Formulation of a nutraceutical derived from carob: β-cyclodextrin encapsulation of antioxidants from carob pod

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Summary
Encapsulation of carob pod antioxidants with β-cyclodextrin has been proposed as simple and sustainable approach to increase the antioxidant activity of carob pod extract (CPE) in food and biological model systems. Combining the process of freeze-drying and encapsulation with β-cyclodextrin (6.4 $g \cdot l^{-1}$) significantly increased yields, provided protection during drying process and improved thermal stability of obtained CPEs. Formation of inclusion complexes of carob antioxidants and β-cyclodextrin was potentiated by removal of soluble carbohydrates prior to extraction step and resulted in significantly improved thermal properties of obtained extracts. Solid CPEs were rich in polyphenolic compounds (up to 59.520 $g \cdot kg^{-1}$ expressed as gallic acid equivalents) and showed antioxidant activity comparable with 1 $mmol \cdot l^{-1}$ butylated hidroxyanisole in β-carotene-linoleate food model system. In biological model systems, analysed CPEs (1 $mg \cdot ml^{-1}$) showed antioxidant activity comparable to 0.1 $mg \cdot ml^{-1}$ of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Results presented in this paper contribute to current efforts in finding sustainable solutions for isolation of valuable compounds from carob biomass and expand possibilities of its utilization in food and pharmaceutical industries.

Keywords
carob biomass; β-cyclodextrin; encapsulation; freeze-drying; antioxidant activity

Carob tree (*Ceratonia siliqua* L.) has been grown since ancient times in most countries of the Mediterranean basin. Currently, the most important economical application of carob is utilization of carob seed in production of carob been gum [1]. For that purpose, carob pod is crushed to separate the seeds from the pulp, leaving behind significant amounts of deseeded carob pods as a low-cost by-product. Extensive research conducted over the last few decades recognized carob kibble as a valuable source of different non-nutritive bioactive compounds with possible health benefits, primarily phenolic acids (4-hydroxybenzoic, coumaric, caffeic and ferrulic) and flavonoids (naringenin, chrysoeriol, tricetin) [2]. Their proven antioxidant, antidiabetic and antiproliferative properties [3, 4] additionally expand the possibilities of utilization of carob biomass in food and pharmaceutical industries but, at the same time, emphasize the need for finding effective and sustainable solutions for their isolation from carob biomass and formulation into stable products. Several methodologies have been used for the extraction of phenolic compounds from carob residues. Acetone-water extraction, Soxhlet extraction using methanol or supercritical CO_2 extraction were claimed to produce higher yields of polyphenols in comparison to water batch extraction at room temperature [5–7]. Although these methods demonstrate certain benefits over classic water batch extraction on

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laboratory scale, their conversion to a larger scale is particularly challenged in terms of expensive equipment and environmental issues emerging from the use of toxic non-polar solvents [8].

The majority of plant extracts are sensitive to degradation, oxidation and polymerization at elevated temperatures, and may have strong taste or smell. Therefore, it is a great challenge to find ways to incorporate them into food products or dietary supplements. Encapsulation provides one way of dealing with the above mentioned problems since it provides an oxygen, light and temperature barrier, and it may minimize the damage and prevent the unpleasant taste [9–11].

The main goal of our work was to develop an efficient and sustainable procedure for obtaining carob pod extract (CPE) and to investigate, for the first time, the impact of different formulation approaches on its technological characteristics, chemical composition and activity in different biological model systems. For that purpose, different sample pre-treatment options were used to optimize the yields of classical water batch extraction, which is a simple, accessible and environmentally friendly approach in terms of equipment, used solvent and possible scale-up. Several encapsulating agents and drying techniques were tested and optimized for obtaining a stable and organoleptically acceptable solid extract.

MATERIALS AND METHODS

Experimental design

In the first phase of investigation, carob kibbles were roasted under optimized time/temperature conditions and used for the preparation of carob kibble extracts using different types and amounts of pharmaceutical carriers, as well as different drying techniques with the aim to achieve maximal yields of bioactive compounds in the final product. Additional optimization of the developed procedure was achieved by removing soluble carbohydrates from the roasted raw material. Finally, extracts obtained under optimized conditions were characterized as antioxidants in different types of chemical, food and biological model systems.

Chemicals

All chemicals were of analytical and spectrophotometric grade. Sodium carbonate 10340 was purchased from Merck (Darmstadt, Germany). β-Cyclodextrin 444041 and γ-cyclodextrin 86575 (βCD and γCD, respectively) were obtained from Wacker Chemie (Munich, Germany). Potassium persulphate 24412, potassium dihydrogen phosphate 516951, sodium chloride 5234, aluminium chloride 24012, sodium nitrite 23668193, sodium hydroxide 14798, iron (III) chloride 24380, iron (II) sulphate 24393, sodium dihydrogen phosphate 23672064, fluorescein 16850, hydrochloric acid 313 and acetic acid 176 were from Kemika (Zagreb, Croatia). All other chemicals were from Sigma–Aldrich Chemie (Steinheim, Germany).

Roasting of carob pods

Ripe carob pods were collected during November–December 2013 from two locations in Northern Dalmatian area. Collected samples were delivered to the laboratory, rinsed with cold water to remove dust and soil particles, dried and kibbled. Kibbles were placed in one thin layer on a perforated metal shelf of the laboratory dryer oven (Inkolab, Zagreb, Croatia) in order to enable circulation of hot air through the sample during roasting. Different time/temperature combinations were used: 130 °C (5, 10, 15, 30 and 45 min); 150 °C (5, 10, 15 and 30 min); 165 °C (5, 10 and 15 min). Roasted carob kibbles were milled in an electric grinder to obtain particles of approximately 2–3 mm in diameter. Sugar-free samples were obtained according to the procedure described by El Batal et al. [12]. Briefly, 100 g of roasted or native carob powder was mixed with 300 ml water at 43 °C for 160 min; sugar extract and sugar-free solid phase were separated by centrifugation at 3000×*g*. Native, roasted, sugar-free samples and samples obtained by the combination of roasting and subsequent removal of sugar were stored in plastic containers at 4 °C until analysis.

