Development and validation of high-performance liquid chromatography assay method for simultaneous determination of Caffeic acid, Vanillin, and Cinnamic acid in Algerian propolis extract

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Abstract

Propolis is a natural bee substance and resinous material produced from various plants, which showed important biological activities. The current study aims to evaluate and validate simultaneous methods of identification and quantification of three phenolic compounds (caffeic acid, vanillin, and cinnamic acid) in propolis extract from different Bejaia regions using high performance liquid chromatography (HPLC). Chromatographic analyses were performed using gradient mode, in are versed-phase C-18 (150 x 4.6 mm, 5 µm) column. The mobile phase contained 0.5% v/v acetic acid in water (solvent A) and acetonitrile (solvent B), using a flow rate of 1 ml/min and by injecting 20 µl, at a wavelength of 290 nm. The method was validated according to ICH guidelines. Specificity, linearity, accuracy, precision, detection, and quantification limits studies were made. The results showed that the correlation coefficient (R2) was > 0.99 for Caffeic acid, Vanillin, and Cinnamic acid, the percentage of relative standard deviation (RSD) of all assays found below 2% and 5% for intra-day and inter-day, respectively, limits of detection were 0.024, 0.008, and 0.009 mg/ml and limits of quantitation was 0.074, 0.025, and 0,029 mg/ml, for Caffeic acid, Vanillin, and Cinnamic acid respectively. According to these various parameters: accuracy, precision, linearity, and specificity, the proposed method was successfully validated in the simultaneous assay of Caffeic acid, Vanillin, and Cinnamic acid.

Keywords: *High-Performance Chromatography, Simultaneous Validation, Natural Product, Polyphenol, Propolis Extract.*

I. Introduction

Propolis is a natural complex mixture collected and synthesized by bees from different plants (resins and waxes) and their salivary enzyme [1, 2]. It is used to construct, protect, and maintain the hives, and to treat many diseases in the poplar medicine [3].

Many studies revealed that propolis shows several pharmacological properties including anti-inflammatory [4, 5], antibacterial [6, 7], antioxidant [8], antiviral [9], and anticancer [10], this is due to the diversity of its chemical composition which depends on the bee species, meteorological conditions, plant, and geographical source [11].

The most important chemical contents of propolis are phenolic compounds which are considered secondary metabolites of plants, and they consist of wide large groups and classes that include flavonoids, terpenes, lignans, stilbene, aldehyde (vanillin), and phenolic acids [12].

Phenolic acids contain two main classes: hydroxycinnamic acids such as caffeic acid, cinnamic acid, and hydroxybenzoic acids like gallic acid [13, 14].

Different analytical methods were studied and applied in the analysis and quality assessment of propolis, among these chromatographic techniques: gas chromatography (MS), high performance liquid chromatography with diode array detection (HPLC-DAD) [15, 16], and high-performance liquid chromatography with Ultra-violet detector (HPLC-UV). Among the analytical methods, high-performance liquid chromatography (HPLC) [17, 18] is one of the most powerful analytical methods for this purpose. However, to ensure that analytical methodology is accurateusing (HPLC) methods, specific and over the specified range that an analyte will be analyzed; an analytical test method validation was completed. This validation, guidelines from the US Pharmacopeia (USP) and International Conference on Harmonization (ICH) [19].

The purpose of the present study was to develop and validate simultaneous method assay of Caffeicacid,Vanillin, and

Cinnamic acid in propolis extract from different Bejaia regions: Adekar, Akfadou, Baccaro, El kseur, Kendira, Kherrata, and Melbou, extracted by ultrasound and agitation methods. A simple HPLC method was used, allowing a good separation and short run time followed by a qualitative and quantitative determination of Caffeicacid, Vanillin, and Cinnamic acid.

II. Material and methods

Chemicals and reagents

Caffeicacid, Vanillin, Cinnamic acid,Alpha-tocopherol (vitamin E), Ascorbic acid (Vitamin C),Cholesterol was purchased from SIGMA-ALDRICH. Saturated phospholipids were purchased from RHONE POULENC (Phospholipon 90H lot: 90060). β-Cyclodextrin (β-CD) was obtained from Roquette Frères. Polyethylene Glycol 6000 (PEG 6000) was purchased from BIOCHEM.

