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Hibiscus sabdariffa (Roselle) Extracts and Wine: Phytochemical Profile, Physicochemical Properties and Carbohydrase Inhibition

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ABSTRACT

Three varieties of Hibiscus sabdariffa were analyzed for their phytochemical content and inhibitory potential on carbohydrate-digesting enzymes as a basis for selecting a variety for wine production. The dark red variety was chosen as it was highest in phenolic content and partially inhibited α–glucosidase (maltase), with delphinidin 3-O-sambubioside, cyanidin 3-O-sambubioside and 3-O-caffeoylquinic acid accounting for 65 % of this activity. None of the varieties significantly inhibited α-amylase. Regarding Hibiscus sabdarifa wine, the effect of fermentation temperature (20 and 30 ˚C) on the physicochemical, phytochemical and aroma composition was monitored over 40 days. The main change in phytochemical composition observed was the hydrolysis of 3-O-caffeolquinic acid and the concomitant increase of caffeic acid irrespective of fermentation temperature. Wine fermented at 20 ˚C was slightly more active for α–glucosidase inhibition with more fruity aromas (ethyl octanoate), but there were more flowery notes (2-phenylethanol) at 30 ˚C.

Keywords: Hibiscus sabdariffa, roselle wine, phytochemicals, carbohydrases, fermentation, aroma compounds

Introduction.

Hibiscus sabdariffa (H. sabdariffa) is an underutilized and rich source of bioactive compounds with potential pharmacological activities.[1] Some of the phytochemicals reported to be present in H. sabdariffa include organic acids, phenolic acids, anthocyanins and flavonoids such as quercetin, luteolin and their respective glycosides.[2-4] In traditional medicine, extracts of H. sabdariffa have been used in various countries to treat a wide variety of diseases.[5-7] Some scientific reports give credence to its use in folk medicine, in the treatment of health conditions like hypertension, hyperlipidemia and recently type 2 diabetes.[1, 8-10] With respect to the management of type 2 diabetes, the inhibition of α-amylase and α-glucosidases by small molecules with acarbose-like activity is considered an alternative strategy, however, only a few studies have reported this activity in extracts of H. sabdariffa.[11-13] Although hibiscus acid and its 6-methyl ester have been identified as active principles for the inhibition of α-amylase, no compound in H. sabdariffa has been linked to the inhibition of α-glucosidases.[12] This warrants further investigation. Based on the reported health benefit of H. sabdariffa and consumer interest in foods that may reduce the risk of diabetes, functional beverages like tea, soft drinks have been developed from H. sabdariffa calyces and some are currently being marketed in the US and Europe.[14,15] In order to diversify the application of the functional properties of this crop and to improve economies in developing countries, its use in the production of wine should be exploited.

Regarding wine, the manufacture of the product from other plant sources entails the same processing steps applicable to grape wine, resulting in significant changes in phenolic compositional data and subsequent bioactivity. In a previous study, wines processed from blueberry and blackberry blends showed inhibition of α-glucosidase activity and this was attributed to the anthocyanin content, which increased after fermentation.[16] While wine has been produced from H. sabdariffa (roselle wine), the concomitant changes in the

phytochemical and physicochemical properties during its processing to the best of our knowledge has not been studied.[17] In addition, although the main aroma compounds present in H. sabdariffa drinks have been identified, the fate of these compounds during roselle wine manufacture is unknown.[18,19]

One of the main factors influencing wine quality is the fermentation temperature.[20,21] Concerning wine aroma, the fermentation process (temperature-dependent) yields hundreds of volatile compounds in concentrations ranging from ng/L to mg/L.[22] These include the esters, alcohols, acids, and terpenes, all contributing to the aroma and flavor attributes of wine.[23] In addition, native phenolic substances during fermentation are transformed into secondary metabolites that might possess a different biological activity from the parent compound.[24] Since fermentation conditions are critical factors influencing wine quality, a study on the impact of this processing step on the phytochemical profile, aroma composition and subsequent bioactivity on starch digestion during roselle wine manufacture is needed to improve the wine processing and to establish quality parameters needed for the development and commercialization of a functional product. Hence, the objectives of this research were two-fold: (1) to analyze the physicochemical properties and phytochemical profiles of three different varieties of H. sabdariffa and their inhibitory potential on carbohydrate-digesting enzymes with the aim of selecting the ideal variety for wine; (2) to study the impact of fermentation on the phytochemical composition and aroma profile of roselle wines alongside its effect on the resultant bioactivity.

Materials and Methods.

Chemicals.

Commercial standards of delphinidin 3-O-sambubioside (DS), cyanidin 3-O-sambubioside (CS), delphinidin 3-O-glucoside, rutin, quercetin, protocatechuic acid, 3-O-caffeoylquinic acid (3-CQA) (Extrasynthase 49915 and a gift from Mike Clifford), catechin and quercetin 3-

O-glucoside (all HPLC grade) were purchased from Extrasynthase, Genay, France, while trifluoroacetic acid, gallic acid, caffeic acid, caftaric acid, Folin-Ciocalteu's reagent, 3,5-dinitrosalicylic acid, potassium sodium tartrate, chromatographically purified human salivary α-amylase type IX-, intestinal acetone powder from rat, glucose assay reagent, sodium mono and dibasic phosphate, maltose, amylose and amylopectin were from Sigma-Aldrich. Co., Ltd., Dorset, UK. All other standards used in the study were either analytical grade, HPLC or GC grade. The Oasis MAX cartridge 3 mL (60 mg) used for Solid Phase Extraction (SPE) was from Waters Corporation Ltd., Milford, Mass, Massachusetts.

Planting and Harvesting.

Three varieties of H. sabdariffa seeds (dark red, light red and white variety) were planted in mid-July, 2013, in the nursery and transplanted as seedlings to the field in August, 2013, at the National Horticultural Research Institute (NIHORT), Ibadan, Nigeria. Harvesting was done in mid-December, 2013, after which the calyces were allowed to dry in a storage chamber with temperatures between 28-30 °C. Samples were then vacuum-packed, transported to the UK and stored in a freezer at -20 °C. The varieties were identified based on the color properties as described in a previously published paper.[25]

Sample Preparation of H. sabdariffa Varieties.

H. sabdariffa extracts for phytochemical analysis and inhibition of starch digestion were prepared by weighing out 2 g of each variety and grinding them into a powder with the use of a pestle and mortar. Extraction was done using 100 mL of distilled water. The sample was immediately transferred to a water bath (GLS Aqua 12 plus) at 50 °C and extraction was carried out for 30 min with stirring intermittently. The sample was then centrifuged (2500 g; 10 min), filtered through a Whatman no.1 filter paper and used for the analysis. The extraction was repeated in triplicate.

Roselle Wine Production from Dark Red Variety

92 H. sabdariffa calyces were sorted, cleaned to remove extraneous material and washed under
93 cold running tap water. The extraction of roselle juice was done using distilled water at 30 °C
94 for 1 h and the ratio of calyces to water was 1:35 (w/v). The juice was then pasteurized
95 (50 °C for 30 min), followed by addition of sodium metabisulphite (60 mg/L). Juice
96 amelioration was performed by addition of brewing sugar (glucose) to raise the soluble solids
97 of the must to 20˚ Brix while yeast nutrient (1 g/L) from Young Home Brew, Bilston, UK
98 were introduced into the must according to the manufacturer's instruction.
99 The fermentation of roselle juice was carried out in 20 L fermentation vats with glass
100 stoppers filled with sodium metabisulphite (100 mg/L) and placed inside incubators (Sanyo-
101 MIR -153, Japan) programmed at 20 and 30 ˚C. Furthermore, the fermentation was allowed
102 to proceed in contact with the calyces (similar to skin contact in grape wine). Dried wine
103 yeast Saccharomyces cerevisiae (1 g/L) from Abbey Brew, Leeds, UK, re-activated by
104 addition of distilled water (50 mL) at 40 ˚C for 30 min, was added to the vats to initiate the
105 primary fermentation process which was considered finished (cessation of bubbles) on day 8.
106 Wines were then racked (removal of yeast lees and calyces), transferred into secondary
107 fermentation vats and kept at the stated temperature conditions until day 40. Samples (50 mL)
108 were taken after stirring the must on the days assigned for the different analysis. The
109 fermentation at each temperature condition was performed in duplicate and independent
110 sampling and analysis from each fermenting vat was conducted in triplicate (n=6).
111 **Physicochemical Analysis of H. sabdariffa Varieties and Roselle Wine.**
112 The pH in H. sabdariffa extracts and roselle wine were measured with the use of a pH meter
113 (HANNA HI2211) calibrated with pH 4.0 and 7.0 buffers, while titratable acidity (TA) was
114 determined by titration with 0.1M NaOH until pH 8.1 and expressed as % malic acid (g/L).
115 The color measurement L* (lightness), a* (redness), b* (blue to yellow), c* (chroma) and h*
116 (hue angle) was performed using a colorimeter (Lovibond RT 100, Tintometer Series II, UK)