Preparation of carob extracts

Liquid carob pod extracts were obtained by cooking 8 g of unprocessed, roasted, sugar-free, roasted and sugar-free carob powder in 250 ml of distilled water under reflux for 60 min, alone or with addition of different amounts of β-cyclodextrin, γ-cylodextrin or mannitol (1.6 $g \cdot l^{-1}$, 3.2 $g \cdot l^{-1}$ and 6.4 $g \cdot l^{-1}$). Type of solvent, temperature and duration of extraction were optimized previously, during preliminary investigation. Aliquots of liquid extracts obtained by using optimized protocols (in terms of the amount and the type of pharmaceutical excipient) were subsequently spray-dried in Büchi 190 mini spray dryer (Buchi Labortechnik, Flawil, Switzerland), or lyophilized in Christ Alpha 1-4 lyophilizer (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). In order to rationally select the optimal drying procedure as well as the type and concentration of the encapsulating agent, relative yield, Trolox equivalent antioxidant capacity

(*TEAC*) and total phenolic content (*TPC*) of the prepared dry extracts were considered as quality parameters of the dry products prepared. Another important parameter was the transfer ratio (*TR*) of *TPC* and *TEAC* from liquid extract to the solid product, calculated according to Eq. 1 or Eq. 2:

$$TR_{TPC} = \frac{TPC_s}{TPC_l} \times 100 \quad (1)$$

$$TR_{TEAC} = \frac{TEAC_s}{TEAC_l} \times 100 \quad (2)$$

where TPC_s and $TEAC_s$ are *TPC* and *TEAC* values of solid extract, respectively, TPC_l and $TEAC_l$ are *TPC* and *TEAC* values of liquid extract, respectively. Transfer ratio was expressed as percentage of olive pomace.

Liquid extracts were dried in a lyophilizer for 24 h, with the temperature of the condenser set at –49 °C, with application of vacuum (3 Pa). In order to increase the surface area and prevent foaming, prior to freeze-drying, samples were placed in thin layer in Petri dishes and frozen. Lyophilized extracts were stored at 4 °C until analysis.

Spray-dried extracts were obtained by processing the 200 ml of cold (4 °C) liquid extract (with or without excipients). The sample flow rate was 7.5 ml·min^{-1} and compressed airflow speed was 400 l·h^{-1}; 0.7 mm nozzle tip diameter was used; inlet and outlet temperatures were (150 ± 1) °C and (80 ± 1) °C, respectively.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) measurements were made on a Pyris 1 thermogravimetric analyser (Perkin Elmer, Waltham, Massachusetts, USA) under a nitrogen atmosphere with a gas flow of 30 ml·min^{-1}. Accurately weighed samples were placed in aluminium crucibles and scanned at a heating rate of 10 °C·min^{-1} over the temperature range of 30–500 °C.

Antioxidant activity in chemical model systems

TEAC assay

TEAC assay was conducted as described by Re et al. [13]. ABTS radical was prepared by mixing equal volumes of 7 mmol·l^{-1} of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2.45 mmol·l^{-1} solution of $K_2S_2O_8$, and leaving the mixture overnight, allowing the complete development of the chromophore radical. The reaction mixture was prepared by mixing 2.5 ml of adequately diluted $ABTS^{\bullet+}$ and 300 μl of adequately diluted sample, and absorbance was measured after 3 min using UV4-100 UV-visible scanning spectrophotometer (Pye Unicam, Cambridge, United Kingdom) at 732 nm. The quenching of initial absorbance was plotted against the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) concentration and the obtained results were expressed as grams of Trolox equivalents (TE) per kilogram of sample.

ORAC assay

An improved oxygen radical absorbance capacity (*ORAC*) assay was used [14], using fluorescein as a fluorescent probe. The reaction mixture was prepared by mixing 1 ml of fluorescein (78 nmol·l^{-1}), 1 ml of adequately diluted sample or phosphate buffered saline (PBS, blank), Trolox 20 μmol·l^{-1} (standard) and 0.5 ml of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 221 mmol·l^{-1}). Florescence was measured immediately after the addition of reagent and then every 5 min until the relative fluorescence intensity was less than 5 % of the initial fluorescence. All solutions and dilutions were prepared using PBS (75 mmol·l^{-1}, pH 7.0) and all the measurements were conducted at 37 °C using Perkin Elmer 3000 fluorescence spectrometer (Perkin Elmer). Area under the curve was plotted against Trolox concentration and the obtained results were expressed as grams of TE per kilogram of sample.

FRAP assay

The ferric reducing power (*FRAP*) assay was performed according to Benzie and Strain [15] with some modifications [16]. Reaction mixture was prepared by mixing 2.7 ml of *FRAP* reagent, 270 μl of distilled water and 150 μl of adequately diluted carob extract. *FRAP* reagent was prepared by mixing 25 ml of 0.3 mol·l^{-1} acetic acid, 2.5 ml of 20 mmol·l^{-1} $FeCl_3$ and 2.5 ml 20 mmol·l^{-1} 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) (dissolved in 40 mmol·l^{-1} HCl). The reaction mixture was incubated at 37 °C for 40 min in the dark and absorbance was measured at 593 nm using UV4-100 spectrophotometer. Obtained values were plotted against $FeSO_4$ concentration and results were expressed as moles of $FeSO_4$ per kilogram of sample.

Antioxidant activity in food and biological model systems

β-Carotene-linoleate model system

Antioxidant activity in β-carotene-linoleate model system was determined by the method of Amarowicz et al. [17] with slight modifications. A stock solution of β-carotene-linoleic acid was initially prepared by dissolving 5 mg of β-carotene in 50 ml of chloroform. A volume of 5 ml of the solution was pipetted into a 100 ml round-bottomed flask and chloroform was removed under vacuum

using a rotary evaporator at 40 °C. Amounts of 40 mg of linoleic acid, 400 mg of Tween 40 (Sigma–Aldrich Chemie) and 100 ml of aerated distilled water were added to the flask with vigorous shaking. The initial absorbance at 470 nm was immediately recorded. A volume of 100 μl of carob extract (prepared by dissolving 25 mg in 100 ml of deionized water for extracts prepared without βCD, and 53.7 mg in 100 ml for extracts with βCD, taking into account mass fraction of βCD in lyophilized extracts) were mixed with β-carotene-linoleic acid emulsion (2.9 ml). Immediately after the addition of the emulsion to each tube, the zero-time absorbance was measured at 470 nm. Subsequent absorbance readings were recorded over a 2 h period at 15 min intervals, keeping the samples at 50 °C in the dark. Butylated hydroxyanisole (BHA, 1 mmol·l^{-1}) was used as a standard for comparative purposes. Degradation rate (*DR*) of β-carotene was calculated according to Eq. 3.

$$DR = \frac{1}{2}\ln\frac{A_0}{A_{2h}} \tag{3}$$

where A_0 is absorbance recorded at the beginning of the experiment, and A_{2h} is absorbance recorded after 2 h of incubation.

