Isolation and Identification of Cyclitols in Carob Pods (*Ceratonia siliqua* L.)

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In the food industry, carob powder is used as a cocoa substitute. It consists primarily of sugars (sucrose, glucose, fructose) in addition to tannins, fibers, etc. D-(+)-Pinitol (3-*O*-methyl-D-*chiro*-inositol), *myo*-inositol, and D-(+)-*chiro*-inositol were isolated from a fermented water extract of carob powder. The concentration of pinitol ranged from 5 to 7.5% of the dry weight of the powder as determined by GC. *myo*-Inositol and *chiro*-inositol were minor components with concentrations of 0.5 to 1% and 0.1%, respectively. As pinitol is not present in cocoa powder, it can be used as a natural marker of carob adulteration of cocoa powder. Further investigations, including GC–MS, revealed traces of ononitol (4-*O*-methyl-*myo*-inositol), sequoyitol (5-*O*-methyl-*myo*-inositol), and bornesitol (1-*O*-methyl-*myo*-inositol) to be present in a fermented water extract of carob powder in addition to sorbitol.

INTRODUCTION

Carob powder is manufactured by grinding deseeded carob pods, the fruits of the carob tree (*Ceratonia siliqua* L.) also known as locust bean or St. John's bread. It has a long history of use, as both a food and a pharmaceutical. Due to its flavor which is enhanced upon roasting, it has found application as a substitute for cocoa. The degree of roasting may be varied to produce a range of colors and flavors similar to cocoa. Because of its low price and the absence of caffeine, carob has been used in various health food products and occasionally also as an adulterant of cocoa powder. A reliable method for the detection and quantification of carob powder in cocoa products (e.g., chocolate) has not been available to our knowledge.

Carob pods are known to be rich in sugars (Binder et al., 1959; Davies et al., 1971; Saura Calixto and Canellas, 1982). The average amount of sucrose, glucose, and fructose ranges from 40 to 50% of the dry matter of the pod, depending on the variety and ripeness of the fruit. As the free sugar content in cocoa is less than 1%, the determination of sugars in cocoa powder has been used to prove the addition of carob powder to cocoa powder; a HPLC method was proposed by Albright et al. (1978). In chocolate products however the detection of reducing sugars is of no value as an indicator of the presence of carob.

Carob and cocoa powders have to date not been analyzed for cyclitols, although they occur in many plant families (Plouvier, 1963). *myo*-Inositol, *chiro*-inositol, and pinitol are commonly found in considerable concentrations in legumes. Pinitol is the most dominant component of the low molecular weight carbohydrate fraction of soybeans, which belong to the same plant family as carob (Honig et al., 1971; Phillips and Smith, 1974; Schweizer et al., 1978; Phillips et al., 1982, 1984). Kindl and Hoffmann-Ostenhof (1966) postulated that *myo*-inositol is the precusor of all methylated inositols and suggested a biochemical pathway from *myo*-inositol through sequoyitol to D-(+)-pinitol and D-(+)-*chiro*-inositol. Dittrich and Korak (1984) showed another possible biosynthesis of D-(+)-pinitol with D-(+)-ononitol as an intermediate. The fourth methyl ether of *myo*-inositol occurring naturally in legumes is D-(−)-bornesitol.

An unknown compound was detected in a fairly high concentration (approximately 6% dry weight) during analysis of the carob powder. It was found to be a cyclitol. A thorough investigation of the unfermentable residue of a carob extract seemed to be desirable and indeed led to the identification of additional polyols.

