JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification and Quantification of Polyphenols in Carob Fruits (*Ceratonia siliqua* L.) and Derived Products by HPLC-UV-ESI/MS^n

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The polyphenolic patterns of carob pods (*Ceratonia siliqua* L.) and derived products were identified and quantified using high-performance liquid chromatography−UV absorption−electrospray ion trap mass spectrometry after pressurized liquid extraction and solid-phase extraction. In carob fiber, 41 individual phenolic compounds could be identified. In addition, spectrophotometric quantification using the Folin−Ciocalteu and vanillin assays was performed, and the antioxidative activity was determined as the 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity. Carob pods contain 448 mg/kg extractable polyphenols comprising gallic acid, hydrolyzable and condensed tannins, flavonol-glycosides, and traces of isoflavonoids. Among the products investigated, carob fiber, a carob pod preparation rich in insoluble dietary fiber (total polyphenol content = 4142 mg/kg), shows the highest concentrations in flavonol-glycosides and hydrolyzable tannins, whereas roasted carob products contain the highest levels of gallic acid. The production process seems to have an important influence on the polyphenolic patterns and quantities in carob products.

KEYWORDS: ***Ceratonia siliqua* L.; polyphenols; tannins; dietary fiber; HPLC-MS; pressurized liquid extraction; PLE; antioxidants; DPPH**

INTRODUCTION

The evergreen carob tree is a Mediterranean leguminosae grown today in many mild and semiarid parts of the world (*1*). The pulp of the carob fruit contains 40−50 wt % of sweet carbohydrates as well as dietary fiber, tannins, and polyphenols (*2*, *3*).

Carob pods have been consumed traditionally as animal and human food (*4*). Industrially, carob is mainly used for locust bean gum production from the seeds. From the pulp different human foods can be derived such as sugar syrups or molasses, unroasted and roasted carob powder used as cocoa substitutes, or especially tannin-rich preparations as antidiarrheic products (*3*, *5*, *6*). A carob pulp product especially rich in insoluble dietary fiber (carob fiber) recently showed promising cholesterol-lowering properties in animal (*7*, *8*) as well as human trials (*9*, *10*). Furthermore, this carob fiber product as well as water extracts from carob pods showed a high antioxidative activity in different in vitro tests (*11*, *12*).

Carob pods contain condensed tannins (proanthocyanidins), composed of flavan-3-ol groups and their galloyl esters (*13*−*15*), gallic acid (*16*, *17*), (+)-catechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate, and quercetin glycosides (*18*). However, detailed analyses of the diverse polyphenolic compounds in carob pods and the influence of processing on the composition of the phenolic constituents have not yet been published. The aim of this study was to identify and quantify the main extractable polyphenols of carob pods and different processed carob products using pressurized liquid extraction and high-performance liquid chromatography coupled to ion trap mass spectrometry. The Folin−Ciocalteu and vanillin assays were used in addition to compare the results from fast and simple spectrophotometric quantification to data from the specific yet laborious high-performance liquid chromatography. The antioxidative activity was determined as the 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity and compared to the quantities of extractable phenolics found.

MATERIALS AND METHODS

Sample Material. Locust bean powder (Rapunzel Naturkost, Legau, Germany), kibbles (carob pod pieces after separation of the seeds; sample from Nutrinova, Frankfurt, Germany), Caromax (a carob fiber product after extraction of carbohydrates from the kibbles), and the resulting sugar syrup (both obtained from Nutrinova) and roasted carob flours were investigated (Carochoc, Euroduna, Barmstedt, Germany;

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# Nutrinova, Nutrition Specialties and Food Ingredients GmbH.

10.1021/jf030660y CCC: $27.50
Published on Web 05/15/2004

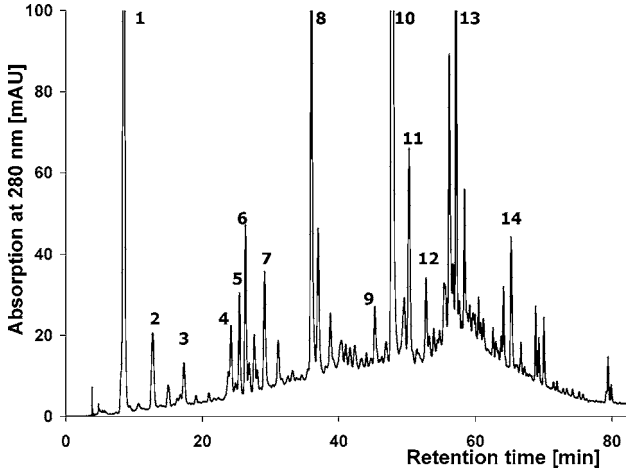

Figure 1. Chromatogram recorded at 280 nm of a carob fiber extract purified by polyamide-SPE. The marked peaks represent the compounds used for the calculation of extraction efficiency: 1, gallic acid; 2, hexose + 2 gallic acid units; 3, hexose + 2 gallic acid units; 4, pentose + 2 gallic acid units; 5, trimeric procyanidin; 6, hexose + 2 gallic acid units; 7, 2 pentoses + 2 gallic acid units; 8, hexose + 3 gallic acid units; 9, myricetin-hexoside; 10, hexose + 4 gallic acid units; 11, myricetin-desoxyhexoside; 12, quercetin-hexoside; 13, quercetin-desoxyhexoside; 14, kaempferol.