HPLC-grade methanol, HPLC-grade acetonitrile, and acetic acid were purchased from BIOCHEM CHEMOPHARMA.

Equipment and Chromatographic conditions

Quantitative analysis of Caffeic acid, Vanillin, Cinnamic acid, vitamin E, vitamin C phospholipids, β-CD, and PEG 6000 were carried out using the high-performance liquid chromatography (HPLC) method, coupled to a UV detector set to 290 nm. HPLC-UV system (UltiMate 3000 RS-Variable Wavelength detector) was equipped with an auto-injector LC 1650, consisting of vacuum degasser, temperature-controlled well-plate autosampler, column thermostat, quaternary pump, and photodiode array detector. Chromatographic analysis was performed using a Hypersil ODS C-18 (150 x 4.6 mm, 5 µm particle size, 80˚A pore size column) from Thermo (Bellefonte, PA, USA). The mobile phase consisted of 0.5% v/v acetic acid in water (solvent A) and acetonitrile (solvent B), with a flow rate of 1 ml/min, injection volume was 20 µl, and the column temperature was 40°C as presented in Box 1. Vanillin, Caffeic acid, Cinnamic acid were identified by retention times and spectral data.

Box 1: Chromatographic condition of HPLC analysis.

Column: A reverse-phase C-18 (150 x 4.6 mm, 5 µm)

Mobile phase: 0.5% v/v acetic acid in water (solvent A), and acetonitrile (solvent B)

Flow rate: 1 ml/min

Detector: UV detector 290 nm

Injection volume: 20 µl

Mode: Gradient (90% solvent (A) at 0 min, 65% of solvent (A) at 5 min, 40% solvent (A) at 10 min, 20% solvent (A) at 15 min, 80% of solvent (A) at 20 min, and 90% of solvent (A) at 30 min)

Temperature: 40°C.

Standard preparation

The standard solution of Caffeic acid, Vanillin and Cinnamic acid were prepared in methanol in different concentrations: 0.0196-0.059 mg/ml, 0.0144- 0.0216 mg/ml, and 0.0392-0.059 mg/ml of Caffeic acid, Vanillin, and Cinnamic acid, respectively. These solutions were used to study linearity, accuracy, and precision. Specificity study was realized by the preparation of different solutions containing Polyethylene glycol 6000, β cyclodextrin, phospholipids 90H, Vitamin E, Cholesterol, and vitamin C.

Samples preparation

Propolis extracted from different regions was prepared in methanol at a concentration of 2mg/ml and analyzed by HPLC.

Method validation

Specificity

Specificity was determined by analyzing samples containing: Cyclodextrins, phospholipids, PEG 6000, methanol, mobile phase, mixer solution (containing all substances), cholesterol, ascorbic acid, and vitamin E and the sample solutions. All chromatograms were examined to determine if Caffeic acid, Vanillin, and Cinnamic acid co-eluted with any other excipient peak.

Linearity and range

Calibration standard was tested in the concentration range: 0.0196-0.059 mg/ml, 0.0144- 0.0216 mg/ml, and 0.0392-0.059 mg/ml of Caffeic acid, Vanillin, and Cinnamic acid, respectively. Five points of calibration were prepared at different concentration levels in methanol. Peaks areas (y-axis) versus drug (percentage) concentrations (x-axis) were plotted and subjected to regression analysis by the least-squares method, calibration equations $y = m x + a$ were obtained.

A method with r values higher than 0.99 can be considered linear. The range was an interval between the highest and lowest concentration analyte where acceptable linearity, accuracy and precision were obtained.

Accuracy

Accuracy was established by recovery studies at five concentrations: 0.0196-0.059 mg/ml, 0.0144- 0.0216 mg/ml, and 0.0392-0.059 mg/ml of Caffeic acid, Vanillin, and Cinnamic acid, respectively. At each level, samples were prepared in triplicate and recovery percentage was determined.

Precision

Precision was validated through intra-day and inter-day testing. The intra-day precision of the assay method was evaluated by carrying out five independent assays of Caffeic acid, Vanillin, and Cinnamic acid. Samples were against qualified reference standards on the same day and these studies

were repeated on three consecutive days to determine inter-day precision. Precision was expressed as % RSD of analyte concentration.