Antioxidant activity (*AA*) was calculated according to Eq. 4 and expressed in percent.

$$AA = \frac{DRc - DRs}{DRc} \times 100 \tag{4}$$

where DR_c is degradation rate of control (BHA), and DR_s is degradation rate of sample.

Inhibition of peroxyl radical-induced DNA scission

Effects of carob extracts on preventing DNA from free radical-induced scission in vitro were evaluated using the procedure of Hu et al. [18] with some modification. AAPH-induced peroxyl radicals were generated in the presence of DNA. Plasmid DNA (pBR322 DNA), AAPH and solid carob extracts were dissolved in 10 mmol·l^{-1} PBS at pH 7.4. A volume 4 μl of DNA solution was mixed with the mixture of carob extract (1 mg·ml^{-1}), PBS and AAPH (sample); Trolox, PBS and AAPH (standard); PBS and APPH (blank); or PBS only (control). Volumes of 10 μl of each reaction mixture were prepared and samples were incubated at 37 °C in the dark for 2 h. The final concentrations of DNA, Trolox and AAPH in reaction mixture were 20 ng·ml^{-1}, 10 μg·ml^{-1} and 10 μmol·ml^{-1}, respectively.

After incubation, the loading dye (mixture of bromophenol blue, xylene cyanol and saccharose) was added to samples and samples were loaded to agarose gel. Gel electrophoresis was performed in a Tris-acetic acid-EDTA buffer at 4 V·cm^{-1} for 1 h using a horizontal gel electrophoresis apparatus model (ThermoFisher Scientific, Waltham, Massachusetts, USA). DNA was stained by 0.5 μg·ml^{-1} ethidium bromide and visualized under ultraviolet light. DNA breakage was quantified as percentage of supercoiled DNA that was nicked by peroxyl radicals. The protective effects of samples were expressed as the retention percentage (*RT*) that was calculated by the following equation:

$$RT = \frac{c_s}{c_c} \times 100 \tag{5}$$

where c_s and c_c represent the concentrations of supercoiled DNA with oxidative radical and without oxidative radical, respectively.

Phenolic compounds

TPC was determined spectrophotometrically by the method of Singleton and Rossi [19] with some modifications. Briefly, carob extract solutions (200 μl) were mixed with 1.35 ml of distilled water and 150 μl of Folin Ciocalteu reagent (Sigma–Aldrich Chemie). After 5 min incubation, 1.5 ml of 6% Na_2CO_3 was added to each reaction mixture and obtained solutions were incubated at 50 °C for 30 min. Absorbance was measured by UV4-100 spectrophotometer at 725 nm and results were expressed as grams of gallic acid equivalents (GAE) per kilogram of sample.

Total flavonoid content was determined by spectrophotometric $AlCl_3$ method [20]. Briefly, 1.5 ml of carob extract (50 mg ± 0.1 mg in 25 ml of deionized water) was mixed with 300 μl $NaNO_2$ (5%); after 5 min, 300 μl of $AlCl_3 \times 6H_2O$ (10%) and 2 ml of NaOH (1 mol·l^{-1}) were added, and the reaction mixture was filled with deionized water to 10 ml. Blank was prepared in the same way but it contained deionized water instead of $AlCl_3$. Calibration curve was prepared with catechine as a standard, and results were expressed as grams of catechine equivalents (CE) per kilogram of carob extract.

Statistical analysis

All analytical measurements were conducted at least in triplicates; the results were averaged and presented as mean ± standard deviation. The significance of differences among obtained results was checked by Student's *t*-test or one way analysis of variance (ANOVA) followed by Tukey's post hoc test. Observed differences were considered significant at $p < 0.05$. Analyses were conducted using Prism software (GraphPad, San Diego, California, USA).

RESULTS AND DISCUSSION

Roasting of carob kibbles

Roasting of kibbled carob pods changes flavour and taste [21] but can also significantly alter the amount and bioavailability of its bioactive compounds [22, 23]. Therefore in this work, time and temperature of roasting were optimized in order to achieve optimal yields of phenolic antioxidants. The highest yields of polyphenols and the highest antioxidant activity were obtained after 5 min of roasting at 165 °C (Fig. 1). Further elongation of roasting, or applying higher temperatures, resulted in a decrease in the antioxidant content. Obtained results are consistent with previous investigations of SAHIN et al. [22] and VITALI et al. [23] who showed that the observed changes are the result of improved polyphenol solubility and formation of early-stage MRPs that possess significant antioxidant activity. Taking into account the obtained results, carob kibbles roasted at 165 °C for 5 min were used as the starting raw material for all further investigations.

Encapsulation of antioxidants

The main objective of encapsulation is to protect and stabilize the core material from degradation [24]. Spray-drying is a well-established and the most widely used encapsulation technique in the food industry. Its major limitation is, in the fact, that high air inlet temperature can cause deterioration of some heat-sensitive materials [25]. Therefore, we also investigated the feasibility of freeze-drying process, a simple technique usually used for encapsulation of water-soluble essences, natural aromas and other thermolabile materials [26].

Considering the efficiency of cyclodextrins in improving solubility, stability, bioavailability and antioxidant activity of botanical extracts, due to inclusion complex formation [27], different concentration levels of β- and γ-cyclodextrins were tested as encapsulation carriers. Mannitol was selected as an inert alternative. Its sweet taste and negative enthalpy of dissolution can also mask the bitter taste of the encapsulated compound [28].

