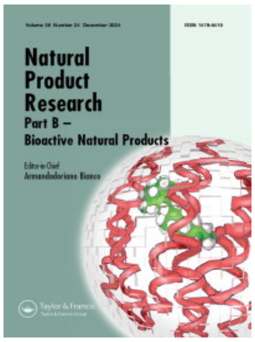

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Detection of polyphenols in carob pods (*Ceratonia siliqua*) from Southern Italy by a lC-HRMS method

Calogero Alfano[a], Elisa Maria Domenica Messina[a], Gaetano Cammilleri[a], Francesco Giuseppe Galluzzo[a], Licia Pantano[a], Maria Drussilla Buscemi[a], Andrea Macaluso[a], Tiziana Bertuglia[a], Andrea Pulvirenti[b], Gianluigi Maria Lo Dico[a], Ursula M. Jacob[c], Tilman Fritsch[d], Vincenzo Ferrantelli[a], Arnold Stern[e] and Vittorio Calabrese[f]

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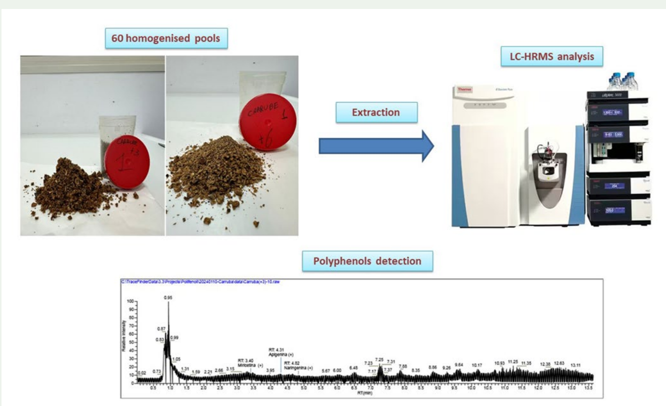
ABSTRACT

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 ± 108 332.05 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.



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1. Introduction

The carob tree (*Ceratonia siliqua*) is a typical Leguminosae component of the Mediterranean basin that usually grows in mild places with dry soil (Tous et al. 2013). Initially intended for the production of animal feed, carob fruit has also proven of use in human consumption for the production of sweets, in particular chocolate bars and as raw material for the production of other food products. They are also used for the extraction of galactomannan from their seeds (Quiles-Carrillo et al. 2019). Global production of carob pods is estimated at approximately 310,000 tonnes, and occurs predominantly in Mediterranean countries (Portugal, Italy, Morocco, Turkey, Greece, Cyprus, and Algeria) (FAOSTAT 2019). In Italy, carob is mostly produced in Sicily (Southern Italy), with a mean production of 28,000 tonnes (FAOSTAT 2019).

Carob pulp is rich in sugars (48–51%) and fibres (18–20%) and low in fats (0.6–0.8%) (Rodríguez-Solana et al. 2021). There are several compounds in the carob fruit, such as amino acids, minerals and insoluble fibres (Ikram et al. 2023), that have beneficial effects in gastrointestinal and cardiovascular diseases (Brassesco et al. 2021).

Extracts from carob pods may have potential chemo-preventive properties due to their specific activity against cancer cells (Gregoriou et al. 2021; Peterle et al. 2023). Carob pods contain high concentrations of phenolic compounds in 3 different forms (soluble free, soluble conjugated and bound) in particular phenolic acids and flavonoids such as gallic acid, chlorogenic acid, catechin and rutin (Chait et al. 2020). Most of the biological effects and health benefits of carob have been associated with the presence of polyphenols, also present in different natural products (Cicero et al. 2023; Di Salvo et al. 2023; Ioannou et al. 2023). However, the composition of polyphenols in carob pods is subjected to variations due to the carob cultivars, the cultivation region, the tree genus and the fruit development stages (Stavrou et al. 2018). Few studies from Italy have focused on the detection of phenolic compounds in carobs (Santonocito et al. 2020); most of them have examined the total polyphenol content with inaccurate methods (Lucci et al. 2017). The analysis of polyphenols in vegetables is relatively complex due to the diverse range of compounds present, varying in polarity and size. The chemical diversity of polyphenols complicates sample extraction, treatment, separation, determination, and identification processes (Lucci et al. 2017). Recent studies on carobs collected in Italy are focused mainly on their seeds as a possible source of bioactive compounds (Santonocito et al. 2020). Liquid Chromatography associated with High Resolution Mass Spectrometry (LC-HRMS) has become a powerful tool for simultaneous quantitative and qualitative analysis of organic pollutants, enabling their quantification and the detection of metabolites and transformation products (Aceña et al. 2015). High-resolution mass spectrometry (HRMS) offers several advantages over classical unit-mass-resolution tandem mass spectrometry: i) differentiation of isobaric compounds; ii) simplification of sample preparation procedures; iii) comprehensive information from a single injection; iv) retrospective analysis (Lucci et al. 2017). This technique offers exceptional sensitivity and specificity, ideal for detecting compounds at low concentrations even in complex matrices. It is more versatile and efficient than techniques such as HPLC or GC-MS, since it does not require derivatization and can analyse non-volatile compounds. LC-HRMS allows the simultaneous analysis of multiple analytes, reducing operating time and costs.