Quantitation Limit and detection limit

Quantitation limit (LQ) is the lowest amount of Caffeic acid, Vanillin, and Cinnamic acid, in a sample, which can be quantitatively determined with suitable precision and accuracy. The detection limit (LD) is the lowest amount of Caffeic acid, Vanillin, and Cinnamic acid in a sample that can be detected, but not necessarily quantitated as an exact value. Quantitation limit (LQ) and detection limit (LD) were determined based on the standard deviation of the response and the slope. The quantitation limit and detection limit are expressed as:

$$LQ = 10\ \sigma / S$$
$$LD = 3\ \sigma / S$$

Where σ is the residual standard deviation of the regression line, and S is the slope of the analyte calibration curve.

III.Results and discussion

HPLC method described was developed for simultaneous Caffeic acid, Vanillin, and Cinnamic acid quantification following International Conference on Harmonization (ICH) guidelines [19]. Specificity, Linearity, accuracy, precision, quantitation, and detection limit were tested to determine if the developed method is suitable for the identification and quantitation of Caffeic acid, Vanillin, and Cinnamic acid in propolis media. Retention time, plate number (N), and peak asymmetry factor (Tailing) were evaluated with the help of a standard chromatogram and shown in Table 1.

Table 1: System Suitability Parameters.

Parameters	Caffeic acid	Vanillin	Cinnamic acid
Tailing factor	1.1	1.36	1.05
No. of theoretical plate	80959	47937	176492
Retention time (min)	10.86	11.77	15.28

Method validation

Specificity

The specificity of the analytical method was determined by injection of 20µl: standard stock solution of Vitamin E and Cholesterol, β-Cyclodextrin, phospholipids 90H, PEG 6000, ascorbic acid, methanol, mobile phase, and solution contain all compounds. Chromatogram of Vitamin E and Cholesterol, β-Cyclodextrin, phospholipids 90H, PEG 6000, methanol, the mobile phase was not shown any significant peak at 10.86 min (caffeic acid retention time), 11.77 min (vanillin retention time), and 15.28 min (cinnamic acid retention time) (Figure 1, 2, and 3).

All compounds solution chromatogram (Figure 4) confirmed the absence of other peaks at the retention time of Caffeic acid, Vanillin, and Cinnamic acid.

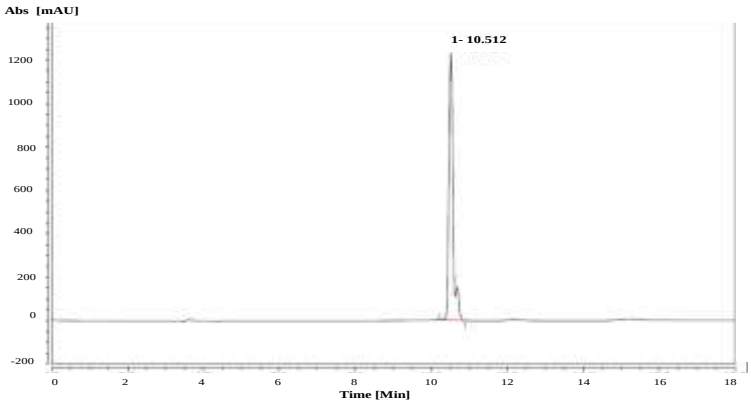

Figure 1: HPLC chromatogram of caffeic acid.

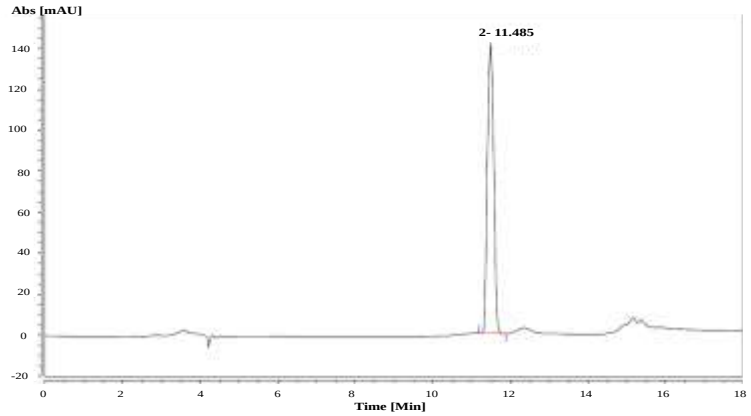

Figure 2: HPLC chromatogram of vanillin.