117 calibrated with the instrument's standard white tile with XYZ color scale values as (X = 81.5,
118 Y = 85.8 and Z = 88.0). Samples (5 mL) were put in a Petri dish placed on a white tile and
119 the parameters recorded in total transmittance mode, illuminant D65, and 10° observer angle.
120 In addition to the rapid color measurement, the following spectrophotometric wine color
121 parameters including: color density (CD), hue tint (HT), polymeric anthocyanin (PA) were
122 evaluated according to the method of [26] using a spectrophotometer (CECIL CE 3021 Series).
123 An aliquot of sample (H. sabdariffa extracts or wine) (200 µL) was placed in a 1 mm
124 pathlength cuvette and the color indices were determined.
125 **HPLC Analysis.**
126 HPLC identification and quantitation of phenolics in H. sabdariffa extracts and roselle wine
127 was carried out using a $UFLC_{XR}$ system (Shimadzu) consisting of a binary pump, a
128 photodiode array with multiple wavelength (SPD-20A), a Solvent Delivery Module (LC-
129 20AD) coupled with an online unit degasser (DGU-20A3/A5) and a thermostat
130 autosampler/injector unit (SIL-20A). The photodiode array detector was set to measure at
131 wavelengths of 265, 280, 320, 360 and 520 nm. A two phase gradient system consisting of
132 0.1 % (v/v) trifluoroacetic acid mobile phase (A) and trifluoroacetic acid/acetonitrile/water
133 (50:49.9:0.1) mobile phase (B) was employed for the analysis. The gradient conditions were
134 as follows: the initial condition started with 92 % A and was increased to 18 % solvent B at
135 3.50 min, 32 % B at 18 min, 60 % B at 28 min, reaching 100 % B at 32 min, held at 100 % B
136 for 4 min, and returning to the initial conditions for 3.5 min for the next analysis. The
137 chromatographic separation was performed on a Phenomenex Gemini C_{18} column (5 µm, 250
138 mm x 4.6 mm) at a flow rate of 1 mL/min. The temperature of the column was maintained at
139 35 °C and the injection volume was 10 µL. Identification of phenolic compounds in H.
140 sabdariffa extracts was done based on comparison with standard phenolic compounds run
141 under similar conditions in terms of the retention time, UV-visible spectrum, spiking of the

sample with the corresponding standard phenolic compound and LC-MS data. The concentration of compounds for which there was no pure reference available as well as unidentified compounds was approximated by using the same calibration graphs as one of the compounds with the most similar and relevant chemical structure. Gallic acid, 5-O-caffeolquinic acid (5-CQA) (Sigma C3878), caffeic acid, quercetin and catechin were used for quantitation of hydroxybenzoic acids, chlorogenic acid isomers, hydroxycinammic acids, flavanols and flavan-3-ols respectively.

LC-MS Analysis.

LC-MS was used to confirm the identity of polyphenols in H. sabdariffa extracts. HPLC (LC-2010 HT) coupled with a 2020 quadrupole mass spectrometer (Shimadzu) fitted with an electro spray ionization source (ESI-MS) operated in single ion monitoring (SIM) was used in positive mode for anthocyanins and negative mode for other polyphenols. The other operating parameters were detector -1.80 kV, DL temperature 250 °C and nebulizing gas flow and drying gas flow set as 1.50 and 15 L/min respectively. The mobile phase A was 0.5 % formic acid in water and mobile phase B was a mixture of acetonitrile/ water/formic acid (50:49.5:0.5). The flow rate was 0.5 mL/min and the total time of analysis was increased to 60 min to make adjustments for the change in flow rate. All other conditions were identical to the HPLC analysis described above.

Total Phenolics in H. sabdariffa Varieties and Roselle wine.

The total phenolic content was determined using Folin–Ciocalteau's method with some modifications.[27] The assay contained 1 mL (H. sabdariffa extracts or roselle wine) diluted with 80 % methanol solution (1:10), 5 mL of diluted Folin-Ciocalteu's phenol reagent (1:10) and 4 mL of 75 g/L sodium carbonate solution. The mixture was then kept in a water bath at 26 °C and the absorbance reading measured at 765 nm with a spectrophotometer after 2 h. The estimation of phenolic content was performed using gallic acid as standard.

HPLC-ELSD determination of sugars in H. sabdariffa Varieties and Roselle Wine.

The UFLC$_{XR}$ system (Shimadzu) indicated above for H. sabdariffa polyphenol quantitation was attached to an evaporative light scattering detector (ELSD) and used for sugar identification and quantitation. The analysis was performed under isocratic conditions with an analysis run time of 30 min using a Grace Davison Prevail Carbohydrate Es column (5 µm, 250 mm x 4.6 mm). The mobile phase was 75 % acetonitrile (v/v) delivered at a flow rate of 0.5 mL/min and the sample volume was 10 µL. The operating conditions of the ELSD were as follows: signal 0.00, gain 4, pressure 350 kPa, with the drift tube temperature set at 40 °C. Peak identification was based on comparison of retention times and spiking with authentic standards, while peak quantitation was based on the external standard method.

HPLC determination of organic acids in H. sabdariffa Varieties and Roselle Wine.

Organic acids in H. sabdariffa and roselle wine were extracted by undertaking a sample clean up using the SPE (Oasis Max 60 mg) cartridge. An aliquot of the sample (1 mL) diluted 1:5 with phosphoric acid (0.005 M) was passed through the cartridge previously conditioned with methanol (1 mL) and 0.005 M phosphoric acid (1 mL). The cartridge was washed with 1 mL of phosphoric acid (0.005 M) and the eluted organic acid fractions pooled together. The HPLC analysis was performed with the same equipment used for polyphenol quantitation using a Thermoscientific Acclaim ^{TM} Organic acid column (5 µm, 250 x 4.6 mm) thermostat at 20 °C. The chromatographic separation of the acids was achieved in 10 min under an isocratic procedure. The operating conditions were as follows: flow rate 0.5 mL/min, eluent 10 mM KH_2PO_4 (pH 2.6), injection volume 5 µL and the detection wavelength set at 210 nm. Peak identification was based on comparison of retention times and spiking with authentic standards, while peak quantitation was based on the external standard method.

Inhibition of α-Amylase by H. sabdariffa Extracts and Roselle Wine.

Prior to the enzyme inhibition assays, sugars were removed from the samples by SPE to prevent any interference with the assay. The efficiency of sugar removal was confirmed by analyzing the sample after SPE under conditions described for sugar analysis. Thereafter the assay was conducted according to the optimized protocol recently published.[28]

Inhibition of α-Glucosidase by H. sabdariffa Extracts and Roselle Wine.

The assay contained 200 µL each of substrate (maltose, final concentration of 3 mM) and enzyme, 50 µL sodium phosphate buffer (10 mM pH 7.0) and 50 µL of potential inhibitor (H. sabdariffa extracts, roselle wine or compounds) at different concentrations. For the control assay, the inhibitor was replaced by an equal volume of the buffer. The enzyme source was an acetone protein extract from rat intestine (10 mg/mL), prepared in sodium phosphate buffer (10 mM pH 7.0) to give a concentration of 4 mg/mL in the assay. The enzyme stock solution and the assay mixture containing the inhibitor, PBS and substrate were pre-incubated at 37 °C in a water bath for 10 min and the reaction was started by adding the enzyme to the assay solution. The reaction was carried out at 37 °C for 20 min with various concentrations of the inhibitor up to 6 mg/mL. The reaction was stopped by placing the samples in a water bath at 100 °C for 10 min, transferred to ice to cool down to room temperature and centrifuged for 5 min. The sample obtained was used for SPE, carried out using Oasis MAX cartridges to remove polyphenols before adding the hexokinase reagent. The resulting sample (50 µL) was added to 250 µL of hexokinase reagent, placed in a 96 well plate and the absorbance recorded at 340 nm. The rate of enzyme inhibition was calculated as a percentage of the control (without inhibitor).

Analysis of Volatile Compounds in Roselle Wine.

Extraction of Headspace Volatiles.

