Brazilian Journal of Analytical Chemistry
Pre-publication – Accepted after peer review
doi: 10.30744/brjac.2179-3425.AR-03-2025

BrJAC

ARTICLE

The Determination of Abietic Acid in Natural Resins and their Derived Products Using Assisted Ultrasonic Sample Preparation and Analysis by Liquid Chromatography

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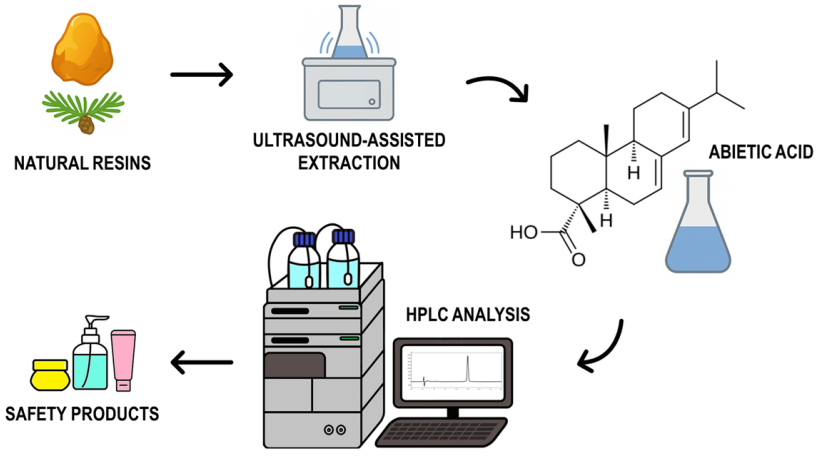


This study focused on the development and validation of a method for determining abietic acid in natural resins and derivative products using High-Performance Liquid Chromatography with spectrophotometric detection. A Pursuit PFP column (150 mm x 4.6 mm, 5.0 µm) was used to separate the abietic acid from other matrix compounds, using methanol:formic acid 0.1% (75:25) as mobile phase in isocratic elution mode at a flow rate of 0.7 mL min^{-1}. The sample analysis volume was set at 10 µL and the abietic acid was detected at a wavelength of 245 nm. The samples were prepared by ultrasonic assisted approach. The developed method showed a good linearity of the calibration curve with determination coefficient equal to 0.999. Validation parameters such as accuracy, precision, specificity, detection and quantitation limits, recovery and matrix effect were evaluated and displayed excellent reliability, accuracy and sensitivity. This method proved to be efficient to identify and quantify abietic acid in natural resins and its derivatives used as raw materials for cosmetic products.

Keywords: abietic acid, natural resins, high-performance liquid chromatography, cosmetic products, validation

INTRODUCTION

Natural resins are complex mixtures primarily composed of organic compounds, including terpenes, acids, and esters, among others. They are amorphous substances, odorless or with a slight aroma, translucent and with a color varying between yellow and dark brown, used for many years by different industries. In

Cite: de Sousa, T. F.; Daniel, D. The Determination of Abietic Acid in Natural Resins and their Derived Products Using Assisted Ultrasonic Sample Preparation and Analysis by Liquid Chromatography. *Braz. J. Anal. Chem.* (Forthcoming). http://dx.doi.org/10.30744/brjac.2179-3425.AR-03-2025

Submitted January 13, 2025; Resubmitted May 02, 2025; 2nd time Resubmitted July 07, 2025; Accepted August 18, 2025; Available online September 2025.

This article was submitted to the BrJAC special issue on the 21st ENQA and 9th CIAQA.

cosmetics, for example, it is present in mascaras, blushes and lipsticks, helping the makeup hold together, under the name “colophonium” by INCI. It is also commonly found in dental floss, sunscreen and depilatory cream.[1,2]

The composition of natural resins can vary depending on the species of plant or tree, environmental conditions and extraction methods, and although there are variations in the composition, the major components are the same. Common components found in all natural resins are the resins acids (90%) and neutral compounds (10%). Resin acids are diterpenoid acids, of which the abietane-type structures (abietic, palustric, levopimaric and dehydroabietic acids) and pimarane-type structures (pimaric, isopimaric and sandarapimaric acids) are the most abundant. Neutral substances are composed of terpenes, terpene alcohols, sesquiterpene and diterpene hydrocarbons, aldehydes and alcohols.[3,4]

Natural resins are a common cause of allergic contact dermatitis due to their widespread usage and skin sensitizing capacity, of which the main allergenic components are abietane-type resin acids oxidized by air exposure.[5] Abietic acid is the major substance present in resins and its oxidation occurs via a free radical chain reaction, producing allergenic compounds, such as 15-hydroperoxyabetic acid, considered the major sensitizer in natural resins.[6,7] Given this situation, it is important to determinate the concentration of abietic acid, especially when you want to incorporate natural resins into a cosmetic formulation, mainly aiming to protect consumer safety.