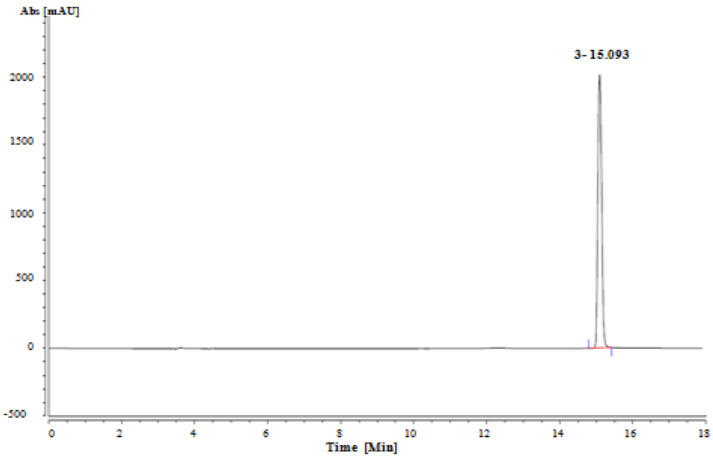

Figure 3: HPLC chromatogram of cinnamic acid.

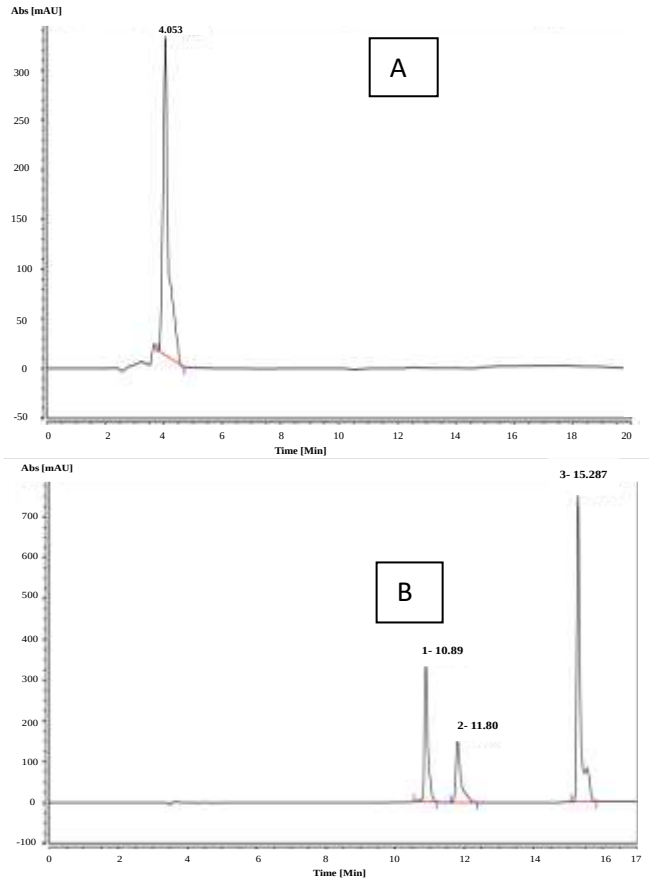

Figure 4: (A) Chromatogram of solution contains all compounds, and Chromatogram of Caffeic acid, Vanillin, and Cinnamic acid.

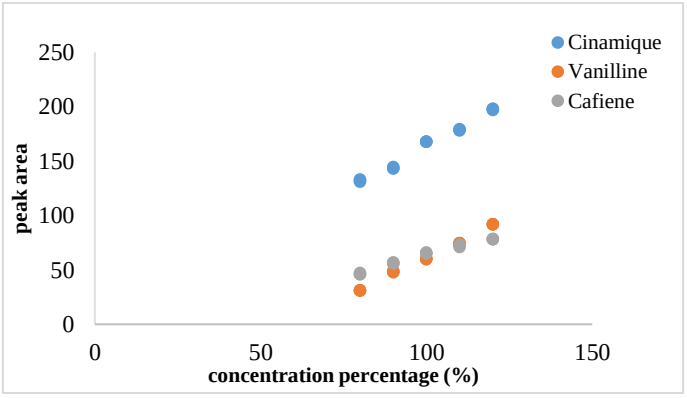

Figure 5: Calibration curve data for Caffeic acid, Vanillin , and Cinnamic acid.