Carobpulver, Rapunzel Naturkost; Carob Pulver, Flügge-Diät, Stuttgart, Germany).

The carob fiber used (Caromax, Nutrinova) is a commercial product rich in insoluble dietary fiber, which is obtained from carob pods according to a patented extraction process (*11*, *19*). In short, carob kibbles are subjected to a cold-water extraction that yields a syrup rich in sweet carbohydrates and gallic acid and also containing phenylcarboxylic acids and flavonol-glycosides. The remaining solids are then dried with hot air at 120 °C, ground to a fine powder, and sieved (particle size < 200 μm, hammer mill). For the roasted carob products, no details on the production process were available.

For identification and quantification, gallic acid monohydrate (>98%), (+)-catechin hydrate (98%), (−)-epicatechin, kaempferol (>90%), quercetin dihydrate, rutin hydrate (>95%), myricetin (~85%), and myricitrin (Sigma, Deisenhofen, Germany) were available as standard reference substances (purity given in parentheses when this information was available).

Extraction of Polyphenols from Solid Samples. Carob kibbles were ground with a ball mill (MM2000, Retsch, Haan, Germany) under cooling with liquid nitrogen to obtain a fine powder. All other samples were already sufficiently finely ground for analysis.

Pressurized liquid extraction (PLE) was carried out using an Accelerated Solvent Extractor (ASE 200, Dionex, Idstein, Germany). One gram of diatomaceous earth (Hydromatrix HM-N, Separtis, Grenzach-Wyhlen, Germany) at the bottom and 1 g of the sample material mixed with 2 g of diatomaceous earth on the top were packed into 11 mL stainless steel extraction cells after insertion of two cellulose filters (Schleicher & Schuell GmbH, Dasseln, Germany). During extraction method development, water, acetone, methanol, water plus acetone, and water plus methanol in different compositions as well as different temperature and time settings were used. The PLE parameters found to be optimal (acetone plus water, 50 + 50, v + v; 60 °C, 5 min, two cycles, 50% flush volume) were used throughout.

For calculation of extraction efficiency during method development, crude extracts were subjected to high-performance liquid chromatographic (HPLC) separation and ultraviolet (UV) detection at 280 nm. From the peak areas of 14 different predominant compounds (**Figure 1**) the extraction efficiency was quantified as the percentage of compound with respect to the amount extracted under the conditions found to be optimal and used for all subsequent analyses.

To compare the extraction of polyphenolic compounds using PLE with extraction methods used by other authors, manual extraction of carob fiber was carried out in a beaker under stirring. One gram of carob fiber powder was mixed with 20 mL of distilled water or water plus solvent mixtures and stirred at a given temperature for a given time. The solution was filtered through a cellulose filter (Schleicher & Schuell) and the extract used directly or after solid-phase extraction (SPE) for HPLC analysis.

Solid-Phase Extraction. SPE was performed using a Gilson ASPEC XL system (Automated Sample Preparation with Extraction Cartridges, Abimed, Langenfeld, Germany) according to a method described previously (*20*, *21*). Polyamide SPE cartridges (1 g of Polyamide 6, 6 mL cartridge, Macherey-Nagel, Düren, Germany) were conditioned with 10 mL of distilled water for 10 min and washed with 5 mL of water. If a water−solvent mixture was used for extraction, the extract was diluted with water to contain 20% v + v of the organic solvent prior to loading onto the cartridge. The cartridge was washed with 15 mL of water to remove matrix interferences, and the phenolic compounds bound to the polyamide were eluted with dimethylformamide plus water, 85 + 15, v + v. While eluting, the first milliliter was discarded because it did not contain compounds of interest, and the next 2.5 mL was collected, containing all of the compounds. These elution samples were then subjected to HPLC.

Identification of Individual Polyphenols by HPLC−Mass Spectrometry (MS). The liquid chromatograph was a Dionex summit system consisting of a Degasys DG-1310 degasser (Uniflows, Tokyo, Japan), a P-580A HPG pump, an ASI-100 T automated sample injector, an STH-585 column oven, and a UVD-340S UV−vis detector equipped with a capillary cell (Dionex, Germering, Germany). The system was controlled using the Chromeleon software package version 6.20 Build 531 (Dionex). The analytical column (Aqua 3 μm C18, 150 mm, 2 mm i.d.) was equipped with a guard column (Security Guard, C18, 4 mm, 2 mm i.d.; both Phenomenex, Aschaffenburg, Germany) and kept at 25 °C. One percent v + v acetic acid in high-purity water (mobile phase A) and 1% v + v acetic acid in acetonitrile (mobile phase B) were used as solvents with a flow rate of 200 μL/min. A gradient elution program was used starting at 5% B with a linear gradient to 20% B after 45 min and to 60% B after 77 min. The column was washed with 100% B for 10 min and reequilibrated for 15 min with the initial conditions. Ten microliters of each sample was injected for analysis and the chromatogram monitored at 200−595 nm with a wavelength of 280 nm for quantification.

An LCQ classic ion-trap mass spectrometer (MS) equipped with an electrospray interface (ESI) and a metal needle kit was coupled to the HPLC and controlled with the Xcalibur software version 1.2 (Thermo Finnigan, Egelsbach, Germany). A flow of 100 μL/min methanol delivered by a System Gold programmable solvent module 116 (Beckman, Unterschleissheim, Germany) was added through a T-union before the HPLC eluent entered the ion source to enhance ionization of very polar compounds. The settings for the MS were as follows: source voltage, 4.5 kV (negative mode); sheath gas flow, 60; auxiliary gas flow, 0; capillary voltage, −45 V; capillary temperature, 200 °C; first octapole offset, +3.0 V; interoctapole lens voltage, +22.0 V; second octapole offset, +7.0 V; ion trap DC offset, +10.2 V.