Extraction of the headspace volatiles was performed with a SPME manual device equipped with a 65 µm PDMS/DVB fiber (Supercool, Bellefonte, PA.). An aliquot of sample (10 mL)

and 2-octanol as internal standard (10 µL) were blended together in a 20 mL magnetic crimp sample vial. To facilitate the release of the volatile compounds, 1.5 g of NaCl was added to the sample vial which was then equilibrated for 30 min at 40 °C with agitation on and off at 30 s intervals. The SPME fiber was then exposed to the head space for 20 min and inserted into the GC injector to desorb the analytes.

GC-MS Analysis.

Volatile compounds were analyzed in a GC (Varian 3800) equipped with an MS detector (Saturn 2200), an automatic sampler (CP-8400) and an autoinjector (CP-8410). The separation was performed on a phenomenex ZB-WAX (30 m × 0.32 mm × 0.50 µm film thickness). The injector temperature was 250 °C and helium was the carrier gas at a flow rate of 1.0 mL/min. The oven programme started at 40 °C (held for 10 min), increased to 100 °C at 15 °C/min (held for 5 min) and finally to 250 °C at 15 °C/ min and held at that temperature for 5 min. The MS was operated in electron ionization mode (70 eV) and scanning was programmed for a m/z range of 29-300. Identification of volatile compounds was achieved by comparison with reference standard, matched spectra from the NIST 2.0 library and fragmentation patterns for compounds reported in the literature. For quantitation of volatiles, stock solutions of standards were dissolved in dichloromethane, and thereafter working concentrations were prepared by diluting to appropriate levels in a model wine solution containing 10 % ethanol, 3.0 g/L malic acid and the pH adjusted to 3.0 with NaOH.

Statistical Analysis.

Statistical analysis was performed by one-way analysis of variance using the Statistical Analysis System (SAS) version 9.4 software. Significant differences were assessed with Least Significant Difference (LSD) test ($p \leq 0.05$).

Results and Discussion

Physicochemical Properties, Sugar and Organic Acid Profiles of H. Sabdariffa Extracts.

Data on the physicochemical properties, simple sugars and organic acids profiles analyzed in H. sabdariffa extracts is presented in Table 1. Fructose, glucose and sucrose were the sugars prevalent in the extracts, while malic acid was the major organic acid identified, followed by succinic acid which was in highest concentration in the white variety ($p \leq 0.05$). The result obtained from the analysis is coherent with data in the literature on organic acids and sugars in H. sabdariffa.[29]

Identification and Quantitation of Polyphenols in H. sabdariffa Varieties.

Different classes of phenolic compounds were identified in H. sabdariffa extracts (Figure 1) and listed in Table 2. These include hydroxybenzoic acids, hydroxycinammic acids, anthocyanins, flavonols and flavan-3-ols, thus confirming earlier studies reporting that H. sabdariffa contains a myriad of phenolic compounds.[30] Hibiscus acid and its glucoside were tentatively identified in the three varieties by their m/z ratio from the LCMS analysis. With respect to the hydroxybenzoic acids in H. sabdariffa, gallic acid and protocatechuic acid were identified in the red varieties, however, for the white variety, gallic acid was not present. The major hydroxycinnamic acid in H. sabdariffa was the 3-CQA, while free caffeic acid was detected in the three varieties. Regarding the flavonols, myricetin 3-arabinogalactoside (M3A), quercetin 3-sambubioside (Q3S), rutin and quercetin 3-O-glucoside were identified in all varieties in various proportions. The white variety contained the highest amount of rutin, while in the light red variety, Q3S concentration was higher than M3A, with the reverse being the case for the dark red. These findings are in good agreement with published data on flavonols found in red H. sabdariffa.[18,31] As expected, DS and CS in the ratio 3:1 were found to be the major compounds responsible for the brilliant red color of red H. sabdariffa extracts, with other minor anthocyanins being present and tentatively identified. Furthermore, flavan-3-ols were only identified in the white variety. Catechin (peak 13) was identified with the use of an authentic standard, while peaks 19, 23 24 and 28 were tentatively identified as

proanthocyanidins from their UV-visible spectra. In summary, the major phenolic compounds present in H. sabdariffa varieties are the anthocyanins (dark and light red only) with hydroxycinnamic acids abundant in all three varieties.

Inhibition of α-Amylase and α-Glucosidase Activities.

An approach to reducing postprandial hyperglycemia is the inhibition of carbohydrate-hydrolyzing enzymes in the digestive system. Consequently these assays are vital for identifying inhibitors with potential to reduce the post-prandial glycemic response.[25] The three varieties of H. sabdariffa extracts exhibited negligible or no inhibition of human α-amylase activity. The highest inhibition value obtained was only 8 % with the dark red variety even at 6 mg/mL. This result apparently contradicts an earlier published study on the inhibition of porcine α-amylase by H. sabdariffa extracts, where IC_{50} values of 187.9 and 90.5 μg/mL were obtained for the red and white varieties respectively.[13] It is possible that the conditions under which the assay was performed could explain this conflicting result, since assay conditions greatly influence the inhibition; e.g. excess enzyme significantly affects measured IC_{50} values leading to incorrect values.[28,32] On the other hand, H. sabdariffa extracts inhibited α-glucosidase in a dose-dependent manner as depicted in Figure 2A, except for the white variety where the inhibition was very low even at the highest concentration tested (6 mg/mL). The IC_{50} values of the dark and light red variety were 4.35 ± 0.07 and 5.90 ± 0.14 mg/mL (dried powder extract) respectively. Although the inhibition of rat intestinal α-glucosidase in red and white H. sabdariffa varieties have been published, the compounds responsible for the effect have not been identified.[11,13] To investigate this, DS, CS and 3-CQA were tested individually and in combination at the concentrations equivalent to their presence in the dark red variety that gave the IC_{50} value (≈ 4 mg/mL of extract). The result presented in Figure 2B shows that DS and CS together provided about half of the inhibition, while the combination of DS, CS and 3-CQA accounted for 65 % of the total inhibition. Thus

we can conclude that these three compounds are mainly responsible for the α-glucosidase inhibition activity of H. sabdariffa extracts. It is noteworthy that CS proved to be a more potent inhibitor than DS as reflected in their IC_{50} values of 543 and 756 µM respectively. Finally, the outcome of the first part of this study showed that the dark red variety was superior in phenolic content and exhibited the highest bioactivity, and consequently it was chosen as the ideal variety for roselle wine production. The result of the changes in the physicochemical, phytochemical and aroma attributes during fermentation of dark red H. sabdariffa juice are presented below.

pH, Titratable Acidity and Sugar Consumption During Fermentation of Roselle Must.

The changes in pH and titratable acidity are presented in Table 3. The pH values on the final day were 3.0 and 3.1 at 20 °C and 30 °C respectively. TA levels increased in the course of fermentation at both temperatures and this can be associated with the production of α-ketoglutaric and succinic acids in the glyceropyruvic pathway during fermentation. In the early stages of fermentation, the yeast by-products tend to be pyruvic acid and glycerol, but not ethanol; pyruvic acid then goes on to form secondary products like α-ketoglutaric acid, succinic acid, diacetyl, and acetoin.[33] Concerning yeast activity, the rate of consumption of reducing sugars (fructose, glucose and sucrose) was faster at 30 °C than at 20 °C up until day 3 (Figure S1). This is possibly due to the delay in attaining maximal population (longer lag phase) at 20 °C compared to 30 °C. Thereafter, sugar consumption proceeded faster at 20 °C (longer stationary phase) until the end of fermentation. The same pattern was observed in another study,[34] where between fermentation temperatures of 15 to 35 °C, yeast cells attained maximal population size much slower at the lower temperatures, thereafter remaining constant throughout fermentation resulting in higher alcohol production.

Color of Roselle Wine during Fermentation.

