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Catechins and Derived Procyanidins in Red and White Sorghum: Their Contributions to Antioxidant Activity

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ABSTRACT

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Flavan-3-ol monomers and derived procyanidins were determined by NP- and RP-HPLC-ESI(-)-MS/MS after extraction with a mixture of acetone/water/acetic acid (70:28:2, v/v). Logically, red sorghum was found to contain much higher amounts of monomers up to hexamers (∑(P1–P6) = 61.4–450.2 mg/kg, (-)epicatechin equivalents) than white sorghum (0.2 mg/kg, (-)epicatechin equivalents). (+)Catechin was identified as its major monomer and B1 turned out to be the main dimer. Thiolysis confirmed that (+)catechin is the major terminal unit (50–88%), while (-)epicatechin is more frequent as extension units (50–86%). The contribution of monomers ((+)catechin and (-)epicatechin) and dimers (B1 and B2) to the total antioxidant activity determined by RP-HPLC-online TEAC was revealed to be very weak.

Key words: Antioxidant activity, beer, flavan-3-ols, procyanidins, sorghum, thiolysis.

INTRODUCTION

Sorghum is an important cereal in Africa for food preparations such as porridge, couscous or local beer production (e.g. Dolo in Burkina Faso, Pito in Ghana and Kaffir in South Africa). Sorghum is reported to contain several polyphenols which provide health benefits such as antitumor, antioxidant and antibacterial activity, reducing the risk for developing chronic diseases such as coronary heart disease, cancer, diabetes or Alzheimer disease[10]. The fact that sorghum is gluten-free increases brewers' interest in using sorghum, as it raises the possibility of producing beers for coeliacs. Probably also due to its very high polyphenolic content, sorghum is recognized by brewers as efficiently limiting the cardboard off-flavour (*trans*-2-nonenal) in aged beers, by inhibiting lipid oxidation during mashing and boiling[9,10,19,21].

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As recently described[4], sorghum contains a wide variety of polyphenols, some of which are rare or absent in other plants. This is the case for anthocyani(di)ns such as apigeninidin and luteolinidin, which lack the hydroxyl group at the 3-position of the C-ring[1]. Other sorghum flavonoids include the flavan-4-ols apiforol (leucoapigeninidin) and luteoforol (leucoluteolinidin)[9,11], the flavones apigenin and luteolin[11], the flavanones naringenin and eriodictyol[12], the flavonol kaempferol[20], the dihydroflavonol taxifolin[15] and the flavan-3-ols (+)-catechin and (-)-epicatechin[2]. The stilbenoids trans-piceid and trans-resveratrol were also recently evidenced, especially in red sorghum[4].

The polyflavans found in sorghum are either proanthocyanidins (polymers of flavan-3-ols, mainly B-type homopolymers of (+)catechin/(-)epicatechin)[14] or pro-3-deoxyanthocyanidins (mainly pro-apigeninidins and pro-luteolinidins)[17]. Dicko et al.[9] have reported large differences in proanthocyanidin concentrations between red and white sorghum grains. Red sorghums showed an average of 9,400 mg/kg, against 1,300 mg/kg for the white ones. In most samples, proanthocyanidins decreased after germination. The proanthocyanidin levels revealed positively correlated to the total phenolic contents, especially in ungerminated grains.

Brandon et al.[3] have also found evidence of heteropolymers with both (epi)catechin and (epi)gallocatechin hydroxylation patterns. In addition, Gujer et al.[15] have described the structure of unique sorghum polyflavan dimers and trimers, glycosylated on the 5-hydroxy group of the extending flavan units and having a flavanone, either eriodictyol or eriodictyol 5-O-β-glucoside, as the terminal unit. More recently, Krueger et al.[17] have confirmed by MALDI-TOF-MS the very high structural heterogeneity of sorghum polyflavans, in terms of repeating monomeric units (flavan, flavan-3-ol and flavanone), pattern of hydroxylation, type of interflavan bonds (A- and B-types), and substitutions with moieties such as glucose. Such a complexity has never been reported in beer. No data allows predicting how these polyflavans could impact the beer colloidal instability.

