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Potentiality of Red Sorghum for Producing Stilbenoid-Enriched Beers with High Antioxidant Activity

Meike Bröhan, Vesna Jerkovic, and Sonia Collin*

Unité de Brasserie et des Industries Alimentaires, Earth and Life Institute, ELIM, Faculté d'Ingénierie Biologique, Agronomique et Environnementale, Université catholique de Louvain, Croix du Sud, 2 bte 7, B-1348 Louvain-la-Neuve, Belgium

ABSTRACT: *trans*-Piceid and *trans*-resveratrol were authenticated for the first time by high-resoution mass spectrometry in red sorghum grains. A 0.4−1 mg/kg amount of *trans*-piceid and up to 0.2 mg/kg *trans*-resveratrol were quantified by reversed phase high-performance liquid chromatography−atmospheric pressure chemical ionization(+)-tandem mass spectrometry. The white sorghum samples contained only traces of *trans*-piceid (up to 0.1 mg/kg), and *trans*-resveratrol was absent. In much lower amounts than procyanidins, stilbenoids are not able to contribute significantly to the exceptional antioxidant activity of red sorghum (ORAC, 83−147 μmol TE/g; AAPH, 0.61−1.79 min/mg kg^{-1}). More than 10 mg/kg of total stilbenoids have been reported in some hop varieties. Yet, as hop is a minor wort ingredient as compared to cereals, red sorghum could be the main source of *trans*-piceid in beer. Hop remains, however, the single source of *cis*-piceid.

KEYWORDS: Sorghum, beer, resveratrol, piceid, stilbenoids, antioxidant activity

INTRODUCTION

In many African countries, sorghum is used for local beer production (e.g., Dolo in Burkina Faso, Pito in Ghana, and Kaffir in South Africa). Sorghum has long been recognized by brewers as efficiently limiting the cardboard off-flavor (*trans*-2-nonenal) in aged beers, by inhibiting lipid oxidation during mashing and boiling.[1−3] The fact that sorghum is gluten-free is another advantage explaining brewers' increasing interest in using sorghum, as it raises the possibility of producing beers for coeliacs.

As depicted in Table 1, sorghum contains a wide variety of polyphenols, some of which are rare or absent in other beer ingredients. When barley malt is used for mashing, around 30% of total beer polyphenols are issued from hop, although added in 100 times lesser quantity than malt.[4] In the case of sorghum-made beers, the cereal contribution to beer polyphenols could be much higher.

Sorghum phenolic acids include hydroxybenzoic (mainly protocatechuic and *p*-hydroxybenzoic acid) and hydroxycinnamic acids (mainly ferulic and *p*-coumaric acid),[5,6] both free and bound as esters. Most of them are found in usual lager beers, issued either from barley malt or from hop.[4]

Sorghum anthocyani(di)ns are unique, as they lack the hydroxyl group at the 3-position of the C ring. These 3-deoxyanthocyani(di)ns such as luteolinidin and apigeninidin are used as natural food colorings, because they are more stable than anthocyanidins in both organic solvents and acidic solutions.[7]

Flavan-4-ols such as apiforol (leucoapigeninidin) and luteoforol (leucoluteolinidin) are other interesting sorghum polyphenols, as precursors of sorghum 3-deoxyanthocyani(di)ns.[1,8,9] Never reported in beer, they have been found at concentrations up to 4200 mg/kg in sorghum.[1]

Other sorghum flavonoids include the flavones apigenin and luteolin,[9] the flavanones naringenin [7,10] and eriodictyol,[9,10] the flavonol kaempferol,[11] the dihydroflavonol taxifolin,[12] and the flavan-3-ols (+)-catechin and (−)-epicatechin.[13] Hop brings similar flavonols and flavan-3-ols to wort, but its flavanones are unique due to prenyl and geranyl substituents (e.g., isoxanthohumol and hopein).[4] Barley malt also contains (+)-catechin but no epicatechin at all.

