Effect of laboratory-scale decoction on the antioxidative activity of Zhenjiang
Aromatic Vinegar: the contribution of melanoidins

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ABSTRACT

Decoction, the characteristic heat-treatment procedure in Zhenjiang Aromatic Vinegar (ZAV) processing, was performed in laboratory to investigate its effect on the antioxidative activity (AA) of ZAV. Owing to the laboratory-scale decoction, DPPH radical scavenging activity, ORAC value and cellular antioxidant activity (CAA) of ZAV increased by 63, 24 and 54% of that of undecocted vinegar, respectively. Enzymatic assay and HPLC determination proved that phenolic content decreased after the decoction, while melanoidins, also with antioxidative activity and recently found in ZAV, were generated. Regardless of the fluctuation in AA of melanoidins during decoction, increased melanoidin content was responsible for the increase in AA of ZAV. At the end of the decoction, melanoidins accounted for 57.1 and 44.1% of the total AA of ZAV, according to DPPH and ORAC assay, respectively. Incorporation of phenolic compounds into melanoidins and degradation of melanoidins might both occur during decoction.

Keywords: Zhenjiang Aromatic Vinegar, antioxidative activity, phenolic compounds, melanoidins

Abbreviations: ZAV, Zhenjiang Aromatic Vinegar; AA, antioxidative activity; CAA, Cellular Antioxidant Activity; RV, raw vinegar; VCEAC, Vitamin C Equivalent Antioxidative Capacity ; HMw, high molecular material; LMw, low molecular material

1. Introduction

Vinegar, a commonly used food seasoning, has functional therapeutic properties, such as antioxidative, antibacterial activity, promoting recovery from exhaustion, and regulating blood pressure and blood glucose (Fushimi et al., 2001; Johnston et al., 2013; Ohnami et al., 1985; Tsujihata et al., 1998; Verzelloni et al., 2007). With increasing interest in the potential health effects of vinegar worldwide, there have been many reports confirming the antioxidative activity (AA) of various vinegars (Budak et al., 2014). Phenolic compounds play an important role in the AA of vinegars (Shimoji et al., 2002; Verzelloni et al., 2007; Wang et al., 2012). Melanoidins, coloured polymers synthesized through Maillard reaction, are able to exert AA and may be the predominant contributors to the AA in severely heat-treated food samples (Daglia et al., 2004; Liu & Kitts, 2011; Morales et al., 2012). Recently, it has been suggested that melanoidins contribute to the AA of vinegars (Lei et al., 2014; Tagliazucchi et al., 2008; Verzelloni et al., 2010).

Zhenjiang Aromatic Vinegar (ZAV) is the most famous and popular vinegar in southern China for its outstanding flavour and long history. ZAV is produced from sticky rice through liquid-state saccharification and alcohol fermentation, solid-state acetic acid fermentation, leaching, decoction and further storage successively (Dengru et al., 2004). Studies illustrated that the AA of ZAV has good correlation with its total phenolic compounds content (Xu, 2005). Other than phenolic compounds,

melanoidins separated from ZAV have been also reported to possess DPPH radical scavenging activity and reducing power (Xu et al., 2007).

The production of ZAV employs a series of characteristic technologies. Decoction, among them, is mainly adopted to pasteurize right before further storage. Specifically, during decoction, raw vinegar (RV) obtained by leaching is mixed with 2% sugar and heated to keep slightly boiling at 102 °C or so for 60 min at least. During the decoction step, ZAV is subjected to high temperatures. Therefore, a series of chemical reactions including Maillard reaction, decomposition, and degradation are likely to occur, which will have significant effect on vinegar quality. As to the AA of ZAV, On the one hand, melanoindins as Maillard products could be developed and act as newly formed antioxidants. As a result, it might enhance the AA of ZAV. On the other, under the heating condition above 80 °C, polyphenolic compounds are highly thermolabile and easily decomposed (Hečimović et al., 2011; Katsube et al., 2009; Larrauri et al., 1997). In addition, high temperature treatment could cause degradation of the formed melanoidins (Adams et al., 2005; Bekedam et al., 2008a; Brudzynski & Miotto, 2011). Thus, decrease in the AA of ZAV probably takes place. Above all, decoction must be emphasized since the AA of ZAV could alter drastically during it. Studies are needed to clearly demonstrate the effect of decoction on AA of ZAV, and to understand the chemical reactions occurring during the process. Moreover, in Chinese vinegar market, development of vinegars with functional therapeutic properties has the greatest economic importance. But commercial vinegar products

