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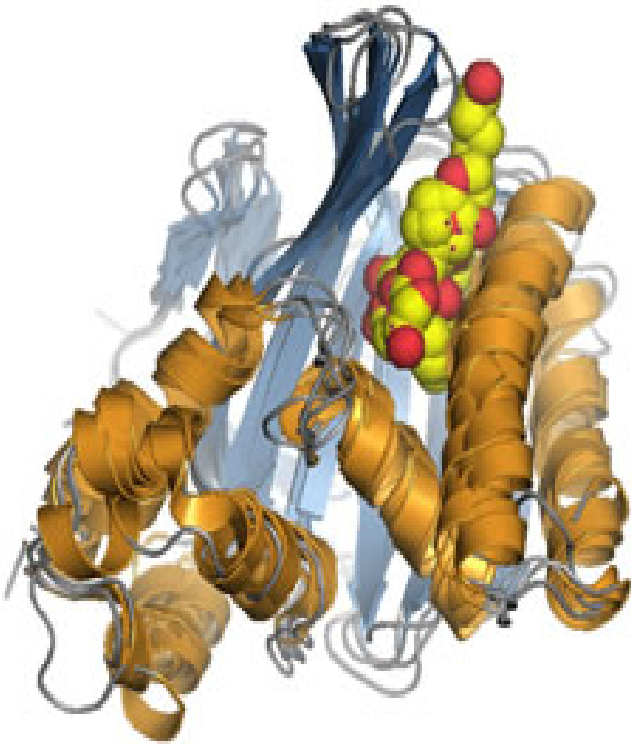

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Determination of caffeic acid and gallic acid in Algerian bee pollen by an HPLC method

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Abstract. *Bee pollen is a fine powder-like material produced by flowering plants pollen, mixed with nectar and bee secretions and gathered by the honey-bees. Pollens are the male reproductive cells of flowers and bees' primary food source, containing concentrations of phytochemicals and nutrients and rich in secondary metabolites. It is known that bee pollen contains lipids, sugars, proteins, amino acids, flavonoid and phenolic acids such as caffeic acid and gallic acid. The aim of our study is to evaluate caffeic and gallic acids from different sorts of bee pollen from Algeria using an HPLC method. Analysis of caffeic acid, and gallic acid was realized with the chromatographic column VP-ODS RP18 type and using a gradient of mobile phase (Acetonitril- 0,1% acetic acid) and spectrophotometric detection. Caffeic acid in the range from 24.734 to 42.182 µg/g and gallic acid from 0.321 to 0.406 mg/g was determined in tested pollen samples.*

***Key Words*:** *Bee pollen, HPLC, phenolic acids, caffeic acid, gallic acid.*

INTRODUCTION

Bee pollen has been used for many years in both traditional medicine and supplementary nutrition, as well as in alternative diets, mainly due to its nutritional properties and health benefits. Bee pollen is the result of the agglutination of flower pollens; it is made by worker honey bees with nectar and salivary substances and stored at the hive entrance [1]. The collection of bee pollen is a relatively recent development, dependent primarily on the basic concept of scraping pollen off of the bees' legs as they enter the hive. When analyzing and studying the nutritional and therapeutic properties of bee pollen, modern science has made it possible to specify its valuable antimicrobial [2], antifungal [3], antioxidant [4], anti-radiation [5], hepatoprotective [6], chemopreventive [7], anticancer [8] and antiinflammatory activities [9].

The major components of bee pollen are carbohydrates, crude fibers, proteins and lipids at proportions ranging between 13 and 55%, 0.3 and 20%, 10 and 40%, 1 and 10%, respectively. Other minor components are minerals and trace elements, vitamins and carotenoids, phenolic compounds, flavonoids, sterols and terpenes [10].
However, the composition of bee pollen depends strongly on the plant source and geographic origin, together with other factors such as climatic conditions, soil type, and beekeeper activities.
During the last decade, interest in the study of phenolic compounds has increased greatly, mainly due to the antioxidant capacity of these substances in scavenging free radicals that are harmful to human health [11]. Among these compounds flavonoids and phenolic acids were suggested to be responsible for the biological activities. Therefore, the content of phenolic acids is considered as an important index for evaluating pollen quality. In general, Bee pollen composition is directly related to that of pollen exudates collected from various floras. A large number of analytical methods have been used for the analysis of phenolic compounds in bee pollen, including spectrophotometry, high performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS), gas chromatography–mass spectrometry (GC–MS), Among these methods, (HPLC) is one of the most-used techniques in the research of bee products, as it is able to analyze complex mixtures because of its high selectivity. [12]
In this study we aimed to evaluate Gallic acid and Caffeic acid from different sorts of bee pollen from Algeria using an HPLC method.