Dicko et al.[1] have reported big differences in proanthocyanidin concentrations between red and white sorghum grains. Red sorghums showed an average of 9400 mg/kg, against 1300 mg/kg for white samples. In most samples, proanthocyanidins decreased after germination. The proanthocyanidin levels were revealed to be positively correlated to the total phenolic contents, especially in ungerminated grains. The ratio of oligomers (until DP 10) to total proanthocyanidins proved to vary considerably according to the variety.[13] Different B type catechin/epicatechin homopolymers have been described in sorghum,[14] while procyanidin B1 is the most common one.[15] Brandon et al.[16] have also found evidence of heteropolymers with both catechin/epicatechin and gallocatechin/epigallocatechin hydroxylation patterns. In addition, Gujer et al.[12] 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 matrix-assisted laser desorption/ionization time-of-flight mass spectrometry 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 allow predicting how these polyflavans could impact the beer colloidal instability.

Beside major polyphenols, usually, lager beers also contain traces of stilbenoids.[18] Even in low concentrations, resveratrol and its glucoside, piceid, seem to exhibit interesting biological effects including antiplatelet, anti-inflammatory, estrogenic, cardioprotective,

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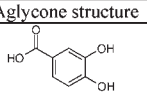
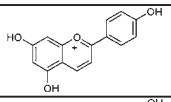
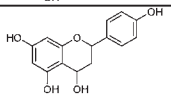
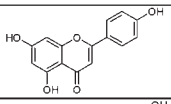
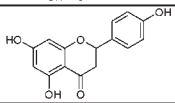
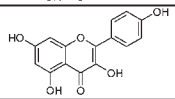
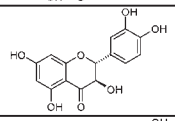
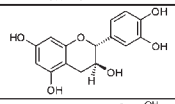
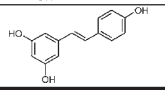
Table 1. Phenolic Compounds Found in Sorghum Grains

Phenolic class	Identified compounds	Concentration	*Aglycone structure
phenolic acids	protocatechuic acid*[a], *p*-hydroxybenzoic acid[b], vanillic acid[c], p-coumaric acid[d] [5,6], ferulic acid[e], gallic acid[f], caffeic acid[g], cinnamic acid[h] [6]	total free phenolic acids: 117-636 mg/kg [5], [a]7-141 mg/kg [5,6], [b]15-34 mg/kg, [c]8-51 mg/kg, [d]86-232 mg/kg, [e]105-343 mg/kg, [f]20-46 mg/kg, [g]26-52 mg/kg, [h]5-20 mg/kg [6]	
3-deoxyanthocyani(di)ns	apigeninidin*[i], luteolinidin[j] [7], 7-methoxyapigeninidin[k], 5-methoxyluteolinidin[l] [10]	[i]300-1000 mg/kg, [j]trace-1500 mg/kg [7], [k]0.4-137.4 mg/kg, [l]0.3-153.5 mg/kg [10]	
flavan-4-ols	apiforol*, luteoforol [9]	total flavan-4-ols: 1700-4200 mg/kg [1]	
flavones	apigenin*[m], luteolin[n] [9,10]	[m]2.8-203.7 mg/kg, [n]2.6-182.2 mg/kg [10]	
flavanones	naringenin*[o] [7,10], eriodictyol[p] [10], eriodictyol 5-glucoside [9]	[o]5.6-48.4 mg/kg, [p]5.6-12.9 mg/kg [10]	
flavonols	kaempferol 3-rutinoside-7-glucuronide* [11]		
dihydroflavonols	taxifolin*, taxifolin 7-glucoside [12]		
flavan-3-ols	catechin*[q], epicatechin[r], procyanidins[s] [13]	[q,r]10-180 mg/kg, [s]1300 mg/kg in white sorghum [1], [s]9400 mg/kg in red sorghum [1], [s]21000-22000 mg/kg in brown sorghum [13]	
stilbenes	*trans*-resveratrol*, *trans*-piceid* [25]	total stilbenes: <1 mg/kg [25]	

antitumor, and antiviral properties.[19−21] Concentrations of *trans*-piceid, *cis*-piceid, and *trans*-resveratrol very close to those reported in grapes have been described in some American low-bitter hop varieties at levels above 10 mg/kg.[22]

Recently, Yu et al.[23,24] have isolated a stilbene synthase gene from sorghum, suggesting the capability of sorghum to produce stilbenoid metabolites as phytoalexins. In sorghum seedlings infected with *Colletotrichum sublineolum*, they found *trans*-piceid as the major stilbene metabolite and an unknown derivative (m/z 475 in negative mode).