Table 1. Mean contents of carob polyphenols (μg/kg). N.d. not detected.

Polyphenols	*N*	Mean ± sd (N > LOD)	Maximum value
Gallic acid	60	N.d.	N.d.
Chlorogenic acid	60	734.27 ± 3211.56 (6)	14377.21
Syringic acid	60	2089.12 ± 3788.31 (21)	13627.34
Rutin	60	N.d.	N.d.
Catechin	60	532.21 ± 2357.74 (3)	10549.13
Caffeic acid	60	28.39 ± 47.38 (15)	181.95
Ellagic acid	60	N.d.	N.d.
Ferulic acid	60	436.03 ± 1873.56 (12)	8394.60
Myricetin	60	24897.92 ± 108332.05 (30)	485084.50
Hesperidin	60	173.48 ± 251.92 (27)	1009.368
Quercetin	60	354.85 ± 1564.57 (3)	7002
Kaempferol	60	10.36 ± 23.98 (3)	112.25
Apigenin	60	51490.22 ± 34399.16 (57)	122517.82
Naringenin	60	1043.91 ± 2417.41 (30)	9026.30

Due to its speed and precision, it is widely used and is an essential technology for modern scientific applications (Aydoğan 2020).

This work aimed at increasing knowledge concerning the content of polyphenols and its by-products in carob pods by the implementation of a reliable LC- HRMS method, with the objective of promoting the cultivation and consumption of carob in the human diet, based on the observation of its multiple beneficial effects.

2. Results and discussion

The linearity test of the LC-HRMS method for the phenolic compounds analysis showed r^2 values > 0.993. The LOD and LOQ values of all the analytes studied were between 10 and 86 μg/Kg, and between 40 and 290 μg/Kg, respectively. Recoveries studies showed values between 71% and 119%. Repeatability analysis demonstrated acceptable outcomes for all the concentration levels, according to metrological standards. The Horrat values for repeatability and reproducibility for the concentration levels considered were below 2, meeting the criteria specified by Commission Regulation No. 836/2011. The content of polyphenols analysed are shown in Table 1.

The mean contents of phenols followed the order: Apigenin > Myricetin > Syringic acid > Naringenin > Chlorogenic acid > Catechin > Ferulic acid > Quercetin > Hesperidin > Caffeic acid > Kaempferol. No Gallic acid, Rutin and Ellagic acid contents were found in all the samples examined.

Apigenin was the most abundant polyphenol, detected in 95% of the samples analysed, with a mean value of 51490.22 ± 34399.16 μg/kg followed by Myricetin and Naringenin. The maximum apigenin value was found in a sample from Catania (122517.82 μg/kg). Interestingly, although myricetin was detected in half of the samples examined, it showed the highest maximum value (485084.50 μg/kg), revealed in a sample collected in Catania. The comparison between sampling areas showed significant differences for naringenin and myricetin contents ($p < 0.05$). Carob pods from Catania showed a greater presence of polyphenols than the samples from Agrigento. Furthermore, caffeic acid was found only in the samples from Catania with a mean of 28.39 ± 47.38 μg/kg. Our results suggested a variability related to the geochemical and climatic conditions of the sampling areas. It was proven that temperature, humidity and soil fertility are parameters that influence the variability of phenolic compounds

(Viljevac Vuletić et al. 2017). In particular, it was found a positive correlation between polyphenols contents and physical parameters such as temperature and illumination. These parameters work synergistically to give plants an effective defence from harmful ultraviolet (UV) radiation, playing a fundamental role as a protective filter (Mansour-Gueddes et al. 2021; Viljevac Vuletić et al. 2017). Correia et al. (2018) demonstrated that two different types of soils can significantly influence the nutritional properties of carob pods (*Ceratonia siliqua*) and the presence of bioactive compounds in their pulp. In particular, the study highlighted that elements such as N, Zn and Mn play a key role in the accumulation of tannins in the pod pulp. Furthermore, the concentration of Mn in the leaves of carob plants grown on non-calcareous soils is positively correlated with greater antioxidant activity in the pods. This relationship could explain the higher content of polyphenols found in carobs collected from Catania than those from Agrigento, probably due to the presence of the Etna volcano in this area and therefore to a volcanic origin soil (Giordano et al. 2003).

