Chapter N 056

CAROB LEAVES EXTRACTS AS NEW INGREDIENTS IN THE FOOD FIELD: EXTRACTION, CHARACTERIZATION AND ANTIOXIDANT FEATURES

**Abstract.** Two Apulian carob cultivar (Selvatica and Amele) leaves have been investigated in terms of their phytochemical profiles. UAE extraction proved to be effective in the recovery of the bioactive compounds in comparison to classical methods. Being equal the solvent, the Selvatica leaves extracts showed higher contents of antioxidants and, among solvents, water showed to possess the most effective extraction capacity leading to the highest yield. LC-DAD analyses revealed remarkable amounts of antioxidants in carob leaves extracts as confirmed by different colorimetric assays. Myricitrin and 4-HBA were showed to be the most abundant compounds in all samples, containing also simple phenols, polyphenols and flavanols. The obtained data demonstrated the suitability of the carob leaves extracts as a promising ingredient during functional foods formulation.

**Keywords.** Carob leaves, Extraction, Antioxidants, Functional Foods, Circular Economy

# Introduction

Increased consumers’ awareness of the importance of a healthy diet in preventing illness and diseases, led to the development of a
healthier food industry, prompting the market of functional foods and gluten-free products. However, a proper formulation of this kind of products poses many issues to be faced, including extraction, analysis, and technology parameters as well as bioavailability, biological activity, sensory features and legal constrains (Banwo et al., 2021). It follows, that the design and the formulation of such a product must be carefully evaluated by choosing proper matrices as ingredients, to confer the novel food acceptable/improved nutritional, technological and organoleptic characteristics (Galanakis, 2017).

To this regard, many efforts have been devoted during the last decades by researchers and a great number of novel foods are now available on the market. Among all possible exploitable bioactive compounds to obtain fortified foods, antioxidant molecules surely play the major role for their established health promoting effects (Koch, 2019). By-products of fruits and vegetables have been proved to be a valuable source of these molecules (Ramirez-Pulido et al., 2021). Their exploitation showed to be particularly convenient for minimizing wastes and reintroducing them into the production chain. In this sense, lowering agri-food wastes and by-products to develop functional foods could limit the resource depletion, the economic loss and the environmental impact in a circular economy approach.

When considering fortified foods, many aspects need to be addressed and the new ingredient must be carefully evaluated, in order to clearly elucidate composition in relation to the desired activity to be conferred. In this context, the research focused on the extraction and characterization of different carob leaf extracts from Selvatica and Amele cultivars. Carob leaves have been selected as they represent a by-product of an ancient cultivation of the Mediterranean basin showing a great food potential (Brasseco et al. 2021). Several extracts were obtained using different extraction techniques and solvents. A complete characterization of each extract by LC-DAD and colorimetric assays was then carried out, to evaluate the suitability of the functional ingredient to be possibly included in a fortified food formulation.

# Material and methods

***1.2.1 Chemicals and reagents***

Chlorogenic acid was purchased from Phytolab (Aprilia, Italy). Gallic acid, 4-hydroxy-benzoic acid (HBA), ferulic acid, 4-hydroxy-coumaric acid (HCA), caffeic acid, syringic acid, catechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, quercitrin, myricitrin, and rutin were purchased from Extrasynthese (Genay, France) and used as HPLC reference standards. Other chemicals and reagents were purchased from Merck (Darmstadt, Germany) and VWR International (Milan, Italy). Formic acid, ethanol, HPLC grade water, and acetonitrile were supplied by Merk Life Science S.r.l. (Milan - Italy). Other solvents and standards were purchased from Merck (Darmstadt, Germany).

***1.2.2 Carob leaves extraction***

The leaves of two carob tree cultivars, i.e. Selvatica (CS), and Amele (CA) were analysed. The extracts obtained using the Ultrasound Assisted Method (UAE) are labelled CSU and CAU, and those using the Soxhlet CSS and CAS. Five solvents, Absolute Ethanol, Water, Acetone, Ethyl Acetate and Dichloromethane, were employed. 1.0g of freeze-dried CS and CA, in 50mL of solvent, were sonicated for 30 minutes at 40°C. The extracts were filtered on Whatman paper N°3, and freeze-dried (CSU5, CAU5) or under vacuum (CSU1-4, CAU1-4). 1.0g of CS and CA, in 100 mL of solvent, were refluxed in a Soxhlet apparatus for 5h. The extracts were filtered on Whatman paper N°3, dried under vacuum and stored at -20 before use.

