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Seasonal variation in Hibiscus sabdariffa (Roselle) calyx phytochemical profile, soluble solids and α-glucosidase inhibition

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Running title: Seasonal variation in Hibiscus sabdariffa

Abstract

Seasonal variations in crops can alter the profile and amount of constituent compounds and consequentially any biological activity. Differences in phytochemical profile, total phenolic content and inhibitory activity on α-glucosidase (maltase) of Hibiscus sabdariffa calyces grown in South Western Nigeria were determined over wet and dry seasons. The phenolic profile, organic acids and sugars were analysed using HPLC, while inhibition of rat intestinal maltase was measured enzymically. There was a significant increase (1.4-fold; $p \leq 0.05$) in total anthocyanin content in the dry compared to wet planting seasons and maltase activity from the dry season were slightly more potent (1.15-fold, $p \leq 0.05$). When the dry was compared to the wet season, fructose (1.8-fold), glucose (1.8-fold) and malic acid (3.7-fold) were significantly higher ($p \leq 0.05$) but citric acid was lower (62-fold, $p \leq 0.008$). Environmental conditions provoke metabolic responses in Hibiscus sabdariffa affecting constituent phytochemicals and nutritional value.

Keywords: Hibiscus sabdariffa, Seasonal variation, Phytochemicals, Anthocyanins, α-Glucosidase inhibition

1. Introduction

Secondary metabolites in edible plants contribute to health benefits and organoleptic properties (Alminger et al., 2014; Jakobek, 2015), and their biosynthesis, although largely controlled by genetics, is also influenced by environmental factors (Rodrigues, Pérez-Gregorio, García-Falcón, Simal-Gándara, & Almeida, 2011; Ren et al., 2017). Within a fruit and vegetable family, the quality and quantity of the phenolic pool may change with the cultivar, growth stage and season (Pérez-Gregorio et al., 2010). Furthermore, these secondary metabolites are reported to accumulate in plants that have been subjected to various forms of stress such as drought, temperature extremes (heat or cold) and other environmental conditions. (Akula and Ravishankar, 2011; Duda et al., 2015). Since the biosynthesis of secondary metabolites in plants is heavily dependent on growth conditions and environmental factors, it is important to determine the seasonal fluctuations in their phytochemical composition and the subsequent effect on potential health benefits (Galasso et al., 2014; Luo et al., 2016). Knowledge of the seasonal variations in phytochemical content of plants is critical as food producers become increasingly interested in manufacturing novel products with an increased level of bioactive compounds to meet consumer demands for healthy foods.

H. sabdariffa belongs to the Malvaceae family and is grown in most tropical and subtropical climates of the world (Borrás-Linares et al., 2015). The calyx, which is the commercially important part of H. sabdariffa, is mostly exported from these regions and is added to jam, juice, jelly, gelatine, syrup, wine, ice cream, pudding and cake. It is a rich source of secondary metabolites, mainly anthocyanins, flavonoids and large quantities of organic acids

(Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014). Anthocyanins may inhibit intestinal α-glucosidase which in turn could have a potential therapeutic effect on post-meal blood glucose levels (McDougall, Shpiro, Dobson, Smith, Blake, & Stewart, 2005). Although the main phytochemicals in H. sabdariffa extracts have been well documented (Borrás-Linares et al., 2015; Ifie, Marshall, Ho, & Williamson, 2016), seasonal variation in the bioactive constituents has not been determined in any detail.

In tropical regions of West Africa, where H. sabdariffa is commonly cultivated, there are two distinct planting seasons: the rainy season that starts in March and lasts until the end of July, with a peak period in June, and the dry season, which starts around October and lasts until around mid-March with a short dry season generally experienced in August lasting for ~3 - 4 weeks. Knowledge on the seasonal variation in phytochemical profiles of H. sabdariffa calyces will help to inform the best agronomical practices during hibiscus planting and cultivation. Hence, the objective of this work was to assess the impact of two planting seasons on the phytochemical profile and bioactivity (maltase inhibition) of H. sabdariffa.