There are several analytical methods in the literature to quantify the abietic acid in different matrices. In the beginning the analysis of abietic acid was carried out by gas chromatography (GC), however the high temperature used in the analysis leads to abietic acid isomerization and consequently to inaccurate results. The high-performance liquid chromatography (HPLC) coupled to ultraviolet or fluorescence detector has been widely developed as a better alternative to GC for high-temperature susceptible analytes, such as abietic acid. To increase the detection limit of HPLC-based methods, it is necessary to preconcentrate or derivatize the samples. However, this approach increases the number of tedious and time-consuming operational steps, raising the possibility of errors due to analyte loss or sample contamination.[8]

Ultrasound-assisted extraction (UAE) enhances the interaction between the solid matrix and the solvent by increasing local pressure, thereby promoting more efficient solvent penetration. As it operates at relatively low temperatures, UAE is suitable for extracting heat-sensitive compounds. Compared to conventional extraction methods, it offers several advantages, including operational simplicity, reduced extraction time, lower solvent consumption, and the capability for simultaneous processing of multiple samples. Additionally, UAE conditions are relatively easy to optimize, as the process depends on a limited number of parameters (primarily matrix moisture content, solvent characteristics, and extraction time) making it a practical and efficient alternative for sample preparation.[9]

Table I presents a comparison of various analytical methods reported in the literature for the determination of abietic acid by HPLC. Although several methods have been previously described, none integrates the same combination of sample selection, sample preparation, and analytical conditions as the method developed in this study. This unique combination results in improved efficiency, reliability, and applicability for routine analysis. Additionally, the proposed method was validated in accordance with INMETRO guidelines and is currently implemented in the routine quality control laboratory of our manufacturing unit, allowing the quantification of abietic acid in natural resins and their derivatives used as raw materials in cosmetic formulations.

Table I. Analytical methods for abietic acid determination reported in literature

Author	Method	Matrice	Sample treatment	Linear Range (ppm)	LOD (ppm)	LOQ (ppm)
Zhu, Y. 2014[7]	HPLC-PAD	Duck meat	SPE-C18*	0.05 – 5.0	0.015	0.05
Liu, J. 2014[8]	HPLC-FLD-MS/MS	Cosmetics	UCSED**	0.075 – 3.0	0.0082	0.0267

(continues on next page)

Table I. Analytical methods for abietic acid determination reported in literature (continuation)

Author	Method	Matrice	Sample treatment	Linear Range (ppm)	LOD (ppm)	LOQ (ppm)
Mitani, K. 2007[10]	LC-MS	Food	On-line SPME	0.0005 – 0.05	0.0003	0.0005
Mckeon, L. 2014[11]	CE-DAD	Rosin	Dissolution	5.0 – 1000.0	0.15	0.5
Lee, B. L. 1994[12]	HPLC-PAD	Adhesive	SPE-C18*	0.025 – 1.0	0.025	0.05
Sarria-Villa, R. A. 2021[13]	HPLC-PAD	Rosin	SPE-C18*	10.0 – 100.0	0.091	0.304
Hroboňová, K. 2006[14]	HPLC-DAD-MS	Propolis	Liquid extraction with methanol	0.2 – 1.0	0.1	0.2
Sakunpak, A. 2024[15]	HPLC-DAD	Oral spray	Dissolution and filtration	31.3 – 1000.0	10.9	33.2
This report	HPLC-DAD	Natural Resins	Ultrasonic extraction and filtration	1.0 -100.0	0.15	0.5

*SPE-C18: Solid-phase extraction using C18 cartridges. **UCSED: Ultrasonic-assisted closed in-syringe extraction and derivatization.

MATERIALS AND METHODS

Materials and chemicals

Abietic acid, ultrapure water and methanol HPLC grade were purchased from Sigma-Aldrich (USA) while formic acid was purchased from Neon (Brazil). To carry out the analysis a stock solution abietic acid standard was prepared diary dissolving 1.0 g of abietic acid in 10 mL of methanol. Working standards for calibration curve were prepared immediately before use in mobile phase.