Identification of individual compounds was conducted by UV spectral and MS^n fragmentation data in samples after PLE and SPE. A UV spectral library setup in the laboratory from standard compounds and the comparison of typical mass fragmentation patterns from the samples and standards were the basis of the identification.

Quantification of Individual Compounds by HPLC-UV-DAD. The apparent lack of standard substances for the majority of polyphenolic compounds makes it necessary to calculate the amounts present in comparison to available standards. Whereas substitution of a hydroxyl group alters the UV spectral properties of the compound, the nature of a non-UV-active substituent (e.g., glycosylation) is of much lesser importance, especially with respect to the absorption coefficient near 280 nm. If a UV-active substituent is present (e.g., gallic acid), the sum of the absorption of the moieties present was assumed as the overall absorption. As has been shown by other authors, available standard substances can be used for the quantification of polyphenols containing the same aglycon and paying attention to glycosylation, methoxylation, and acylation patterns (*21*, *22*).

Analytes were first identified using the UV spectral data and the molecular weight and fragmentation pattern from the mass spectrometer.

The peak area from the UV chromatogram at 280 nm was used together with the molecular weight to calculate the amount of the identified compounds with respect to solutions with known amounts of the standard substances.

In some cases, the presence of compounds can be determined unambiguously by LC-ESI/MS^n, whereas quantification by UV absorption is not possible due to very small, distorted, or overlapping peaks. This has been designated in the tables by "nq" (not quantifiable). This is the case in the samples investigated only for compounds with low concentration, giving only a small deviation in the calculation of the total amount of phenolic constituents. For the calculation of the mean value of an individual phenolic compound, only the samples in which the compound could be quantified were taken into account. The mean value of the total amount of phenolics was calculated as the mean of the total amount in each sample.

Spectrophotometric Quantification. For best comparison with the quantification of individual polyphenols by HPLC, the same extracts were used for the spectrophotometric assays.

The total polyphenolic content of the extracts was determined according to the Folin−Ciocalteu colorimetric method as described elsewhere (*23*). In short, 100 μL of test sample was added to 5 mL of distilled water and 500 μL of Folin−Ciocalteu color reagent (Fluka, Buchs, Switzerland) and vigorously shaken. After 3 min, 1 mL of a saturated solution of sodium carbonate was added and the sample made up with distilled water to a volume of 10 mL. Absorbance was measured after 60 min at 720 nm using a Lambda2 UV−vis spectrometer (Perkin-Elmer Instruments GmbH, Rodgau-Jügesheim, Germany). Total phenols were expressed as grams per kilogram of gallic acid equivalents.

Catechins and proanthocyanidins were analyzed according to the vanillin method as described by Bradhurst and Jones (*24*). In short, 100 μL of test sample was added to 2 mL of distilled water and 4 mL of the vanillin reagent (1% vanillin in 7 M H_2SO_4), shaken, and cooled on ice for 5 min. After 10 min at room temperature, the absorbance was measured at 500 nm. Condensed tannin contents were expressed as grams per kilogram of (+)-catechin equivalents.

Antioxidant activity was determined by scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (*12*). One hundred microliters of test sample was reacted with 2.5 mL of ethanolic DPPH solution (100 μM; Sigma-Aldrich, Steinheim, Germany) for 30 min in the dark, and the absorbance was measured at 517 nm. Results were expressed as IC_{50} values; the IC_{50} value is the concentration of test sample calculated to be needed to reduce the color of the DPPH solution by 50%. Trolox (Fluka) was used as positive control and reference.

RESULTS AND DISCUSSION

Liquid Extraction and Solid-Phase Extraction of Polyphenols. As the first step in analysis, the liquid extraction of polyphenols from a solid matrix is of utmost importance. The choice of method, solvent, temperature, and time largely determines the extraction selectivity and efficiency of the different sample compounds. Due to the antioxidant properties and reactivity of phenolic compounds, the extraction procedure has to be rapid and careful to avoid artifact formation.

Previous studies mainly used water as extraction solvent (*16*−*18*). Samples were ground and heated or boiled in water, and the extract was analyzed after filtration. Unfortunately, the apparent lack of a standardized extraction method compromises the quantitative comparison of data between studies from different researchers, and alteration of sample compounds during extraction is not accounted for.

In this work, the relatively new technique of pressurized liquid extraction was applied to solid carob product samples. PLE uses conventional extraction solvents or solvent mixtures at elevated temperature and pressure (*25*). This results in fast and efficient recovery of analytes from solid samples with a minimized use of solvent. This technique has been successfully applied during recent years to environmental analysis and has been shown to be of advantage in the analysis of different analytes from food and plant samples (*20*, *26*−*29*).

