RESEARCH ARTICLE

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Determination of sugars and organic acids in diverse carob genotypes using HPLC techniques

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Abstract
Carob fruit is widely known for their abundance of health-boosting compounds like polyphenols, L-ascorbic acid, organic acids, and micronutrients. These compounds offer various benefits, including antioxidative, antimicrobial, antidiabetic, liver-protective, anti-inflammatory, anticancer, and heart-protective effects. In this research, High-Performance Liquid Chromatography (HPLC) methods were employed to assess sugar and organic acid levels in mature fruit pods of three distinct carob genotypes from the Kahramanmaras region in Turkey. The findings revealed that genotype-2 had the highest concentrations of glucose (1301 mg/100g) and fructose (875 mg/100g), genotype-3 exhibited the highest level of xylose (1548 mg/100g), while genotype-1 displayed the highest levels of sucrose (9183 mg/100g) and total sugars (12457 mg/100g). Regarding organic acids, genotype-1 exhibited the highest levels of oxalic acid (17.62 mg/100g), citric acid (612.50 mg/100g), and fumaric acid (8.0 mg/100g), while genotype-3 showed the highest levels of malic acid (234.92 mg/100g) and succinic acid (1089.76 mg/100g); however, genotype-2 had the highest amount of L-ascorbic acid (8.17 mg/100g). In conclusion, genotype-1 demonstrated the most favorable performance in terms of having the highest levels of total sugar and organic acids compared to the other two genotypes.

Keywords: Carob, Genotype, Sugars, Organic Acids, HPLC

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INTRODUCTION

Carob (*Ceratonia siliqua* L.) is a member of the *Leguminosae* family within the *Rosales* order and is extensively grown in the Mediterranean region (Battle et al. 1997). Turkey is recognized as one of the native habitats for both wild and cultivated carob, with its growth primarily in the Mediterranean and Aegean regions (Correia et al. 2004). The four main genetic groups of carobs are Southern Spain; South Morocco; Central Mediterranean (genotypes from Algeria, Portugal, Sicily, Sardinia, France, and the Balearic Islands; Eastern Mediterranean (Cyprus, Greece, and Lebanon) (Viruel et al., 2019). The tree grows to a height of 8-17 m and has a broad hemispherical brown crown, a thick trunk with rough bark, and a robust branch structure (Tous and Antoni, 2013). It yields fruit resembling an edible pod, commonly referred to as a grasshopper bean. The pods form clusters and remain green until they reach maturity, at which point they measure 10-25 cm in length. Variations in pod characteristics, such as size, weight, shape, density, color, and seed rate, were observed among different varieties and under various climatic conditions, as reported by Nasar-abbas et al. (2016). Carob seeds exhibit distinct features, including their brown color, significant hardness, measuring approximately 10 mm in length, and weighing roughly 0.2 grams each, with

Table 1. Global carob statistics for the years 2015 to 2018

Countries	Portugal	Italy	Morocco	Turkey	Greece	Cyprus	Algeria	Spain	World
Harvested area (ha)	13427	5600	10224	3099	2410	1004	808	2292	41593
Yield (kg/ha)	29.393	56,385	21.532	45.776	59.377	71.571	45.762	8382	32.839
Production (MT)	39.387	31.577	22.013	14.195	12.819	7.179	3.701	1.916	136.613
Production (%)	28.83	23.11	16.11	10.39	9.38	5.25	2.71	1.40	100

Source: (FAOSTAT, 2020; Brassesco et al., 2021)

their composition comprising 30-33% husk, 42-46% endosperm, and 23-25% seed material (Tous and Antoni, 2013). Between 2015 and 2018, Portugal (28.83%), Italy (23.11%), Morocco (16.11%), and Turkey (10.39%) were the primary global producers of carob pods, with their production and area harvested statistics detailed in Table 1.