Spray-drying resulted in approximately 40 % lower yield compared to that obtained by the freeze-drying procedure (Tab. 1), except for the case of products prepared using mannitol as a carrier, where the reduction of the production yield up to 97 % was observed. The spray-dried products demonstrated pronounced affinity for the adsorption to the glass part of the spray-drier, so they were more difficult to collect. Also, the loss of the smallest particles through the exhaust of the instrument, which was not equipped with a suitable trap to recover such particles, contributed to the observed result. Secondly, *TPC* and the corresponding antioxidant capacity, as well as *TPC* and *TEAC* transfer ratio of the spray-dried products, were always significantly lower than those of the corresponding lyophilized product. Such result could be attributed to the thermally induced deterioration of antioxidants extracted from the carob kibble during the drying procedure, regardless the type and concentration of the carriers used. The attempts to perform spray-drying procedure at inlet air temperature lower than 150 °C were not successful, because high energy input was

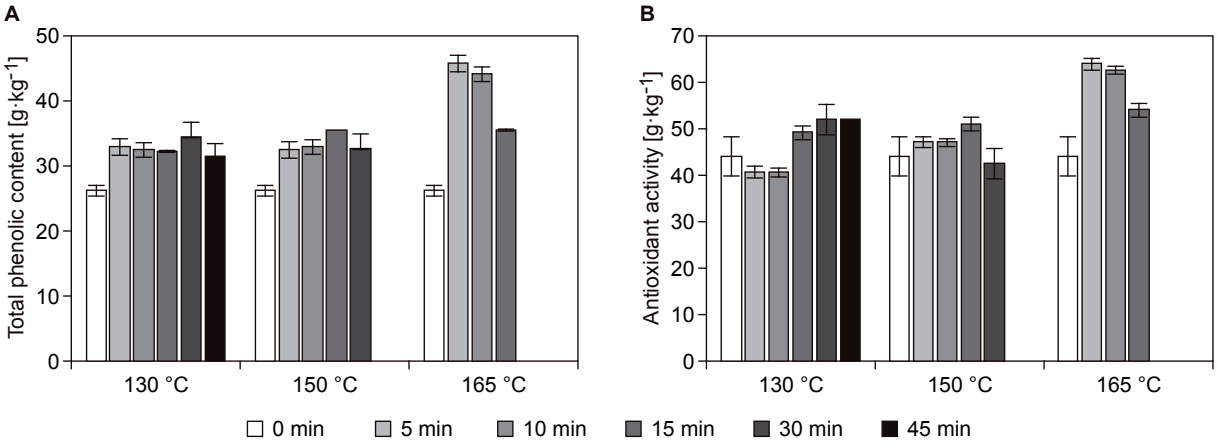

Fig. 1. Optimization of roasting conditions.

A – Impact of roasting on total phenolic content; B – Impact of roasting on antioxidant activity.
Total phenolic content is expressed as grams gallic acid equivalents per kilogram of sample. Antioxidant activity is expressed as grams of Trolox equivalents per kilogram of sample.

necessary in order to obtain the proper particle formation and drying from an aqueous spray-drying feed.

The selected carriers significantly affected all investigated parameters of the extracts prepared, depending on the type and concentration of the carrier used (Tab. 1). In spray-dried products, an increase of the relative yield, *TPC* and *TEAC* was observed as a function of the concentration of both CD derivatives used. The spray-dried product prepared using 6.4 $g \cdot l^{-1}$ of γCD contained by approximately 15% higher amount *TPC* and, consequently, higher *TEAC* than the corresponding product with βCD, while the amount of *TPC* was by more than 30% higher than in the spray-dried product prepared without any carrier. However, βCD was more efficient in protecting the extracted antioxidants from the carob pods during the drying procedure, as could be seen from the corresponding *TPC* and *TEAC* transfer ratio (Tab. 1). Mannitol was not a suitable carrier for the spray-drying procedure, due to formation of a glassy product, which could not be collected from the glass parts of the instrument, leading to decreased yields.

Compared to spray-drying, freeze-drying of the carob pod aqueous extract without any carrier resulted in much higher *TPC* and *TEAC* ratio, indicating that this drying procedure could be considered as the method of choice for the purpose of this study. Its efficiency was further improved by application of encapsulating agents. In this regard, βCD appeared to be the most efficient carrier, providing high protection of the extracted antioxidants during the freeze-drying procedure, as could be concluded from *TPC* and *TEAC* transfer ratios, which were close to or even higher than 100% in some cases. Although the yield of the lyophilized products prepared with γCD as a carrier was comparable to that of βCD extracts, the ability of γCD to protect the extracted antioxidants during the drying procedure was less pronounced (Tab. 1). It seems that the molecular dimensions of βCD central cavity are of more appropriate size compared to that of γCD, which allows inclusion of phenolic compounds present in the carob pods, thus protecting them more efficiently from deterioration during the drying procedure. The preferential steric compatibility of βCD over γCD for different polyphenols and other natural antioxidants was stated in the literature

Tab. 1. Carob pod extract optimization.