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MATERIALS AND METHODS

Carob beans were broken, deseeded (kibbled), and roasted. The kibbles were ground and extracted with 5 times their weight of water while being stirred at 50 °C for 2 h. 0.01% (w/v) of dried yeast (*Saccharomyces bayanus*) was added to the lukewarm, turbid extract. After 7 days at 30 °C, the fermentation was completed and the extract was filtered and concentrated to 50° Brix. Remaining sugars were removed from the cyclitols on a Dowex 1-X8 anion-exchange resin, 100–200-mesh column (120 × 1.5 cm) in the hydroxide ion form. The eluate was evaporated to dryness, and pinitol crystals were obtained by recrystallization from ethanol. The mother liquid of the crystals contained traces of different cyclitols besides *myo*-inositol and *chiro*-inositol. They were separated on a Dowex 50W-X4, 50–100-mesh cation-exchange resin in lithium cation form and eluted with 87% ethanol (Kretz, 1973). Four fractions were collected and analyzed by thin-layer chromatography (TLC), gas chromatography (GC), and gas chromatography–mass spectrometry (GC–MS). *myo*-Inositol and *chiro*-inositol were obtained in pure fractions and crystals through recrystallization of the dried fraction with ethanol.

For GC and GC–MS studies trimethylsilyl (Me_3Si) derivatives of cyclitols and sugars oximes were prepared by a modification of the method described by Zürcher et al. (1975). Dry syrup samples of 0.5–2.0 mg of sugars were dissolved in 1 mL of pyridine, containing 0.5 mg of xylitol as internal standard and 3 mg of hydroxylamine hydrochloride. After reaction at 90 °C for 30 min, 100 μL of this reaction mixture was silylated by adding 10 μL of trimethylchlorosilane and 100 μL of MSFBHA [*N*-methyl-*N*-(trimethylsilyl)heptafluorobutyramide] and analyzed on a 4% OV 17 on Chromosorb G column (2 m × 2 mm) and a SE 54 fused silica column of 25 m. The runs on the packed column were programmed from 130 to 270 °C, increasing 5 °C/min. Nitrogen was used as a carrier gas at 20 mL/min. Separations on the capillary column were made with a temperature program from 120 to 270 °C, increasing 5 °C/min with helium as a carrier gas at the pressure of 0.45 bar, split 1:20. Injector and detector were set at 300 °C. *chiro*-Inositol and ononitol were separated by changing the temperature program to 3 °C/min with a 5-min holding time at 200 °C. GC–MS studies were performed on a Carlo Erba/MS 80 RF mass spectrometer. Two microliters was injected splitless on the SE 54 column with the helium pressure of 1.17 bar and a temperature

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Table I. R_f Values and Retention Times of the Different Substances (RR_t Relative to Xylitol)

compd	GC RR_t 4% OV 17	GC RR_t SE 54	TLC R_f
pinitol	1.21	1.19	0.45
ononitol	1.42	1.38	0.22
sequoyitol	1.42	1.34	0.22
bornesitol	1.52	1.45	0.11
chiro-inositol	1.36	1.38	0.09
myo-inositol	1.59	1.58	0.04
sorbitol	1.32	1.36	0.19
xylose	1.13	1.08[a]	0.57
fructose	1.36	1.38[a]	0.36
glucose	1.46	1.44[a]	0.39
sucrose	2.30		0.29
maltose	2.56		0.22

[a] Main peak, in the case of multiple peaks.

program from 100 to 260 °C at 5 °C/min. MS was performed as electron ionization mass spectrometry (EIMS) at 70 eV and chemical ionization mass spectrometry (CIMS) at 90 eV with ammonia as the reagent gas.

The identity of all crystals was additionally verified by MS, optical rotation, and the melting point.

Throughout the whole study, results were also monitored by TLC. It was carried out on silica gel with the solvent system water–ethyl acetate–2-propanol (6:11:83, v/v/v). The substances were detected with 0.5% (w/v) potassium permanganate in a 1 N solution of sodium hydroxide. To specifically detect sugars, a solution of 1% aniline and 1% diphenylamine in 100 mL acetone was mixed with 10 mL of concentrated sulfuric acid and sprayed on the plate before heating it to 110 °C for 10 min (Lewis and Smith, 1967).