It is widely used in the preparation of cold drinks, syrups, and liqueurs due to its high nutritional value (Youssef et al., 2013). Historical records indicate that ancient Egyptians fed their animals carob shells and utilized carob gum as an adhesive for mummies, while in modern Egypt, carob pods find extensive use in cookies, cakes, beverages, and different snacks. The Arabs adopted the carob seed as a measure of weight, referring to its kernel as a "carat," and the established weight of the carob seed served as the standard unit for measuring precious metals (Viruel et al., 2019). Carobs are well recognized as fruit rich in multiple health-promoting bioactive compounds, including polyphenols, ascorbic acid, organic acids, and micronutrients, which contribute to a broad range of bioactivities, such as antioxidant, antimicrobial, antidiabetic, liver protective, anti-inflammatory, anticancer, and cardioprotective activities (Goulas et al., 2016). Research has documented the presence of D-pinitol, a carbohydrate with properties resembling insulin, in carob (Bates et al., 2009). This sugar alcohol has been derived from various plant sources, including soybeans (Smith and Phillips, 1982), bougainvillea flowers (Narayanan et al., 1987), and ice plants (Vernon et al., 1993; Zunft et al., 2001). Studies have indicated that carob can have a positive impact on nutraceutical elements and blood LDL cholesterol levels, contributing to the reduction of postprandial blood sugar in individuals with Type II diabetes mellitus (Kang et al., 2006). According to Nasar-abbas et al. (2016), carob pods consist of sugar content ranging from 45% to 52%. However, up to this point, there hasn't been any prior scientific documentation concerning the analysis of various sugars and organic acids in carob pods using the HPLC method. Therefore, the primary objective of this study is to determine the sugar and organic acid levels in three distinct carob genotypes grown in the Andırın district, Kahramanmaraş region using the HPLC method. The study aims to contribute to a better understanding of carob's potential nutraceutical and related properties, as well as its potential beneficial effects.

MATERIALS AND METHODS

Plant materials

Fully ripe pods of carob fruit, weighing approximately 1.5-2 kg for each of the three carob genotypes (genotype-1, genotype-2, and genotype-3) (three replications from each genotype), were harvested from a commercial orchards in the Andırın district, Kahramanmaraş province of Turkey (Latitude: 37°34'59.99"N Longitude: 36°20'59.99"E) during the August 2020 growing season and subsequently conveyed to the Instrumental Analysis laboratory at the Department of Horticulture, Faculty of Agriculture, Çukurova University, Turkey, maintaining cold chain conditions.

Experimental procedure for sugar analysis of dry carob fruit

Alterations in the levels of glucose, fructose, sucrose, xylose, and total sugars in the homogenized carob samples were assessed using the HPLC method as described by Crisosto (1997). Prior to analysis, frozen samples were dissolved at 25 °C, and 1 g of dried fruit was mixed with 4 mL of ultrapure distilled H_2O (Millipore Corp., Bedford, MA, USA). The mixture was introduced into an ultrasonic bath and subjected to sonication at room temperature for 15 min, followed by centrifugation for 15 min at 5500 rpm. Prior to HPLC analysis, the centrifuged solution underwent filtration using Whatman nylon syringe filters with a 0.45 µm pore size and a 13 mm diameter. Sugar levels were assessed through a process involving three repetitions, utilizing HPLC equipment (Shimadzu LC-20A system, Kyoto, Japan Kyoto) with a RID (Refractive Index) detector and a Coregel-87C column (7.8 x 300mm). The separations were conducted at a temperature of 70 °C, while maintaining a 0.6 mL flow rate per min. Isocratic ultrapure water was employed in the elution process. The specific sugar was quantified based on their respective standards and presented as a percentage of the fresh weight. For the reference materials, calibration curves were created, and by referring to these calibration curves, the content was calculated.

