. Method validation revealed good analyte linearity with a correlation coefficient (r^2) of 0.9995, an accuracy of 92.37% to 107.35%, and high precision (RSD ≤ 1.05%). Our findings in this study could make an important contribution to establishing a data profiling procedure for characterizing chestnut honeys produced in different regions and may also provide basic data for the identification of functional honey.

Acknowledgments

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