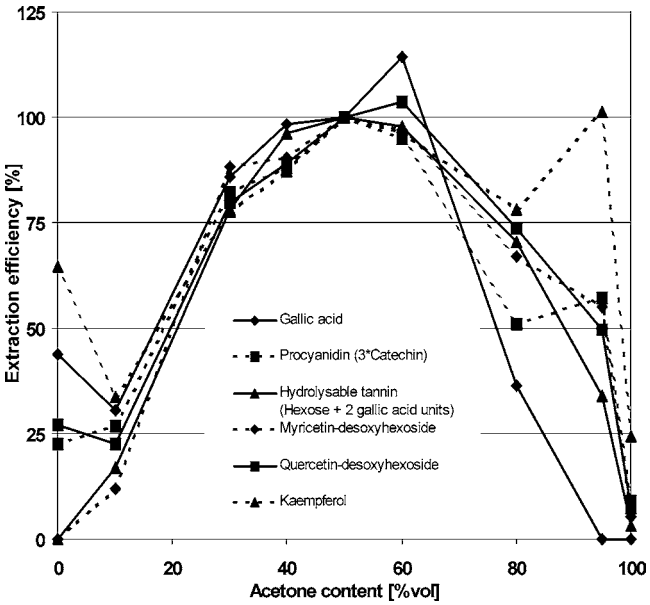

Figure 2. Extraction efficiency versus acetone–water percentage with PLE. The main components used for the calculation of extraction efficiency are shown.

Carob fiber was used as sample for the evaluation of extraction parameters applied to PLE. The choice of solvent was found to be the key factor for extraction efficiency (**Figure 2**). Acetone plus water mixtures showed the highest efficiencies, whereas methanol plus water mixtures had lower extraction yields. Using water alone even at the optimal time and temperature setting led to significantly lower extraction yields. Extraction time and temperature are of significant influence if a nonoptimal solvent composition is used. If an optimal solvent composition is chosen, however, temperature and time are of marginal influence. Generally, a longer extraction time results in a higher efficiency, whereas with higher temperatures compounds are thermally degraded. Thus, the extraction time and temperature should be kept low using an optimal solvent composition.

The impact of these parameters on PLE of polyphenols is comparable to those found for barley malt (*20*) and hops (*26*). The impact of the acetone content in the extraction solvent depends on the polarity of the compounds. Extracts with very low acetone content have a higher gallic acid and hydrolyzable tannins to flavonol-glycosides ratio and vice versa.

PLE was compared to a manual extraction procedure comparable to the methods described by other authors (*16*−*18*). Manual extraction had significantly lower efficiency than PLE when the same solvent, time, and temperature were used, except for when the acetone plus water, 50 + 50, v + v, mixture found to be the optimal setting in PLE was used, resulting in comparable extraction efficiency even with manual extraction at ambient temperature. This result indicates the benefits of pressure applied in PLE, which helps in the penetration of the solvent into the solid sample matrix. This delivers highly concentrated extracts, using smaller volumes of solvents compared to manual extraction. The automated PLE also gives better reproducibility than manual extraction.

The PLE extracts contain a high amount of coextracted matrix compounds such as sugars, colored substances, lipids, and resins. Polyphenols are minor constituents of plant material, and the identification and quantification of compounds benefit from a concentration and purification process.