Experimental procedure for organic acid analysis of dry carob fruit

The analysis of organic acids of carob fruit extract was conducted using an HPLC method established by Bozan et al. (1997) with some adjustments. The alterations in the levels of citric, malic, fumaric, L-ascorbic, succinic, and

oxalic acids within carob fruit samples were assessed. To extract organic acids, 1 g of working sample was combined with 4 mL metaphosphoric acid (3%) solution. The concoction was nestled in a soothing ultrasonic water ballet for a quarter-hour in ambient conditions, where it waltzed to the harmonious tune of sonication before gracefully pirouetting in the centrifuge at 5500 rpm for another 15 min. Subsequently, the concoction underwent a straining process, passing through Whatman nylon syringe filters with a 0.45 µm pore size and a 13 mm diameter. A Shimadzu LC 20A VP HPLC device from Kyoto, Japan, equipped with a UV detector, namely the Shimadzu SPD 20A VP, and an 87 H column (5 µm, 300 × 7.8 mm, Transgenomic), was employed for the analysis of organic acids. Sulphuric acid (0.05 mM) was used as a solvent. The operational parameters included a column temperature of 40 °C, an injection volume of 20 µL, detection at a wavelength of 210 nm, and a flow rate of 0.8 mL per min. Identifying organic acids and pinpointing peak values is dependent on aligning peak retention times and cross-referencing spectral data with established standards. The appropriate standard calibration curves were employed to evaluate the recognized acids.

Statistical analysis

The JMP software was employed for data analysis using analysis of variance (ANOVA) to scrutinize the results (JMP Start Statistics, 1996). The data is presented in the form of the mean ± standard deviation of the samples (n=3). The difference among the three carob fruit species is considered significant at a level of $p<0.05$.

RESULTS AND DISCUSSION

The objective of this study was to determine the sugars and organic acids content from 100 g of samples for three carob genotype mature dry pod fruit, excluding seeds. The chemical analysis of sugar compounds and organic acids for the three genotypes is presented in Table 1 and Table 2, respectively.

Sugar analysis of dry carob fruit

Genotype-1 exhibited notably greater sucrose (9183 mg/100g) and total sugar (12457 mg/100g) levels than genotype-2 and genotype-3, with the lowest levels of these sugars observed in genotype-3 pods, as indicated in Table 2. The genotype-2 exhibited notably higher glucose (1301 mg/100g) and fructose (875 mg/100g) contents compared to genotype-1 and genotype-3, with the lowest levels of glucose and fructose observed in the pods of genotype-3 (Table 2). Interestingly, genotype-3 exhibited significantly higher levels of xylose (1548 mg/100g) than genotype-1 and genotype-2, with the lowest levels of xylose (1213 mg/100g) observed in the pods of genotype-2 (Table 2). The composition of sugar compounds in carob fruit may vary depending on geographical diversity (Würsch et al., 1984; Saura-Calixto, 1988; Avallone et al., 1997). According to Karkacier and Artik (1995), carob fruits are considered ripe for harvesting when they contain 91-92% total dry matter and 62-67% total soluble solids, including 34-42% sucrose, 10-12% fructose, and 7-10% glucose. Based on prior research conducted in Turkey and other countries, sucrose, glucose, xylose, and fructose have been detected and measured in carob. Avallone et al. (1997) studied the carob genotypes containing sucrose (27-40%), glucose (3-5%) and fructose (3-8%). Ayaz et al. (2007), reported that sucrose in carob fruit is 43.73%, was the major sugar and followed by glucose and fructose. Biner et al. (2007) recorded that sucrose is a high amount of sugar in carob fruit with smaller amounts of glucose and fructose. Gubbuk et al. (2010), studied that the sugar compound of carobs significantly changed allowing the genotypes which reported sucrose (27.7-43.8%) and glucose (10.8-17.4%). Fructose (0.54-1.4%) was identified as the lowest amount of sugars in the carob and was determined to be the main sugar in carob pods.