***1.2.3 Chemical composition and antioxidant performances of the extracts***

*1.2.3.1 Characterization of the extracts by HPLC-DAD*

HPLC 1260 (Agilent Technologies, Palo Alto, USA), with a degasser, quaternary pump solvent delivery, thermostated column compartment, and diode array detector was exploited for the polyphenols determination. The extracts (3 μL) were injected onto a reversed stationary phase column, Zorbax SB-C18 (Agilent Technologies, Palo Alto, USA) 3.5 μm (150 x 4.6 mm i.d.), protected by a pre-column, Gemini C18 (Phenomenex, Torrance, CA, USA) 5 μm (4 x 2 mm i.d.), and maintained at 40 °C. Water/formic acid (99.9:0.1, v/v) (solvent A) and acetonitrile (solvent B) were adopted as mobile phase through a binary gradient with a total run time of 35 min. 5 min were added to restore the starting conditions. The flow was maintained at 0.8 mL/min. DAD was between 190 and 400 nm, and absorbance was recorded at 360, 330, and 280 nm. Positions of absorption maxima (λmax.), absorption spectra profile, and retention times (RT) were matched with those of pure standards for identification. External calibration was carried out for quantification. The method was validated in terms of usual figures of merit (LOD, LOQ, linearity, precision, and analytical sensitivity).

*1.2.3.2 Polyphenols’ total content and Antioxidant performances*

The amount of total phenolic content (TPC), expressed in milligrams of gallic acid (GA) per gram of sample (mg GA/g sample), was determined using the Folin-Ciocalteu assay (Restuccia et al., 2019). Free radical scavenging properties of the extracts were estimated towards DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,20-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radicals and expressed in terms of IC50 (Restuccia et al., 2019).

# Result and Discussion

* + 1. ***Extraction techniques and parameters***

It’s now well established, that the extraction technique applied for bioactive compounds recovery from plant matrices, is a crucial step for further analysis and characterization. In table 1 and 2 the analytical parameters related to UAE and Soxhlet extraction are reported. As can be seen, the classical method generally offers higher yields; however, the Soxhlet procedure is time-consuming, requires relatively large quantities of solvents, and the examined active components may degrade or oxidize due to the long processing times and high temperatures used.

**Table 1.** Ultrasounds assisted extraction of Carob Selvatica (S) and Amele (A) carob leaves.

|  |  |  |
| --- | --- | --- |
| **Samples** | **Extraction Conditions** | **Yield**  |
| *label* | *Amount (g)* | *Solvent* | *Volume (ml)* | *T (°C)* | *t (h)* | *w**(g)* | *%* |
| CSU1 | 1.0 | Ethanol | 50 | 40 | 0.5 | 0.070 | 7.0 |
| CSU2 | 1.0 | Acetone | 50 | 40 | 0.5 | 0.040 | 4.0 |
| CSU3 | 1.0 | Ethyl Acetate | 50 | 40 | 0.5 | 0.034 | 3.4 |
| CSU4 | 1.0 | Dichloromethane | 50 | 40 | 0.5 | 0.034 | 3.4 |
| CSU5 | 1.0 | Water | 50 | 40 | 0.5 | 0.302 | 30.2 |
| CAU1 | 1.0 | Ethanol | 50 | 40 | 0.5 | 0.147 | 14.7 |
| CAU2 | 1.0 | Acetone | 50 | 40 | 0.5 | 0.112 | 11.2 |
| CAU3 | 1.0 | Ethyl Acetate | 50 | 40 | 0.5 | 0.038 | 3.8 |
| CAU4 | 1.0 | Dichloromethane | 50 | 40 | 0.5 | 0.014 | 1.4 |
| CAU5 | 1.0 | Water | 50 | 40 | 0.5 | 0.024 | 2.4 |

On the contrary. UAE is a simple. efficient. inexpensive. and environment friendly method of extraction. In this case. the disruption of the cell walls and the mass transfer. are linked to acoustic cavitation leading to both thermal and mechanical effects (Stavrou et al. 2018). All considered. UAE shows good extraction yields. significantly reduced processing times and lower temperatures. thus representing a convenient alternative for the extraction of thermally unstable molecules.