MATERIALS AND METHODS

Chemicals: Methanol (99%), was purchased from Sigma-Aldrich Co. 3,4,5-Trihydroxybenzoic acid (gallic acid; GA) (99%), (caffeic acid; CA) (99%), (Acetic acid; Ac) and Acetonitrile HPLC grad were procured from Alfa Aesar (Etats-Unis). Aluminium chloride ($AlCl_3$) and sodium carbonate (Na_2CO_3) were purchased from Prolabo (Etats-Unis). The Folin-Ciocalteu reagent (FCR), methanol (MeOH) and hexane were obtained from BIOCHEM chemopharma Co (FRANCE). High purity water, which was used in all experiments. All other reagents used were of analytical grade.

Pollen origin: Five dehydrated bee pollen samples were collected by beekeepers from five locations in North and south Algeria (Table 1), during the period from 2011 to 2012. After collection, the bee pollen was sent to the laboratory and each sample was separately crushed in commercial blender, homogenized and stored in freezer for later analysis.

Table 1 .Algerian bee pollen samples used in this study on the basis of date of harvest, geographical origin.

Sample code	Date of harvest	Place of production
P01	2011	Tlemcen
P02	2012	Algiers
P03	2012	Blida
P04	2011	Tipaza
P05	2012	Tizi Ouzou

Methanolic extract of pollen (EEP): 5 g bee pollen samples were extracted twice (30 min.) in an ultrasonic bath with 100 ml of methanol, at room temperature to obtain the extract. Each of the extract was filtered through Whatman NO.4 filter paper and evaporated with rotary evaporator at 45 ^{o}C. The extracts so obtained were weighed and stored in a brown bottle at 4 ^{o}C until further use.

Determination of total phenolic content (TPC): Total phenolic content was determined using Folin- Ciocalteu reagents according to the method of Kumazawa et al [13], briefly described as 0.5 ml of Folin and Ciocalteu's phenol reagent was mixed with 100 µl extract solution. After 3 min, 2 ml of 20% aqueous sodium carbonate solution was added to the mixture. The reaction was kept in the dark for 30 min, after which the absorbance was read at λ = 760 nm. [14]
Gallic acid was used as the standard to produce the calibration curve (0.03-0.3 mg/ml). The mean of three readings was used and the total phenolic content expressed in mg of gallic acid equivalents (GAEs) (mg/100g).

Instrumentation and chromatographic conditions: Spectrophotometric measurements were performed on an UV-1800 Schimadzu Spectrophotometer (double-beam) equipped with 1 cm quartz cuvettes. Ultrasonic bath (J.P. SELECTA,s.a.). A high performance liquid chromatography system, Shimadzu LC 20 AL equipped with universal injector (Hamilton 25 µL) SPD 20A, UV-VIS detector SPD 20A (Shimadzu) was used.

Preparation of mobile phase: The mobile phase was a mixture of acetonitrile and acetic acid 0,1%. The contents of the mobile phase were filtered before use through a 0.45µm membrane filter, sonicated and pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. A linear gradient was used for elution as described below (Table 2).

Table 2 . Gradient programme for elution of phenolic acids

Time	CH_3COOH (0,1%)	ACN
0	90	10
6	86	14
16	83	17
23	81	19
28	77	23
30	90	10

The effluent was detected at 300 nm.
The column temperature was maintained at room temperature level and the volume of injection was 20 µL. Prior to injection of analyte, the column was equilibrated for 40- 50 min with the mobile phase.

Preparation of standard solution: Phenol standards including caffeic acid and Gallic acid were dissolved in mobile phase as 1mg/ml concentrations. The solutions were filtered through a 0.45 μm membrane filter and stored in darkness. Standards are prepared biofreshly and immediately injected to HPLC column. Evaluation of each standard was repeated three times.

Preparation of Calibration graph: The stock solution of Gallic Acid and Caffeic Acid was diluted to four different concentrations between 0.5-8 % of working concentration. These were injected in triplicate for the preparation of calibration graph. The calibration graph was plotted by using the concentrations versus average peak area at 300 nm.

RESULTS AND DISCUSSION

Total phenolic content of methanolic extract from pollen: The Folin-Ciocalteu's assay is fast and simple methods which rapidly determine a content of phenolic in samples. Many of the phenolic have been shown to contain high levels of antioxidant activities. The total phenolic content of methanolic extract of bee pollen was presented in Table 3.
The total phenolic were found to be higher in sample P01 (32.01mg GAE/g extract) followed by sample P02 (31.52 ± 2,47mg GAE/g), P04 (30.83±0.98 mg GAE/g), P03 and P05 respectively. It has been investigated in many plant species that the total phenolic could significantly contribute to the antioxidant capacity of that species. Therefore, the higher amount of phenolic in bee pollen can be taken as a good indication for its higher antioxidant capacity.