Pine and Mastic naturals resins are commercially sold and were acquired from local producers or imported. The raw material for cosmetics produced from these natural resins were manufactured by the company Aqia Química Inovativa Ltda.

Apparatus

An LC-2030 HPLC system (Shimadzu) with DAD detector set at the wavelength of 245 nm and a Pursuit PFP column (150 mm x 4.6 mm, 5.0 µm, Agilent) was used to analyze the abietic acid. The best composition of the methanol:formic acid mobile phase was determined based on tests varying the concentration of formic acid (0.05 - 1%) and different proportions between the solvents (70:30, 75:25 or 80:20). To determine the best analysis conditions, flow rates (0.5 - 0.7 mL min^{-1}), temperatures (25 ºC - 50 ºC) and injection volumes (1 - 50 µL) were tested. Data acquisition was performed on LabSolution® software. A SSBuc 10L ultrasonic bath (SolidSteel, Brazil) was used for the extractions of the samples.

Sample preparation

In this study the abietic acid was determined in two natural resins (pine and mastic resin) and six cosmetic raw material samples. The natural resin samples and their derivative products were prepared before the HPLC analysis by ultrasonic-assisted liquid extraction. A precisely weighed mass of 100.0 mg of sample was added into 15-mL centrifuge tube, followed by addition of 10 mL methanol. The tube was capped and shaken in an ultrasonic batch. For the extraction of abietic acid from diverse samples, different times into ultrasonic batch were investigated (2, 5, 10, 20 and 30 minutes) to determine which would promote better extraction. After appropriate dilution with methanol (1:10, v/v), samples were filtered through a 0.45 µm nylon syringe filter prior to injection into HPLC system.

Method validation

The validation of the analytical method for determination of abietic acid in natural resins and their derivatives products was performed through range, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ) and matrix effect.

RESULTS AND DISCUSSION

Chromatographic conditions

Abietic acid is a weak organic acid and its retention on reverse phase HPLC-based method can be improved by using some additives in mobile phase, such as formic acid, acetic acid, phosphoric acid, etc., as well by its composition. In the present study, 03 different concentrations of formic acid, ranging from 0.05 to 1% were evaluated at same mobile phase composition (75:25, v/v). The best compromise between retention time and peak area was obtained for concentration of 0.1% of formic acid.

To choose the best mobile phase composition, mixtures of methanol and formic acid 0.1% were tested in different proportions: 80:20 (v/v), 75:25 (v/v) and 70:30 (v/v), and the better response considering the retention time and peak shape was obtained using a solution methanol/formic acid 0.1% in the proportion of 75:25 (v/v), as can be seen in Figure 1A.

Flow rate, ranging from 0.5 to 0.7 mL min^{-1}, temperature, from 25 to 50 °C, and injected sample volume, from 1 to 20 µL, were also parameters evaluated in the chromatographic method optimization, and the chromatograms obtained are shown in Figures 1B, C and D. The comprehensive evaluation of chromatographic parameters enabled the identification of the best analytical conditions for the determination of abietic acid, summarized in Table II, resulting in improved sensitivity, accuracy, analysis time, and overall robustness of the developed method.