315 Wine color is derived from a complex chain of reactions controlled by factors such as the
316 type and amount of flavonoids within the raw material, their extraction efficiencies during
317 fermentation and their stability in the course of ageing.[35] The evolution of color indices in
318 roselle wine is reflected in Table 3. The data from the tintometer implies that the wine color
319 was darker and more saturated on day 40, which can be directly linked to the polymerization
320 of anthocyanins. Furthermore, the spectrophotometric data on wine color parameters are also
321 presented in Table 3. In red young wines, the color density is mainly due to monomeric
322 anthocyanins and a direct correlation between anthocyanin content and color density of wines
323 has been reported.[36,37] However, as wine matures, the color is highly dependent on more
324 stable polymeric compounds formed through polymerization with other flavonoids and
325 tannins. The levels of DS, CS and polymeric anthocyanins recorded on day 0 justifies why
326 CD readings were least for the wines at both temperatures. Thereafter, the increase observed
327 is mainly due to the increased extraction of these anthocyanins and the formation of some
328 polymerized anthocyanins occurring simultaneously during fermentation. The CD values
329 were highest on day 3 and this is similar to published data on wine color density where
330 maximum values were attained between day 2 and 3 after the onset of fermentation.[36] For the
331 hue tint (browning index), the values increased (excluding day 0) for both temperatures as
332 fermentation progressed due to polymerization of anthocyanins. The degradation of
333 anthocyanins during pasteurization of the juice might explain the apparent contradiction
334 observed on day 0. Moreover, the decrease in individual anthocyanins (Table 4) after
335 attaining maximum levels and the concomitant increase in polymeric anthocyanins agrees
336 with a previous study on color evolution during wine processing.[38]

337 **Main Phenolic Compounds of Roselle Wine.**

338 The changes in the concentration of selected phenolic compounds during fermentation is
339 shown in Table 4. These compounds were chosen on the basis of their relative amounts in the

extracts. As anticipated, the results showed increases in quantities of phenolic compounds as fermentation progressed as contact between the liquid and solid phase (fermenting must and calyces) provides for more extraction of phenolics from the solid calyces into the fermenting must. DS and CS contents peaked on day 3 at 20 °C, while their concentrations were highest on day 2 for wines fermented at 30 °C. The subsequent decrease observed in the anthocyanin content after day 8 is a combination of polymerization and oxidation reactions, adsorption by yeast cells/fixation on solid portions, and enzymatic activity by microbial glucosidases.[39] Gallic acid levels increased steadily from the onset achieving highest concentrations on day 21 at both temperatures. The increase in gallic acid can be the result of increased extraction and also the breakdown of hydrolysable tannins arising from yeast activity. Perhaps the major transformation in phenolic compounds (irrespective of fermentation temperature) was the rapid decrease in 3-CQA levels. The reduction was followed by the simultaneous increase in caffeic acid, indicating the hydrolysis of 3-CQA into caffeic and quinic acids. By day 40, 3CQA content had dropped by over 80% from their initial contents, while caffeic acid levels increased by ≈ 84 %. This occurrence could be significant as caffeic acid has been shown to be a more potent inhibitor of both α-amylase and α-glucosidase than chlorogenic acid in vitro.[40] Furthermore, while caffeic acid is readily absorbed into the bloodstream, most of the chlorogenic acid consumed reaches the colon intact where the quinic acid moiety is cleaved off by the action of the colonic microbiota to release caffeic acid.[41] M3A and Q3S concentrations peaked around day 3 which was followed by a gradual decrease as fermentation progressed at both temperatures. Their reduction can be attributed to hydrolysis, oxidation and precipitation reactions occurring during the winemaking process.[24] Altogether, the data on compositional changes in phenolic compounds in roselle wine showed that fermentation temperature had no major impact on the phytochemical profiles of the wine. In summary, the proportion of DS, CS, M3A, and Q3S decreased after achieving a maximum

concentration, while GA content increased as fermentation progressed. Moreover, the increase in caffeic acid levels occasioned by fermentation makes roselle wine a rich source of this bioavailable compound.[39]

Roselle Wine Phenolic Content and α-Glucosidase Inhibition of Roselle Wine.

The soluble phenolic content in roselle wine increased from 743 mg/L (day 0) to 1260 and 1363 mg/L on the final day for wines fermented at 30 and 20 °C respectively. The concentration in roselle wine is within the range of 971-1753 mg/L obtained for fruit wines processed from cranberry, raspberry and elderberry.[42] With respect to the inhibition of α-glucosidase by roselle wine phenolics, this activity increased as fermentation progressed in wine fermented at 20 °C, while at 30 °C, the inhibition increased from day 0 to 8, thereafter declining and remaining constant until day 40 (Figure 2C). In blueberry wines, fermentation at room temperature (20 - 22°C) and 4 °C showed no difference in their inhibition of α-glucosidases.[43] A possible explanation to this occurrence in this experiment is that the secondary compounds derived from anthocyanin breakdown (after day 8) at 20 °C were better inhibitors than the compounds produced at 30 °C.

Profile of Organic Acids.

The changes in the organic acids profile of roselle wine during fermentation are shown in (Figure S2). In general as fermentation progresses, yeast cells make adjustments physiologically to the changing medium and the organic acid content is generally affected.[44] Malic acid levels in wines at both fermentation temperatures increased from day 0 to day 3. Thereafter, the levels of malic acid reduced minimally possibly arising from bacterial activities and reached a concentration of ≈ 1.65 g/L on day 40 at both temperature conditions. Generally, the standard wine yeast Saccharomyces cerevisiae is not able to degrade malic acid efficiently as it does not possess an active malate transport system and the production of

lactic acid registered on day 3 supports this line of reasoning.[45] Although wine yeast produces small amounts of acetic acid as metabolites during fermentation, elevated amounts in wine are detrimental to wine quality and are mainly due to the oxidation of ethanol by acetic acid bacteria.[21] In this study, we found higher amounts at 30 °C and this agrees with an earlier study that showed increased acetic acid production with higher fermentation temperature.[46] Nevertheless in this work, the levels of acetic acid (0.02-0.04 g/L) are within the permitted levels of up to 1.5 g/L for red wine. One of the major organic acid metabolites produced by yeast which contributes to the salty-bitter acid taste of wines is succinic acid and there were higher levels (1.18 g/L) produced in wine fermented at 30 °C. In a similar study, succinic acid production was 0.92 and 0.89 g/L for fermentations conducted at 30 and 20 °C respectively.[31] Citric acid levels in wine can fluctuate as it is both synthesized by yeast cells and later taken in and used up for other metabolic processes.[33] In this study, the final concentrations of citric acid were 0.03 and 0.04 mg/L for wines at 20 and 30 °C respectively.

Volatile Composition of Roselle Wine.

The main volatiles/aromas compounds synthesized by yeast during fermentation include the higher alcohols, fatty acids, acetate and ethyl esters, amongst others.[47] The production of higher alcohols occurs via amino acid metabolism or by reduction of related aldehydes during yeast fermentation, while esters are mainly formed from lipid and acetyl-CoA metabolism.[21] In this study, the major aroma compounds quantified on day 40 as depicted in Figure 1C were the fatty acids (hexanoic, octanoic and decanoic) and their ethyl esters, ethyl acetate, isoamyl alcohol, 2-phenyl ethanol and 1-hexanol. The pre-fermentation volatiles detected in roselle musts that persisted throughout fermentation were 1-hexanol and eugenol. In addition, linalool detected in the must might have been esterified to produce ethyl linalyl ether. These three compounds had previously been identified in H. sabdariffa extracts.[18] The concentrations of yeast-derived volatile compounds at the end of alcoholic fermentation are

414 presented in Table 5 and revealed that the concentrations of ethyl esters responsible for fruity
415 aromas were ≈ 2-fold higher at 20 °C than at 30 °C. On the other hand, higher amounts of
416 higher alcohols mainly responsible for flowery and fusel notes in wines were synthesized at
417 30 °C. Three medium chain volatile fatty acids were investigated in this study (hexanoic,
418 octanoic and decanoic) being the main precursors for ethyl ester biosynthesis. These volatiles
419 presented higher concentrations at 20 °C than at 30 °C; hence it is not surprising that higher
420 amounts of their corresponding ethyl esters were formed at 20 °C. This result is consistent
421 with published data on grape wines where fermentation at 13 °C resulted in higher synthesis
422 of fusel alcohol acetate esters, fatty acids and their corresponding ethyl esters, compared to
423 fermentation at 25 °C which favored the production of fusel alcohols and increased volatile
424 acidity.[48] With respect to ethyl acetate and diethyl succinate, the higher contents found at
425 30 °C can be linked to higher lactic acid bacteria activity at that temperature.[49] Although our
426 data on the influence of fermentation temperature on volatiles monitored in this research is in
427 accordance with some published results,[48,50] it is important to state that the concentrations of
428 these volatiles in wine is not totally dependent on temperature. Additional variables such as
429 the raw material, aeration of fermenting medium, amino acid composition and yeast strain
430 have also been proven to influence the content of these volatiles in wine.[47,48]
431 In summary**,** our experiments confirm that the dark red variety is highest in phenolic content.
432 We tested if the reported anti-diabetic properties of H. sabdariffa could be partly through
433 inhibition of α-glucosidase (maltase) and α-amylase. The inhibition of these enzymes was
434 modest, even though the inhibition of the latter has been reported in the literature, and so we
435 can conclude that this mechanism is unlikely to be responsible for the health effects of
436 Hibiscus. Concerning roselle wine processing and technological application, lower
437 temperature fermentation is desirable to enhance the production of desirable aroma attributes.