The aim of the present work was to compare the structures of catechins and flavan-3-ol derived procyanidins in red and white sorghums. After specific acidified acetone-water extractions, NP- and RP-HPLC-ESI(-)-MS/MS al-

lowed us to quantify monomers and oligomers, while thiolysis gave us new data concerning the extension and terminal units in the latter ones. The antioxidant activity of these extracts was measured by the HPLC-online TEAC procedure recently proposed by Leitao et al.[18] and compared to previously published global antioxidant activities[4] applied on methanolic extracts.

MATERIALS AND METHODS

Sorghum samples

Seven red sorghum samples (I-VII) and two white sorghum samples (VIII-IX), all harvested in 2007, were bought on different markets in Burkina Faso. Sorghum I was grown in Léo, sorghum II in Komsilga, sorghum III in Kaya, sorghum IV and VII were cultivated in Koukoulgho and sorghum VIII in Koupéla. The origins of sorghum V, VI and IX are unknown. All samples were stored in the dark at 4°C under an inert atmosphere.

Chemicals

Acetone (99.9%), (+)-catechin (98%), (-)-epicatechin (98%), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{\bullet+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and potassium persulphate (di-potassium peroxdisulphate) were supplied by Sigma-Aldrich (Bornem, Belgium). Cyclohexane (99.5%), dichloromethane (99.8%), ethanol (99.8%), methanol (99.8%), and acetonitrile (99.9%) were from VWR (Leuven, Belgium). Acetic acid (100%) was obtained from Merck (Darmstadt, Germany). Formic acid (99%) was supplied by Acros Organics (Geel, Belgium). Toluene-α-thiol (99%) and ammonium acetate (99%) were from Fluka (Buchs, Switzerland). Hydrochloric acid was obtained from Fisher Scientific (Loughborough, UK). Procyanidin B2 (>90%) was supplied by Extrasynthèse (Lyon, France). The compounds 3,4-β-epicatechin benzylthioether, 3,4-α- and β-catechin benzylthioether and 3,4-α- and β-gallocatechin benzylthioether were synthesized as described by Callemien et al.[6] Aqueous solutions were made with Milli-Q (Millipore, USA) water.

Extraction of catechins and flavan-3-ol derived procyanidins

This method has been adapted from the one developed in our laboratory for the analysis of flavanoids in chocolate[7]. Sorghum ground samples (5 g) were delipidated three times with 50 mL of cyclohexane, each time for 10 min under gentle stirring. At the end of each step, the samples were centrifuged for 10 min at 2,500 × *g* and the supernatant was eliminated. After the last step, the powder was dried under vacuum to get rid of solvent residues. Delipidated sorghum powder (2 g) was extracted three times with 50 mL of acetone/water/acetic acid (70:28:2, v/v), each time for 1 h under shaking at room temperature. Previous data[7] have shown that of three different solvents (acetone, ethanol and methanol) mixed with water and acetic acid, the acetone/water/acetic acid mixture (70:28:2, v/v) is the best for extracting flavan-3-ol derived procyanidins. After each extraction, the suspension was centrifuged for 10 min at 2,500 × *g* and the supernatants were collected. The combined supernatants were concentrated by rotary evaporation to obtain ~50 mL of extract and afterwards residual particles were removed by filtration. In order to purify this extract, a 10 g C18 Sep-Pak cartridge (Waters, Millipore) was preconditioned with 200 mL of methanol and 300 mL of water. The extract was loaded on the cartridge and sugars were removed with 50 mL of water. Flavanoids were finally eluted with 50 mL of acetone/water/acetic acid (70:28:2, v/v). The eluates were concentrated by rotary evaporation and freeze-dried. A 20 mg aliquot of the obtained extract was dissolved in 2 mL of methanol and stored under nitrogen at –80°C in the dark until used.