In 2007, by using reversed phase high-performance liquid chromatography−atmospheric pressure chemical ionization (+)−tandem mass spectrometry (RP-HPLC-APCI(+)-MS/MS), our group did not find any stilbenoids in barley malt and adjuncts like rice and corn but detected *trans*-resveratrol and *trans*-piceid in a sample of red sorghum grains.[25]

The first aim of the present work was therefore to confirm the presence of stilbenoids in sorghum grains by high-resolution mass spectrometry (HRMS)/MS. *trans*-Resveratrol and *trans*-piceid were further quantified by RP-HPLC-APCI(+)-MS/MS in various red and white sorghums. For all samples, stilbenoid amounts were related to both the antioxidant activity and the total polyphenolic level.

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 was grown in Komsilga, sorghum III was grown in Kaya, sorghums IV and VII were cultivated in Koukoulgho, and sorghum VIII was grown in Koupéla. The origins of sorghums V, VI, and IX are unknown. All samples were stored in the dark at 4 °C under an inert atmosphere.

Determination of Total Polyphenols and Interferences. The optimized Folin−Ciocalteu method described by Georgé et al.[26] was used. Sorghum ground samples (0.5 g) were mixed with 50 mL of acetone/water (70:30, v/v) during 30 min. The supernatants were recovered by filtration and constituted the raw extracts (RE). Solid-phase extraction cartridges (Oasis HLB, 6 cm^3, 200 mg) from Waters (Milford, MA) were conditioned with 4 mL of methanol and 2 × 4 mL of water and then loaded with 2 mL of diluted REs (1:10). Interfering water-soluble compounds (sugars and ascorbic acid) were eluted with 2 × 2 mL of water, and the recovered volume of the washing extract (WE) was measured. Water-diluted (1:10) Folin−Ciocalteu reagent (2.5 mL) was added to 0.5 mL of diluted RE (1:10), 0.5 mL of WE, 0.5 mL of water (blank), or 0.5 mL of calibration solution (gallic acid). The mixture was incubated for 2 min at room temperature before adding 2 mL of sodium carbonate solution (75 g/L). Afterward, an incubation of the mixture for 15 min at 50 °C followed, whereas the Folin−Ciocalteu reagent was reduced by phenols to blue oxides of tungstene and molybdene. After the mixture was cooled in a water−ice bath, the absorbance at 760 nm was immediately measured. The results were expressed as gallic acid equivalents (GAE), and the total polyphenol contents were determined by subtracting the concentrations of WE from the ones of RE.

Antioxidant Activity Measurements—2,2′-Azobis(2-amidinopropane) Dihydrochloride (AAPH) and Oxygen Radical Absorbance Capacity (ORAC) Assays. Ground sorghum