2. Materials and methods

2.1 Chemicals

Commercial standards of delphinidin 3-O-sambubioside, cyanidin 3-O-sambubioside, delphinidin 3-O-glucoside, protocatechuic acid, 3-O-caffeoylquinic acid (Extrasynthase 49915 and a gift from Mike Clifford), were purchased from Extrasynthase, Genay, France, while maltose, fructose, glucose, sucrose, gallic acid, caffeic acid, Folin-Ciocalteu's reagent, trifluoroacetic acid, citric acid, malic acid, oxalic acid, tartaric acid, succinic acid, sodium mono and dibasic phosphate, intestinal acetone powder from rat, and glucose assay reagent

were from Sigma-Aldrich, Dorset, U.K. The 3 mL (60 mg) Oasis MAX cartridges used for Solid Phase Extraction (SPE) were from Waters Corporation Ltd., Milford, Massachusetts.

2.2 Cultivation of H. sabdariffa plant

H. sabdariffa seeds (dark red variety) were planted within two consecutive growing seasons, dry and wet, in 2013-2014. The hibiscus variety used in this study was obtained from the genebank at the National Horticultural Research Institute, Ibadan. The hibiscus seeds planted in August 2013 were planted at the onset of the seasonal rain break in August and the calyx was harvested in mid-December 2013, which falls in the dry season. The seeds planted in early March 2014 at the onset of the rains while the calyx was harvested before the break of rain in mid July 2014. The study was carried out at the National Horticultural Research Institute (NIHORT), Ibadan, South-Western Nigeria (Latitude 7^{o} 23' and 7^{o} 25'N and longitude 3^{o} 50' and 3^{o} 52'E). The experiment was conducted in two non-organic plots, within the same vicinity to ensure similar soil conditions. The design used was a Randomized Complete Block Design (RCBD) with four replicates. Each plot consisted of 6 rows of 5 m length, with 75 cm inter-row spacing and 50 cm intra-row spacing. The total plot size was 22.5 m^2 and the net harvested plot size was 12 m^2 (4 middle rows of 4 m length). Seeds (4 - 5) were planted in each hole, and seedlings were thinned to 2 plants per hole, 2 weeks after planting. Each row contained 20 plants. Weeding was performed by hand twice per season and care was taken to ensure similar agrotechnical conditions (weeding and harvesting were carried out at the same stage of growth) in both planting seasons. After harvesting, the calyces were dried to a moisture content of 12 % in a storage chamber at 28 - 30 °C. Samples were then vacuum-packed, transported to the UK and stored in a freezer at -20 °C.

2.3 Sample preparation

Samples of H. sabdariffa extracts for phytochemical analysis and for inhibition of maltase (α-glucosidase) from each planting season were prepared by weighing out 2 g of hibiscus calyces and grinding them into a powder with the aid of a pestle and mortar. Distilled water (100 mL) was introduced into the sample and the extraction was carried out in a water bath at 50°C for 30 min with intermittent stirring. 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.

2.4 Identification and quantification of phenolics in H. sabdariffa

The polyphenols in H. sabdariffa extracts (gallic acid, protocatechuic acid, 3-O-caffeoylquinic acid, caffeic acid, myricetin 3-O-arabinogalactoside, quercetin 3-O-sambubioside, delphinidin 3-O-sambubioside, delphinidin 3-O-glucoside, cyanidin 3-O-sambubioside) were identified and quantified by HPLC with diode array detection and by LCMS using authentic standards as presented in detail previously (Ifie et al., 2016). Briefly, compounds were separated on a Phenomenex Gemini C18 column maintained at 35 °C. (5 µm, 250 mm × 4.6 mm). A gradient elution prepared from mixtures of 0.1% (v/v) trifluoroacetic acid mobile phase (A) and trifluoroacetic acid/acetonitrile/water (50:49.9:0.1) mobile phase (B) was used for the analysis at a flow rate of 1mL/min. The gradient programme started with 92 % A, solvent B was then increased to 18 % at 3.50 min, 32 % B at 18 min, 60 % B at 28 min, reaching 100 % B at 32 min. The composition was held at 100 % B for 4 min, before returning to the starting conditions for 3.5 min in preparation for the next analysis. The sample injection volume was 10 µL.