NP-HPLC-ESI(-)-MS/MS analysis of catechins and flavan-3-ol derived procyanidins

A SpectraSystem equipped with an SCM degasser, an AS3000 autosampler, and a P4000 quaternary pump was used. The system was controlled with the Xcalibur software version 1.2 (Thermo Fisher, San Jose, CA, USA). Five microlitres of flavanoid extract was injected onto a 250 mm × 2.1 mm, 5 µm Alltima HP Silica 5u column (Grace, Deerfield, IL, USA) at a flow rate of 200 µL/min. Elution was carried out with a linear gradient from A (dichloromethane) to B (methanol) and a constant 4% level of C (acetic acid/water, 1:1, v/v). Gradient elution was as follows: from 82% A to 72% in 20 min, 72% to 61% in 30 min, 61% to 10 % in 5 min, isocratic for 5 min and return to the initial conditions for 15 min. Mass spectra were acquired using an LCQ mass spectrometer equipped with an ESI source (Thermo Fisher). The following ESI inlet conditions in negative mode were as follows: source voltage, 4.9 kV; capillary voltage, –4 V; capillary temperature, 200 °C; sheath gas, 39 psi. Collision-induced dissociation spectra were recorded at relative collision energies of 30, 35 and 40% respectively for singly charged $[M-H]^-$ ions of monomers, dimers and trimers to hexamers. A post-column addition of ammonium acetate (10 mM in methanol) at a flow rate of 0.05 mL/min was used to improve sensitivity.

The flavanoid extracts were also analyzed by RP-HPLC-ESI(-)-MS/MS using the method described here under for thiolysis measurements.

Thiolysis of flavan-3-ol derived procyanidins coupled to RP-HPLC-ESI(-)-MS/MS

This method is adapted from Guyot et al.[16] In a polypropylene vial, 40 µL of the catechin/procyanidin extract, 40 µL of methanol containing 3.3% HCl (v/v) and 80 µL of toluene-α-thiol (5% v/v in methanol) were mixed together. The vials were incubated for 30 min at 40°C. To ensure complete B-type procyanidin degradation, the reaction medium was further kept at room temperature for 10 h. In order to quantify the monomeric native structures of the extract, 80 µL of toluene-α-thiol (5% v/v in methanol) was replaced by 80 µL of methanol and treated as described above. Five microlitres of the sample was injected onto a reversed phase C18 Prevail column (150 × 2.1 mm, 2 µm) at a flow rate of 200 µL/min. A linear gradient from water with 1% acetonitrile and 0.1% formic acid (A) to acetonitrile (B) was applied. Gradient elution

was from 97% to 91% A in 5 min, 91% to 84% in 10 min, 84% to 50% in 30 min, 50% to 10% in 3 min, isocratic for 3 min and return to the initial conditions for 15 min. For the ESI source, the following inlet conditions were applied: source voltage, 4.9kV; capillary voltage, –4V; capillary temperature, 200 °C; and sheath gas, 40 psi. Collision-induced dissociation spectra were recorded at 30%. Identification was done by three methods: comparison of retention times, mass spectra obtained by ESI(-)-MS/MS and pseudo molecular ions by full scan ESI(-)-MS with those of commercial and isolated products. Calibration curves obtained for each standard (commercial or synthesized) were used for quantifying the terminal and extension units, using MS/MS on *m/z* 289 for (+)-catechin or (-)-epicatechin and *m/z* 305 for (-)-gallocatechin or (-)-epigallocatechin (the same ion had been selected for free and nucleophile-bounded flavan-3-ols). The mean degree of polymerization (mDP) was obtained with the following equation: mDP = (terminal units + extension units)/terminal units.

RP-HPLC-UV/vis-online TEAC

RP-HPLC-online TEAC was performed by using the method recently described by Leitao et al.[18], with slight modifications. Briefly, 10 mg ABTS was mixed with 2.6 ml of a potassium persulphate solution (2.5 mM in water) to obtain a final concentration of 7 mM $ABTS^{\bullet+}$. This $ABTS^{\bullet+}$ stock solution was kept in the dark at room temperature for 12–16 h to ensure the stabilization of the radical[23]. It was then diluted 200 times with ethanol to reach an absorbance of 0.77 ± 0.02 at 412 nm (working solution).