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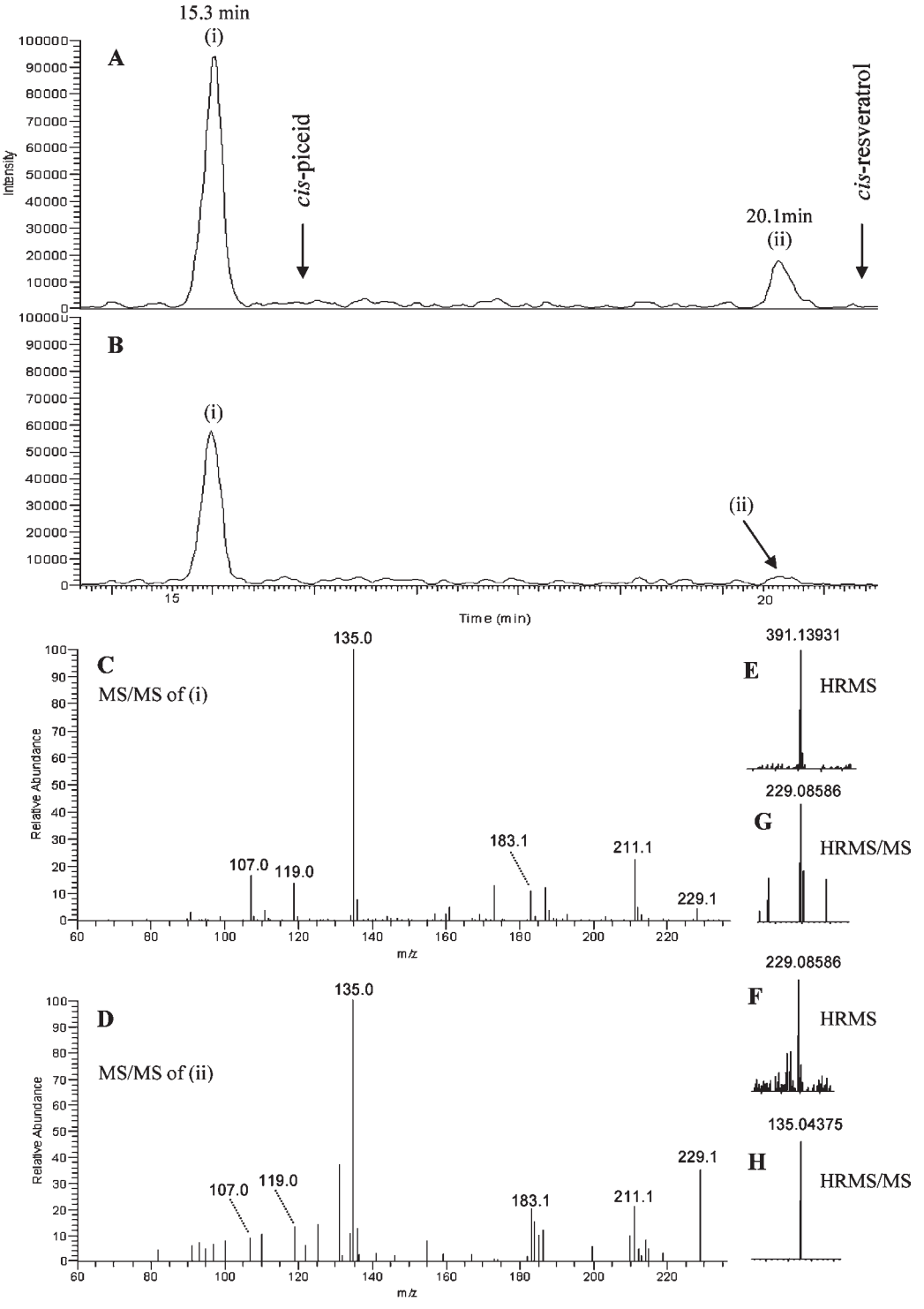

Figure 1. (A and B) RP-HPLC-APCI(+)-MS/MS (*m*/*z* 229) chromatogram of the red sorghum VI extract spiked or not with *trans*-piceid (i) and *trans*-resveratrol (ii). (C and D) MS/MS spectrum with *m*/*z* 229 on peaks i and ii. (E and F) Major ion in the HRMS spectrum on peaks i and ii. (G and H) HRMS/MS spectrum with *m*/*z* 391 on peak i and *m*/*z* 229 on peak ii.

samples (1 g) were extracted with methanol as described by Liégeois et al.[27] In the AAPH assay developed in our laboratory,[27] the oxidation of linoleic acid is induced by AAPH in an aqueous dispersion in the absence or presence of antioxidant. The rate of oxidation at 37 °C was monitored by recording the increase of absorption at 234 nm caused by the formation of conjugated diene hydroperoxides. A Shimadzu UV−visible 240 spectrophotometer (Antwerp, Belgium) equipped with an automatic sample positioner allowed the analysis of six samples. In all cases, the measurements were run in duplicate against the buffer and compared with a separate AAPH-free control to check for any spontaneous oxidation. The inhibition time (T_{inh}) was estimated with Microsoft Excel software as the point of intersection between the tangents to the