Table 2: Calibration curve data for Vitamin E and Cholesterol.

Regression parameters	Caffeic acid	Vanillin	Cinnamic acid
Regression coefficient (R^2)	0.99	0.99	0,99
Slope	1	1	2
Concentration range (mg/ml)	0.0196-0.059	0.0144- 0.0216	0.0392-0.059
Number of points	5	5	5

Linearity and range

A plot of peak area response against concentration is shown in Figure 5. Linearity was evaluated by calibration curves over the analytical range of 0.0196-0.059 mg/ml, 0.0144- 0.0216 mg/ml, and 0.0392-0.059 mg/ml of Caffeic acid, Vanillin, and Cinnamic acid, respectively (Table 2). Linear regression analysis for two reference compounds was performed by the external standard method. The correlation coefficient (R2) was found to be > 0.99 for Caffeic acid, Vanillin, and Cinnamic acid, indicating suitability for Caffeic acid, Vanillin, and Cinnamic acid, quantification. A great linear relationship was found for the two components (Caffeic acid, Vanillin, and Cinnamic acid). Results of linearity were presented in Table 2.

Accuracy

To evaluate the accuracy and reliability of the method, recovery studies were carried out in the range of 80–120% concentration. Recoveries percentages were found to be average of 99.97%and 100% for Caffeic acid, Vanillin, and Cinnamic acid respectively. This method showed suitable accuracy. The obtained results are presented in Table 3.

Precision

The precision study has comprised the evaluation of the intra-day precision of the assay method. Five injections of target levels of calibration standard for Caffeic acid, Vanillin, and Cinnamic acid were performed. These studies were also repeated on three consecutive days to determine inter-day precision. The percentage of relative standard deviation (RSD) of six assay values was calculated and found to be below 2% and 5% for intra-day and inter-day, respectively. The results obtained are presented in Table 3. Thus, it concluded that it assures the precise HPLC method.

Table 3: Linearity, Precision and Recovery data for Caffeic acid, Vanillin andCinnamic acid**.**

Samples	Added standard (mg/ml)	Linearity (R2)	Accuracy (%recovery)	Precision (%RSD)		LD (mg/ml)	LQ(mg/ml)
				Intra-day	Inter-day		
Caffeic acid	0,039	0.997	97,81	0,670	9,298	0,024	0,074
	0,044		100,78				
	0,049		102,11				
	0,054		100,64				
	0,059		98,54				
Vanillin	0,032	0.997	99,57	0,049	3,116	0,008	0,025
	0,036		101,43				
	0,04		99,28				
	0,044		99,04				
	0,048		100,69				
Cinnamic acid	0,014	0.998	100,97	0,209	9,637	0,009	0,029
	0,016		97,60				
	0,018		102,25				
	0,019		98,98				
	0,022		100,21				

