SUPPLEMENTARY MATERIAL

Detection of polyphenols in carob pods (*Ceratonia siliqua*) from Southern Italy by a LC-HRMS method

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Abstract

The presence of polyphenols was examined in carob pods (Ceratonia siliqua) from Southern Italy 90 days after harvest by the validation of a reliable LC-HRMS method. A greater abundance of Apigenin (51490.22±34399.16 μg/Kg) and Myricetin (24897.92±108332.05 μg/Kg) compared to previous research works conducted in Mediterranean countries. Significant differences in the polyphenol content between sampling areas ($p<0.05$) were observed, particularly differences in hesperidin and myricetin. These differences confirmed the role of geochemical and climatic conditions in the variation of polyphenol content. This study is a first regarding the phenolic content of carob pods from Southern Italy, confirming the presence of these substances even long after harvest and that carob pods are valuable sources of phenolic substances that may be useful in the prevention of diseases related to oxidative stress.

Keywords: *polyphenols; flavones; carob pods; Southern Italy*

3. Experimental

3.1 Reagents and gases

The standard solutions of gallic acid, catechin, caffeic acid, syringic acid, rutin, ellagic acid, hesperidin, ferulic acid, myricetin, quercetin, apigenin, naringenin, and kaempferol, each with a purity exceeding 99.9%, were procured from Sigma-Aldrich S.r.l. (Milan, Italy), whereas chlorogenic acid was purchased from HWI Analytik GmbH (Rülzheim, Germany). 10 mg of each standard in powder was diluted with 10 mL of methanol, obtaining a concentration of 1000 mg L^{-1}. Notably, only apigenin and kaempferol were dissolved in an aqueous solution with a pH greater than 8 (Cammilleri et al. 2024). Ultrapure deionized water was obtained by a Milli-Q® Integral water purification system from Millipore (Bedford, MA, USA). All reagents used were of HPLC grade, with acetone, acetonitrile, and formic acid purchased from Sigma Aldrich (Amsterdam, Holland), and hydrochloric acid purchased from Carlo Erba (Milan, Italy).

3.2 Sample collection and extraction

Carob pods were collected from organic farms of Agrigento and Catania provinces (Sicily, Southern Italy) during 2023. Carob pods were collected from each carob tree in order to obtain pools of approximately 100 g. A total of 60 pools, equally distributed for each province considered, were collected for the phenolic compounds detection. Considering the lack of studies on the concentrations of phenolic compounds in carob pods long after harvest, we decided to store the carob pods at room temperature in a controlled humidity environment for 90 days before the samples extraction.

The extraction protocol for the detection of phenolic compounds was performed according to Puigventos et al. (2014), for the detection of phenolic compounds in fruit extracts. In brief, each pool of carob pods was deseeded and kibbled by a vertical mixer B-400 (Büchi, Flawil, Switzerland). 0.1 g of the kibbled samples were introduced into a 10 mL solution composed of acetone, water, and hydrochloric acid in a volumetric ratio of 70:29:0.1 v/v/v and subjected to 30 minutes of sonication. Then, centrifugation was performed for 15 minutes at 3500 rpm, and the resultant extract was cryogenically stored at -4°C. Prior to analysis, filtration with 0.45 μm nylon filters was implemented on the extract.

3.3 LC-HRMS analysis and method validation

Chromatographic separations were carried out using a Raptor C18 column (2.1 mm × 100 mm, 1.7 μm) according to Cammilleri et al. (2023 table S1). The mobile phase comprised eluent A (H2O+formic acid 1%) and eluent B (acetonitrile+formic acid 1%), with a total run time of 14

minutes and a flow rate of 0.3 mL min^{-1}. The chromatographic run initiates with 95% of A and 5% of B. Subsequently, there is a decrease in A and an increase in B by 25% within 0.33 minutes. Over the next 1.63 minutes, there is a continuous increase in B up to 100%. This condition is maintained for approximately 9 minutes. Finally, there is an increase in A and a decrease in B until the initial conditions are restored at 11.63 minutes. The operating conditions were in accordance with. Polyphenols quantification was conducted employing a Q-Exactive Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in both positive and negative polarity modes (Tables S2 and S3). Polyphenol concentrations were expressed as μg/Kg. The method underwent validation for linearity, recovery, repeatability, and reproducibility within the laboratory, following ISO/IEC 17025:2005, the Commission Regulation 836/2011 and the decision 657/2002/EC of the European Union (Cammilleri et al. 2019; Galluzzo et al. 2021 Istamina; Mauro Diclofenac 2022). Limits of detection and quantification (LODs and LOQs) were determined using the 3σ and 10σ approach (Lo Dico miele; Bacchi Rane).