**Table 2.** Extraction process by Soxhlet of Selvatica (S) and Amele (A) carob leaves.

|  |  |  |
| --- | --- | --- |
| **Samples** | **Extraction Conditions** | **Yield** |
| *Label* | *Amount**(g)* | *Solvent* | *Volume (ml)* | *T (°C)* | *t (h)* | *w**(g)* | *%* |
| CSS1 | 1.0 | Ethanol | 100 | 70 | 5 | 0.132 | 13.2 |
| CSS2 | 1.0 | Acetone | 100 | 50 | 5 | 0.175 | 17.5 |
| CSS3 | 1.0 | Ethyl Acetate | 100 | 70 | 5 | 0.105 | 10.5 |
| CSS4 | 1.0 | Dichloromethane | 100 | 30 | 5 | 0.098 | 9.8 |
| CAS1 | 1.0 | Ethanol | 100 | 70 | 5 | 0.177 | 17.7 |
| CAS2 | 1.0 | Acetone | 100 | 50 | 5 | 0.109 | 10.9 |
| CAS3 | 1.0 | Ethyl Acetate | 100 | 70 | 5 | 0.074 | 7.4 |
| CAS4 | 1.0 | Dichloromethane | 100 | 30 | 5 | 0.038 | 3.8 |

Considering the food application of the extract and the data evaluation. CSU5 seemed the best extract to be considered for the new ingredient design and application. This choice. made because of the highest extraction yield. had to be confirmed by the total phenolic content and the antioxidant properties of each extract as obtained by colorimetric assays (Table 3).

As can be seen. being equal the solvent. Selvatica derived extracts showed better performances as compared to Amele *cv* extracts. whereas among solvents. water showed highest TPC and good IC50 values. Poor values for each experiment were recorded using dichloromethane which were not exploited any further. All considered. antioxidant data confirmed CSU5 as the better extract to apply. as already hypothesized by previous data.

**Table 3.** Antioxidant features of Selvatica (S) and Amele (A) carob leaves extracts. *.\*IC30*

|  |  |  |
| --- | --- | --- |
| **Sample** | **TPC**(mg AG/g extract) | **IC50** (mg mL-1) |
| **DPPH** | **ABTS** |
| **CSS1** | 60.0±0.4 | 0.0138 ± 0.0006 | 0.0050 ± 0.0001 |
| **CSS2** | 67.1±0.3 | 0.0180 ± 0.0007 | 0.0037 ± 0.0001 |
| **CSS3** | 37.4±0.2 | 0.0179 ± 0.0007 | 0.0034 ± 0.0001 |
| **CSS4** | 14.3±0.2 | 0.2510 ± 0.0074 | 0.1610 ± 0.0054 |
| **CAS1** | 61.9±0.3 | 0.0132 ± 0.0005 | 0.0018 ± 0.0001 |
| **CAS2** | 69.0±0.4 | 0.0170 ± 0.0007 | 0.0023 ± 0.0001 |
| **CAS3** | 26.8±0.2 | 0.0510 ± 0.0006 | 0.0210 ± 0.0007 |
| **CAS4** | 5.3±0.1 | 0.2010 ± 0.0061 | 0.1110 ± 0.0051 |
| **CSU 1** | 29.2±0.2 | 0.0330± 0.0001 | 0.0037 ± 0.0001 |
| **CSU 2** | 28.1±0.2 | 0.0372± 0.0001 | 0.0046 ± 0.0015 |
| **CSU 3** | 42.5±0.3 | 0.0440± 0.0001 | 0.0106 ± 0.0007 |
| **CSU 4** | 18.0±0.1 | 0.0904± 0.0016 | 0.0530 ± 0.0006 |
| **CSU 5** | 313.0±0.4 | 0.0420± 0.0014 | 0.0072 ± 0.0002 |
| **CAU 1** | 58.7±0.3 | 0.0160± 0.0007 | 0.0080 ± 0.0001 |
| **CAU 2** | 23.0±0.2 | 0.0405± 0.0014 | 0.0125 ± 0.0007 |
| **CAU 3** | 15.2±0.1 | 0.0720\*± 0.0015 | 0.0355 ± 0.0013 |
| **CAU 4** | 12.4±0.1 | 0.0780± 0.0016 | 0.0380 ± 0.0016 |
| **CAU 5** | 174.4±0.4 | 0.0180± 0.0006 | 0.0039 ± 0.0006 |

To this regard. LC-DAD data further confirmed the choice of CSU5 as the best extract to be used as a functional ingredient (Table 4). The result of this study showed that carob leaves contain varieties of individual components from several classes: simple phenols. polyphenols. free flavonoids and glycosylated flavonoid (Stavrou et al. 2018).