	No carrier	β-Cyclodextrin		γ-Cyclodextrin			Mannitol		
		1.6 $g \cdot l^{-1}$	3.2 $g \cdot l^{-1}$	6.4 $g \cdot l^{-1}$	1.6 $g \cdot l^{-1}$	3.2 $g \cdot l^{-1}$	6.4 $g \cdot l^{-1}$	1.6 $g \cdot l^{-1}$	3.2 $g \cdot l^{-1}$	6.4 $g \cdot l^{-1}$
Spray drying										
Relative yield [g]	40.7 ± 2.85	47.39 ± 3.97	51.76 ± 4.58	55.50 ± 5.11	43.15 ± 4.56	44.87 ± 1.42	57.24 ± 2.57	33.93 ± 19.50	22.101 ± 9.68	1.66 ± 0.18
TPC [$g \cdot kg^{-1}$]	12.14 ± 2.05	14.73 ± 0.75	16.12 ± 0.43	17.72 ± 0.79	14.69 ± 2.65	14.97 ± 1.57	20.40 ± 1.69	10.92 ± 5.57	7.10 ± 3.00	0.50 ± 0.10
TEAC [$g \cdot kg^{-1}$]	24.18 ± 0.24	27.39 ± 3.00	32.10 ± 7.61	33.48 ± 3.80	26.00 ± 3.46	29.07 ± 0.43	40.33 ± 2.17	19.64 ± 1.20	13.87 ± 6.87	1.09 ± 0.15
TR_{TPC} [%]	27.8	35.5	66.8	62.6	43.1	46.0	57.2	32.2	20.9	1.4
TR_{TEAC} [%]	37.9	43.1	52.0	56.0	39.9	47.4	62.3	31.1	21.5	1.6
Freeze drying										
Relative yield [g]	69.74±0.51	77.53 ± 1.77	87.44 ± 0.50	89.72 ± 4.21	74.73 ± 6.17	80.43 ± 6.53	84.77 ± 8.25	85.16 ± 5.30	88.16 ± 5.30	78.90 ± 5.00
TPC [$g \cdot kg^{-1}$]	16.67±1.37	21.37 ± 2.57	27.38 ± 7.00	24.97 ± 2.74	24.52 ± 2.54	17.33 ± 8.57	28.42 ± 5.34	29.53 ± 3.22	31.47 ± 3.63	30.65 ± 2.51
TEAC [$g \cdot kg^{-1}$]	38.79±1.86	45.15 ± 4.71	58.11 ± 2.79	57.48 ± 4.25	43.38 ± 4.80	41.74 ± 3.36	53.3 ± 7.32	49.97 ± 0.31	53.26 ± 4.74	57.68 ± 4.37
TR_{TPC} [%]	38.1	51.5	113.5	88.1	71.9	53.3	79.7	87.0	92.4	85.7
TR_{TEAC} [%]	60.8	71.1	94.2	96.2	66.5	68.1	82.4	79.2	82.6	83.7

TPC – total phenolic content (expressed as grams of gallic acid equivalents), TEAC – antioxidant capacity (expressed as grams of Trolox equivalents), TR_{TPC} – transfer ratio of TPC, TR_{TEAC} – transfer ratio of TEAC.

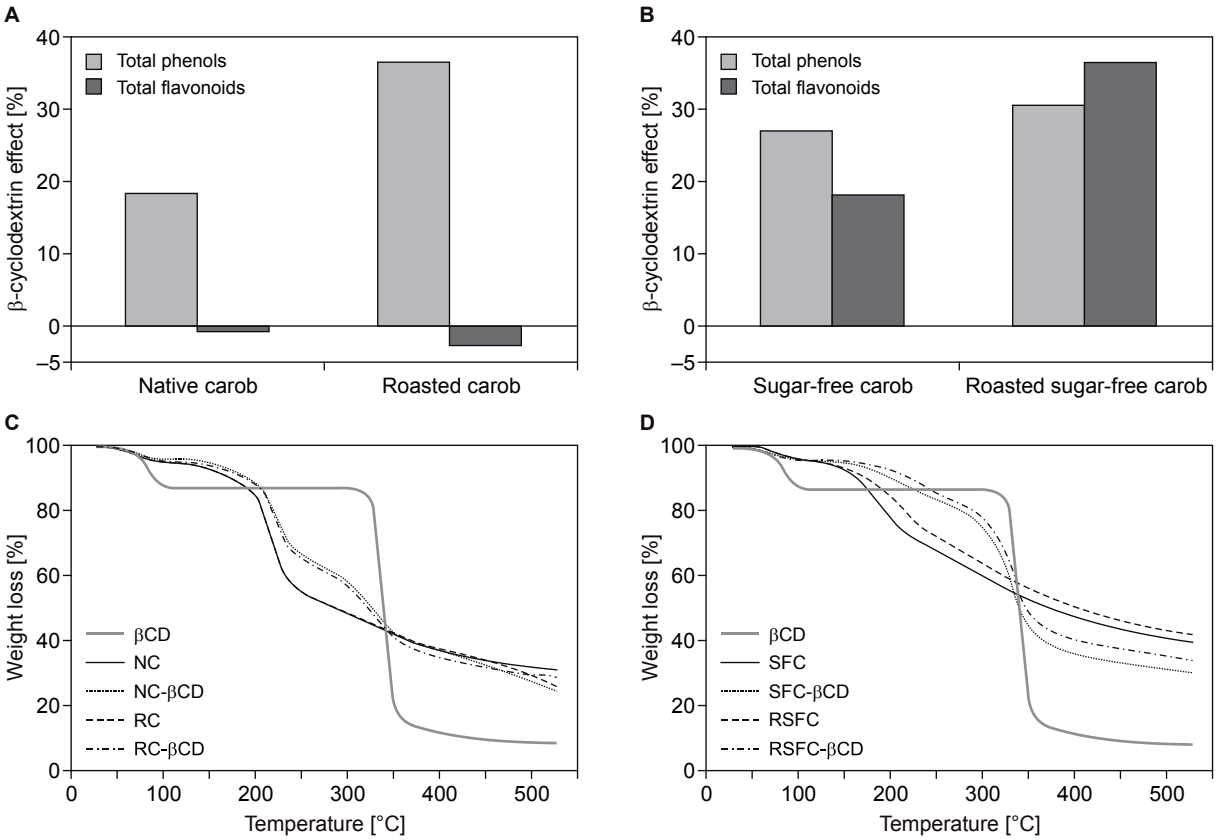

Fig. 2. Impact of β-cyclodextrin on polyphenol yields and product stability in original and sugar-free samples.

A – Impact of β-cyclodextrin on polyphenol yields in native and roasted samples; B – Impact of β-cyclodextrin on polyphenol yields in sugar-free samples; C – Thermogravimetric curves of extracts obtained from native carob; D – Thermogravimetric curves of extracts obtained from sugar-free carob.
βCD – β-cyclodextrin, NC – native carob, NC-βCD – native carob with β-cyclodextrin, RC – roasted carob, RC-βCD – roasted carob with β-cyclodextrin, SFC – sugar-free carob, SFC-βCD – sugar-free carob with β-cyclodextrin, RSFC – roasted sugar-free carob, RSFC-βCD – roasted sugar-free carob with β-cyclodextrin.