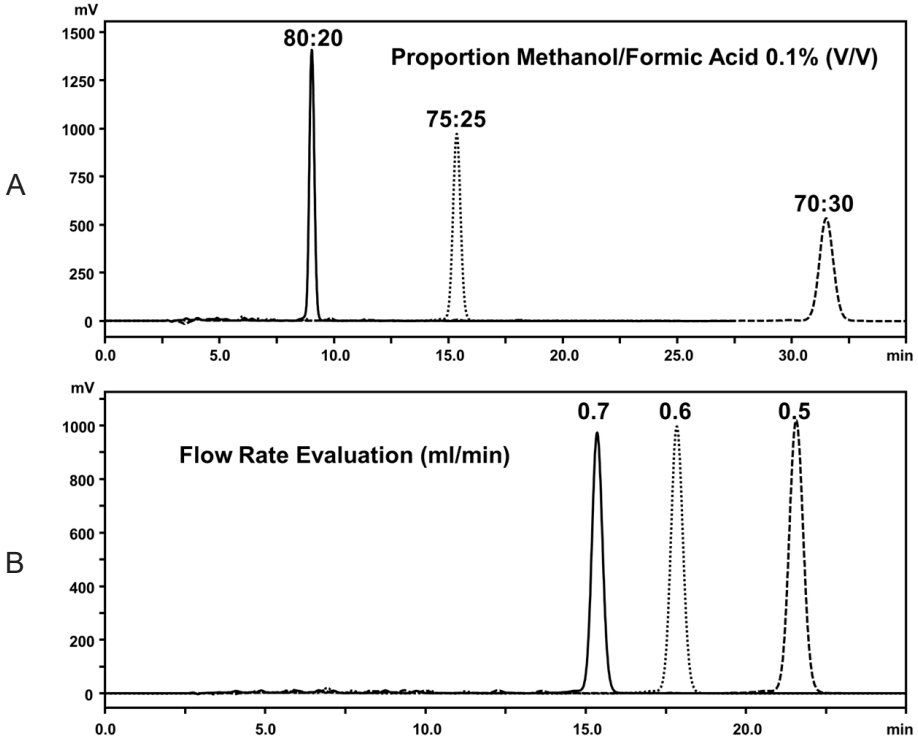


Figure 1. Influence of chromatographic parameters on method performance: (A) Mobile phase composition; (B) Flow rate; (C) Temperature and (D) Injection volume. (continues on the next page)

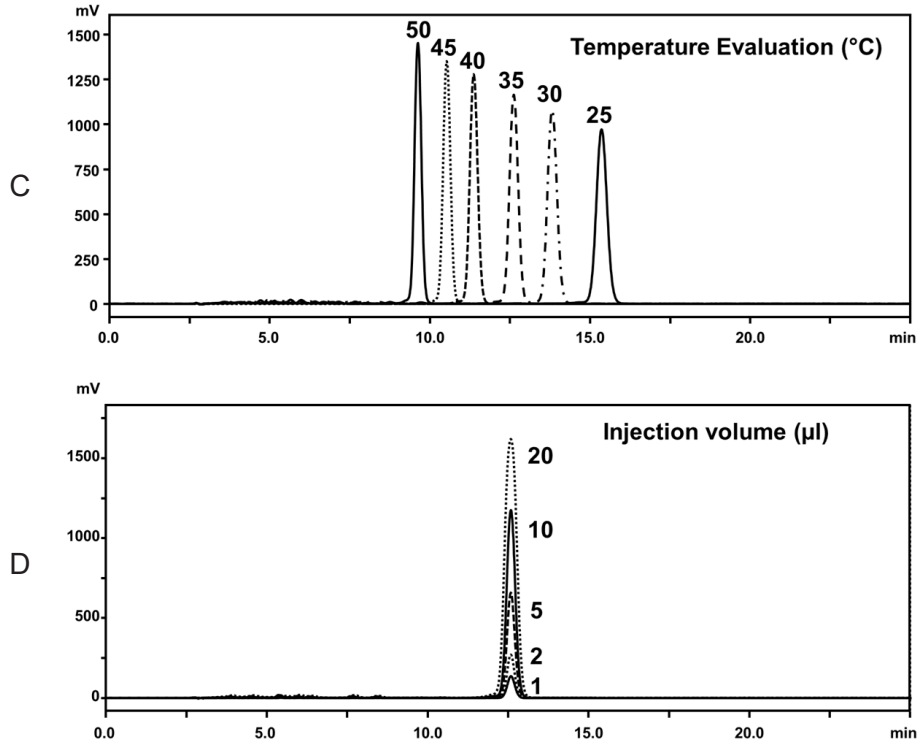


Figure 1. Influence of chromatographic parameters on method performance: (A) Mobile phase composition; (B) Flow rate; (C) Temperature and (D) Injection volume. (continuation)

Table II. Chromatographic conditions of the developed method for determination of abietic acid

Instrument	Shimadzu LC-2030
Column	Pursuit PFP (150 mm x 4,6 mm, 5,0 µm)
Detector	UV, 245 nm
Flow rate	0.7 mL min^{-1}
Temperature	35 ºC
Injection volume	10 µL
Mobile phase	Methanol:formic acid 0.1% (75:25)
Elution	Isocratic
Run time	20 minutes

Sample preparation

The ultrasonic-assisted liquid extraction of abietic acid from natural resins was investigated submitting the samples to times ranging from 2 to 30 minutes in an ultrasonic bath, monitoring the peak area result. Pine resin samples in methanol were analyzed in triplicate and results are presented in Figure 2. Just ten minutes provided the best abietic acid extraction, and it was adopted as analysis parameter.