The HPLC system (Waters, Milford, MA, USA) consisted of a 1525 binary pump and a 2489 UV/vis detector set to 280 nm. Five microlitres of the samples was injected onto the C18 Prevail column here-above described for RP-HPLC-ESI(-)-MS/MS, at a flow rate of 200 µL/min. The linear gradient from water with 1% acetonitrile and 0,1% formic acid (A) to acetonitrile (B) was slowed down compared to the MS experiments, in order to improve resolution at the UV detectors : from 97% to 91% A in 5 min, 91% to 85% in 50 min, 85% to 70% in 25 min, 70% to 10% in 10 min, isocratic for 3 min and return to the initial conditions in 17 min. The data were analyzed using the Breeze 2 software (Waters). At the detector exit, the $ABTS^{\bullet+}$ working solution was added to a reaction coil (stainless steel, 8m x 0,25 mm) by a T-valve at a flow rate of 100 µL/min. A second 2487 UV/vis detector (Waters) set to 412 nm allowed for the measuring of the decolourization of the working solution when $ABTS^{\bullet+}$ was captured by a compound in the eluate. The negative peaks were recorded by a C-R8A integrator (Shimadzu, Columbia, MD, USA). The antioxidant activity of each compound was calculated as trolox equivalents (TE).

Statistical analysis

All analyses were carried out in duplicate. Mean values and standard deviations are reported. Statistical analysis was done using SAS software version 9.2, and significant differences in mean performance were tested by Tukey's test; $P < 0.05$ implies significance.

RESULTS AND DISCUSSION

Nine sorghum samples from Burkina Faso, previously investigated by our group for their stilbenoid and total polyphenol content, as well as their global antioxidant activity (methanolic extracts)[4], were here extracted to evidence potential differences in catechins and flavan-3-ol derived procyanidins between red and white samples.

Flavan-3-ol monomers to hexamers were analyzed by NP-HPLC-ESI(-)-MS/MS (Table I and Fig. 1a). In all red samples, monomers (P1), dimers (P2), trimers (P3), tetramers (P4), pentamers (P5) and hexamers (P6) were present, with trimers being the major compounds. In white sorghum, only monomers were detectable. Flavan-3-ol monomers and oligomers were determined in much higher amounts in the red samples ($\sum$(P1–P6) = 61.4–450.2 mg (-)epicatechin equivalents/kg) than in the white ones (0.2 mg EE/kg). However, these values are still 15 to 30 times lower than those detected in cocoa or chocolate samples[8]. As depicted by the absence of correlation between the $\sum$(P1–P6) levels and total polyphenol amounts (R^2 = 0.10), other polyphenolic fractions present in sorghum (3-deoxyanthocyanidins, flavan-4-ols, flavones, unconventional polyflavans and phenolic acids) could contribute much more to the antioxidant activity than flavan-3-ol derived procyanidins. The previously published global antioxidant activity data[4], obtained on methanolic extracts (ORAC and AAPH assays), was also found to correlate poorly with the flavan-3-ol (P1-P6) content (R^2 = 0.19 and 0.16 for the ORAC and AAPH assays). For instance, samples III and VII did not emerge as richer in P1 to P6 than samples I and VI, despite their higher global antioxidant activity (Table I). On the opposite, in cocoa liquors, Counet et al.[8] found a strong correlation (R^2 = 0.94) between AAPH values and P1 to P8 concentrations.

Individual monomers and dimers were further analyzed by RP-HPLC-ESI(-)-MS/MS (Table II). In red sorghum (Fig. 1b), the major monomer (62-98%) proved to be (+)catechin (6.2–84.7 mg/kg), while only 0.8-16.9 mg/kg (-)epicatechin was found. Neither (-)gallocatechin nor (-)epigallocatechin was detected in our sorghum samples. Much lower concentrations of flavan-3-ol monomers were found in white sorghum (Fig. 1c) with (-)epicatechin being the major isomer (0.2 mg/kg).

In red sorghum (Fig. 1b), the major flavan-3-ol dimer (60-82%; 57.8–144.5 mg/kg, B2 equivalents) was identified as procyanidin B1 [(-)-epicatechin-(4β-8)-(+)-catechin], according to its retention time and mass spectrum[5]. Procyanidin B2 [(-)-epicatechin-(4β-8)-(-)-epicatechin] was also identified by comparing its retention time and mass spectrum with that of a commercial standard. Its concentration ranged from 0.3 to 7.0 mg/kg, which accounted for <1–6% of total dimers. A third dimer (Bx; 17–39%; 22.3–55.6 mg/kg, B2 equivalents) was suspected to be the C_4-C_6-analogue of B1 (procyanidin B7), when its relative retention time was compared with that mentioned in the work of Poupard[22]. In white sorghum (Fig. 1c), only two dimers were detected. Procyanidin B2 was the major dimer (57–100%), although at lower concentration (0.2–0.8 mg/kg) than in most red sorghums. In sample VIII, another dimer (By; 0.6 mg/kg, B2 equivalents) was detected, and we assume that it could be the C_4-C_6-analogue

of B2 (procyanidin B5). Procyanidins B1 and Bx were absent in both white sorghums.

