J Appl Biol Chem (2023) 66:10, 67–72
https://doi.org/10.3839/jabc.2023.010

Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Bioactive Materials

Development of an analytical method for the quantification of oleanonic acid from mastic gum using HPLC/PDA

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Received: 11 January 2023 / Accepted: 5 February 2023 / Published Online: 21 February 2023

Abstract A simple and accurate method was developed for the quantitative analysis of oleanonic acid (OA) from mastic gum. The analysis was carried out using reverse-phase high-performance liquid chromatography combined with a photodiode array detector (HPLC/PDA). Our optimized method was validated by measuring various parameters, using an INNO C18 column fitted with a gradient elution system. The results revealed limits of detection and quantification of 0.34 and 1.042 μg/mL, respectively. The OA calibration curve exhibited excellent linearity over the concentration range of 0.0625 to 2.0 mg/mL, with r^2 =0.9996. Accuracy tests revealed a high recovery rate of 99.44-103.66%, with precision values below 0.15%. These results suggest that the present analytical method can identify and quantify OA in mastic gum with high precision. The HPLC approach developed in this study might be applied to routine analyses and large-scale extraction procedures for OA content quantification.

Keywords High-performance liquid chromatography combined with a photodiode array detector · Mastic gum · Method development · Oleanonic acid · Validation

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Introduction

Pistacia lentiscus, commonly known as mastic tree, is a widespread evergreen shrub native to the eastern Mediterranean [1], and a Greek island (Chios Island) at Aegean Sea is the sole natural growth location for this tree. Its entire production originates from various villages (“Mastichochoria” in Greek), and the cultivation of *P. lentiscus* and sap harvesting are part of the cultural legacy of this region [2]. In 2014, the methods and knowledge of mastic trees cultivation in Chios Island have been registered in the UNESCO Representative List of the Intangible Cultural Heritage of Humanity [3]. Many attempts have been made to transfer the cultivation of mastic trees to other regions, but eventually failed because of the deteriorated quality of the resin produced [4]. The thick calcareous soil of “Mastichochoria” provides optimal conditions for *P. lentiscus* growth and resin production [5].

Mastic gum (MG), an aromatic resin and natural sap, starts to be produced in the fifth year of the tree’s life and reaches a maximum yield of 1 kg after the 12th year [5]. MG collected from incisions in branches and trunks of mastic trees has been utilized to cure oral problems, such as halitosis and tooth decay [6]. MG has been utilized in Greek medicine for almost two millennia, and numerous ancient Greek physicians have praised its curative qualities [6]. Previous studies have explored its antioxidant, anti-inflammatory, anti-bacterial, anti-atherogenic, hepatoprotective, cholesterol-lowering, and glucose lowering properties [7-12] along with its widely used for the treatment of gastrointestinal disorders [13].

Constituent analyses of isolated MG showed that it contains simple phenols and triterpenoids. In detail, the main components of MG are oleanonic acid (OA), isomasticadienonic acid, isomasticadienolic acid, masticadienonic acid, masticadienolic acid, and moronic acid [10, 14–16], and is mainly composed of triterpenic acids, among which OA is the most common and easily commercially available species.

Pentacyclic triterpenoids, which are biosynthesized in plants through squalene cycling, are found throughout the vegetable kingdom and are an important component of many medicinal herbs used in traditional oriental medicine for over 2000 years. Due to their low toxicity, low cost, and accessibility, substances derived from plants have recently garnered considerable attention in the field of health supplement. The remarkable biological and pharmacological properties of several natural triterpenoids sparked interest in the health benefits afforded by these plant-derived substances, including anti-tumor, anti-bacterial, anti-diabetic, anti-inflammatory, hepatoprotective, and immuno-modulatory effects [17-22]. In addition to continuous extraction and studies of natural triterpenoids, new synthetic derivatives with lower toxicity and greater medicinal potential are being developed.

Triterpenoid-containing supplements are becoming increasingly prevalent in the pharmaceutical and functional food industries. Crossoptines A and B from *Crossopteryx febrifuga*, triterpenes glycosides from *Ilex paraguariensis*, and pulchinenoside triterpenes from *Pulsatilla* species have all been patented for a variety of pharmacological uses, including anti-inflammatory and labor pain relief [23]. Several additional triterpenes produced from natural products, as well as their synthetic derivatives, have been patented as potential novel analgesic and anti-inflammatory drugs or nutritional supplements [23]. Therefore, the current investigation was aimed to establish an effective and practical method for quantifying marker compounds in MG by using high-performance liquid chromatography combined with a photodiode array detector (HPLC/PDA). The optimized method was validated by HPLC-PDA analysis of OA in MG. These results could contribute to the development of high value-added MG-derived healthcare products.