Exploring the impact of yeast strain and other processing conditions on roselle could be the direction of future research aimed at improving the final product quality.

Supporting Information

Changes in organic acids during the fermentation of roselle wine (Figure S1) and sugar consumption kinetics during fermentation of roselle wine (Figure S2).

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642 **Figure captions**

643 **Figure 1**. Identified phenolic compounds from (A) dark red and (B) white H. sabdariffa
644 extracts analyzed by HPLC-PDA. The numbering of the peaks corresponds to those listed in
645 Table 3. (C) GC MS chromatogram of identified volatile aroma compounds in roselle wine
646 after alcoholic fermentation. (1) ethanol, (2) ethyl acetate, (3) isoamyl alcohol, (4) 1-hexanol,
647 (5) ethyl hexanoate, (6) hexanoic acid, (7) 2-phenyl ethanol, (8) ethyl octanoate, (9) octanoic
648 acid, (10) ethyl decanoate and (11) decanoic acid. The chromatogram inserted amplifies the
649 volatiles detected at low concentrations in the wine.

650 **Figure 2**. (A) Dose-dependent inhibition of α–glucosidase by extracts of H. sabdariffa
651 varieties and (B) Effect of polyphenols in dark red extract on α–glucosidase inhibition.
652 Compounds were tested at their respective concentrations in the 4 mg/mL samples. Acarbose
653 IC_{50} (0.4 µM) was used as a positive control, DS IC_{50} (756 µM), CS IC_{50} (543 µM) and
654 H.sabdarifa at 4mg/mL. The results are expressed as means ± SD (n = 3) and values with
655 different letters indicate statistically significant differences at $p \leq 0.05$.

656 (C) Impact of fermentation temperature on α–glucosidase inhibition of roselle wine. The
657 results are expressed as mean ± SD and values with different letters indicate statistically
658 significant differences at $p \leq 0.05$.

Table 1: Physicochemical Properties, Contents of Sugars and Organic Acids Quantified in H. sabdariffa Varieties.

Properties	variety		
	dark red	**light red**	**white**
Physicochemical properties			
Ph	3.16 ± 0.06 [a]	3.08 ± 0.16 [a]	3.07 ± 0.15 [a]
CIEL	14.6 ± 0.2 [c]	36.4 ± 0.2 [b]	48.5 ± 0.9 [a]
CIEa	34.8 ± 0.3 [a]	24.1 ± 0.2 [b]	nd
CIEb	22.4 ± 0.1 [a]	14.2 ± 0.1 [b]	10.8±0.4 [c]
color density	9.60 ± 0.06 [a]	4.70 ± 0.01 [b]	1.10 ± 0.02 [c]
Sugars (mg/g)			
fructose	16.5 ± 1.7 [a]	11.9 ± 0.9 [b]	15.0 ± 0.9 [ab]
glucose	22.9 ± 2.7 [a]	18.2 ± 3.1 [ab]	15.3 ± 1.0 [b]
sucrose	11.7 ± 2.5 [a]	7.3 ± 0.4 [b]	12.7 ± 0.7 [a]
Organic acids (mg/g)			
oxalic	0.06 ± 0.02	trace	trace
Malic	45.59 ± 6.77 [a]	41.72 ± 4.46 [a]	40.68 ± 8.94 [a]
Citric	0.45 ± 0.39 [a]	0.58 ± 0.37 [a]	0.73 ± 0.59 [a]
succinic	0.80 ± 0.30 [b]	0.85 ± 0.16 [b]	2.27 ± 0.98 [a]
tartaric	0.11 ± 0.05 [a]	0.11 ± 0.04 [a]	0.06 ± 0.02 [a]

Values with similar letters within row are not significantly different at $p \leq 0.05$ (n = 3). nd: not determined for white variety.

Table 2. Identification and Quantitation of Polyphenols in H. sabdariffa Varieties According to Their Retention Time, UV- Vis and Mass Spectral Characteristics

compound	HPLC-PDA			LC-MS data m/z (+ /-)	dark red variety (mg/100g)	light red variety (mg/100g)	white variety (mg/100g)
	peak no	retention time	λmax				
hibiscus acid glucoside[a]	1	4.3	256	351	25.8 ± 4.0 [a]	18.1 ± 2.2 [b]	21.7 ± 1.4 [ab]
hibiscus acid[a]	2	4.6	265	189	21.8 ± 4.5 [ab]	24.9 ± 2.9 [a]	17.8 ± 0.3 [b]
gallic acid[c]	3	6.0	271	169	23.2 ± 3.1 [a]	15.9 ± 4.7 [b]	nd
unidentified	4	6.8	282		27.7 ± 0.8 [a]	13.3 ± 0.6 [b]	6.9 ± 2.4 [c]
unidentified	5	7.4	283		nd	nd	8.1 ± 1.4
protocatechuic acid[c]	6	8.8	262	153	16.4 ± 4.2 [a]	17.9 ± 1.5 [a]	5.9 ± 0.7 [b]
Unidentified	8	9.86	282		nd	nd	8.7 ± 1.8
Unidentified	9	10.53	273		nd	nd	11.1 ± 2.6
Total Hydroxybenzoic Acids					**115 ± 12 [a]**	**90 ± 7 [b]**	**80 ± 6 [b]**
3-O-caffeoylquinic acid[c]	7	9.2	324	353	319 ± 22 [b]	381 ± 30 [a]	64 ± 10 [c]
4-O-caffeoylquinic acid[a]	10	10.8	324	353	18 ± 2 [a]	12 ± 6 [a]	nd
cis/trans caftaric acid[b]	11	11.6	309		57 ± 12 [a]	66 ± 4 [a]	36 ± 4 [b]
cis/trans caftaric acid[b]	12	12.05	309		193 ± 32 [b]	291 ± 34 [a]	27 ± 6 [c]
chlorogenic acid isomer[a]	14	13.15	325	353	nd	nd	32 ± 7 [c]
5-O-caffeoylquinic acid[c]	15	13.31	324	353	69.5 ± 2.3 [a]	41.2 ± 5.4 [b]	34.7 ± 7.2 [b]
caffeic acid[c]	18	15.22	323	179	29.8 ± 4.5 [a]	19.7 ± 2.5 [b]	25.3 ± 4.1 [a]
chlorogenic acid isomer[a]	22	16.37	330	353	nd	nd	13.5 ± 1.9
cinamic acid	26	20.2	322		9.5 ± 0.6 [b]	13.5 ± 1.9 [a]	
coumaric acid[b]	27	20.8	316		7.8 ± 0.7 [a]	8.7 ± 2.2 [a]	5.8 ± 1.5 [a]
ferulic acid[b]	29	22.3	329		11.4 ± 1.6 [a]	11.4 ± 2.2 [a]	5.9 ± 1.3 [b]
Total hydroxycinnamic					**714 ± 57 [b]**	**844 ± 7 [a]**	**244 ± 32 [c]**
myricetin 3-arabinogalactoside[a]	25	19.7	354	611	28.5 ± 1.8 [a]	19.4 ± 3.3 [b]	9.1 ± 2.6 [c]
quercetin 3-sambubioside[a]	27	22.4	349	595	20.9 ± 0.9 [b]	34.1 ± 3.1 [a]	12.2 ± 2.9 [c]
rutin[c]	30	23.9	348	463	10.7 ± 0.7 [a]	8.2 ± 0.5 [b]	30.3 ± 3.7 [b]
quercetin 3-O-glucoside[c].	31	24.8	348	609	9.9 ± 0.4 [a]	10 ± 0.3 [a]	10.5 ± 1.2 [a]
Total Flavonols					**70 ± 2 [a]**	**72 ± 5 [a]**	**62 ± 5 [a]**
delphinidin 3-O-sambubioside[c]	16	13.8	526	597	2116 ± 216 [a]	535 ± 37 [b]	nd
delphinidin 3-O-glucoside[b]	17	14.6	526		76 ± 8 [a]	38 ± 1 [b]	nd
cyanidin 3-O-glucoside[a]	20	15.79	526		24.1 ± 3.9 [a]	18.6 ± 1.3 [a]	nd
cyanidin 3-O-sambubioside[c]	21	16.3	517	581	517 ± 42 [a]	136 ± 19 [b]	nd
Total Anthocyanins					**2732 ± 260 [a]**	**727 ± 55 [b]**	
catechin[b]	13	12.88	279		nd	nd	18.0 ± 4.2
Unidentified	19	15.60	285		nd	nd	29.8 ± 1.4
Unidentified	23	17.04	271		nd	nd	32.4 ± 3.3
Unidentified	24	18.80	279		nd	nd	23.2 ± 2.3
Unidentified	28	21.51	279		nd	nd	26.9 ± 3.3
Total Flavan-3-ols							**130 ± 10**
Total phenolics (HPLC) mg/100g					**3632 ± 235 [a]**	**1733 ± 74 [b]**	**516 ± 50 [c]**
Total phenolics from Folin's					**3801 ± 195 [a]**	**2260 ± 190 [b]**	**994 ± 150 [c]**