Thiolysis hyphenated to RP-HPLC-MS/MS-ESI(-) was used to determine the mean degree of polymerization (mDP) and the chemical composition of the terminal and extension units of B-type flavan-3-ol procyanidins. In red sorghum (Table III and Fig. 2a), (+)catechin proved to be the main unit at terminal positions (79–88%, 53.1–175.2 mg/kg), but a minor unit at extension positions (28–43%, 339.0–1,266.4 mg/kg). The (-)epicatechin was found to account for only 12–21% (10.8–46.4 mg/kg) of the terminal units, but for 50–66% (634.7–1,471.8 mg/kg) of the extension units. Gu et al.[13], who have analyzed brown sorghum bran, also determined the majority of terminal units being (+)catechin (88.5%) and the majority of extension units being (-)epicatechin (100%). We also found the presence of (-)gallocatechin in extension units (5–9%, 61.6–205.0 mg/kg), while (-)epigallocatechin was not present at all. An mDP of 10–20 was calculated for red sorghum samples. These thiolysis-depolymerized proanthocyanidins amounted to 1,145–3,165 mg/kg, which is much lower than the values for total polyphenols obtained previously[4] by the Folin-Ciocalteu assay (7,000–25,000 mg/kg). This confirmed the occurrence of unusual flavanoid oligomers and polymers in sorghum, without the hydroxyl group at the 3-position of the C-ring, and/or glycosylated on the 5-hydroxy group (not analyzed in the present work). Moreover, as for hops and beer[6], more complex flavan-3-ol derived structures not degraded by thiolysis should be present in sorghum.

White samples displayed a similar profile for their terminal and extension units (Fig. 2b), although at much lower concentrations and an mDP of 5–12.

In order to assess the relative contribution of flavan-3-ol monomers and dimers to the antioxidant activity of sorghum, one red (VI) and one white (VIII) of the catechins/procyanidins extracts were analyzed by RP-HPLC-UV/vis-online TEAC. As shown by the total antioxidant activity (total areas measured on the TEAC chromatograms, Table IV), red sorghum VI logically emerged as much more antioxidant than the white one.

In red sorghum, (+)catechin and (-)epicatechin peaks only accounted for 2.2% and 0.6% of the total antioxidant capacity, respectively (total antioxidant capacity = total area at 412 nm). The major dimer B1 contributed to 3.0%. Polyphenolic compounds eluting after 60 min (region where various flavanones, flavones, flavan-4-ols and hydroxycinnamic acids elute) were revealed to be responsible for the most part of the antioxidant activity in the acidified acetone/water extracts (absorption at 412 nm in Fig. 3a).

In white sorghum, compounds c (t_R = 80.8 min) and d (t_R = 84.9 min) accounted together for 60% of the total antioxidant capacity (Fig. 3b). No antioxidant activity was detected at the retention time of (+)catechin, (-)epicatechin, and procyanidins B1 and B2. Two well resolved peaks a and b (t_R = 26.3 min and 30.0 min) eluting between (+)catechin and (-)epicatechin brought another 24%. According to their retention times, they could be phenolic acids (standards of chlorogenic acid, vanillic acid and caffeic acid eluted at 27.4 min, 28.6 min and 30.9 min, respectively).

In conclusion, and contrary to what was observed for cocoa, the contribution of flavan-3-ol procyanidins (P1–P6) to the total antioxidant activity of sorghum is very weak. Complementary data are required to identify the polyphenolic fractions eluting after 60 min, which contribute to the high antioxidant activity of red sorghum.

ABBREVIATIONS USED

AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS$^{\bullet+}$, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid
EE, (-)epicatechin equivalents
ESI, electrospray ionization
GAE, gallic acid equivalents
HPLC, high performance liquid chromatography
MS/MS, tandem mass spectrometry
NP, normal phase
P1–P6, procyanidin monomers to hexamers
RP, reversed phase
TE, trolox equivalents
TEAC, trolox equivalent antioxidant capacity

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