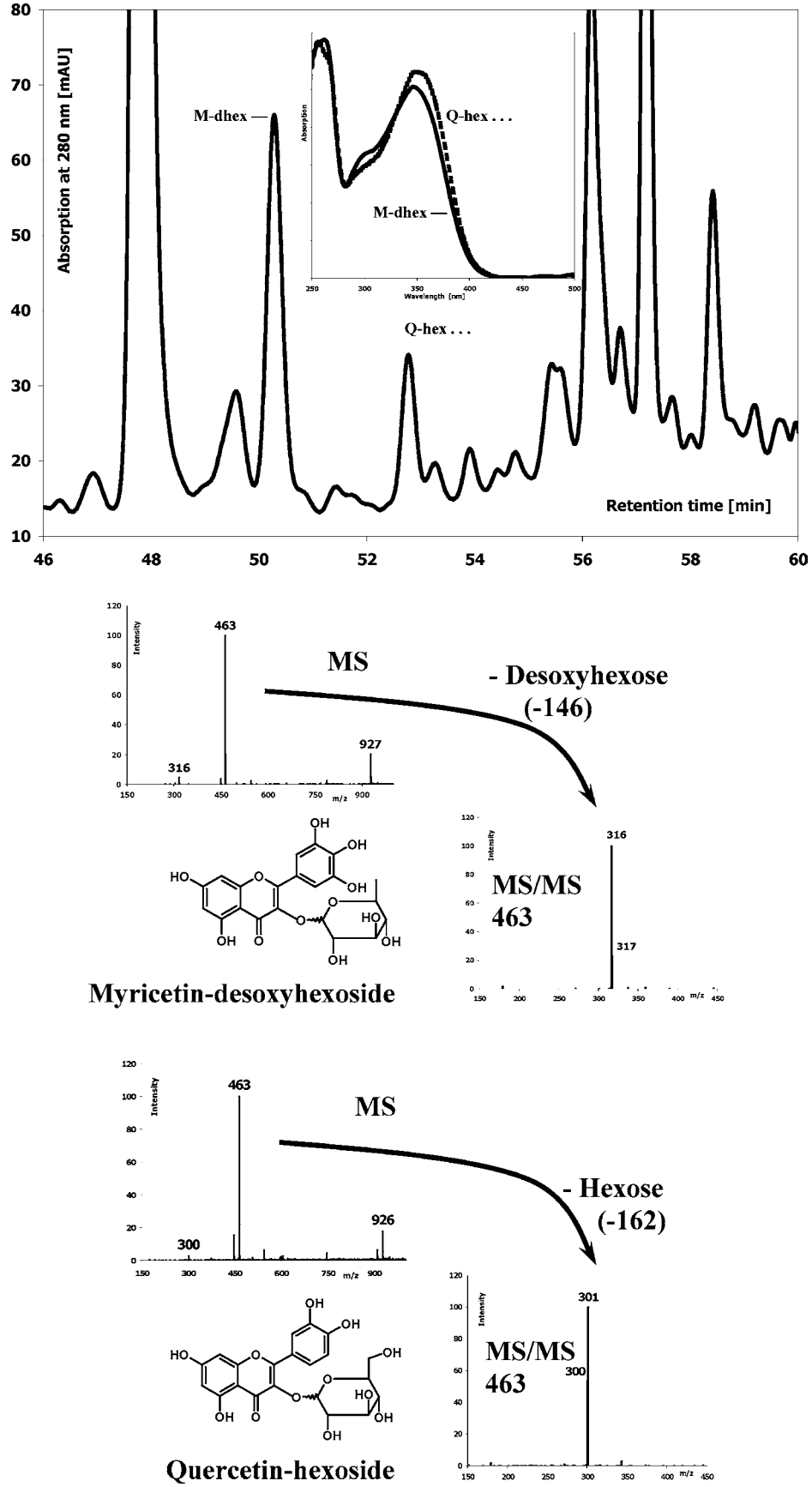

Figure 3. Chromatogram, UV spectra, and MS^2 fragmentation pattern of a quercetin-hexoside and a myricetin-desoxyhexoside from a carob fiber extract. Molecular structures are possible examples, as the nature of the sugar and the position of substitution cannot be derived from the experiments.

Polyamide is often used for SPE in polyphenol analysis as it exhibits a selective retention mechanism to polyphenols. In previous work (*20*), an automated method for polyamide SPE of polyphenols was developed and optimized, which overcomes

Table 1. Quantitative Data of the Extractable Phenolics As Identified by HPLC-UV-ESI/MS^n in Four Different Lots of Carob Fiber

RT[a] (min)	compound[b] (peak no.[c])	lot A (mg/kg)	lot B (mg/kg)	lot C (mg/kg)	lot D (mg/kg)	mean[d] (mg/kg)
8.6	gallic acid (1)	287.7	214.6	266.6	165.8	233.7
9.6	(epi)gallocatechin + 4 gallic acid units	2.2	2.7	nq[e]	nq	2.5
10.8	prodelphinidin dimer (2*GC[f])	11.0	24.9	7.9	nq	14.6
12.9	hexose + 2 gallic acid units (2)	48.6	59.0	44.4	nq	50.7
13.9	prodelphinidin trimer (1*C, 2*GC)	nq	7.1	4.3	nq	5.7
15.0	(epi)gallocatechin	42.7	41.0	nq	nq	41.9
17.5	hexose + 2 gallic acid units (3)	23.7	20.0	18.0	nq	20.6
19.2	(epi)gallocatechin + 3 gallic acid units	4.3	1.8	7.1	nq	4.4
22.4	procyanidin dimer	nq	5.1	3.2	nq	4.2
24.4	pentose + 3 gallic acid units (4)	34.2	49.3	37.6	46.1	41.8
25.6	procyanidin trimer (5)	166.5	62.2	nq	nq	114.3
26.4	hexose + 2 gallic acid units (6)	75.5	52.9	43.2	31.3	50.7
26.5	hexose + 2 gallic acid units	nq	103.0	64.8	83.8	83.9
27.8	hexose + 3 gallic acid units	27.7	25.0	15.9	17.3	21.5
29.3	2 pentoses + 2 gallic acid units (7)	74.5	127.9	83.0	163.5	112.2
31.3	hexose + 3 gallic acid units	20.8	29.6	22.4	23.7	24.1
33.6	(epi)gallocatechingallate	nq	nq	nq	4.3	4.3
36.2	hexose + 3 gallic acid units (8)	222.4	312.8	283.2	246.6	266.3
37.2	hexose + 3 gallic acid units	70.9	85.0	74.4	60.8	72.8
38.9	hexose + 3 gallic acid units	28.2	28.0	23.3	28.1	26.9
40.2	hexose + 3 gallic acid units	nq	25.8	9.9	24.0	19.9
41.2	2 pentoses + 2 gallic acid units	13.8	15.4	13.6	12.6	13.8
43.5	hexose + 3 gallic acid units	6.2	nq	8.4	5.6	6.7
45.5	myricetin-hexoside (9)	114.1	157.7	144.9	96.3	128.2
46.9	myricetin-pentoside	nq	nq	nq	77.1	77.1
48.0	hexose + 4 gallic acid units (10)	573.3	557.4	543.1	596.2	567.5
49.6	myricetin-pentoside	173.3	nq	nq	164.7	169.0
50.5	myricetin-desoxyhexoside (11)	369.3	581.9	498.4	412.8	465.6
53.0	quercetin-hexoside (12)	111.2	193.1	158.2	120.9	145.9
54.9	quercetin-pentoside	19.0	38.3	34.1	21.0	28.1
55.7	quercetin-hexuronic acid	nq	179.3	nq	nq	179.3
55.8	quercetin-pentoside	nq	nq	81.5	nq	81.5
56.4	hexose + 5 gallic acid units	98.2	nq	138.0	146.1	127.4
57.4	quercetin-desoxyhexoside (13)	435.5	848.5	765.8	549.2	649.8
58.5	kaempferol derivative	nq	39.3	nq	48.7	44.0
60.7	kaempferol-desoxyhexoside	23.7	43.2	35.4	33.0	33.8
65.5	kaempferol (14)	67.5	123.7	75.4	70.4	84.3
66.9	quercetin-derivative	18.3	39.0	32.6	nq	30.0
70.2	kaempferol + methyl group	42.5	nq	38.6	27.2	36.1
72.1	kaempferol +3 methyl groups	9.1	8.2	8.2	3.6	7.3
79.6	methoxykaempferol derivative	39.3	nq	61.2	nq	50.2
	total amount	3255.2	4102.9	3646.6	3280.6	4142.4