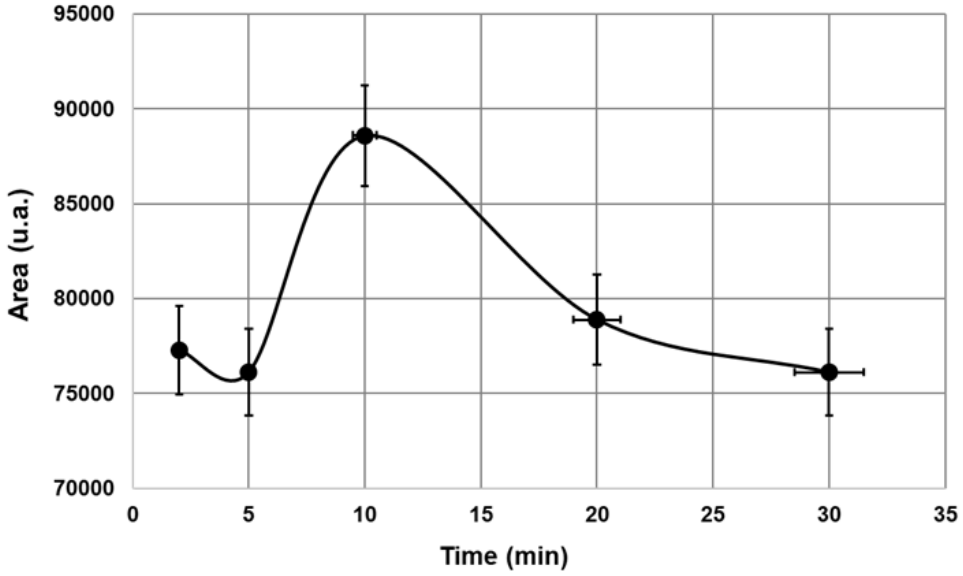


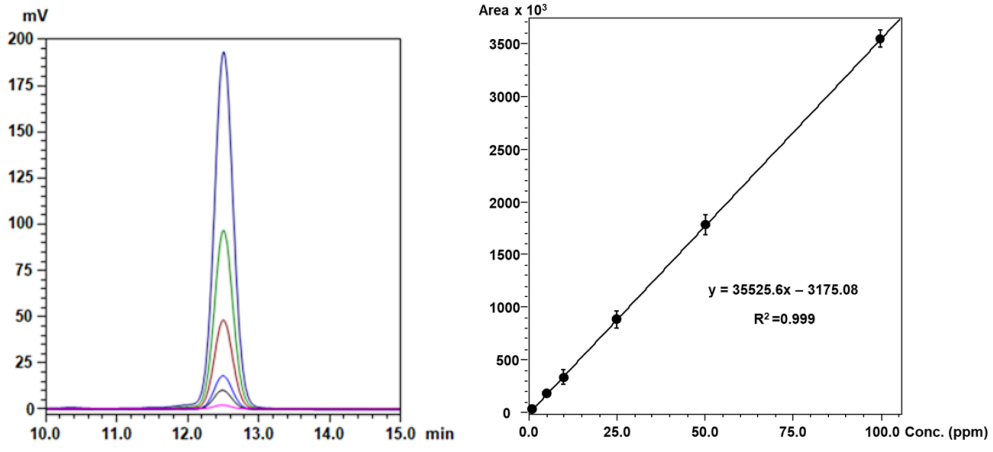
Figure 2. Ultrasonic-assisted liquid extraction time of abietic acid in pine vs peak area.

Method validation

Linearity, Matrix Effect and Limits of detection and quantitation

The linearity was determined in triplicate at six different concentration levels, range 1 – 100 ppm, prepared in methanol. The different concentrations of abietic acid were plotted against their respective peak areas. The linear regression equation, obtained by the least squares method, is y = 35525.6x – 3175.08 with determination coefficient (r^2) equal to 0.999. The chromatograms and linearity curve are presented in Figure 3, while the ANOVA results (at 95% confidence level) for the calibration curves are presented in Table III. The p-value obtained was greater than 0.05, indicating that there is no significant difference between the calibration curves. The residual analysis, used to assess homoscedasticity, revealed a random distribution of the residuals, confirming that the homoscedasticity assumption was satisfied and that the linear model is adequate.