RESULTS AND DISCUSSION

The analysis of the trimethylsilyl ethers of cyclitols and sugar oximes of carob powder by GC showed the presence of sucrose, fructose, glucose, pinitol, and *myo*-inositol in the concentrations of 25–40%, 3–8%, 2–6%, 5–7%, and 0.5–1%, respectively. The yeast fermentation and consequent purification by anion-exchange chromatography led to a solution with three major substances: pinitol, *myo*-inositol, and *chiro*-inositol, in the concentration ratio of 50:4:1. *chiro*-Inositol has the same RR_ts as fructose on the 4% OV 17 and the SE 54 column (Table I) and therefore required identification with TLC. Its concentration in the fermented extract corresponds to 0.1% in the dry matter of the pod. The melting point of the recrystallized pinitol was 186–187 °C (lit. mp 185 °C) and was not decreased when mixed with reference material. The optical rotation was +64.5° (H_2O, *c* 5.0 g/mL; lit. $[\alpha]^{20}_D$ + 65°) and indicated the dextrorotary form, D-(+)-pinitol (3-*O*-methyl-D-*chiro*-inositol). The mass spectrum of the crystalline and the silylated material showed the same ratios as those reported by Balabanova et al. (1982) and Binder and Haddon (1984b), respectively. As in the EIMS the molecular ion (*m/e* 194) had only a relative intensity of 0.2% and *m/e* 195 was prominent (1.0%), CIMS was also measured and the molecular ion (*m/e* 212) corresponding to $[M + NH_3]^+$ showed the relative intensity of 100% (Figure 1).

The mother liquid of the pinitol crystals contained traces of other cyclitols in addition to *myo*- and *chiro*-inositol (Figure 2). The fractions of the concentrated residue, isolated after cation-exchange chromatography, were analyzed by GC and GC–MS. *chiro*-Inositol and *myo*-inositol were recrystallized from pure fractions with ethanol. EIMS and melting points were determined as well as the optical rotation and the CIMS of *chiro*-inositol. The

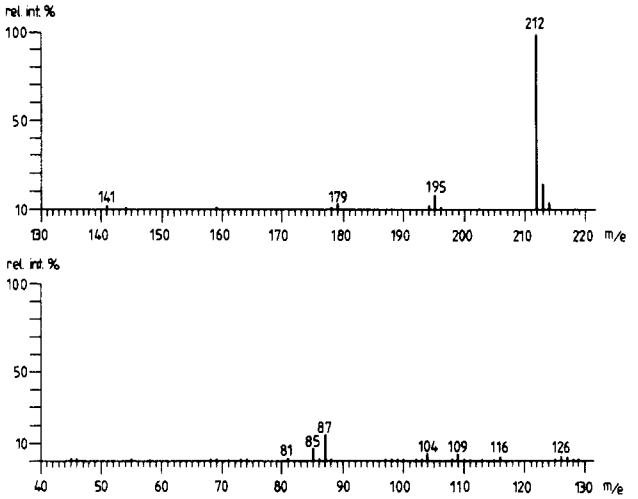

Figure 1. CIMS profile of D-(+)-pinitol (for experimental details, see text).