[a] Retention time in minutes. [b] As identified by UV spectrum and MS^n fragmentation. [c] Corresponding peak number for calculation of extraction efficiency. [d] Calculated from samples when quantifiable. [e] Not quantifiable; compound present but quantification not possible due to overlapping peaks. [f] C, catechin or epicatechin; GC, gallocatechin or epigallocatechin.

some limitations of previous manual SPE methods (e.g., the detection of oligomeric proanthocyanidins). This method was found to be readily applicable to the purification and concentration of PLE extracts from carob samples and other material investigated in this work.

Identification of Polyphenols. The large number of different polyphenols found in plant material complicates the identification of individual compounds. Comparison of the retention time and the ultraviolet spectrum of a compound in question and a known reference alone does not provide enough information to unambiguously distinguish individual compounds (**Figures 1** and **3**). The coupling of UV-diode array detection with MS/MS enables partial structural elucidation and identification of polyphenols (*21*, *30*−*35*). However, this is of key importance when the bioavailability or physiological action of these substances is assessed. The bioavailability of polyphenols is known to be dependent on the type of sugar residues and their position and varies remarkably between similar derivatives (*36*−*38*).

In this work, after HPLC separation, the UV spectrum of an unknown compound was compared to spectra of reference substances from a database. In addition, the results from subsequent mass spectrometrical fragmentation experiments were used to identify sugars and aglycons. Combination of these data provides a structural verification of the compound in question. **Figure 3** shows an example of the identification of flavonol-glycosides. From their similar retention times and UV spectra, these compounds can be identified as flavonols; however, if the peaks are small and the UV spectra therefore distorted, no unambiguous distinction is possible between kaempferol-, quercetin-, and myricetin-*O*-glycosides. The molecular masses of both compounds still are the same, but fragmentation of the quasi-molecular ion leads to the aglycon, enabling the clear identification as a myricetin-desoxyhexoside and a quercetin-hexoside. The position of glycosylation, the nature of the sugar moieties, and the interglycosidic linkage, however, can only partly be derived from the MS^n fragmentation data (*35*, *39*−*42*).

In similar fragmentation experiments, the identity and sequence of the flavan-3-ol units forming oligomeric proanthocyanidins can be elucidated (*21*), as well as the composition of the galloyl esters representing hydrolyzable tannins.

Table 2. Results of the Spectrophotometric Assays: Total Phenolics, Condensed Tannins, and Antioxidative Activity of the Investigated Sample Materials after Pressurized Liquid Extraction and Solid-Phase Extraction

sample material	total phenolics as gallic acid equiv (g/kg)	condensed tannins as catechin equiv (g/kg)	antioxidative activity as IC_{50} DPPH (g/L)	antioxidative activity as Trolox equiv
		Raw Materials		
kibbles	8.31	1.00	33.26	204.05
syrup	3.94	1.32	26.33	161.53
		Carob Fiber (Caromax)		
lot A	8.23	1.30	10.86	66.63
lot B	14.36	2.38	8.57	52.58
lot C	19.37	2.60	6.76	41.47
lot D	18.00	2.67	6.51	39.94
mean	14.99	2.24	8.18	50.16
		Carob Flours (Roasted Carob Products)		
product A	24.52	2.52	7.04	43.19
product B	14.55	1.51	9.96	61.10
product C	23.73	2.12	7.13	43.74
mean	20.93	2.05	8.04	49.34

The application of this technique enabled the identification of 41 different polyphenols in carob fiber in this work (**Table 1**). The substances identified comprise gallic acid and hydrolyzable tannins, proanthocyanidins, flavonol-glycosides with a large amount of quercetin and myricetin derivatives, and traces of isoflavonoids and flavanol galloyl esters. However, we could not detect appreciable amounts of some compounds reported to be present in carob by other investigators using only UV detection and retention time for identification (*16*−*18*). This is especially true for the galloyl esters of flavanols (epicatechin gallate, epigallocatechin gallate, catechin gallate, and gallocatechin gallate).

Quantification of Polyphenols. The quantitative data for the sample materials analyzed are shown in **Tables 1**−**5**.

It is quite obvious that the results obtained by HPLC and by the spectrophotometric assays vary. Photometric assays are prone to under- or overestimation by cross-reactions, whereas with the HPLC quantification a number of minor compounds might not be taken into account, if they are below their quantification limit. Nevertheless, photometric assays allow fast screening of different samples for the quantity and quality of polyphenols and antioxidant components. **Table 2** shows that carob fiber and carob flours show significantly higher levels of total extractable phenols than native carob and much higher antioxidative activity expressed in a high DPPH radical scavenging activity. Furthermore, carob fiber demonstrated this high activity at even lower total phenol levels than carob flour, suggesting a specifically higher radical scavenging activity of the compounds present in the carob fiber extracts.