The limit of detection (LOD) and quantitation (LOQ) of the analytical method were calculated according to INMETRO guidance (DOQ-CGCRE-008),[16] based on the standard deviation of the response and slope of the calibration curve. LOD and LOQ for abietic acid was found 0.3 ppm and 1.0 ppm, respectively.



(A) (B)

Figure 3. (A) Chromatograms of abietic acid standards. (B) Linearity of abietic acid determined at 245 nm.

Table III. Results of ANOVA test for the calibration curves data

Source of Variation	DF	SS	MS	*F* ratio	Prob > *F*
Between groups	5	29,008,809,914,920.3	5,801,761,982,984.1	1952.85	< 0.0001
Within group	12	35,651,109,807.3	2,970,925,817.28		
Total	17	29,044,461,024,727.6			

DF = degrees of freedom; SS = sum of squares; and MS = mean square; $p < 0.05$.

To evaluate matrix effects on the quantification of abietic acid, matrix-matched curves calibration curves were prepared at the same concentrations levels as those prepared in solvent. For this purpose, abietic acid standards were spiked into natural pine and mastic resin samples prior to ultrasound-assisted extraction. The matrix effect (ME) was calculated using Equation 1:

$$ME\ (\%) = 100 \times \left[\left(\frac{Slope\ matrix}{Slope\ solvent}\right) - 1\right] \qquad \text{Equation 1}$$

The matrix effect was determined to be 2.6% for mastic resin and 1.9% for pine resin. These values do not significantly impact on the accuracy and precision of the analytical results and can therefore be considered negligible. Consequently, sample quantification was performed using a calibration curve prepared in methanol.

Accuracy and Precision

The accuracy of the analytical method was evaluated through recovery tests by spiking samples with known amount of abietic acid standard at four different concentrations levels (1, 10, 50 and 100 ppm), analyzing in triplicate and then calculating the recovery percentage. The values of the recovery rate were above 96% to all concentration levels, in accordance with INMETRO acceptance criteria (DOQ-CGCRE-008).[16]

The precision was evaluated through intra-day and interday precision. Each sample was analyzed five times within a day (intra-day) and three consecutive days (inter-day). The precision was calculated as percent relative standard deviation (% RSD). The relative standard deviation values of the abietic acid were less than 7.3%. The results demonstrating the accuracy and precision of the proposed method are presented in Table IV.

Table IV. Intra- and inter-day accuracy and precision of abietic acid at four concentration levels (n=5)

Concentration (ppm)	Intra-day		Inter-day		Recovery (%)
	Mean+SD	Precision (RSD%)	Mean+SD	Precision (RSD%)	
1	0.95 ± 0.1	6.8	0.96 ± 0.1	7.3	96
10	10.37 ± 0.1	0.7	10.72 ± 0.4	3.9	107
50	49.38 ± 0.2	0.5	50.95 ± 1.9	3.7	102
100	103.94 ± 1.4	1.3	107.27 ± 3.0	2.8	107

Application of the method

The abietic acid standard and the samples were analyzed by HPLC established method. The retention time of abietic acid was 12.5 minutes, which allowed its unequivocal identification in the samples. The chromatograms of the standard abietic acid and samples are shown in Figure 4. As expected, the concentration of abietic acid in pine resin is higher than in raw material obtained from it, while mastic gum doesn't contain any trace of abietic acid its composition.[17] The results obtained are summarized in Table V.