[a] compounds tentatively identified by LC-MS data and/or from available information from the literature. [b] compounds were identified by authentic standards, retention time and spiking. [c] compounds identified by authentic standards, retention time, spiking and confirmed with LC-MS analysis. nd: not detected. Values with non-italic similar letters within rows are not significantly different $P \leq 0.05$ (n = 9). Some compounds could not be identified in the LCMS due to their low concentrations in the extracts.

Table 3. Changes in Physicochemical Properties During Fermentation of Roselle Wines

attribute/fermentation temperature (°C)	changes in physicochemical attributes during fermentation of roselle wine				
	day 0	day 3	day 8	day 21	day 40
pH					
20	3.09 ± 0.04	2.96 ± 0.01	2.97 ± 0.04	2.97 ± 0.01	3.00 ± 0.04 [a]
30	3.09± 0.04	2.98 ± 0.04	3.06 ± 0.02	3.06 ± 0.01	3.07 ± 0.02 [a]
titratable acidity (g/L)					
20	2.13 ± 0.18	5.6 ± 0.01	5.4 ± 0.04	5.74 ± 0.05	5.74 ± 0.16 [a]
30	2.13 ± 0.18	5.7 ± 0.04	5.5 ± 0.16	5.40 ± 0.24	5.45 ± 0.17 [b]
color density					
20	13.4 ± 3.3	31.3 ± 2.2	21.8 ± 0.5	23.5± 0.1	21.8 ±0.2 [a]
30	13.4 ± 3.3	25.0 ± 0.4	17.1 ± 0.1	19.6 ± 0.1	18.9 ± 0.1 [b]
hue tint					
20	0.35 ± 0.02	0.29 ± 0.03	0.33 ± 0.01	0.34± 0.03	0.36 ±0.02 [b]
30	0.35 ± 0.02	0.33 ± 0.02	0.35 ± 0.02	0.37± 0.01	0.39 ± 0.01[a]
polymeric anthocyanin (%)					
20	6.6 ± 1.5	5.5 ± 1.4	9.6 ± 0.5	11.7 ± 0.2	12.7 ± 0.8 [a]
30	6.6 ± 1.5	8.2 ± 0.2	11.0 ± 0.3	11.8 ± 0.2	11.5 ± 0.1 [b]
CIEL					
20	28.6 ± 3.3	19.4 ± 0.1	23.7 ± 0.1	17.5 ± 0.2	16.7 ± 0.1 [b]
30	28.6 ± 3.3	22.2 ± 0.2	23.8 ± 0.1	20.6 ± 0.3	19.6 ± 0.2 [a]
chroma (C)					
20	54.9 ± 3.1	51.5 ± 0.3	54.8 ± 0.2	47.3 ± 0.3	45.6 ± 0.1 [b]
30	54.9 ± 3.1	53.6 ± 0.2	53.8 ± 0.1	50.1 ± 0.1	48.5 ± 0.1 [a]
hue (H)					
20	38.4 ± 2.8	35.2 ± 0.3	38.7 ± 0.4	33.2 ± 0.4	32.8 ± 0.4 [b]
30	38.4 ± 2.8	38 ± 0.34	38.0 ± 0.2	36.3 ± 0.5	35.1 ± 0.6 [a]

Mean values ± standard deviation. Day 0 represents the average in vats prior to fermentation and values with different letters on the same column indicate statistically significant differences at $p \leq 0.05$.

Table 4. Changes in Phenolic Compounds During Fermentation of Roselle Wine

compound/fermentation temperature (°C)	content in mg/L during days of fermentation						
	day 0	day 1	day 2	day 3	day 8	day 21	day 40
gallic acid							
20	4.3 ± 0.2	7.3 ± 0.5	8.2 ± 0.7	8.4 ± 0.1	9.2 ± 0.1	9.6 ± 0.1	9.6 ± 0.04 [a]
30	4.3 ± 0.2	6.9 ± 0.3	8.3 ± 0.2	8.6 ± 0.1	9.2 ± 0.1	9.9 ± 0.4	9.6 ± 0.10 [a]
3-O-caffeoylquinic acid							
20	81 ± 9	80.3 ± 2.5	60.3 ± 2.3	48.3 ± 7.2	24 ± 0.1	16.1 ± 0.2	8.4 ± 0.6 [a]
30	81 ± 9	51.3 ± 0.4	33.2 ± 3.5	24.5 ± 4.4	9.9 ± 1.4	6.6 ± 0.1	5.0 ± 0.4 [b]
caffeic acid							
20	15.2 ± 0.7	54.0 ± 1.8	56.3 ± 0.8	62.0 ± 1.6	73.0 ± 1.4	91.0 ± 3.7	93.0 ± 4.7 [a]
30	15.2 ± 0.7	52.7 ± 2.0	63.2 ± 1.3	66.0 ± 0.8	70.5 ± 2.2	108.0 ± 0.5	98.1 ± 1.7 [a]
DS							
20	243 ± 29	413 ± 17	428 ± 13	452 ± 6	407 ± 3	350 ± 2	318 ± 4 [a]
30	243 ± 29	392 ± 18	400 ± 13	381 ± 10	344 ± 20	290 ± 2	266 ± 2 [b]
CS							
20	61 ± 8	106 ± 5	116 ± 3	129 ± 2	119 ± 2	113 ± 7	112 ± 12 [a]
30	61 ± 8	110 ± 4	116 ± 2	112 ± 2	102 ± 3	97 ± 13	82 ± 3 [a]
M3A							
20	4.4 ± 0.5	5.7 ± 0.2	7.1 ± 0.3	7.3 ± 0.7	5.9 ± 0.2	6.4 ± 0.5	5.8 ± 0.4 [a]
30	4.4 ± 0.5	5.2 ± 0.3	6.6 ± 0.3	6.4 ± 0.4	6.2 ± 0.3	5.5 ± 0.4	4.4 ± 0.2 [a]
Q3S							
20	3.4 ± 0.3	4.3 ± 0.1	5.4 ± 0.1	5.3 ± 0.5	4.4 ± 0.1	4.8 ± 0.8	5.3 ± 0.1 [a]
30	3.4 ± 0.3	3.9 ± 0.1	5.2 ± 0.1	5.3 ± 0.1	5.2 ± 0.2	4.2 ± 0.1	4.9 ± 0.3 [a]
TPC (Folin's)							
20	743 ± 34	888 ± 12	872 ± 76	962 ± 20	1130 ± 60	1332 ± 40	1363 ± 44 [a]
30	743 ± 34	864 ± 90	886 ± 79	1080 ± 35	1103 ± 30	1270 ± 30	1260 ± 13 [b]