2.5 Analysis of organic acids, soluble sugars and total phenolic content

The organic acids and sugars in H. sabdariffa extracts (citric acid, malic acid, oxalic acid, tartaric acid, succinic acid, fructose, glucose and sucrose) were analysed and quantified as described previously in detail by Ifie et al. (2016). A Thermoscientific Acclaim Organic acid column (5 µm, 250 × 4.6 mm) set at 20 °C was used for the separation of organic acids. The analytical conditions were as follows: eluent 10 mM KH_2PO_4 (pH 2.6), flowrate 0.5 mL/min, injection volume 5 µL and the detection wavelength set at 210 nm. The separation of sugar was performed on a Grace Davison Prevail Carbohydrate Es column (5 µm, 250 mm × 4.6 mm). The mobile phase was 75 % acetonitrile (v/v) applied at a flow rate of 0.5 mL/min. Total polyphenolic content was evaluated using the Folin and Ciocalteu reagent as described by Ifie et al. (2017).

2.6 Inhibition of α-glucosidase

Any residual sugars in samples were removed by solid phase extraction to prevent any interference with the assay, and the inhibition assessed using maltose as substrate with a protein extract from rat intestine as enzyme source (Ifie et al., 2016).

2.7 Statistical methods

Statistical analysis was done using the Statistical Analysis System (SAS) version 9.4 software. The t-test was used to calculate the least significant difference (LSD) and values of $p < 0.05$ were considered to be significantly different.

3.0 Results and discussion

3.1 Seasonal variation in phenolic profile

Anthocyanins are the main phenolic compounds present in H. sabdariffa calyces (Figure 1) and cyanidin 3-O-sambubioside, delphinidin 3-O-glucoside and total anthocyanins were

156 significantly higher during the dry season compared to the wet season (Table 1). Anthocyanin
157 accumulation in plants is generally up-regulated by various environmental stresses, such as
158 drought, UV, blue light, high intensity light, wounding, pathogen attack and nutrient
159 deficiency (Akula and Ravishankar, 2011; Kassim et al., 2009), including drought-stressed
160 grapevines (Vitis vinifera), where the majority of genes committed to the flavonoid pathway
161 were also increased (Castellarin et al., 2007). Similarly, Stagnari, Galieni, Speca, & Pisante
162 (2014), reported an increase in both total phenolic content and betalains in red beet under
163 drought-induced stress conditions. In contrast, the concentrations of 3-O-caffeoylquinic acid
164 and myricetin 3-O-arabinogalactoside were higher in the wet season than the dry season. The
165 concentration of caffeoylquinic acid derivatives and flavonoids diminished in a generalized
166 way in five cherry tomatoes cultivars under water stress (Sánchez-Rodríguez, Moreno,
167 Ferreres, del Mar Rubio-Wilhelmi, & Ruiz, 2011). Furthermore, the impact of water and cold
168 stress treatment on some polyphenols in hartworn plants (Crataegus laevigata and
169 Crataegus. monogyna) was evaluated, and the polyphenols behaved differently (increase and
170 decrease) to both stress conditions (Kirakosyan et al., 2004). Studies on the effect of drought
171 and osmotic stress on polyphenols in different species indicate that polyphenols could
172 increase or decrease depending on the species, the type and intensity of stress (Popović,
173 Štajner, Ždero-Pavlović, Tumbas-Šaponjac, Čanadanović-Brunet, & Orlović, 2016).

174 **3.2 Seasonal variation in organic acids and sugars**

175 Seasonal variation in organic acid and sugars (Figure 2A &B) was quantified in H. sabdariffa
176 calyces (Table 2), suggesting that overall the content of simple sugars and organic acids were
177 higher during the dry season. Fructose, glucose and malic acid were significantly higher ($p \leq$
178 0.05) for planting done in the dry season, while citric acid was dramatically higher during the

179 rainy season. In peaches cultivated under water stress conditions, sugars and organic acids
180 were higher (Rahmati, Vercambre, Davarynejad, Bannayan, Azizi, & Génard, 2015), and
181 total soluble solids were higher in Nicotiana langsdorffii plants grown under water stress
182 compared to the untreated controls (Ancillotti et al., 2015). Although most studies report an
183 increase in sugar content in fruit and vegetables when exposed to water stress, the results are
184 less clear with organic acids. For instance, titratable acidity was found to be higher in apples,
185 but lower in berries from non-irrigated trees than in fruits from well-watered trees (Wu,
186 Genard, Lescourret, Gomez, & Li, 2002). One mechanism could include higher import rates
187 to the fruit, linked with possible osmotic adjustment or simply through the effect of
188 concentration (Rahmati et al., 2015).