Organic acids analysis of dry carob fruit

In terms of organic acids, genotype-1 exhibited significantly higher levels of oxalic acid (17.62 mg/100g), citric acid (612.50 mg/100g), and fumaric acid (8.0 mg/100g) compared to genotype-2 and genotype-3, while the pods of genotype-3 and genotype-2 had the lowest levels of oxalic (15.13 mg/100g) and citric acid (356.92 mg/100g), respectively (Table 3).

Genotype-3 displayed significantly higher levels of malic acid (234.92 mg/100g) and succinic acid (1089.76 mg/100g) than the other two genotypes, while genotype-1 had the lowest levels of malic acid (200.24 mg/100g) and succinic acid (662.65 mg/100g) (Table 3). Genotype-2 contained a significantly higher amount of L-ascorbic acid (8.17 mg/100g) compared to genotype-1 and genotype-3, where genotype-3 contained the lowest

Table 2. Sugar compounds are analyzed from three genotypes of the mature dry carob fruit

mg/100 g	Genotype-1	Genotype-2	Genotype-3
Sucrose	9183±560^{a}	9001±81^{b}	6894±109^{b}
Glucose	1119±84^{b}	1301±41^{a}	1025±109^{b}
Xylose	1399±122^{b}	1213±33^{c}	1548±90^{a}
Fructose	754±40^{a}	875±31^{a}	481±103^{b}
Total sugars	12457±52^{a}	12389±182^{b}	9948±195^{b}

The data is presented as Mean ± Standard deviation (n=3), and values sharing the same letter within the line indicate no statistically significant difference ($p < 0.05$).

Table 3. Organic acid compounds analyze from three genotypes of the mature dry carob fruit

mg/100 g	Genotype-1	Genotype-2	Genotype-3
Ascorbic acid	6.72±0.50[a]	8.17±1.08[a]	6.22±0.25[a]
Oxalic Acid	17.62±3.97[a]	15.98±3.60[a]	15.13±6.29[a]
Citric Acid	612.50±92.71[a]	356.94±60.85[b]	431.05±85.03[b]
Malic Acid	200.24±9.17[b]	202.16±16.33[b]	234.92±4.59[a]
Succinic Acid	662.65±14.47[b]	779.03±275.82[ab]	1089.76±85.17[a]
Fumaric Acid	8.00±0.66[a]	3.12±2.26[b]	3.12±0.07[b]

The data is presented as Mean ± Standard deviation (n=3), and values sharing the same letter within the line indicate no statistically significant difference ($p < 0.05$)

amount of L-ascorbic acid (6.22mg/ 100g) (Table 3). According to Ashoor and Knox (1982), organic acids, free sugars, and amino acids are normal constituents of fruit and vegetables, playing an important role in preserving quality and defining nutritional value. As per Ayaz et al. (2007), the fruit of the pods contained malic acid at a level of 2.4 mg/g dry weight, while citric and L-ascorbic acids were not present in detectable quantities. It can be concluded that a significant variation in the levels of sugars and organic acids was observed across distinct carob genotypes originating from Kahramanmaras, Turkey, with genotype-1 displaying notably higher concentrations of the analyzed sugars and organic acids compared to the other genotypes

CONCLUSION

In conclusion, this study has produced some significant outcomes. The results presented here indicate substantial variations in sugar and organic acid contents among carob genotypes originating from Kahramanmaras, Turkey. Based on the findings, genotype-1 displayed notably elevated levels of the examined sugars and organic acids in comparison to the other genotypes. The results of this study could prove valuable to researchers studying the nutritional content of food products and to the related industry. Further investigations should focus on exploring the potential health implications of diverse carob genotypes' nutritional profiles, particularly their impact on human metabolism and health outcomes.

COMPLIANCE WITH ETHICAL STANDARDS

Peer-review

Externally peer-reviewed.

Conflict of interest

The authors declare that they have no competing, actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the text, figures, and tables are original and that they have not been published before.

Ethics committee approval

Ethics committee approval is not required. This article does not contain any studies with human participants or animals performed by any of the authors.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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