Materials and Methods

Materials

MG produced by the Chios Gum Mastic Growers Association (Chios, Greece) in the form of small tears and ground into powder was provided by Vitalabs Co., Ltd. (Seoul, Korea).

Instruments, reagents, and chemicals

Analyses were conducted utilizing an HPLC system (Waters Alliance 2695 Separations Module, MA, USA) with an autosampler, pump, and photodiode array detector (996 PDA Detector, MA, USA). HPLC-grade water, methanol (MeOH), and acetonitrile (ACN) solvents were obtained from J. T. Baker (Phillipsburg, PA, USA). OA (purity: 97.86%) was purchased from the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea (Fig. 1).

Preparation of stock solutions for HPLC

MG powder was ultrasonically extracted at room temperature for 20 min using methanol (MeOH), and the final concentration was adjusted to 10 mg/mL and filtered through a syringe filter (0.45 μm) to prepare sample stock solutions. Standard stock solutions were prepared by dissolving 10 mg of OA to a concentration of 2 mg/mL in MeOH. Calibration curves were generated by analyzing working solutions prepared by diluting stock solutions to the appropriate concentrations.

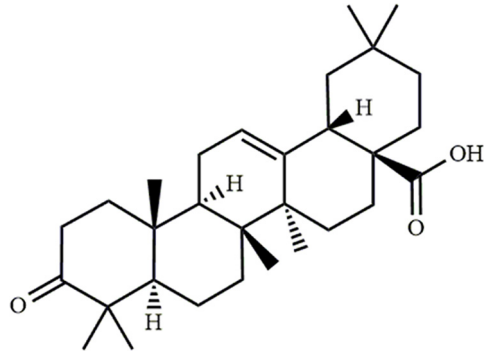

Fig. 1 Chemical structure of OA

HPLC conditions

HPLC analysis was carried out using a reverse-phase HPLC system outfitted with an INNO C18 column (4.6 mm×150 mm, 3 μm). The detection wavelength and injection volume were 205 nm and 4 μL, respectively. The analyses were conducted at a flow rate of 0.8 mL/min and 30 °C, using gradient elution. The mobile phase consisted of water (A) and ACN (B). The elution profile was as follows: 50% A at 0 min, 10% A from 0 to 25 min, 0% A from 25 to 26 min, 0% A maintained until 36 min, 50% A from 36 to 40 min, and then 50% A until 50 min.

Method validation

Validation of the optimized HPLC method was performed by evaluating the linearity, specificity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). The HPLC method was validated in accordance with the criteria set by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [24]. The specificity of the method was analyzed to assess whether there was any interference between the retention times and UV spectra of the OA and MG powder extracts, as well as whether the peaks indicated the existence of the same compound. The accuracy of the HPLC method was determined by conducting recovery tests in which sample extracts were spiked with three standard solutions of varying concentrations and calculating the percentage of the compound recovered in the sample extract. To evaluate the method precision, three different sample extract concentrations were employed for intra-day (replicability) analysis, while only one concentration was used for inter-day (intermediate precision) analysis in quintuplicate. The intra- and inter-day precisions were estimated by calculating the percent relative standard deviation

(RSD) of OA in the sample solutions on the same day and on three separate days, respectively. The linearity was evaluated against six different concentrations of standard solution (0.0625-2.0 mg/mL) and three injections. A calibration curve was constructed based on the concentration *vs.* peak area chromatogram of the six standard solutions at 205 nm. The standard curve of OA was analyzed using linear least-squares regression, and the correlation coefficient (r^2) of the regression equation was used to assess the linearity. The LOD and LOQ parameters were calculated using the standard deviations of the intercept (σ) and slope (S) values from the calibration curve. The LOD is the smallest amount of analyte in a sample that can be reliably distinguished from the baseline with a given analytical method, whereas the LOQ is the smallest amount of a substance that can be quantified with sufficient repeatability and precision. The LOD and LOQ values were calculated as 3.3 (σ/S) and 10 (σ/S), respectively.