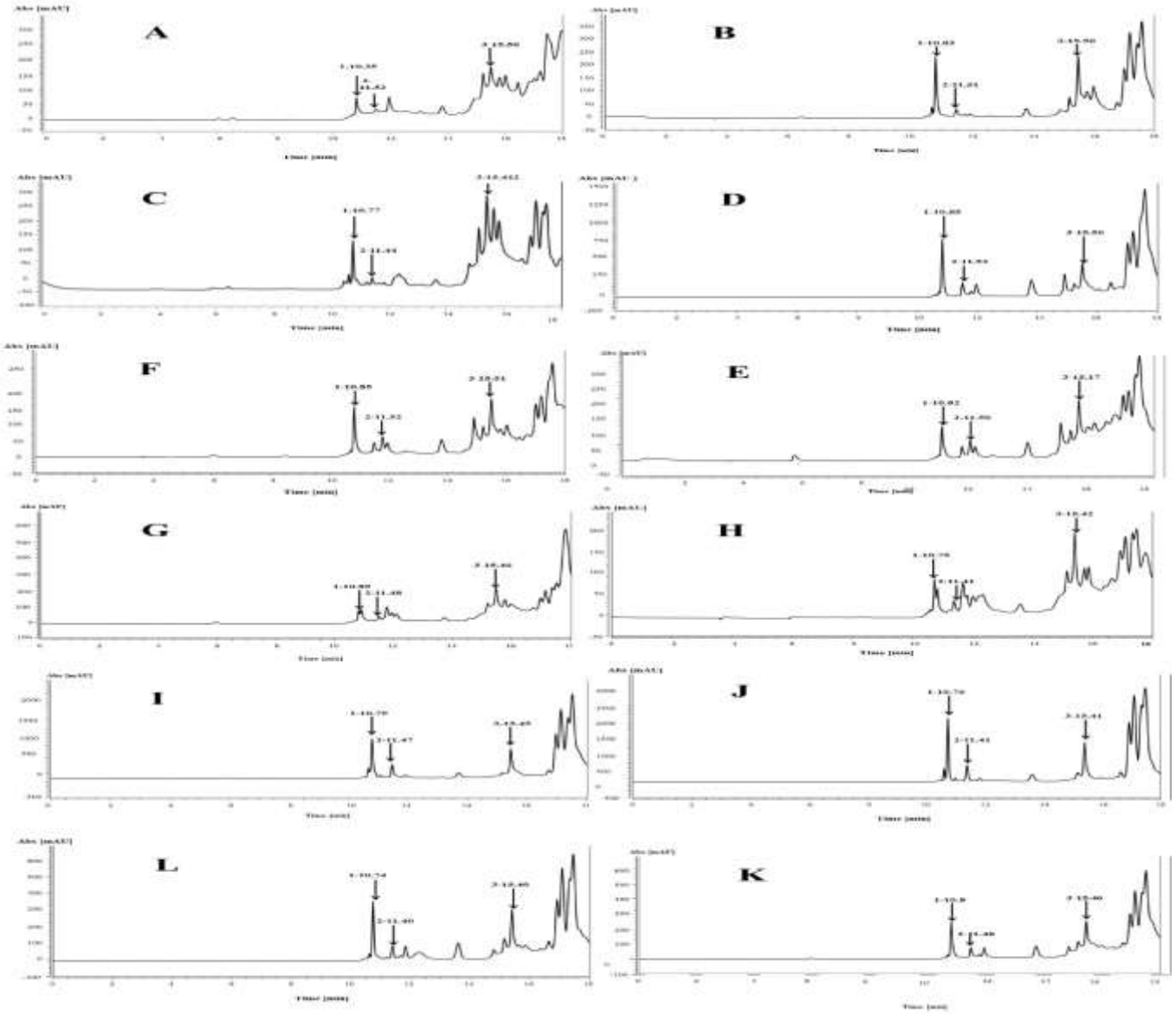

Figure 6: HPLC chromatograms of propolis extract from different Bejaia regions [A-Adekar(ultrasoundmethod),B-Akfadou (ultrasound method),C- Akfadou (agitataion method), D- Baccaro (ultrasound method),E- El kseur (ultrasound method), F- El kseur (agitataion method),G- Kendira (ultrasound method), H- Kendira (agitataion method), I- Kherrata (ultrasound method), J- Kherrata (agitataion method),K- Melbou (ultrasound method),L-Melbou(agitataionmethod),1-Caffeicacid,2-Vanillin,3-Cinnamicacid].

Detection and Quantitation Limits

Limits of detection and quantification were determined according to ICH, based on the standard deviation of the response and the slope. Caffeic acid, Vanillin, and Cinnamic acid presented limits of detection of 0.024, 0.008, and 0.009 mg/ml and limits of quantitation of 0.074,0.025, and 0,029 mg/ml, respectively. The results obtained are presented in Table 3 and indicated that the sensitivity of this HPLC method was suitable for the quantitative determination of Caffeic acid, Vanillin, and Cinnamic acid.

I.V. Conclusions

Analytical HPLC simultaneous assay of Caffeic acid, Vanillin, and Cinnamic acid was developed and validated satisfactorily for various parameters: accuracy, precision, linearity, and specificity as per ICH guidelines. This method shows simple, rapid, high precision and accuracy and offers the advantage of simultaneous assay of Caffeic acid, Vanillin, and Cinnamic acid in propolis extract from different Bejaia regions. Besides, this work offers an excellent alternative to methods already existing for Caffeic acid, Vanillin, and Cinnamic acid determination in propolis.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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