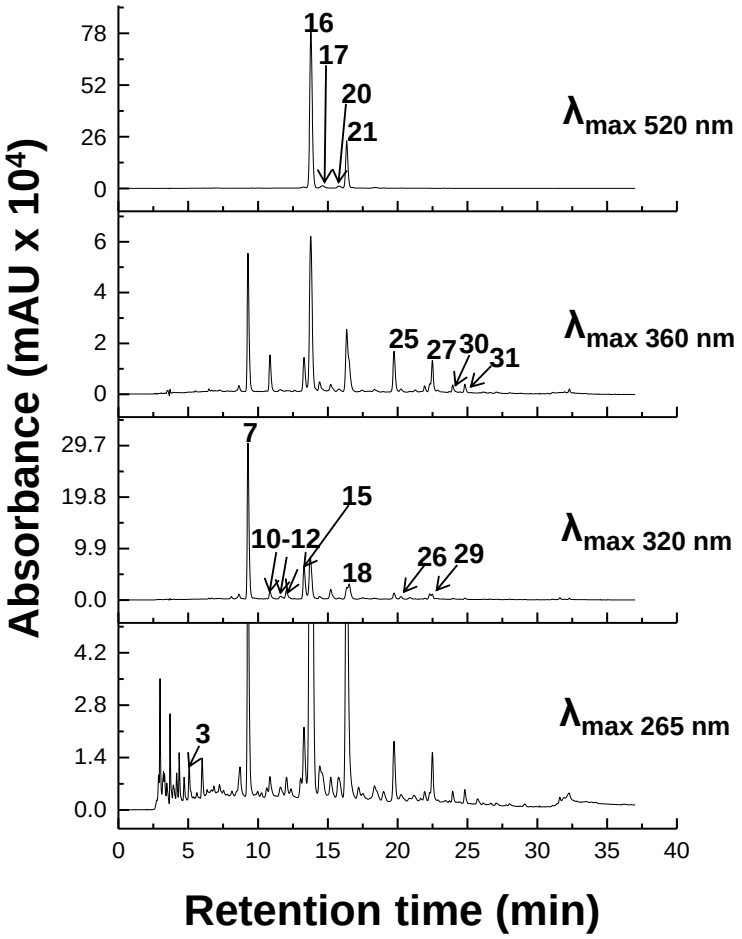
Mean values ± standard deviation. Day 0 represents the average in vats prior to fermentation and values with different letters on the same column indicate statistically significant differences at $p \leq 0.05$.

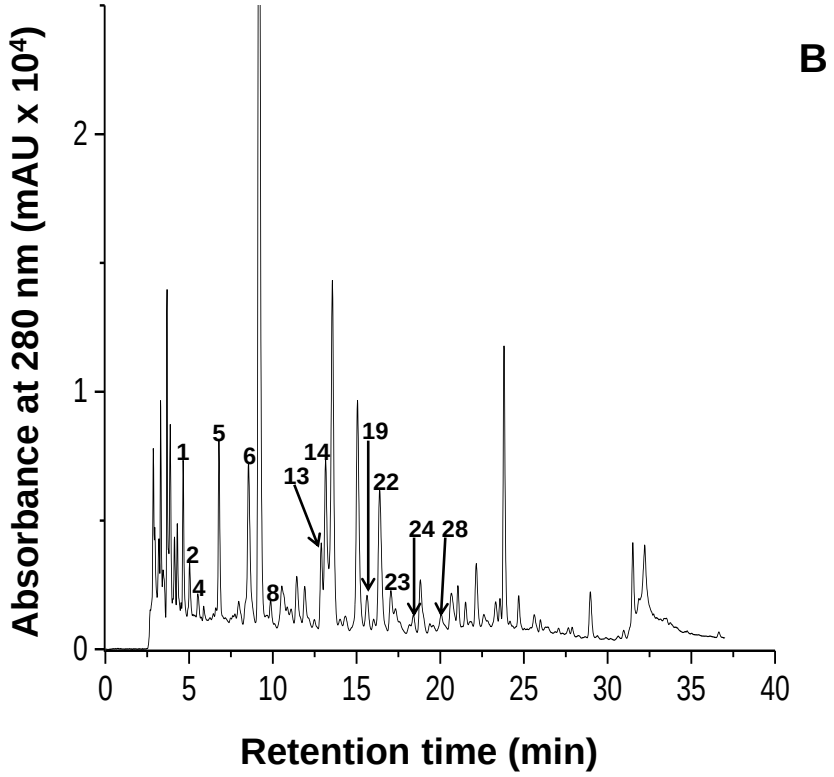
Table 5: Content of Volatile Compounds in Roselle Wine Fermented at 20 °C and 30 °C Determined Using HS-SPME-GCMS.

volatile compound	manufacturer[a]	identification[b]	qualifying ions	concentration (ug/L)	
				20 °C	30 °C
ethanol (% v/v)	Sigma-Aldrich	**AS**	**45**; 43; 74	11.53 ± 1.04 [a]	10.32 ± 1.38 [a]
2-phenylethanol	Sigma-Aldrich	**AS**	**91**; 92; 122	527 ± 63 [b]	1163 ± 114 [a]
isoamyl alcohol	Sigma-Aldrich	**AS**	**71**; 43; 55	130539 ± 21943 [b]	209777 ± 7645 [a]
1-Hexanol	Alfa Aesar	**AS**	**56**; 43; 69	248 ± 103 [a]	258 ± 60 [a]
ethyl acetate	Sigma-Aldrich	**AS**	**43**; 61; 70	156 ± 32 [b]	371 ± 27 [a]
ethyl hexanoate	Sigma-Aldrich	**AS**	**88**; 99; 145	3487 ± 363 [a]	1644 ± 90 [b]
ethyl octanoate	Alfa Aesar	**AS**	**88**; 101; 172	1339 ± 135 [a]	740 ± 33 [b]
ethyl decanoate	Sigma-Aldrich	**AS**	**88**; 101; 200	966 ± 95 [a]	639 ± 53 [b]
hexanoic acid	Sigma-Aldrich	**AS**	**73**; 60; 87	104 ± 20 [a]	65 ± 2 [b]
octanoic acid	Alfa Aesar	**AS**	**73**; 60; 101	99 ± 10 [a]	80 ± 7 [b]
decanoic acid	Sigma-Aldrich	**AS**	**73**; 60; 129	71 ± 7 [a]	61 ± 6 [b]
diethyl succinate[c]	Alfa Aesar	**AS**	**101**; 129; 55	53.92 ± 5.91 [a]	62.74 ± 11.13 [a]
isoamyl acetate[c]	Sigma-Aldrich	**AS**	**70**; 41; 88	69.07 ±12.72 [a]	51.19 ± 7.59 [b]
benzaldehye[c]	Sigma-Aldrich	**AS**	**105**; 77; 51	0.38 ± 0.06 [b]	0.61 ± 0.09 [a]
eugenol[c]	Alfa-Aesar	**AS**	**164**; 149; 103	1.25 ± 0.08 [b]	2.07 ± 0.16 [a]
ethyl linalyl ether[c]		**MS**	**99; 71; 43**	0.53 ± 0.03	NQ
2-phenyl acetate[c]		**MS**	**104**; 91; 65	7.08 ± 1.35 [a]	1.14 ± 0.11 [b]
ethyl linalyl ether[c]		**MS**	**99**; 71; 43	0.53 ± 0.03	NQ
ethyl dodecanoate[d]		**MS**	**88; 101; 228**	231 ± 36 [a]	112 ± 25 [b]

The values are the mean ± SD. Numbers bolded were used as the quantitative ion for aroma compounds;. [a] Manufacturers: Sigma-Aldrich.Co., Ltd., Dorset, UK and Alfa Aesar, Lancashire, UK. [b] Identification based on AS (authentic standard and mass spectra) and MS (tentatively identified by mass spectra only) [c] Compounds measured by semi-quantitation; relative peak areas (compound: internal standard) were used to calculate means and standard deviation. [d] Quantified as ethyl decanoate equivalent. NQ: detected but could not be quantified because of the low MS signal at the quantitative ion.

Figure 1


A
Absorbance (mAU x 10⁴)
78
52
26
0
16
17
20
21
λmax 520 nm
6
4
2
0
25
27
30
31
λmax 360 nm
29.7
19.8
9.9
0.0
7
15
10-12
18
26
29
λmax 320 nm
4.2
2.8
1.4
0.0
3
λmax 265 nm
0
5
10
15
20
25
30
35
40
Retention time (min)


B
Absorbance at 280 nm (mAU x 10⁴)
Retention time (min)
1
2
4
5
6
8
13
14
19
22
23
24
28