Table 3. Global yield and total phenolic content of pollen extract obtained

Sample	Extraction yield (%)	Total phenolic content (mg/g)
P01	68.79	32.01 ± 0.00
P02	68.30	31.52 ± 2,47
P03	66.02	30.76±0.49
P04	64.00	30.83±0.98
P05	66.54	22.81±0.85

*Values are means ± SD (n = 3).

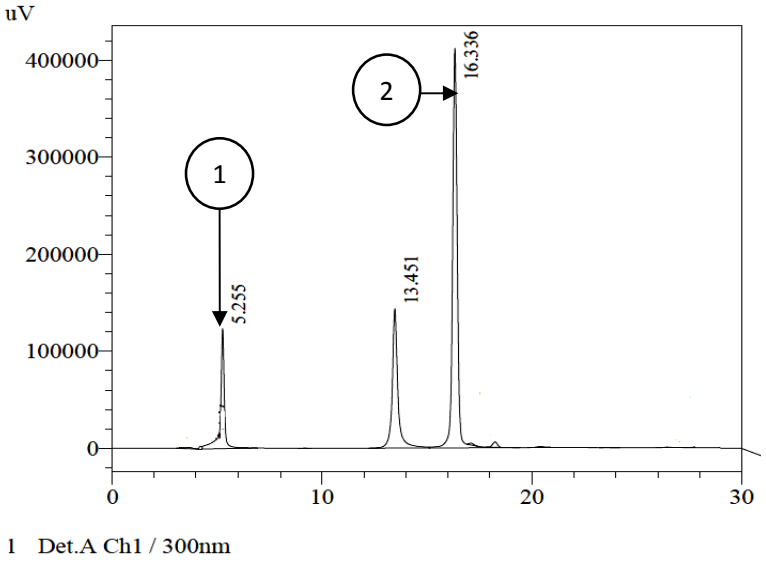

Fig 1. HPLC Chromatogram for standards: 1 – gallic acid; 2 – caffeic acid.

HPLC chromatogram of standards is presented in figure 1. The typical separation of Gallic and caffeic acids is shown in table 4 and expressed in retention times.

The chromatographic profiles at 300 nm were similar for all the methanolic extracts studied, independent of the sample location, consistent with the relationship between the phenolic profile and the surrounding apiary flora. [15]

Table 4. Standards retention times

Peak No	Rt (min)	Compound
1	5.25±0.18	gallic acid
2	16.3±0.14	caffeic acid

Rt: Retention time. *Values are means ± SD (n = 3).

Based on calibration curves and considering the dilutions made, we established the content in Caffeic acid and Gallic acid for all bee pollen samples.
The Gallic acid and Caffeic acid content from the bee pollen samples determined by HPLC method is listed in table 5.

Table 5. Caffeic acid and Gallic acid content, in Algerian bee pollen

Sample	Gallic acid (µg/g)	Caffeic acid (µg/g)
P01	406.43±1.54	28.78±0.41
P02	321.35±1.12	42.18 ±0.65
P03	353.77±0.91	35.6±0.23
P04	324.12±0.87	27. ±0.08
P05	340.78±1.05	24.734±0.17

Gallic acid was detected in all investigated bee pollen samples ranged from 406.43±1.54 to 321.35±1.12 µg/g. Caffeic acid was detected in all investigated samples of bee pollen from Algeria with concentrations ranging from 42.18 ±0.65 to 24.734±0.17 µg/g.

In the experimental conditions of this study, we established calibration curves for standards and regression coefficients (**Fig. 3.**). The linearity of calibration curves for Gallic and Caffeic acid was very good (R^2>0.99).