with enhanced AA are all manufactured by addition of Chinese medicine materials. To thoroughly understand the effect of decoction on the AA of ZAV is expected to help build a new, cost-effective and convenient way to increase the AA of ZAV.

However, up to now, only Xu et al. (2007) reported the influence of decoction on the AA of ZAV expressed as % DPPH radical scavenging activity, and inconsistent results were obtained when different volumes of samples were employed. Thus, further studies are necessary to clarify the change of AA of ZAV induced by decoction. To gain more stable data in detail, RV samples acquired from factory were then decocted in laboratory under much more controllable conditions than those in factory.

Along with DPPH and ORAC, cellular antioxidant activity (CAA) assay, a new approach to quantify AA at physiological conditions, was performed to determine the AA of vinegar samples. Compared with the chemical antioxidative activity assays, CAA assay takes into account the bioavailability, uptake, and metabolism of the antioxidants (Wolfe & Liu, 2007). The CAA assay has been widely used for fruits and vegetables recently (Song et al., 2010; Wolfe et al., 2008), but not yet for vinegars. In addition, with the incorporated *in vitro* digestion, CAA assay in food samples will provide further information close to the real physiological conditions (Huang et al., 2014). Particularly, phenolic compounds and melanoidins are two categories of antioxidants reported in ZAV, and foods containing either or both of them tend to exert modified AA after digestion (Faller et al., 2012; Huang et al., 2014;

Rufián-Henares & Morales, 2007). Thus, the combination of CAA assay and *in vitro* digestion was applied to further assess the AA of vinegar samples.

Moreover, to elucidate the reason behind the change pattern of the AA of ZAV, we evaluated the quantity variation of phenolic compounds and melanoidins during decoction for the first time. Although the existence of melanoidins in ZAV and the AA of them have been reported before, none has shed light on how important melanoidins are to ZAV. Thus, our main goal of this study is to estimate the contribution of melanoidins to the whole AA of ZAV.

2. Materials and methods

2.1. Chemicals

HPLC-grade standards, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azobis(2-amidinopropane) (ABAP), 20,70-dichlorofluorescein diacetate (DCFH-DA) and all other chemicals were purchased from Sigma Co. (St. Louis, MO, USA), except $K_2Fe(CN)_6$ and $Zn(OAc)_2$ of analytical grade and hydrogen peroxide (30% w/v) obtained from Beijing Chemical Reagent Co.(Beijing, China).

HepG2 human liver cancer cells were purchased from the Cancer Hospital Chinese Academy of Medical Sciences (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM) and Hanks' Balanced Salt Solution (HBSS) were purchased from Beijing Solarbio Science & Technology Co. (Beijing, China). Fetal bovine serum (FBS) was obtained from Gibco Co. (Grand Island, NY, USA).