Calibration curve

The concentrations of the standard solutions were plotted against the respective peak areas to generate the calibration curve. The linearity of the fitted line was determined using the correlation coefficient (r^2) of the calibration curve, and the OA concentration in the sample was then calculated using the calibration curve. The peak area (Y), concentration (X, mg/mL), and mean standard deviation (n =3) were used to determine the calibration functions.

Results and Discussion

The current investigation was carried to evaluate a qualification of OA in MG powder extracts. The results of the HPLC/PDA analysis of the standard OA and MG extracts are shown in Fig. 2. The retention time for OA was 29.4 min, indicating an excellent separation. A wavelength of 205 nm provided the most effective response for OA quantification in a single run, including all impurities. No co-eluted peaks were observed during OA quantification, indicating that the method is highly reliable. The HPLC chromatograms and UV spectra show adequate separation and detection of OA, respectively (Fig. 2). The specificity of the present method was determined by comparing the chromatograms of the OA and MG sample solutions. The corresponding retention times and UV spectra indicate that the MG powder extract contained OA. The excellent chromatographic peak resolution for OA demonstrates the high specificity of the present HPLC method.

The OA quantification parameters were evaluated under the HPLC conditions described above. The calibration curve for OA was generated by integrating the peak areas obtained from HPLC analyses of OA solutions with various concentrations. Linear regression was used to fit the data and generate calibration curves, and additional statistical parameters were employed to evaluate the linearity of the regression by analyzing six solutions with OA concentrations ranging from 0.0625 to 2.0 mg/mL (n =3). In order to obtain the regression equation, the peak areas (Y) were plotted against the OA concentrations (X) in mg/mL. A correlation coefficient (r^2) of 0.9996 was obtained, demonstrating a strong linear correlation between OA concentration and peak area (Table 1).

The obtained LOD and LOQ values were 0.344 and 1.042 μg/mL, respectively. These data demonstrate a highly sensitive analytical method was developed in this study for the quantification of OA derived from MG powder extracts (Table 1).

A previous study [25] obtained an r^2 value for OA of 0.9993, along with LOD and LOQ values of 10.63 and 32.22 μg/mL, respectively. Compared to these results, our LOD and LOQ values were significantly lower, demonstrating that the present method provides a more precise and accurate detection and quantification of OA, even at trace concentrations.

To determine the accuracy of the present analytical method, an MG powder extract (5 mg/mL) was spiked with standard solutions

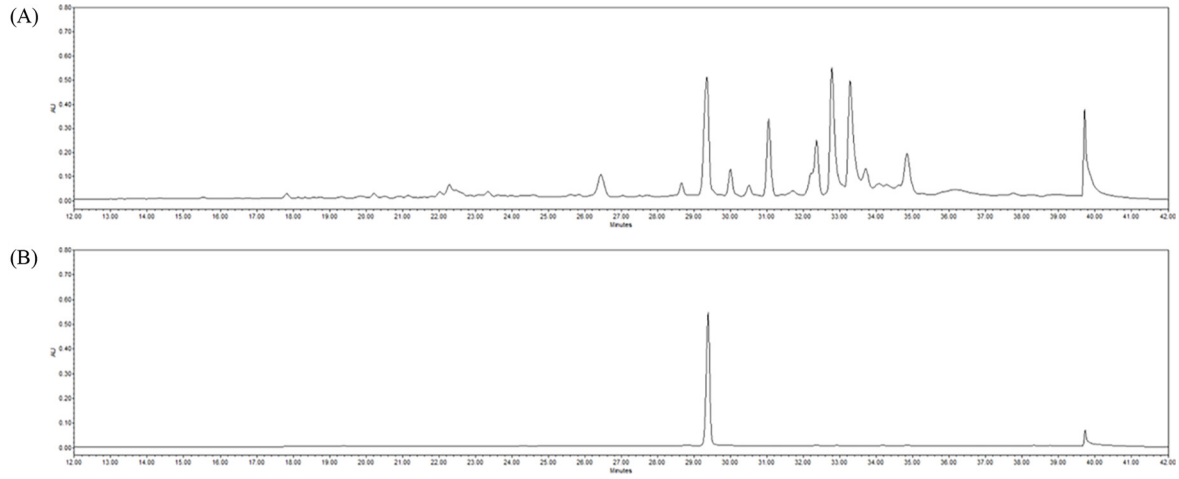

Fig. 2 HPLC chromatograms of MG powder (A) and OA (B)