189 **3.3 Seasonal variation in α-glucosidase (maltase) inhibition**

190 Following food ingestion, starch is digested into maltose and other maltooligosaccharides by
191 α-amylase, and then to glucose by intestinal α-glucosidases. The inhibition of these enzymes
192 is one possible target to help manage glucose excursions during type 2 diabetes. Inhibitors of
193 carbohydrate-digesting enzymes from natural resources represent a promising strategy to
194 attenuate post-prandial blood glucose spikes as they are usually without the adverse side
195 effects sometimes associated with drugs (Etxeberria, de la Garza, Campión, Martinez, &
196 Milagro, 2012). Extracts from H. sabdariffa inhibited rat intestinal maltase activity, derived
197 from 2 intestinal brush border enzymes, sucrase-isomaltase and maltase-glucoamylase, by up
198 to ~50% (Figure 3). There was a small difference between extracts from the wet and dry
199 seasons, but this was statistically significant only at the lowest concentration tested (Figure
200 3). Delphindin 3-O-sambubioside and cyanidin 3-O-sambubioside had previously been
201 implicated as the main compounds responsible for the inhibitory activities of H. sabdariffa on

maltase, and since they increase during the dry season, then the change in maltase inhibition is consistent with the difference in anthocyanin content (Ifie et al., 2016).

4.0 Conclusion

This is the first study to show how dry and wet seasons affect the amounts of anthocyanins and other constituents in H. sabdariffa. The increase in anthocyanins is reflected in higher inhibition of maltase activity. These results also suggest that it is possible to increase the content of anthocyanins in the calyces by manipulating agricultural techniques such as exposing the plant to controlled water stress conditions. Anthocyanins have several other proposed biological activities and these could be optimized by selection of growing conditions or other exogenous factors such as stress conditions to enhance potential health benefits beyond basic nutrition. In addition, the bitterness in hibiscus extracts can be reduced by exposing the plant to controlled water stress, since malic acid enhances sucrose perception while citric and quinic acids mask the perception of sugars. Future studies could evaluate the impact of other climatic factors such as controlled temperature, humidity or exposure to sunlight on the phenolic composition, organic acid content and sugars profiles in H. sabdariffa and their subsequent effect on nutritional properties.

Figure legends

Figure 1: HPLC chromatogram (λ =520 nm) showing anthocyanins identified in H. sabdariffa extracts grown in (A) wet season and (B) dry season.

H. sabdariffa extract (20 mg/mL, 0.01 mL) was loaded on the column and the anthocyanins identified relative to standards: (1) Delphinidin 3-O-sambubioside, (2) Delphinidin 3-O-glucoside and (3) Cyanidin 3-O-sambubioside.

Figure 2: Chromatographic analysis of organic acids and sugars in H. sabdariffa

(Panel A) HPLC chromatogram (λ =210 nm) showing the organic acids identified in H. sabdariffa extracts: (1) oxalic acid (2) tartaric acid, (3) malic acid, (4) citric acid (5) succinic acid. H. sabdariffa extract (20 mg/mL, 0.01 mL) was injected for each run.

(Panel B) HPLC-ELSD chromatogram of simple sugars identified in H. sabdariffa extract. H. sabdariffa extract (20 mg/mL, 0.01 mL) was injected for each run.

Figure 3: Dose-dependent inhibition of α–glucosidase (rat intestinal maltase) by aqueous extracts of H. sabdariffa (2, 4 and 6 mg/mL dried powder) prepared from two different seasons.

The results are expressed as mean ± SD and values with different letters indicate statistically significant differences at $p < 0.05$.

Table 1 Content (mg/100 g dry calyx) of the main phenolic compounds in H. sabdariffa grown in two different seasons.

Compound	Season	
	dry	wet
gallic acid	23.2 ± 3.1 [b]	34.5 ± 2.5 [a]
protocatechuic acid	17.9 ± 1.5 [a]	14.4 ± 0.5 [b]
3-O-caffeoylquinic acid	319 ± 22 [b]	490 ± 44 [a]
caffeic acid	29.8 ± 4.5 [a]	35.4 ± 2.6 [a]
myricetin 3-O-arabinogalactoside	28.5 ± 1.8 [b]	34.9 ± 1.0 [a]
quercetin 3-O-sambubioside	20.9 ± 0.9 [a]	23.8 ± 2.1 [a]
delphinidin 3-O-sambubioside	2120 ± 216 [a]	1610 ± 467 [a]
delphinidin 3-O-glucoside	76.3 ± 8.0 [a]	41.5 ± 10.9 [b]
cyanidin 3-O-sambubioside	517 ± 42 [a]	306 ± 26 [b]
Total Anthocyanins (HPLC)	2710 ± 261 [a]	1957 ± 502 [b]
Total phenolics from Folin assay (mg/100 g)	3800 ± 195 [a]	3604 ± 87 [b]

Values with similar letters within row are not significantly different at $p \leq 0.05$ ($n = 9$).