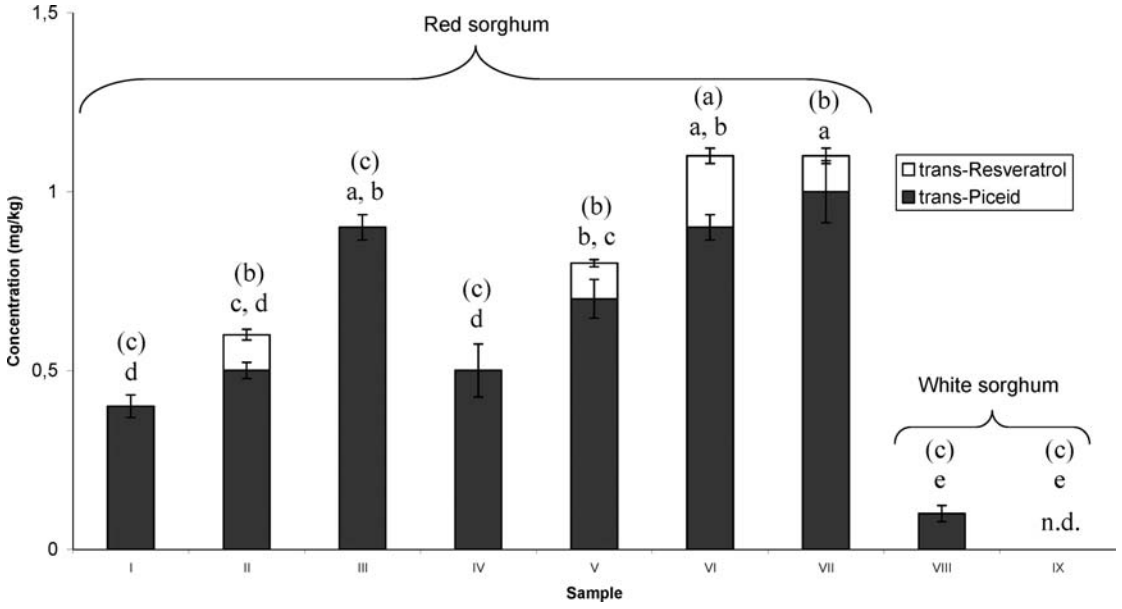

Figure 2. Concentrations and standard deviations of stilbenes in sorghum samples. Values for *trans*-resveratrol in parentheses or for *trans*-piceid that do not share a common letter are significantly different (Tukey's test, $p < 0.05$).

inhibition and propagation phase curves, under precise oxidation conditions.

The methanol extracts were also submitted to the ORAC assay, which was adapted from the procedure described by Huang et al.[28] A 96-well microplate fluorometer (Ascent F.L. Fluorocsan, Labsystem, Finland) was used, and fluorescent filters of 485 nm (excitation wavelength) and of 520 nm (emission wavelength) were applied. The plate reader was controlled by Ascent Software 2.6. All samples were analyzed in duplicate at three different dilutions in phosphate buffer (pH 7.4), and Trolox was used as a calibration standard. Twenty-five microliters of blank (phosphate buffer), diluted sample, or Trolox standard was mixed with 250 μL of fluorescein (55 nM). The microplate was incubated for 10 min at 37 °C before automatic injection of 25 μL of an AAPH solution (153 mM). The fluorescence intensity was measured each minute for 50 min. The final ORAC values were calculated by using the net area under the decay curves and were expressed as Trolox equivalents (TE).

Extraction of Stilbenoids. The method developed in our laboratory for the analysis of stilbenoids in hop[29] has been adapted to analyze stilbenoids in sorghum. All extraction steps have been done in duplicate, with protection against day light. Sorghum ground samples (5 g) were delipidated with cyclohexane and extracted with a mixture of ethanol/water (80:20, v/v) at 60 °C as described by Callemien et al.[29] For the standard addition method, 5 g of milled sorghum was spiked with increasing amounts of *trans*-resveratrol and *trans*-piceid (0.25, 0.5, 0.75, and 1 mg/kg) before delipidation.

RP-HPLC-APCI(+)-HRMS and -HRMS/MS for Stilbenoid Identifications. An Accela system (Thermo Fisher, San Jose, CA) controlled with the Xcalibur software version 2.0.7. was used. Ten microliters of stilbenoid extract was injected onto a 150 mm × 2.1 mm, 2 μm C18 Prevail column (Grace, Deerfield, IL) and eluted with a linear gradient using two solvents: A (water/acetonitrile/formic acid, 98.9:1:0.1, v/v) and B (acetonitrile). Gradient elution was as follows: from 95 to 55% A in 23 min, 55 to 0% in 7 min, and isocratic for 10 min and return to the initial conditions for 23 min at a flow rate of 200 μL/min. HRMS analysis was carried out using an LTQ-Orbitrap-XL (Thermo Fisher) mass spectrometer equipped with an APCI source. Applied were the following APCI inlet conditions in positive mode: vaporization temperature, 470 °C; capillary voltage, 3 V; capillary temperature, 175 °C; sheath gas, 40 psi; auxiliary gas, 7 psi; and discharge current, 5 μA. Collision-induced dissociation spectra were recorded at 37% relative collision energy.