2.2. Preparation of decocted vinegar

During liquid-state saccharification and alcohol fermentation, sticky rice was ground and mixed with tap water, *koji* (encompassing various moulds and saccharolytic enzymes) and yeast which were both prepared in Jiangsu Hengshun Vinegar Industry Co., Ltd. (Zhenjiang, China). The resulting milky rice wine (about 9% ethanol, v/v) was mixed with wheat bran, rice hull, and so-called vinegar seeds (inocula containing plenty of viable acetic acid bacteria) which were obtained from the last successful acetic acid fermentation and functioned as starter culture to initiate the solid-state acetic acid fermentation. After the acetic acid fermentation was terminated by addition of salt at about 2% of vinegar substrate weight, vinegar substrate was mixed with Parched Rice (about 5%, w/w), a characteristic additive of ZAV, to gain an inviting brownish black color. Parched Rice is thick liquid produced by parching white rice into black at about 800 °C and then, to acquire the thick liquid, dissolving the black rice in boiled water to boil for over 10 min. The subsequent leaching procedure was the progress of adding water to vinegar substrate to dissolve out soluble components such as acids, amino acids, sugar and other metabolites. Fresh raw vinegars (RV), the leached solution, with titratable acidity of about 5.0 were collected directly in Jiangsu Hengshun Vinegar Industry Co., Ltd. (Zhenjiang, China) and stored at -20 °C until decoction in laboratory.

Undecocted vinegars were obtained by dissolving 2% sugar (sucrose content $\geq$ 99.8%) into RVs. 400 mL fresh-made undecocted vinegar samples in 500 mL flasks

were first heated to 90 °C by the same electrothermal furnace, and then transferred in a 105 °C oil bath to slow down temperature rise and to further boil samples. Similar to the production followed at the factory, slow boiling conditions were maintained. Boiled vinegar samples were picked out after they had been slightly boiling (102 °C) for 0 min, 30 min and 60 min, respectively and then cooled in water. This heating process, which is decoction, was performed in triplicate. Vinegar samples were placed in -20 °C fridge before analysis.

Undecocted or boiled vinegars (2 mL) were dried in an oven at 103-105 °C to a constant weight and weighed as the content of total solids. pH was measured by pH meter (PB-10, Sartorius, Gottingen, Germany). Titratable acidity calculated as acetic acid was determined by titration with 0.5 M sodium hydroxide solution until the pH of mixture reached to 8.2 as described by Chen et al. (2013). The absorbance of the vinegar samples at 420 nm was recorded to estimate their melanoidins contents, and samples were diluted to ensure that the absorbance was between 0.1 and 1.0.

2.3. High molecular weight melanoidins preparation

High molecular weight melanoidins were obtained according to the method described by Tagliazucchi et al. (2010) with a slight modification. After filtered through a Whatman no. 4 filter paper, 4 mL aliquots of vinegar samples were diluted to about 10 mL with deionized water and subjected to ultrafiltration with Amicon Ultra-15 (MW cutoff 10kDa, Millipore, Milford, MA, USA) at 3500g for 60 min at

4 °C. The retentate was refilled with deionized water and rewashed three times. The retentate and the filtrate were lyophilized, yielding high molecular weight materials (HMw) and low molecular weight materials (LMw), respectively. HMw would be referred as melanoidins after validation by $K_{mix\ 420nm}$ and UV-VIS spectra. HMw and LMw were both kept at -20 °C until analysis.

2.4. $K_{mix\ 420nm}$ and UV-VIS spectrum scan of HMw

According to the method described by Bekedam et al. (2006), the specific extinction coefficient (K_{mix}) of HMw was introduced to quantify melanoidin level, which was calculated using the law of Lambert-Beer: $Abs (-) = K_{mix} (L\ g^{-1}\ cm^{-1}) \times concentration (g\ L^{-1}) \times length\ of\ light\ path (cm)$. The use of the specific extinction coefficient (K) was preferred over the molar extinction coefficient (ϵ) because the molecular weight of melanoidins is unclear and probably variable. The wavelength used was 420 nm, which was commonly used to estimate melanoidins content in vinegars (Falcone & Giudici, 2008).

The UV-VIS spectra of HMw (0.5 mg/mL in deionized water) were determined in the range 200-700 nm. Spectroscopic analyses were performed using a Shimadzu UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan).