[27]. Taking that into consideration, the freeze-drying procedure using 6.4 $g \cdot l^{-1}$ βCD as a carrier was selected as the method of choice and was further developed.

Removal of sugar and impact on stability

Regarding the use of carob kibble as the starting material for the production of highly concentrated source of polyphenolic antioxidants, removal of sugar is a logical step during process development. However, it is important to take into account the fact that polyphenols tend to form complexes with other macronutritive compounds, including carbohydrates. This is why the removal of carbohydrates from natural matrix can affect polyphenols' solubility, formation of complexes with encapsulation materials, stability, activity and the drying process [29–31]. It was therefore necessary to investigate the impact of sugar removal on the observed efficiency of βCD and also on the stability of obtained lyophilized extracts (Fig. 2). The obtained results indicated that removal of sugar did not affect the content of total phenols in the freeze-dried extract but that it significantly enhances the ability of βCD to form complexes with naturally present carob flavonoids.

Based on the obtained results, we could conclude that, in the absence of natural sugars, the higher amount of flavonoids was available for complexation with βCD. Also, it seemed that the removal of sugar increased thermal stability of the freeze-dried extracts, as was obvious from the obtained TGA curves. The TGA curves of all lyophilized carob extracts showed the first event in the temperature range between 30 °C and 96 °C, with a mass loss ranging from 35.5 $g \cdot kg^{-1}$ to 45.8 $g \cdot kg^{-1}$, which might correspond to water loss. Generally, lyophilized carob extracts with native sugars removed prior to extraction contained lower amounts of the moisture, regardless the CD

presence. The thermal degradation of the lyophilized extracts took place at temperatures higher than 140 °C and it might be divided in three different stages: the first, rather slow one in the temperature range between 140 °C and 190 °C, followed by a faster one in temperature range between 190 °C and 350 °C, while the third stage in temperature range between 350 °C and 500 °C again was a slower one (Fig. 3). By comparing the TGA curves of lyophilized carob extracts, it can be concluded that removal of native sugars prior to extraction provided samples with significantly improved thermal stability. On the contrary, thermal treatment prior to the extraction had practically no effect on the thermal stability of the lyophilized extracts. For example, at 350 °C, the lyophilized extract of native carob and extract of roasted native carob presented the mass loss of about 56.3%, while, in corresponding samples where native sugars were removed prior to extraction, the mass loss at the same temperature was 451.8 g·kg^{-1} and 414.5 g·kg^{-1} of the initial weight, respectively. The addition of βCD significantly improved the thermal stability of all extracts, especially at temperatures lower than 318 °C, at which a rapid thermal degradation of the carrier occurs. The most pronounced stabilizing effect of βCD was observed in case of lyophilized extracts of native carob without sugars and roasted carob without sugars, clearly demonstrating that removal of natural sugars and the use of a suitable carrier molecule is a valid approach, which would gain samples with superior thermal stability.

Antioxidant activity

Phenolic content and antioxidant activity in chemical assays

In this research, three different in vitro approaches were used for the assessment of the applicability of different drying methods and encapsulation materials during formulation of carob kibble extract. Since the methods measure different aspects of in vitro antioxidant activity, the combination of results obtained in each of the assays provided more credible insight into the actual antioxidant activity of the obtained extracts. Analysed extracts were those obtained from pure native carob (NC), roasted carob (RC), sugar-free carob (SFC), roasted sugar-free carob (RSFC), or their combinations with βCD (NC-βCD, RC-βCD, SFC-βCD, RSFC-βCD). *TPC*, flavonoid content and different types of antioxidant activity of analysed CPEs are presented in Tab. 2.

TPC ranged from 27.06 g·kg^{-1} to 59.2 g·kg^{-1} (expressed as GAE), the determined values being significantly higher than the yields of CBE phenolic compounds reported in literature [29]. For example, the extract obtained by 8–20 min decoction of untreated carob kibbles ranged from 15.5 g·kg^{-1} to 39.6 g·kg^{-1} [29]. Although the higher content of phenolic compounds in the analysed extracts can partially be attributed to the loss of weight by the extraction of sugars (approximately 40–50% of kibbles weight), it is obvious that significant improvement of the extract's quality was achieved by roasting of the raw material, encapsulation of target bioactive compounds with βCD, or the combination of the two. When comparing the investigated samples it is clear that the same trends were observed for polyphenol yields and all investigated types of antioxidant activity: the lowest values were determined in the reference

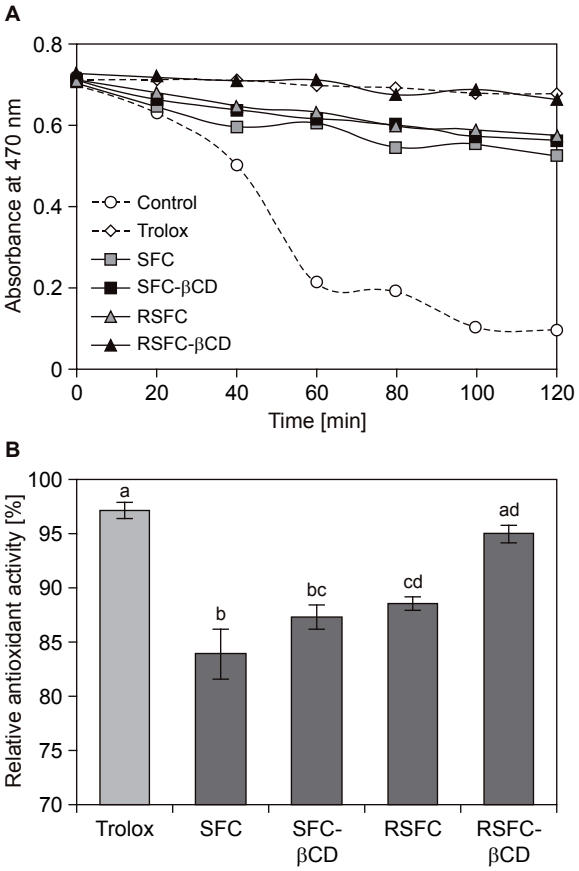

Fig. 3. Antioxidant activity of carob extracts in β-carotene bleaching assay.