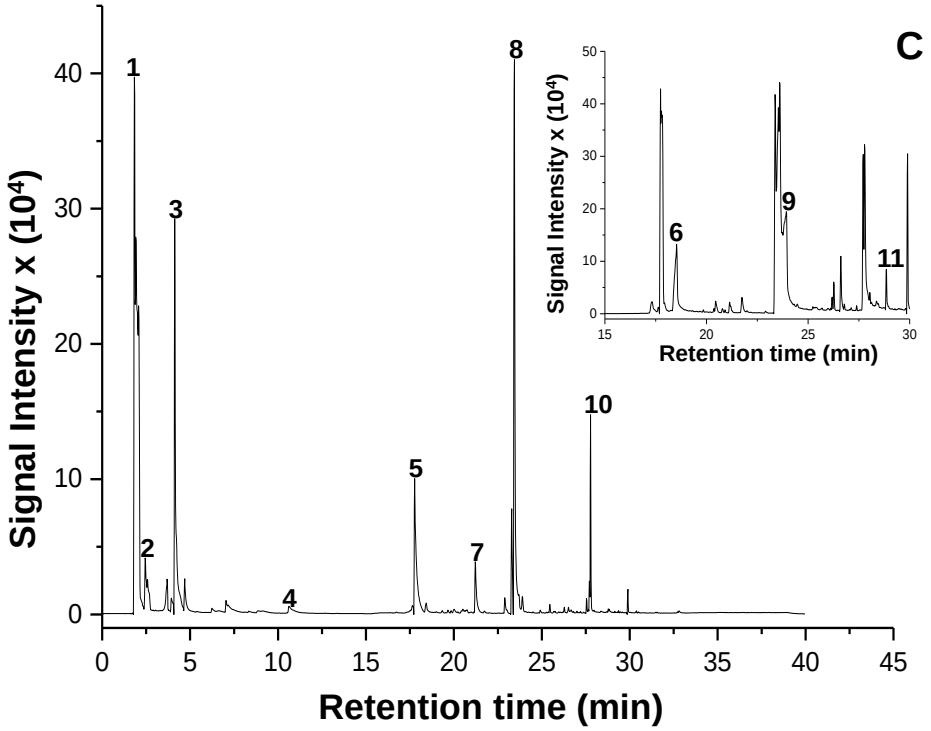
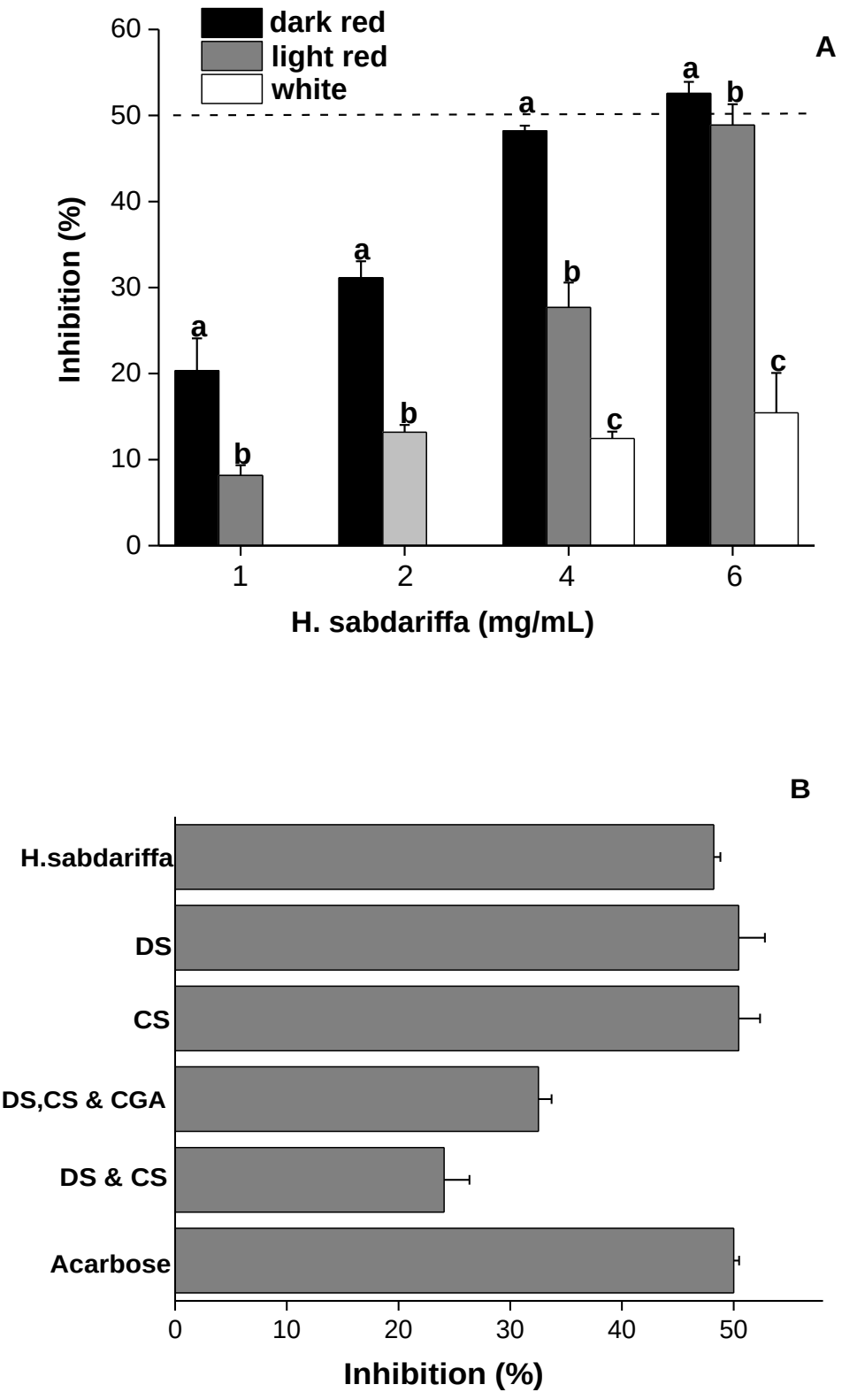
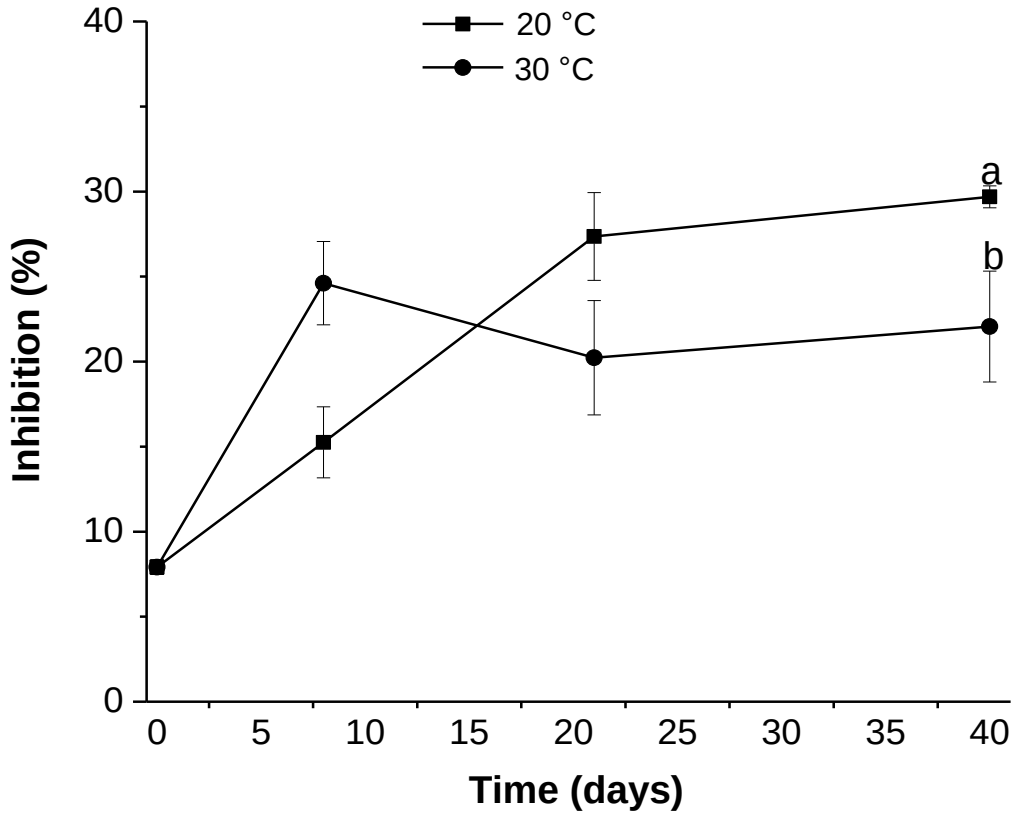

C
Signal Intensity x (10⁴)
Retention time (min)
1
2
3
4
5
6
7
8
9
10
11

Figure 2


A
dark red
light red
white
Inhibition (%)
H. sabdariffa (mg/mL)
B
H.sabdariffa
DS
CS
DS,CS & CGA
DS & CS
Acarbose
Inhibition (%)


20 °C
30 °C
a
b
Inhibition (%)
Time (days)
0
10
20
30
40
0
5
10
15
20
25
30
35
40