Tabe 2 Content (mg/100 g dry calyx) of organic acids and sugars in H. sabdariffa grown in two different seasons

Properties	Season	
	Dry	Wet
Sugars (mg/g)		
fructose	16.5 ± 1.7 [a]	9.30 ± 0.20 [b]
glucose	22.9± 2.7 [a]	12.9 ± 1.7 [b]
sucrose	11.7 ± 2.5 [a]	11.6 ± 2.5 [a]
Organic acids (mg/g)		
oxalic	0.060 ± 0.020	trace
tartaric	0.11 ± 0.05 [a]	0.18 ± 0.01 [a]
malic	45.6 ± 6.8 [a]	12.2 ± 3.40 [b]
citric	0.45 ± 0.39 [b]	27.7 ± 4.40 [a]
succinic	0.80 ± 0.30 [a]	0.57 ± 0.13 [a]

Values with similar letters within rows are not significantly different at $p \leq 0.05$ ($n = 3$).

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Figure 1

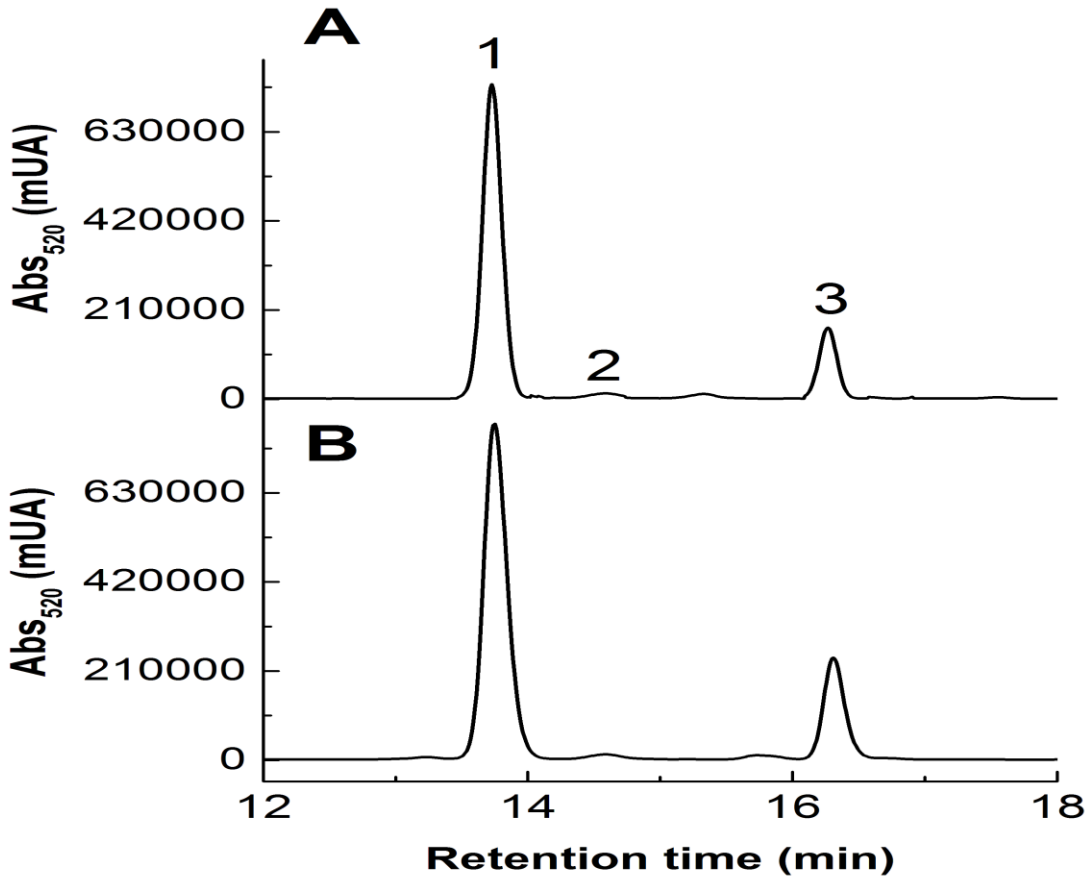
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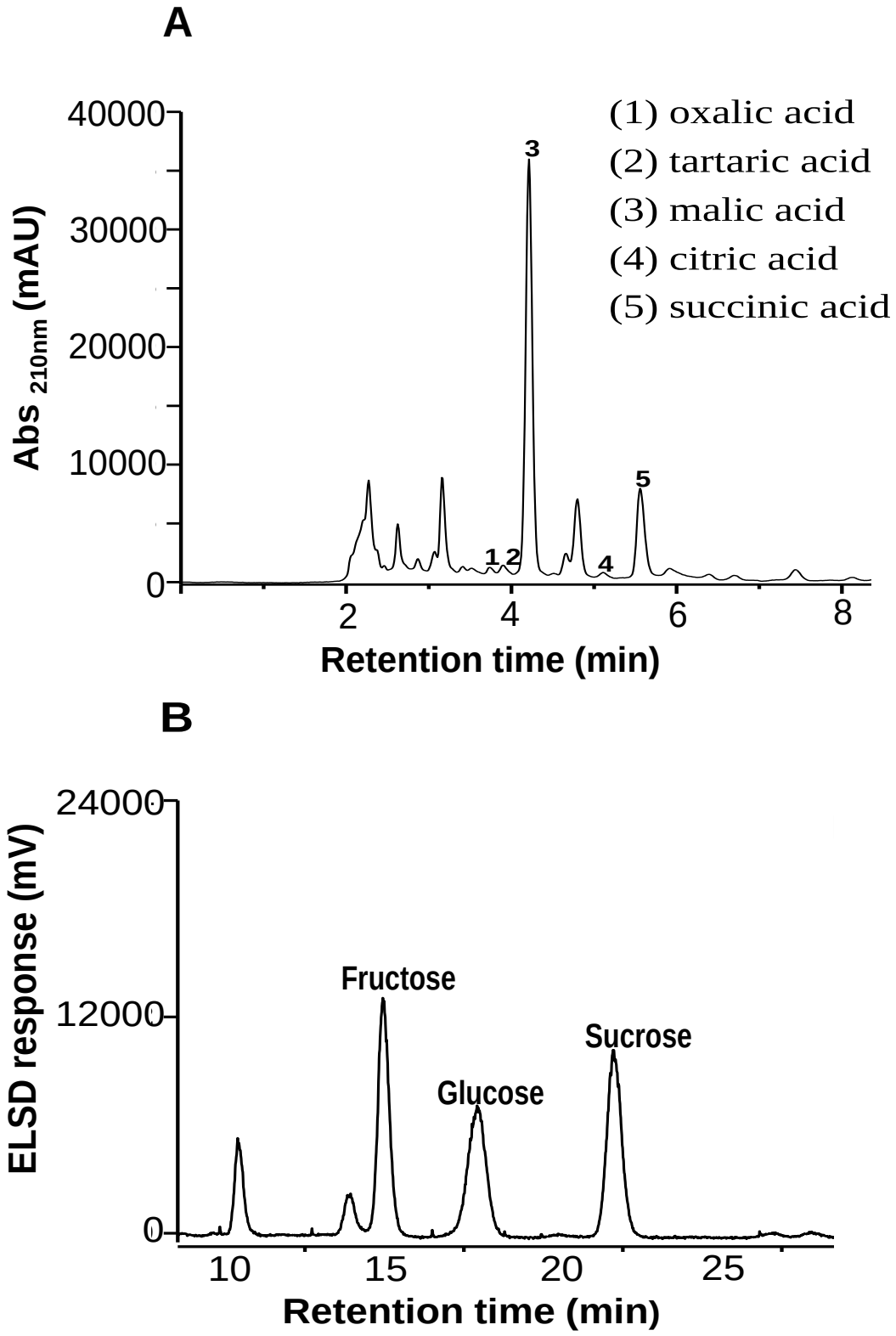
Figure 2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390 **Figure 3**

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