RP-HPLC-APCI(+)-MS/MS for Stilbenoid Quantifications. RP-HPLC was performed on a SpectraSystem equipped with an SCM degasser, an AS3000 autosampler, and a P4000 quaternary pump, as described here above. The system was controlled with the Xcalibur software version 1.2 (Thermo Fisher). Mass spectra were acquired using a LCQ mass spectrometer equipped with an APCI source (ThermoFisher). The same APCI inlet conditions were applied as described here above. After the first monitoring on the m/z 229, collision-induced dissociation spectra were recorded at 37% relative collision energy.

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

Stilbenoids were extracted from nine sorghum samples (I−VII red sorghum and VIII−IX white sorghum) from Burkina Faso (harvest 2007) with a mixture of ethanol/water (80:20, v/v) at 60 °C. RP-HPLC-APCI(+)-HRMS applied to extracts VI and VII allowed us to authenticate *trans*-piceid and *trans*-resveratrol, with the elemental formulas of, respectively, $C_{20}H_{23}O_8$ (experimental m/z: 391.13931; theoretical m/z: 391.13874; δ = 0.566 ppm, well within the variation range of the apparatus) and $C_{14}H_{13}O_3$ (experimental m/z: 229.08586; theoretical m/z: 229.08592; δ = 0.061 ppm) (Figure 1E,F for HRMS and G,H for HRMS/MS).

Neither *cis*-piceid nor *cis*-resveratrol has been detected in sorghum (Figure 1A,B, retention times = 16.6 and 21.1 min, respectively). This is the case for cocoa liquor also.[30] On the opposite, grape and hop contain significant amounts of *cis*-piceid. As recently shown by Jerkovic and Collin,[31] *cis*-resveratrol is absent in fresh hop cones or pellets but can be released from *cis*-piceid during storage.

RP-HPLC-APCI(+)-MS/MS (m/z 229) was used on all nine sorghum samples for quantification. As depicted in Figure 1C,