**Table 4**. Concentration (mg/g) of polyphenols in carob leaves extracts.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Compound** | **CSU1** | **CSU2** | **CSU3** | **CSU5** | **CSS1** | **CSS2** | **CSS3** | **CAU1** | **CAU2** | **CAU3** | **CAU5** | **CAS1** | **CAS2** | **CAS3** |
| Gallic Acid | 0.67 | 2.09 | 0.023 | 2.46 | 2.27 | 1.16 | 0.085 | 0.95 | 1.63 | tr | 1.28 | 2.36 | 1.20 | 0.083 |
| Epigallocatechin | 0.40 | 0.26 | n.d. | 1.04 | 0.87 | 1.00 | n.d. | 0.58 | 0.20 | n.d. | 0.59 | 0.90 | 1.03 | n.d. |
| Chlorogenic Acid | n.d. | n.d. | n.d. | 0.023 | n.d. | 0.014 | n.d. | tr | n.d. | n.d. | 0.013 | n.d. | 0.014 | n.d. |
| Catechin | 0.44 | n.d. | 0.033 | 0.025 | 0.89 | 0.49 | n.d. | 0.64 | n.d. | 0.013 | 0.013 | 0.92 | 0.50 | n.d. |
| 4-HBA | 27.15 | 27.61 | 2.96 | 35.34 | 30.50 | 18.12 | 6.96 | 29.91 | 20.71 | 1.2 | 20.14 | 42.20 | 19.47 | 6.74 |
| Epigallocatechin Gallate | 0.058 | 0.038 | n.d. | 0.39 | 0.058 | 0.028 | n.d. | 0.084 | 0.029 | n.d. | 0.21 | 0.060 | 0.029 | n.d. |
| Syringic Acid | 0.066 | 0.066 | n.d. | 0.59 | 0.079 | 0.27 | n.d. | 0.095 | 0.050 | n.d. | 0.34 | 0.082 | 0.28 | n.d. |
| 4-HCA | tr | 0.010 | n.d. | 0.054 | 0.039 | 0.11 | n.d. | 0.031 | tr | n.d. | 0.030 | 0.040 | 0.11 | n.d. |
| Rutin | 0.23 | 0.15 | 0.041 | 0.82 | 0.43 | 1.49 | 0.32 | 0.33 | 0.11 | 0.018 | 0.48 | 0.45 | 1.54 | 0.31 |
| Myricitrine | 11.38 | 8.50 | 1.66 | 37.80 | 10.80 | 24.16 | 23.65 | 6.39 | 6.38 | 0.71 | 20.79 | 21.51 | 25.68 | 22.89 |
| Epicatechin Gallate | 0.38 | 0.35 | n.d. | 0.60 | 0.29 | 0.27 | n.d. | 0.55 | 0.26 | n.d. | 0.34 | 0.30 | 0.28 | n.d. |
| Ferulic Acid | tr | tr | n.d. | tr | tr | 0.015 | n.d. | 0.014 | n.d. | n.d. | n.d. | Tr | 0.015 | n.d. |
| Quercitrine | 1.59 | 1.72 | 0.38 | 6.99 | 3.01 | 1.59 | 6.53 | 2.29 | 1.29 | 0.16 | 4.05 | 3.13 | 1.91 | 6.35 |

Among them. the most abundant was the flavonol glycoside myricitrin (3-O-*α*-L-rhamnopyranoside of myricetin). claimed to exert antiallergic.[https://www.liebertpub.com/doi/10.1089/jmf.2018.4341 - B5](https://www.liebertpub.com/doi/10.1089/jmf.2018.4341#B5)anti-inflammatory. antioxidant. antifibrotic. and antiatherogenic effects as well as anti-obesity activity (Semwal et al.. 2016). High concentrations of 4-HBA were also found in carob leaves extracts. This molecule has been proved to be highly effective as an antimicrobial. antialgal. anti-inflammatory. antiviral. and antioxidant agent widely exploited as a preservative in many drugs. cosmetic products. pharmaceuticals. food and beverages (Banwo et al.. 2021).

**1.4 Conclusions and future perspectives**

The agri-food sector originates throughout the whole food supply chain many by-products and wastes needing proper handling and disposal. within the current bioeconomy and sustainability framework. To this regard. the recovery of bioactive molecules from carob leaves represents a very challenging task under the analytical and the technological point of view. In particular. the lack of a standardized extraction method underlines the importance of a proper choice of the extraction solvent and the applied temperature. In this sense. data collected in this study. indicated that water was the most efficient solvent. At the same time. the ultrasound-assisted extraction. avoiding extreme conditions. demonstrated the more representative results. Owing to its composition and antioxidant features. the extract of Selvatica carob leaves (CSU5) represents a promising ingredient to be added during food production in relation to the many carob-related health promoting effects (Rtibi et al.. 2017). However. the sensory quality of enriched food products must be investigated since the addition could lower the product sensory features and consumer acceptance Moreover. the bioaccessibility and bioavailability of bioactive compounds should be assessed to estimate the nutraceutical potential of enriched products.

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