Comparing our findings with those found by Christou et al. (2022), conducted in carob pods collected from Cyprus we reported higher values of myricetin, chlorogenic acid and caffeic acid, but lower values of naringenin, catechin, ferulic acid, quercetin, kaempferol and gallic acid. Another study (Santonocito et al. 2020) conducted in Southern Italy using carob seeds collected in Catania at different degrees of ripeness, showed higher values of Catechins and Flavonols and presence of gallic acid. However, their study is focused exclusively on the detection of polyphenol groups (flavonols, flavanols, gallic acid and derivatives and hydrolysable tannins) in seeds. Nevertheless, these discrepancies could be attributable to variations in the degree of ripeness of the carob pods during harvest. Another study (Brahim et al. 2016) that investigated the polyphenols content in carob from Morocco, showed different values depending on the matrix (dry powder and fresh pulp) supporting what was found in literature regarding the correlation between degree of ripeness and polyphenols content. A recent work conducted by Ioannou et al. (2023), in different carob matrices (pulp and powder) from Cyprus using HPLC method found lower myricetin, catechin, quercetin and caffeic acid values, but higher gallic acid and rutin contents confirming a variation on polyphenols contents related to the harvest area.

It is clear from Table 1 that apigenin was the most abundant polyphenols in our carob extract; it is an important phenolic compound which has many interesting pharmacological activities and nutraceutical potential such as anti-cancer activity due to its capacity to induce cell apoptosis (Salehi et al. 2019); Imran et al. (2020) and hyperlipemia and hyperglycaemia regulation (Ren et al. 2016). Furthermore, Ali et al. (2017) verified the ability of this compound to inhibit the activity of β-secretase, the main enzyme responsible for the release of the β-amyloid fragment in Alzheimer's disease. Among the polyphenols most found there is also myricetin, belonging to the flavonol class. Oxidative stress contributes to the progression of Alzheimer's disease through three main pathways: macromolecule peroxidation, Aβ metal ion redox potential, and mitochondrial dysfunction. These processes collectively impact cell homeostasis, leading to the generation of reactive oxygen species (ROS) and the up-regulation of amyloid-beta (Aβ) and phosphorylated tau (p-tau) formation. According to the study conducted by Karunakaran et al. (2019), myricetin appears to protect β cells from the process of programmed cell death caused by high glucose, by blocking one of the pathways through which the cells activate the apoptosis mechanism. Myricetin, at a concentration of 20 μM, was observed to be protective for β cells, reducing the process of programmed cell death (apoptosis) in both INS-1 cells and rat pancreatic islets. Furthermore, myricetin appears to activate the AMPK/Nrf2-HO-1 signalling pathway, which plays a key role in the protection of liver cells (Lv et al. 2020).

Extracts derived from carob pods and leaves were tested to evaluate the effect on the inhibition of cell proliferation in the mouse hepatocellular carcinoma cell line (T1) confirming a marked slowing of T1 cell proliferation in a dose-dependent way, with the maximum effect achieved at a concentration of 1 mg/mL. In another study, pre-treatment of experimental rats by intraperitoneal injection for 8 days with ethyl acetate extract of *C. siliqua* leaves showed significant protection from CCl4-induced liver and kidney disorders (Dahmani et al. 2023).

A previous study conducted on the presence and bioaccessibility of carob's polyphenols after oral, gastric and intestinal digestion (Chait et al. 2020), reported that phenols are assimilated in different quantities depending on the digestive tract and on the relative form (free, conjugated and bound). Regarding the bioaccessibility of phenols, apigenin, myricetin, catechin and chlorogenic acid showed, respectively, a bioaccessibility of 242.4%, 200%, 558.3% and 485.4% in their soluble free forms, syringic acid 66.6% in its soluble conjugated form and ferulic acid about 30% in all three forms. According to this study, the bioaccessibility values greater than 100% are imputable to the differences between free and conjugated form after *in vitro* digestion. As far as we know, there are no data available regarding the recommended daily intake of individual phenols; this means that there are no specific guidelines for optimal intake of each phenolic compound. The lack of such recommendations stresses the need to conduct further studies to better understand the role of these nutrients in the human diet and to determine which natural products, including carobs, reveal the highest concentrations.

3. Experimental

See the supplementary material for the experimental section.

4. Conclusions

The present study proposes a reliable LC-HRMS method with satisfactory validation results in terms of linearity, repeatability, and recovery for the detection of carob polyphenols. Our finding suggests that various factors influence the content of polyphenols like ripeness and collecting zone. In addition, the plant's growth in volcanic soil appears to be positively correlated with a higher content of polyphenols in its fruit. High presence of apigenin, myricetin and syringic acid were found in all the examined samples. As far as we know, this is the first study carried out on the detection of polyphenols in carob pods from Sicily (Southern Italy). In view of emerging data literature supporting the important health benefits of phenols, our results encourage the consumption of carob pods, stressing further studies to create appropriate guidelines for proper nutrient intake, especially because, as far as we know, data on the recommended daily intake are not available.

Author contributions

Conceptualisation, G.C., C.A. and E.M.D.M.; methodology, E.M.D.M., C.A. and G.C.; data curation, G.C., E.M.D.M., F.G.G. and L.P.; writing-original draft preparation, C.A., E.M.D.M. and G.C.; visualisation and validation, V. C., G.M.L., A.S. and A.P.; writing review and editing, U.M.J., T.F. and am Formal analysis, L.P., M.D.B., T.B. Project administration, V.F. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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