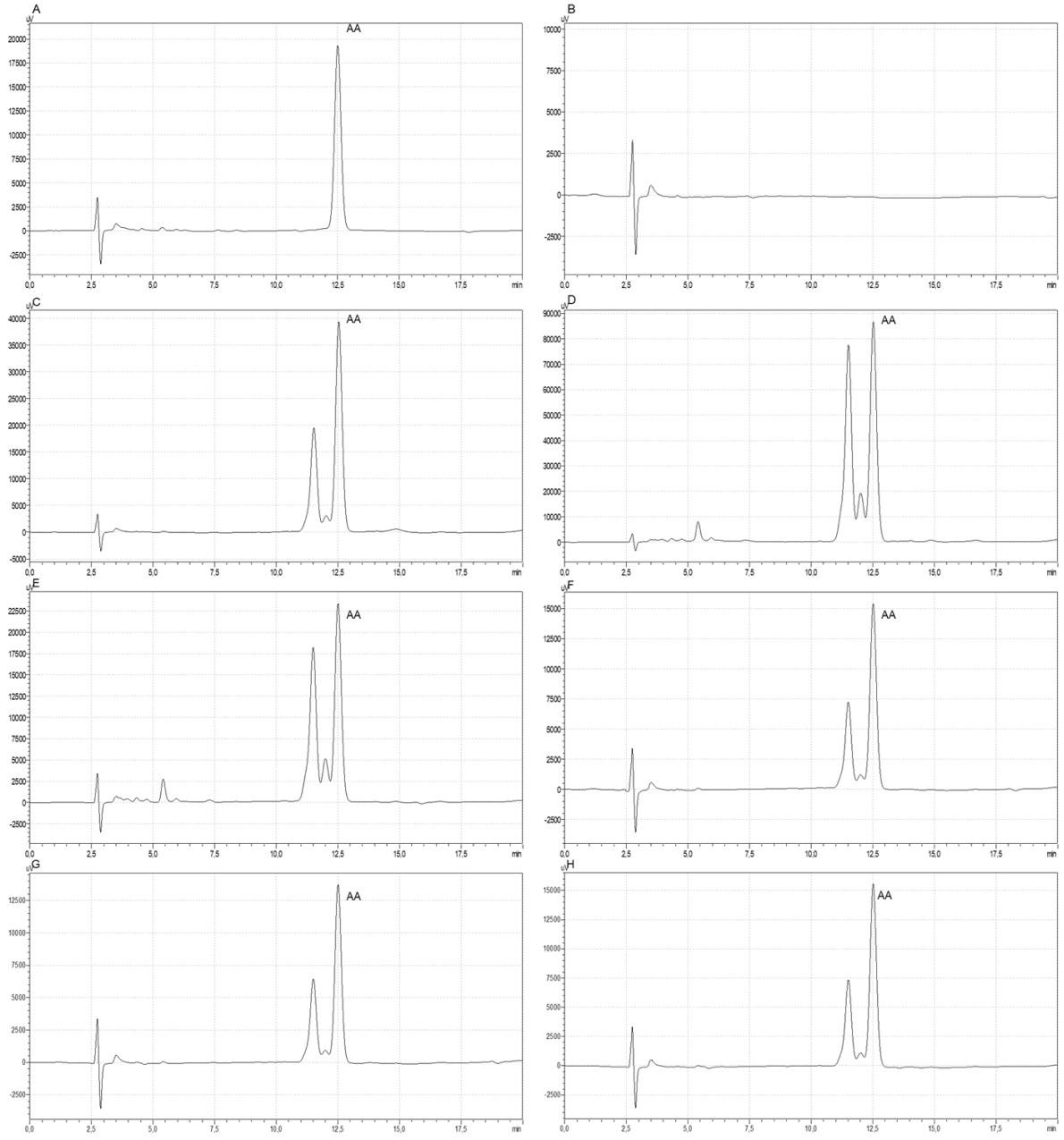


Figure 4. Chromatogram of abietic acid (**AA**). Standard solution **(A)**, mastic resin **(B)**, pine resin **(C)** and cosmetic raw material **(D – H)**.

Table V. Concentration of abietic acid in natural resins and cosmetic raw materials

Sample	Concentration (ppm)
Pine resin	145.0 ± 4.3
Mastic gum	ND
Raw material 1	39.5 ± 1.2
Raw material 2	108.9 ± 3.3
Raw material 3	75.9 ± 2.3
Raw material 4	68.4 ± 2.0
Raw material 5	77.4 ± 2.3

CONCLUSIONS

The proposed method for analyzing the concentration of abietic acid in natural resins and raw material was successfully developed and validated. The method is simple, selective, with good resolution, excellent linearity (> 0.999), and low limit of detection (0.15 ppm). Furthermore, accuracy, precision, recovery, and repeatability for the determination of abietic acid meet the INMETRO criteria for validation of analytical methods. Although several HPLC methods for the determination of abietic acid have been described in previous studies, none of them offer the same combination of cost-efficiency and rapid analysis as the method presented in this study.

The developed method was effectively applied to determination of abietic acid in seven samples of interest, including natural resins and cosmetic raw material, proving to be a useful tool for monitoring abietic acid and in the quality control of cosmetic raw material.

Conflicts of interest

The authors declare that there are no conflicts of financial interest or otherwise.

Acknowledgements

This work was supported by the company AQiA Química Inovativa, Ltda.

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