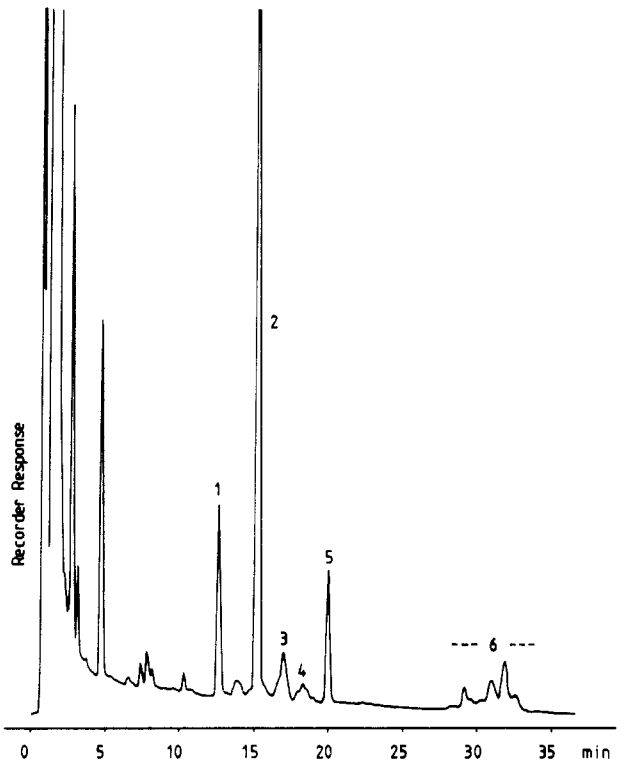

Figure 2. Fermented carob extract after recrystallization of pinitol, analyzed by GC as oxime trimethylsilyl derivatives: 1, xylitol (internal standard); 2, pinitol; 3, sorbitol and *chiro*-inositol; 4, sequoyitol, ononitol, and bornestiol; 5, *myo*-inositol; 6, unidentified disaccharides. For experimental details to the program of the OV 17 column, see the text.

mass spectra of the inositols correlated exactly with the data of our reference spectra and the published spectra (Binder and Haddon, 1984b). *chiro*-Inositol melted at 224 °C (lit. mp 240 °C) but was not decreased when mixed with reference material. It is not clear why the isolated *chiro*-inositol showed this relatively low melting point compared to the literature value. The optical rotation of *chiro*-inositol was $[\alpha]^{20}_D$ +65° (H_2O, *c* 0.001 g/mL; lit. $[\alpha]^{20}_D$ +65°) and verified the dextrorotary form (D-(+)-*chiro*-inositol) as expected as precursor of D-(+)-pinitol. The CIMS showed a major peak of *m/e* 198, corresponding to the $[M + NH_3]^+$ ion. *myo*-Inositol melted at 225 °C, which was cited in literature.

By comparison with standard substances and published data (Binder and Haddon, 1984b), the other compounds

are identified as ononitol (4-*O*-methyl-*myo*-inositol), sequoyitol (5-*O*-methyl-*myo*-inositol), bornesitol (1-*O*-methyl-*myo*-inositol), and sorbitol. Except the last, none of these substances are known to be a metabolic product of yeast. One of the fractions was shown to contain ononitol, sequoyitol, and bornesitol by capillary GC, but on the packed column the presence of *chiro*-inositol was also evident. By using a slower temperature program *chiro*-inositol was additionally detected as a shoulder on the ononitol peak. Because the chromatographic properties of trimethylsilyl ethers of carbohydrates are very similar, the identification was only successful by different analytical methods. Ononitol, bornesitol, and sorbitol were obtained in minute quantities and were not separated from other chiral substances; thus, their optical rotation could not be measured precisely.

Only D-(−)-bornesitol and D-(+)-ononitol were reported to be present in legumes (Plouvier, 1963). Therfore, they are likely of the same chiral form in carob. The same combination of cyclitols was recently found in soybean (Binder and Haddon, 1984a), but carob is the first species in which a large concentration of sugars is also present.

Pinitol was already mentioned in 1922 in context with carob. Charuax (1922) identified the exudate occasionally found on carob trees as almost pure D-(+)-pinitol. The carbohydrate composition of carob pods has been studied several times, and carbohydrates other than sucrose, fructose, and glucose have been reported. Angelidis (1954) demonstrated by preparative paper chromatography the presence of maltose. Wallenfels and Lehmann (1957) isolated two reducing disaccharides with the same R_f values as maltose and lactose and claimed them to be primverose and ceratose; a new fructosylglucose. They could not confirm maltose but postulated the presence of three higher oligosaccharides and xylose. Tinner (1960) reported the presence of xylose and four oligosaccharides, one of which was identified as a xylosidoglucose; a disaccharide not identical with primverose. The hydrolysis of the three other oligosaccharides gave xylose, glucose, and fructose. According to our determinations, xylose and maltose were not present. However there were several other disaccharides in addition to sucrose detected by GC. Presumably these were disaccharides consisting of inositol or pinitol and galactose and similar to the galactocyclitols reported recently from different plants (Beveride et al., 1977; Schweizer and Horman, 1981; Quemener and Brillouet, 1983; Nicolas et al., 1984).

Since no pinitol was found in raw cocoa mass and two commercial, defatted cocoa powders, pinitol could therefore be used as a natural marker to determine the presence of carob in cocoa products.

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