A – Bleaching rates of β-carotene in the presence of carob kibble extracts; B – Antioxidant activity of investigated carob extracts.
Bars marked with the same letter belong to the same statistical group ($p > 0.05$).
SFC – sugar-free carob, SFC-βCD – sugar-free carob with β-cyclodextrin, RSFC – roasted sugar-free carob, RSFC-βCD – roasted sugar free carob with β-cyclodextrin.

Tab. 2. Total phenolic content and antioxidant activity of freeze-dried carob pod extracts.

	TPC [g·kg^{-1}]	Total flavonoids [g·kg^{-1}]	*TEAC* [g·kg^{-1}]	*FRAP* [mol·kg^{-1}]	*ORAC* [g·kg^{-1}]
SFC	27.06 ± 0.22	155.97 ± 6.09	353.31 ± 0.82	3.97 ± 0.13	15.60 ± 0.51
SFC-βCD	33.14 ± 0.42	214.35 ± 0.38	409.23 ± 2.37	5.06 ± 0.07	23.23 ± 0.08
RSFC	37.69 ± 0.19	227.22 ± 1.67	422.02 ± 4.13	5.29 ± 0.26	23.30 ± 0.52
RSFC-βCD	59.52 ± 0.29	328.58 ± 13.58	653.27 ± 1.12	7.60 ± 0.03	30.90 ± 1.31

Data are expressed per kilogram of carob extract corrected with respect to the mass fraction of β-cyclodextrin.
TPC – total phenolic content (expressed as grams of gallic acid equivalents), *TEAC* – antioxidant activity determined by *TEAC* assay (expressed as grams of Trolox equivalents), *FRAP* – ferric reducing power (expressed as moles of $FeSO_4$) *ORAC* – antioxidant activity determined by *ORAC* assay (expressed as grams of Trolox equivalents).
SFC – sugar-free carob; SFC-βCD – sugar free carob with β-cyclodextrin; RSFC – roasted sugar-free carob; RSFC-βCD – roasted sugar-free carob with β-cyclodextrin.

sample, in the pure extract obtained from SFC kibbles; the addition of βCD during extraction/freeze drying (SFC-βCD) increased the antioxidant yields and the observed effects were similar to the effects of kibble roasting under optimized conditions (RSFC). The combination of both treatments (RSFC-βCD) resulted in a cumulative effect on both polyphenol content and on the antioxidant activity, which resulted in twice as high values in comparison to SFC. As previously explained, the positive impact of roasting on phenolic content and antioxidant activity of carob kibble can probably be attributed to the increased solubility of phenolic compounds and formation of early-stage Maillard reaction products [19, 20], which were successfully extracted by the proposed procedure and resulted in significantly higher *TPC* as well as flavonoid content of the final product (39.3% and 45.3%, respectively). Similar improvement was observed for all investigated types of antioxidant activity (19.4% for *TEAC*, 33.3% for *FRAP* and 48.9% for *ORAC*) indicating that roasting of carob kibbles under the proposed conditions is a simple but highly useful pre-treatment approach in the development of the carob biomass-derived antioxidant. The use of βCD as extraction enhancer and encapsulation agent also significantly improved the yields of total phenols and flavonoids in relation to SFC (22.5%, and 45.7%, respectively) and showed higher reduction power and antiradical efficiency (15.8% for *TEAC*, 27.5% for *FRAP* and 32.8% for *ORAC*). Although this is the first time that βCD was used as the extraction enhancer and encapsulation agent for carob polyphenols, previously it has been shown useful for encapsulation of polyphenols of different origin. For example, Mantegna et al. [32] showed that resveratrol extract obtained with a βCD water solution maintains the same radical-scavenging activity and antioxidant capacity as the methanol extract obtained under the same conditions. βCD was also successfully applied for encapsulation of purified St. John's wort [33]. Ratnasooriya et al. [34] successfully applied βCD for the extraction of bioactive phenolic compounds from grape pomace. Our findings support these previously obtained results and indicate that aqueous βCD has a potential to be used for recovering phenolics from fruit processing by-products and subsequent formulation of powder forms for functional food and nutraceutical applications. Our results show that, in the case of carob pod biomass, the beneficial effects of βCD can be additionally potentiated by previous roasting of kibbles.

Antioxidant activity of β-carotene-linoleate model system

Since antioxidants behave differently when used in different media and their efficacy in water may not necessarily reflect that in oil-in-water emulsions, the antioxidant effectiveness of CPE was also tested in β-carotene-linoleic acid emulsion system (Fig. 3). Such high surface-to-volume ratio emulsions resemble natural conditions, common to real foods and biological systems and, therefore, the obtained results might be considered as a more reliable indicator of actual antioxidant efficiency of the extracts. The obtained results showed that substantial and rapid oxidation of β-carotene took place in the control, while the presence of all investigated antioxidants significantly reduced the discoloration rate of β-carotene in the emulsion. Similar efficiency was observed for three investigated types of carob kibble extracts (SFC, SFC-βCD and RSFC), RSFC-βCD extract showing a significantly higher antioxidant activity than 1 mmol·l^{-1} BHA. Although the obtained results resemble those from chemical-based assays, certain discrepancies were noticed. Namely, it was noticed that the addition of βCD to the extrac-

tion system had a much more pronounced effect on antioxidant activity in β-carotene-linoleic acid emulsion system than roasting. Although roasting decreased the discoloration rate of β-carotene, the observed changes were not statistically significant. On the other hand, addition of βCD significantly improved antioxidant activity, while optimal results were obtained by the combination of roasting and βCD encapsulation. This inconsistency with the results of radical-scavenging activity and reducing power might be explained by the fact that the capacity of a particular antioxidant to reach the place where free radicals are being generated is also a critical aspect [35]. This is why antioxidants with high in vitro antioxidant capacity are not necessarily efficient neutralizers of radicals in compartmentalized systems such as β-carotene-linoleic acid emulsion system. In this context, our results can be explained by the formation of inclusion complexes between carob polyphenols and βCD, which increases their apparent hydrophobicity and efficiency in emulsion. Although the antioxidant activity of carob polyphenols was previously investigated in different types of chemical assays [5, 6, 8, 22], this is the first time that carob extract was tested in this type of emulsion model system. The fact that 0.5 mg·ml^{-1} RSFC-βCD had the same effect on the rate of β-carotene bleaching as 1 mmol·l^{-1} BHA indicates that it could be used as an effective natural alternative to the synthetic BHA, which is associated with possible health hazards.