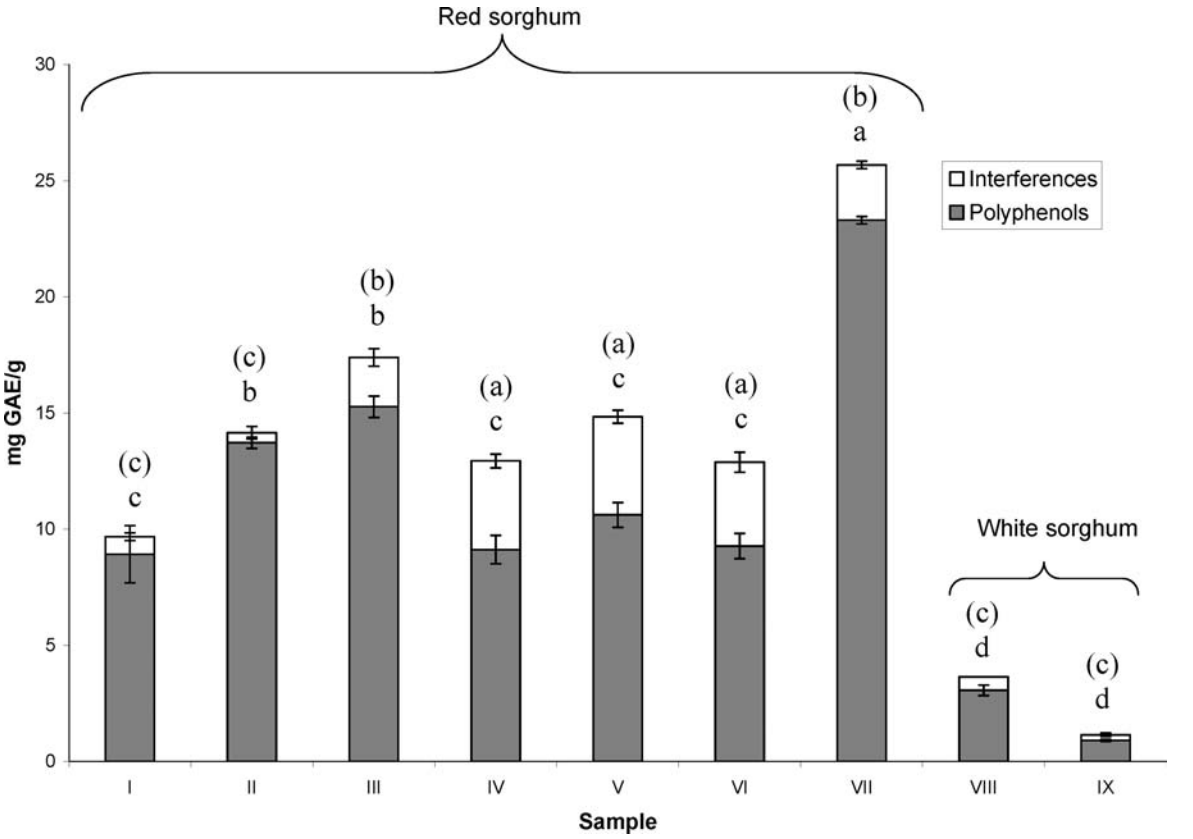

Figure 3. Concentrations and standard deviations of total polyphenols and interferences in sorghum samples. Values for interferences in parentheses or for total polyphenols that do not share a common letter are significantly different (Tukey's test, $p < 0.05$).

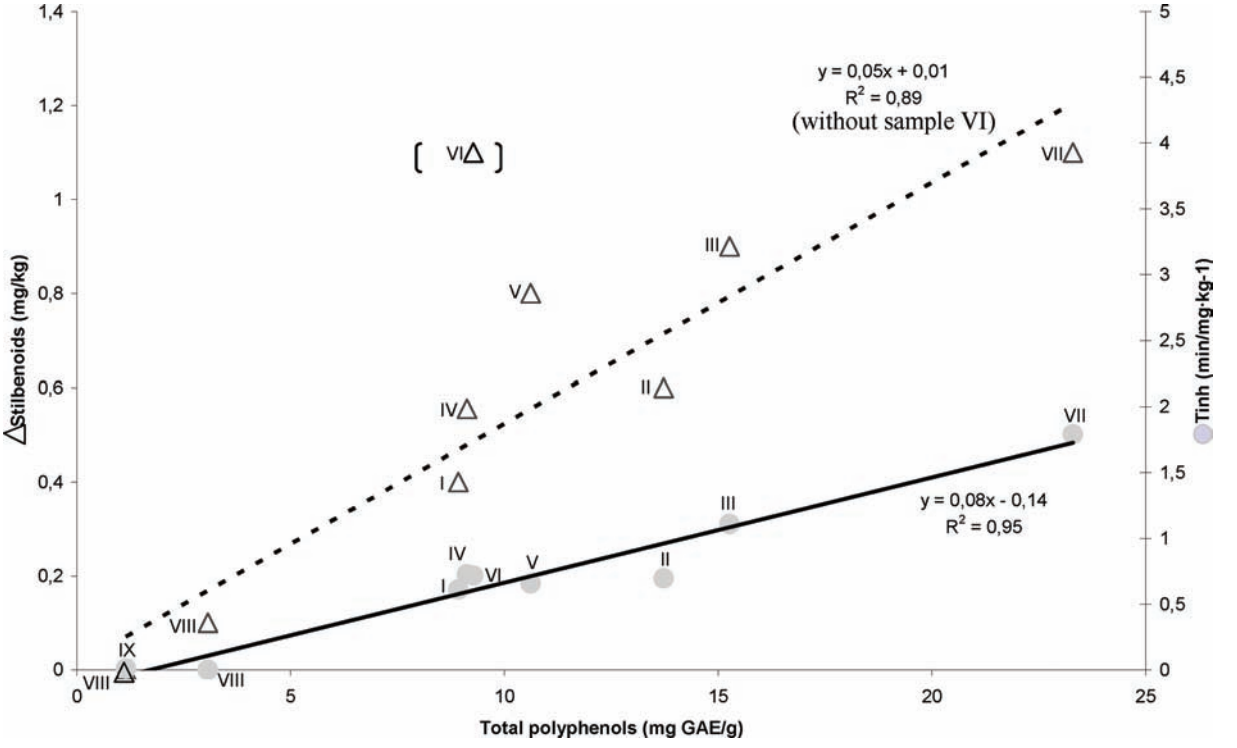

Figure 4. Correlation between stilbenoids or antioxidant activity (AAPH assay, T_{inh}) and total polyphenol contents.