A number of factors in sample preparation may influence the quantification of the compounds examined. The extraction process might lead to the release of gallic acid by the hydrolysis of tannins (and to a lesser degree the formation of more stable galloyl esters), resulting in an overestimation of gallic acid. This complicates the comparison of both qualitative and quantitative data from different sources that have used a variety of extraction methods. Furthermore, especially the more apolar flavonol-glycosides are not completely released if water is used as the extraction solvent.

Distribution of Compounds in the Carob Fruit. The comparison of the polyphenolic patterns in carob kibbles, locust bean powder, sugar syrup, and carob fiber (**Tables 1** and **4**) shows the distribution of extractable polyphenols across the fruit. The pod contains the majority of polyphenolic substances in the fruit. In locust bean powder, only a few different phenolic compounds could be identified and the amount is comparably low. These compounds might be restricted to the outer, dark brown seedcoat, but due to the low overall amount this was not investigated in detail. In the sugar syrup, mainly gallic acid is detected, representing the difference in the polyphenolic pattern of kibbles and carob fiber. Hydrolyzable tannins could not be detected in the sugar syrup; these are thought to be hydrolyzed during the sugar concentration process.

Comparison of Phenolic Pattern and Content in Different Carob Fiber Lots. To account for differences in the phenolic pattern and content due to seasonal or annual changes, variety, and region as well as variables in the production process, four different lots of carob fiber were quantified for their phenolic constituents and the results compared (**Table 1**). The data show that the tested lots have a comparable pattern of constituents, especially with regard to the major polyphenols. The main differences are seen in the content of gallic acid, the percentage

Table 3. Amount of Phenolics by HPLC Quantification in Compound Groups

sample material	gallic acid (mg/kg)	hydrolyzable tannins (mg/kg)	condensed tannins (mg/kg)	myricetin derivatives[a] (mg/kg)	quercetin derivatives[a] (mg/kg)	kaempferol derivatives[a] (mg/kg)	total phenolics[b] (mg/kg)
			Raw Material				
kibbles	174.1	26.3	14.8	171.1	53.3	8.6	448.2
syrup	1012.6	nq[c]	9.0	11.2	0.9	nq	1084.9[d]
			Carob Fiber (Caromax)				
lot A	287.7	1317.9	226.7	656.7	584.1	182.1	3255.2
lot B	214.6	1491.3	144.8	739.5	1298.2	214.5	4102.9
lot C	266.6	1423.0	22.5	643.3	1072.3	218.8	3646.6
lot D	165.8	1485.7	4.3	750.8	691.1	182.9	3280.6
mean	233.7	1506.8	191.8	839.9	1114.5	255.7	4142.4
			Carob Flours (Roasted Carob Products)				
product A	685.6	275.7	34.2	129.7	154.6	nq	1279.8
product B	320.3	483.4	5.5	73.9	88.7	6.6	978.4
product C	264.1	231.7	16.4	92.9	105.2	13.2	723.6
mean	423.3	330.3	18.7	98.8	116.2	6.6	1207.8

[a] Amounts of flavonol-glycosides are given for the different aglycons as the glycosides. [b] Total phenolics are calculated from the mean values of the individual compounds as described under Materials and Methods. [c] Compound not quantifiable. [d] Includes 51.2 mg/kg cinnamic acids.

Table 4. Phenolic Constituents in Carob Kibbles, Locust Bean Gum, and Carob Syrup As Identified and Quantified by HPLC-UV-ESI/MS^n

RT[a] (min)	compound[b]	kibbles (mg/kg)	locust bean (mg/kg)	syrup (mg/kg)
8.8	gallic acid	174.1	nd[c]	1012.6
10.1	2 hexoses + 1 gallic acid unit	nq[d]	nd	nd
15.3	(epi)gallocatechin	nq	nd	nd
22.6	hexose + 2 gallic acid units − water + acetate	nq	nd	nd
22.8	procyanidin dimer	nq	nd	nd
24.4	hydrolyzable tannin	nq	nd	nd
25.6	hexose + 2 gallic acid units	nq	nd	nd
27.2	catechin	14.8	23.8	9.0
29.9	caffeic acid	nd	nd	2.5
30.2	(epi)gallocatechin	nq	nd	nd
30.6	quercetin-desoxyhexoside + water	24.0	nd	nd
31.7	procyanidin dimer	nq	nd	nd
34.6	coumaric acid	nd	nd	9.9
35.0	quercetin-desoxyhexoside	nq	nd	nd
36.0	hexose + 3 gallic acid units	nq	nd	nd
40.7	ferulic acid	nd	nd	14.6
45.4	myricetin-hexoside	22.3	nd	nd
46.3	hexose + 3 gallic acid units + water	26.3	nd	nd
47.8	kaempferol-hexoside + water	nq	nd	nd
47.8	hexose + 4 gallic acid units	nq	nd	nd
47.9	kaempferol-dihexoside	nd	7.0	nd
48.8	hexose + 2 gallic acid units + water	nq	nd	nd
50.3	myricetin-desoxyhexoside	148.8	nd	11.2
52.6	quercetin-hexoside	24.6	4.8	nd
57.2	quercetin-desoxyhexoside	nq	44.7	0.9
59.8	kaempferol derivative	nd	1.9	nd
60.6	kaempferol-desoxyhexoside	8.6	1.2	nd
65.4	kaempferol	nq	nd	nd
66.6	quercetin	4.7	nd	nd
69.8	cinnamic acid	nd	nd	24.2
70.2	kaempferol + methyl group	nq	nd	nd
	total amount	448.2	83.4	1084.9

[a] Retention time in minutes. [b] As identified by UV spectrum and MS^n fragmentation. [c] Compound not detected in this sample. [d] Compound present but quantification not possible due to overlapping or very small peaks.