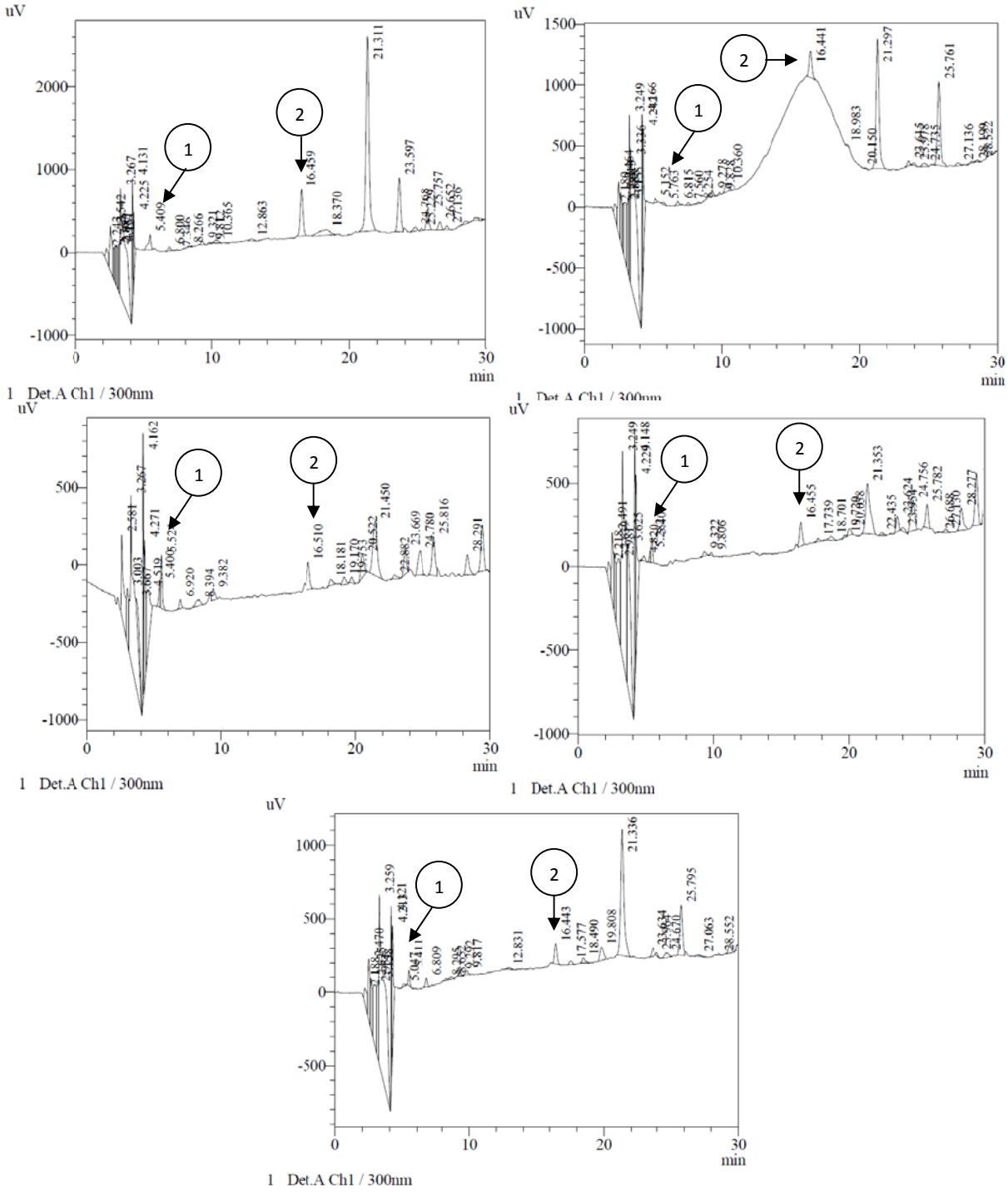

Fig. 2. HPLC Chromatogram of pollen methanolic extract samples : 1 – gallic acid; 2–caffeic acid.

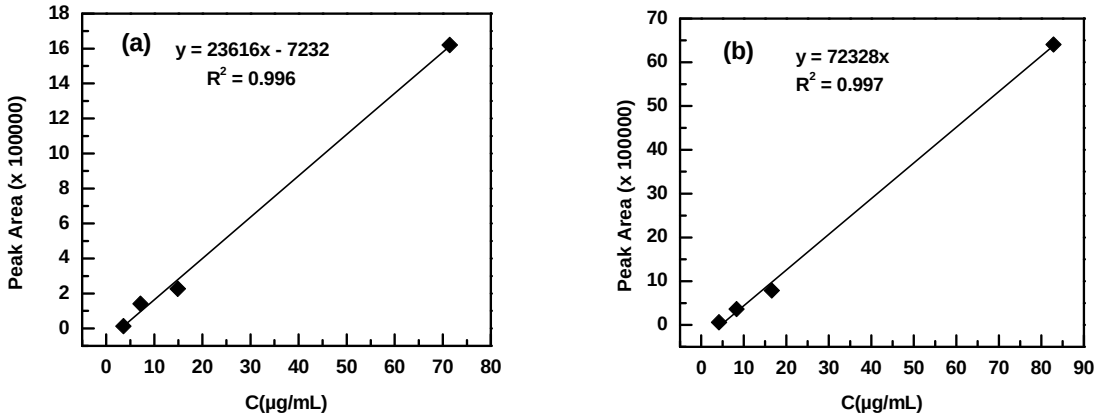

Fig. 3. Calibration curves and regression coefficients for (a) Gallic acid and (b) Caffeic acid.

CONCLUSIONS

In conclusion, caffeic acid and gallic acid of the bee pollen extracts can be successfully separated and identified by reversed phase HPLC (RP-HPLC) with a gradient elution mode. The method proposed allows a simultaneous determination of the naturally occurring organic acids in different sorts of bee pollen, providing an acceptable limit of detection.
In the present study it is found that the methanolic extract of Algeria pollen contains substantial amount of gallic acid and caffeic acid.

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