2.5. Determination of phenolic content

2.5.1. Enzymatic method

Prior to determination, vinegar samples were clarified by Carrez's solutions

$K_2Fe(CN)_6$ (0.3 M) and $Zn(OAc)_2$ (1.0 M) to exclude high molecular weight compounds such as melanoidins using the adapted method described by Perrone et al. (2012). The phenolic contents of vinegar samples were determined by enzymatic method (Stevanato, 2004). Briefly, 4 mL of vinegar samples were mixed with 0.2 mL of each Carrez's solutions and final volume was fixed to 5 mL with deionized water. After 15 min, the colloidal suspension was centrifuged to obtain the supernatant. 200 μ L supernatant was added to 2.5 mL of a 0.1 M PBS (pH=8.0) containing 3 mM 4-aminophenazone, 2 mM H_2O_2 , and 0.33 μ M horseradish peroxidase. The absorbance was read at 500 nm after 15 min. The absorbance values of both reagent blank using deionized water and sample blank were subtracted. Phenolic content was expressed in mg of gallic acid (GA) equivalents per 100 mL of product.

2.5.2. HPLC

The supernatants obtained from vinegar samples after clarification and centrifugation were also subjected to a HPLC analysis according to the method of Chou et al. (2015) adapted from Yang et al. (2010). The HPLC system is composed of a Shimadzu LC-20AT pump system and a Shimadzu SPD-20A UV–VIS detector (Shimadzu SCL-20A system controller module, Kyoto, Japan). A CAPCELL PAK C18 column (250 $\times$ 4.6 mm, 5 μ m; SHISEIDO Co., Ltd. Tokyo, Japan) and a gradient solvent system consisting of MeOH (solvent A) and Milli-Q water with 9% glacial acetic acid (solvent B) were used for separation of components whose spectra were recorded from 220 to 450 nm. Phenolic acid compounds: gallic chlorogenic,

p-hydroxybenzoic, vanillic, caffeic, p-coumaric, ferulic, sinapic and flavonoid standards: protocatechuic, catechin, rutin, quercitrin, daidzein, quercetin were also run on the HPLC as standards to verify chemical compounds of vinegar samples.

To investigate the free phenolic compounds content left in HMw, the materials were dissolved in water (20-40 mg/mL). Then after clarification with Carrez's solutions (1:50, v/v) and centrifugation, the supernatant was analysed by HPLC as described above.

2.6. DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to Xu et al. (2007) with slight modifications. Briefly, 1 mL of a 1.4×10^{-4} M DPPH• ethanol solution was mixed with 1 mL of a test sample dissolved in 100 mM Tris-HCl buffer (pH 7.4). The mixture was then shaken up vigorously and left for 30 min in the dark prior to the measurement at 517 nm by spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan). IC₅₀ (concentration required to inhibit DPPH radical formation by 50%) values were determined from Probit analysis of dose-response curves. IC₅₀ values were further transformed into vitamin C equivalent antioxidative capacity (VCEAC) as mg of Vc equivalents/mL of sample or mg of Vc equivalents/g of sample using the following equations (Kim et al., 2002; Lim et al., 2007)

$$\text{VCEAC} = \text{IC}_{50(\text{Vc})} / \text{IC}_{50(\text{sample})}$$

2.7. ORAC

The ORAC assay was based on the method described by Dávalos et al. (2003,2005). Briefly, the reaction was carried out in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μ L) contained fluorescein (120 μ L, 70 nM final concentration), ABAP (60 μ L, 12 Mm final concentration), and antioxidant (20 μ L either trolox [2–8 μ M, final concentration] or appropriately diluted samples). The reaction was performed at 37 °C and fluorescence was recorded every minute for 80 min. A blank (control) using phosphate buffer instead of the antioxidant was carried out in each experiment. All reaction mixtures were prepared in duplicate. ORAC results were in μ M of Trolox equivalents/mL of vinegar or μ M of Trolox equivalents/g of HMw.