Table 5. Phenolic Constituents in Three Different Roasted Carob Preparations As Identified and Quantified by HPLC-UV-ESI/MS^n

RT[a] (min)	compound[b]	product A (mg/kg)	product B (mg/kg)	product C (mg/kg)	mean[c] (mg/kg)
8.6	gallic acid	685.6	320.3	264.1	423.3
10.9	procyanidin-dimer	21.2	nq[d]	nq	21.2
13.0	hexose + 2 gallic acid units	18.6	32.5	10.8	20.6
15.3	(epi)gallocatechin	6.5	nq	10.6	8.6
16.0	prodelphinidin dimer (1*GC, 1*C[e])	nq	nq	5.8	5.8
17.5	hexose + 2 gallic acid units	8.7	4.5	5.7	6.3
19.1	prodelphinidin dimer (2*GC) + hexose	6.5	nq	nq	6.5
24.3	pentose + 3 gallic acid units	15.2	17.7	6.5	13.2
25.6	hexose + 2 gallic acid units	13.3	nq	8.5	10.9
26.6	hexose + 2 gallic acid units	23.9	16.9	18.6	19.8
27.1	catechin	nq	5.5	nq	5.5
28.0	hexose + 3 gallic acid units	8.5	14.7	6.7	9.9
29.3	2 pentoses + 2 gallic acid units	23.4	82.5	15.6	40.5
31.3	hexose + 3 gallic acid units	7.4	7.3	4.0	6.2
36.0	hexose + 3 gallic acid units	nq	36.8	27.4	32.1
37.2	hexose + 3 gallic acid units	18.4	23.3	15.5	19.1
39.0	hexose + 3 gallic acid units	8.1	6.6	6.7	7.2
41.2	2 pentoses + 2 gallic acid units	7.3	nq	nq	7.3
43.9	desoxyhexose + 2 gallic acid units	3.5	nq	nq	3.5
45.3	myricetin-hexoside	29.3	13.1	16.0	19.5
47.9	hexose + 4 gallic acid units	119.4	233.9	nq	176.7
48.0	myricetin + 1 desoxyhexose + 2 hexoses	nq	nq	97.5	97.5
49.5	hexose + 4 gallic acid units	nq	6.7	nq	6.7
50.3	myricetin-desoxyhexoside	100.5	60.8	76.9	79.4
52.8	quercetin-hexoside	29.4	19.8	nq	24.6
55.7	quercetin-pentoside	nq	nq	17.3	17.3
56.3	hexose + 5 gallic acid units	nq	nq	8.2	8.2
57.2	quercetin-desoxyhexoside	125.2	64.1	87.9	92.4
60.7	kaempferol-desoxyhexoside	nq	4.5	8.7	6.6
66.7	quercetin derivative	nq	4.8	nq	4.8
67.0	kaempferol	nq	nq	4.5	4.5
70.2	kaempferol + methyl group	nq	2.1	nq	2.1
	total amount	1279.8	978.4	723.6	1207.8

[a] Retention time in minutes. [b] As identified by UV spectrum and MS^n fragmentation. [c] Calculated from samples when quantifiable. [d] Compound present but quantification not possible due to overlapping or very small peaks. [e] C, catechin or epicatechin; GC, gallocatechin or epigallocatechin.

of flavonol-glycosides, and the relation of myricetin and quercetin derivatives.

Fate of Polyphenols in Carob Pod Processing. The processing of carob pods during preparation of carob fiber or roasted products has a great effect on the polyphenolic pattern and the extractable quantities, as can be seen from the data presented in **Tables 1**, **3**, and **5**.

Carob fiber has a higher amount of phenolics than kibbles, which can partly be attributed to the loss of weight by the extraction of carbohydrates, accounting for 40−50% of the kibbles' weight. However, this cannot fully explain the higher content of polyphenols in carob fiber. Due to the processing steps of carob fiber the matrix is thought to be ruptured and bound phenolics to be released, thus easing subsequent extraction of phenolics in the analytical process. The change in the phenolic pattern shows a higher percentage of gallic acid and polar hydrolyzable tannins in the kibbles, which are partly coextracted with the carbohydrates in the carob fiber preparation process. Consequently, the resulting sugar syrup contains mainly gallic acid.

The preparation of roasted carob flours shows a large impact on the hydrolyzable tannins and gallic acid. These compounds are altered by hydrolyzation processes, increasing the content of gallic acid in the product and lowering the amount of tannins.

In comparison with the other carob products, carob fiber shows the highest overall amount of phenolic constituents and a diverse pattern of flavonol-glycosides, whereas the other products contain mainly gallic acid and hydrolyzable tannins.

ACKNOWLEDGMENT

We thank Christoph Henke and Claudia Wagenbach for skillful technical assistance while contributing to this work.

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Received for review September 20, 2003. Revised manuscript received March 19, 2004. Accepted March 21, 2004. Nutrinova is gratefully acknowledged for the financial support in this project.

JF030660Y