Table 2. Antioxidant Activity Determined by the AAPH (T_{inh}) and ORAC Assays[a]

sorghum	T_{inh} (min/mg kg^{-1})	ORAC (μmol TE/g)
I	0.61	83
II	0.70	94
III	1.11	147
IV	0.73	85
V	0.66	101
VI	0.72	93
VII	1.79	112
VIII	0.00	13
IX	0.01	12

[a] Experiments in duplicates. Variation coefficients under 6%.

fragmentation of the glucoside was characterized by loss of the sugar, leading to the *trans*-resveratrol M + 1 ion and its usual fragments (Figure 1D). Therefore, both *trans*-stilbenes were quantified by selecting m/z 135. In all cases, *trans*-piceid emerged as the major stilbenoid (up to 1 mg/kg), free *trans*-resveratrol being found only in trace amounts (Figure 1B and Figure 2). These values are much lower than those previously reported in hop.[21,32] Yet, as hop is a minor wort ingredient as compared to cereals (added in 100 times lesser quantity), red sorghum could be the main source of *trans*-piceid in beer. Hop remains, however, the single source of *cis*-piceid.

Both white sorghums strongly differed from the seven red samples by much lower concentrations (up to 0.1 mg/kg *trans*-piceid). The total polyphenol level was determined by the optimized Folin−Ciocalteu method after an acetone/water (70:30, v/v) extraction.[26] The red samples proved to contain between 9 and 23 mg GAE/g polyphenols, as opposed to 1−3 mg GAE/g in the white sorghum samples (Figure 3). Dicko et al.,[1] who have screened 50 sorghum varieties, found values in the same range (6.0−30.1 mg GAE/g total polyphenols for the red grains and 4.5−13.8 mg GAE/g for the white ones). Sample VII was revealed to exhibit the highest values for both *trans*-piceid and total polyphenols, while sample IX showed the lowest concentrations. Nevertheless, the total stilbenoid level was found to correlate poorly (Figure 4) with the total polyphenol level, mainly due to sample VI (R^2 = 0.65; R^2 = 0.89 without sample VI).

The antioxidant activity was measured on a methanolic extract, by both the ORAC and the AAPH assays (Table 2). Samples VII and III exhibited the highest antioxidant activities (AAPH assay: 1.79 and 1.11 min/mg kg^{-1}; ORAC method: 112 and 147 μmol TE/g). In much lower amounts than flavanoids (especially proanthocyanidins, see Table 1), stilbenoids are not able to contribute significantly to the exceptional antioxidant activity of sorghum. On the other hand, the total polyphenol level logically correlates very well with the AAPH values (R^2 = 0.95).

As already mentioned, hop also displays a very high intrinsic antioxidant activity. The Saaz variety, usually recognized as the best one for flavor, shows total polyphenol contents up to 38 mg GAE/g and AAPH values 2−3 times higher (4.73 min/mg kg^{-1})[33] than the best here-investigated red sorghum samples.

In conclusion, as compared to hop, red sorghum is an interesting source of stilbenoids and antioxidant activity for brewers, taking into account the relative ratio of both ingredients in wort. Hop will be, however, the only source of *cis*-piceid. Complementary experiments are required to determine how malting will impact the stilbenoid profile of red sorghum.

AUTHOR INFORMATION

Corresponding Author

*Tel: +32 10 47 29 13. E-mail: sonia.collin@uclouvain.be.

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ABBREVIATIONS USED

RP, reversed phase; HRMS, high-resolution mass spectrometry; APCI, atmospheric pressure chemical ionization; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; GAE, gallic acid equivalents; TE, Trolox equivalents.

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