Table 1 Linearity range, LOD, and LOQ values of OA analysis method

Compound	t_R	Range (mg/mL)	Calibration equation[a]	r^2 [b]	LOD (μg/mL)	LOQ (μg/mL)
OA	29.4	0.0625-2.0	Y = 3797.3X − 9692.8	0.9996	0.344	1.042

[a] Y = peak area, X = standard concentration (μg/mL)
[b] r^2 = correlation coefficient for five data points used for calibration (n = 3)

Table 2 Accuracy of OA content determination

Compound	Spiked amount (mg)	Recovery (%)					Average (%)	RSD (%)
		1st	2nd	3rd	4th	5th		
OA	0.125	97.45	100.48	99.82	99.48	99.98	99.44	1.18
	0.25	98.26	99.56	100.39	101.90	99.43	99.91	1.35
	0.5	104.05	102.34	103.72	104.18	104.00	103.66	0.73

Table 3 Intra-day precision of OA content determination

Compound	MG Concentration (mg/mL)	Intra-day (n=3)	
		Measured content (mg/g)	RSD (%)
OA	2.5	122.90	0.87
	5.0	123.13	0.07
	10.0	123.36	0.52

Table 4 Inter-day precision of OA content determination

Compound	MG Concentration (mg/mL)	Inter-day (n=5)	
		Measured content (mg/g)	RSD (%)
OA	5.0	126.03	0.10
		125.83	0.08
		125.97	0.15

containing known amounts of OA (0.125-0.5 mg). The recovery percentage of each compound was calculated by comparing the detected and original (spiked) amounts. These accuracy tests were repeated five times with three concentrations. The recovery ranged from 99.44 to 103.66%, as shown in Table 2. Each of the obtained data was within an acceptable range, indicating that the analysis procedure was extremely precise.

The precision of the optimized HPLC method was verified by comparing intra- and inter-day precision values, as shown in Tables 3 and 4. For the intra-day precision tests, the coefficient of variation of the OA precision was between 0.07 and 0.87%, whereas in the inter-day precision tests it ranged between 0.08 and 0.15%. These values are much lower than 2%, meeting the ICH-recommended threshold. These results demonstrate the high reliability of the present analytical method for the quantification of OA produced from MG.

In addition, minor differences were observed between the intra- and inter-day RSD mean values, with the inter-day values being slightly lower. This suggests that this compound remained relatively stable after being dissolved.

The OA content in the MG analyzed using the present method was 123.19 mg/g, which was much higher than the amount measured in another study [25]. This shows that the present manufacturing process of the MG product is highly beneficial in terms of OA content, and confirms the effectiveness and reliability of the analytical method established in this study.

Various studies have been conducted on triterpenic acids such as OA. These compounds belong to a group of terpenoids that exhibit various bioactive effects, including anti-inflammatory, antioxidant, anti-viral, anti-carcinogenic, anti-tumoral, and anti-bacterial properties [26-30]. Furthermore, our previous studies primarily focused on validating various marker compounds found in therapeutic plant extracts [31,32].

In this study, we optimized an HPLC analytical method for quantifying OA as a marker compound for MG. Our validation tests aimed to develop and evaluate a method for quantifying the compounds of interest. The present HPLC/PDA method was shown to be an efficient and practical analytical approach with sufficient accuracy and precision for routine analysis.

Consuming bioactive chemicals obtained from plant-based foods has emerged as a promising strategy for enhancing immunity and preventing diseases. As mentioned in the previous sections, triterpenic acids such as OA have a variety of health benefits. These bioactive properties imply that these compounds have a considerable potential for treating a wide range of medical conditions. This stimulates additional research on OA and MG,

especially because of the lack of studies on these compounds as components of health-functional foods. The HPLC/PDA analytical method developed in this study was validated by applying it to the quantification of OA from MG in powder form. The validation analysis yielded acceptable results in terms of accuracy, precision, and specificity. The analysis demonstrated that the present method could reliably identify and quantify OA in MG not only in routine analyses but also in large-scale extraction processes. Furthermore, the optimized method developed in this study can support the initial phases of the production process of health-related products and pharmaceuticals from MG.

Acknowledgments This study was funded by a grant of Vitalabs Co. Ltd, Seoul, Republic of Korea.

Author contributions HDL, investigation and formal analysis; CDL, investigation; SYC, experimental design and funding; SL, writing-review and editing.

Conflict of interest There are no competing or conflicts of interest to declare.

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