3.4 Data collection and statistical analysis

All statistical analyses were performed using the R® 4.2.3 software and the results were expressed as μg/Kg. All the results below the LOD of the method were considered as half of the LOD for the statistical analysis (Durkalec et al. 2023). As the data did not meet the assumptions of normal distribution and homogeneity of variance, a Wilcoxon test was used to assess whether there were significant differences in the levels of phenolic compounds among sampling areas.

Step	Start	A%	B%
1	0.00	95.0	5.0
2	0.33	70.0	30.0
3	1.63	-	100.0
4	9.63	-	100.0
5	11.63	95.0	5.0

Table S1. Chromatographic conditions for the separation of the polyphenols in the carob pods samples.

PARAMETER	VALUE
dd-MS^2/dd-SIM	
Resolution	35.000
AGC target	$2x10^{-5}$
Maximum IT	100 ms
Isolation window	2.0 m/z
Full MS	
Resolution	70.000
AGC target	$3x10^{-6}$
Maximum IT	200 ms
Scan range	100 to 1000 m/z

Table S2. HRMS conditions in negative and positive polarity modes.

Mass Exact [m/z]	Formula [M]	Species	CS [z]	Polarity	Start [min]	End [min]	Analyte
319.04484	C15H10O8	+ H	1	Positive	0	13.00	Myricetin
303.01354	C14H6O8	+ H	1	Positive	0	13.00	Ellagic acid
171.02880	C7H6O5	+ H	1	Positive	0	13.00	Gallic acid
199.06010	C9H10O5	+ H	1	Positive	0	13.00	Syringic
195.06519	C10H10O4	+ H	1	Positive	0	13.00	Ferulic acid
287.05501	C15H10O6	+ H	1	Positive	0	13.00	Kaempferol
273.07575	C15H12O5	+ H	1	Positive	0	13.00	Naringenin
271.06010	C15H10O5	+ H	1	Positive	0	13.00	Apigenin
611.16066	C27H30O16	+ H	1	Positive	0	13.00	Rutin
355.10236	C16H18O9	+ H	1	Positive	0	13.00	Chlorogenic acid
611.19705	C28H34O15	+ H	1	Positive	0	13.00	Hesperidin
181.04954	C9H8O4	+ H	1	Positive	0	13.00	Caffeic acid
291.08631	C15H14O6	+ H	1	Positive	0	13.00	Catechin
303.04993	C15H10O7	+ H	1	Positive	0	13.00	Quercetin
317.03029	C15H10O8	- H	1	Negative	0	13.00	Myricetin
300.99899	C14H6O8	- H	1	Negative	0	13.00	Ellagic acid
169.01425	C7H6O5	- H	1	Negative	0	13.00	Gallic acid
197.04555	C9H10O5	- H	1	Negative	0	13.00	Syringic
193.05063	C10H10O4	- H	1	Negative	0	13.00	Ferulic acid
285.04046	C15H10O6	- H	1	Negative	0	13.00	Kaempferol
271.06120	C15H12O5	- H	1	Negative	0	13.00	Naringenin
269.04555	C15H10O5	- H	1	Negative	0	13.00	Apigenin
609.14611	C27H30O16	- H	1	Negative	0	13.00	Rutin

353.08781	C16H18O9	- H	1	Negative	0	13.00	Chlorogenic acid
609.18249	C28H34O15	- H	1	Negative	0	13.00	Hesperidin
179.03498	C9H8O4	- H	1	Negative	0	13.00	Caffeic acid
289.07176	C15H14O6	- H	1	Negative	0	13.00	Catechin
301.03538	C15H10O7	- H	1	Negative	0	13.00	Quercetin

Table S3. Identification parameters of the polyphenols analysed by LC-HRMS.