Effect of carob pod extract on plasmid DNA scission induced by peroxyl radical

The majority of published data regarding the antioxidant activity of carob were obtained from simple chemical-based assays [5, 6, 8, 22]. In order to further establish the biological significance of CPE, its protective effect was investigated in a more sophisticated in vitro assay based on measuring the extent of scission of DNA as one of major biological targets of free radicals. As presented in Fig. 4, native DNA was present in nicked circular form, and the addition of AAPH radical resulted in its significant scission, leading to linear DNA. Addition of Trolox (10 μg·ml^{-1}) retained the plasmid DNA in its circular form. The results showed that the treatment with all analysed extracts (1 mg·ml^{-1}; corrected with respect to βCD content in the extract) did not result in changes in plasmid DNA conformation (Fig. 4). These observations suggest that the analysed extracts did not cause DNA chain breakage.

On the contrary, investigated CPEs also protected DNA against peroxyl-induced DNA cleavage and showed the activity similar to that observed with Trolox. The DNA protective efficiency differed significantly among tested extracts; the best results were obtained with the extract prepared by the combination of roasting of kibbles and subsequent βCD encapsulation (RSFC-βCD). Applying only roasting or only βCD encapsulation resulted in a significantly lower DNA protection efficiency, while the native CPE (SFC) produced the weakest effect. Probable mechanism by which investigated extracts protect DNA is their ability to scavenge reactive oxygene species. This premise is supported by a strong correlation of results obtained in DNA scission test and the results from chemical radical-scavenging assays (*TEAC* and *ORAC*). Considering their abundance in carob pod, it is logical to assume that the main active compounds in the analysed CPEs are polyphenols, in particular those with ortho-dihydroxy structure in B-ring, such as catechin and luteolin, known for their high scavenging activities. Positive impact of roasting on radical-scavenging activity of CPE can

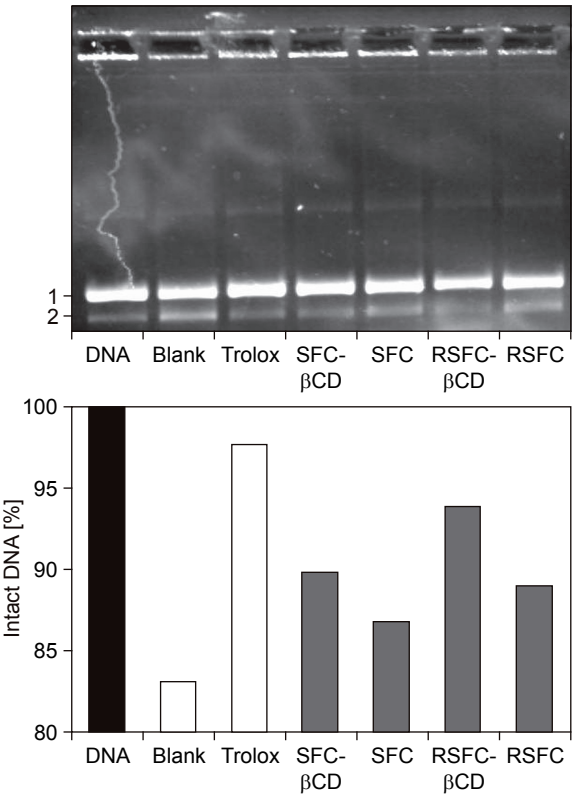

Fig. 4. Protective effect of carob extracts against peroxyl-induced DNA scission.

1 – circular DNA; 2 - linear DNA. SFC-βCD – sugar-free carob with β-cyclodextrin, SFC – sugar-free carob RSFC-βCD – roasted sugar-free carob with β-cyclodextrin, RSFC – roasted sugar-free carob.

probably be attributed to the increased solubility of phenolic compounds (formation of aglycons) but also to the formation of early-stage Maillard reaction products [22, 23]. Enhancement of DNA protection observed in βCD extracts was probably mediated by improved stability and yields of encapsulated bioactive substances. The obtained results emphasize the need for further investigation of activity and mechanism of action of CPE in protection of biomolecules. Considering the complex chemical composition of carob extracts and limited knowledge about the metabolic pathways of major carob polyphenols, available data on high bioacessibility index of carob phenolic acids, flavonoid aglycons and flavonoid glucosides are encouraging [36].

CONCLUSION

In this work, an efficient and sustainable procedure for the formulation of CBE was proposed. Roasting for 5 min at 165 °C and removal of sugar were found to be simple and efficient pre-treatment options that positively affected thermal stability and the content of bioactive compounds in the obtained extracts. Compared to spray-drying, freeze-drying of aqueous CBE resulted in much higher *TPC* and *TEAC* transfer rates, and was selected as a method of choice for CBE preparation. Additional and more pronounced improvement of the extraction procedure in terms of antioxidant yields and thermal stability was achieved by βCD encapsulation. In β-carotene-linoleic acid emulsion system, the addition of βCD showed much more pronounced effect on antioxidant activity than roasting, indicating that formation of inclusion complexes between carob polyphenols and βCD increased their apparent hydrofobicity and antioxidant efficiency in emulsion. Results showed that, among investigated CBS, 0.5 $mg \cdot ml^{-1}$ RSFC-βCD had the same effect on the rate of β-carotene bleaching as 1 $mmol \cdot l^{-1}$ BHA. Testing CPEs in a plasmid DNA scission model showed that the extracts in concentrations up to 1 $mg \cdot ml^{-1}$ did not cause DNA chain breakage but protected DNA against peroxyl-induced scission. The efficiency differed significantly among the tested extracts, the best results being obtained with the extract prepared by the combination of roasting of kibbles and subsequent βCD encapsulation (RSFC-βCD). The obtained results emphasize the potential of CPE as an antioxidant and stress the need for further detailed investigation of its specific biological activities and possible applications.

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