2.8. CAA with *in vitro* digestion

2.8.1. *In vitro* digestion

Undeocoted and decocted vinegars were lyophilized before digestion, because lyophilisation allowed CAA values to be determined at concentrations without cytotoxicity against HepG2 cells (Seki, 2008). *In vitro* digestion was conducted by the protocol described by Faller et al. (2012) with slight modifications. Briefly, 2 g lyophilized vinegar sample was mixed with saline (140 mM NaCl, 5 mM KCl) to create a final volume of 10 mL. The mixture was placed in an orbital shaker at 4 °C till lyophilized sample was dissolved completely. Then, for gastric digestion, it was treated with 0.5 mL of pepsin solution (0.2 g of pepsin in 5 mL of 0.1 M HCl) at pH

2.0 acidified by 0.1 M/2 M HCl. The solution was incubated in a shaking water bath at 37 °C for 1 h. After gastric digestion, the pH of the digestate was adjusted to 6.9 with 0.1 M/1 M NaHCO₃. Further intestinal digestion was performed with the addition of 2.5 mL of pancreatin-bile solution (0.45 g of bile extract and 0.075 g of pancreatin in 37.5 mL of 0.1 M NaHCO₃) and then the incubation in a shaking water bath at 37 °C for 2 h. The digestate was centrifuged at 2700g for 10 min and the supernatant was collected and diluted to 30 mL with PBS (pH=7.2-7.4). The supernatant was stored at -70 °C until analyses.

2.8.2. *Cell culture*

HepG2 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 100 U/mL penicillin-streptomycin. The cells were cultured at 37 °C in a humidified 5% CO₂ incubator.

2.8.3. *Cytotoxicity*

Cytotoxicity was measured using the method of Wolfe et al. (2007). HepG2 cells were plated in a 96-well plate at a density of 4×10^4 /well in 100 µL of growth medium and incubated for 24 h at 37 °C. The growth medium was removed and the cells were washed with PBS. After addition of treatment medium (DEME, phenol free, FBS free) containing the digested sample, cells were incubated for another 24 h. Then cell counting was conducted by the methylene blue assay (Felice, et al., 2009). Concentrations of samples that caused a 10% reduction of absorbance at 570 nm

compared to the control were considered to be cytotoxic.

2.8.4. Cellular antioxidant activity (CAA)

Cellular antioxidant activity was determined according to the method of Wolfe et al. (2007) with a slight modification. HepG2 cells were plated in a 96-well plate at a density of 5×10^4 cells/well. After incubation for 24 h, triplicate wells were treated for 1 h with 100 μ L of quercetin or digested samples plus 25 μ M DCFH-DA dissolved in the treatment medium (DEME, phenol free, FBS free). Then wells were washed twice with 100 μ L of PBS and 600 μ M ABAP was applied to the cells in 100 μ L of HBSS. Fluorescence was measured (excitation at 485 nm and emission at 520 nm) with an Infinite 2000 Pro plate reader (Tecan Co. Salzburg, Austria) at 37 °C every 5 min for 1 h. Final CAA values were calculated using the CAA unit at each concentration of quercetin and samples and expressed as μ mol of quercetin equivalents (QE)/g of lyophilized vinegar. CAA values of vinegars before lyophilisation were obtained by further multiplying those of lyophilized counterpart by lyophilized vinegar yields (Table S1).

2.9. Statistical Analyses

The effect of decoction on the chemical composition and AA of vinegar and HMw was evaluated using analysis of variance (ANOVA) with Duncan multiple comparison test. Considering that data were normally distributed, Pearson correlation analysis between A420 of vinegars and AA measured by DPPH and ORAC assay was

performed. All statistical analyses were performed using SPSS Statistics software for Windows, version 20 (IBM Software, Inc.). Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Preparation and characterization of Vinegar Samples

The RVs with titratable acidity of about 5.0 were obtained since they were produced massively in the factory and were further processed into various vinegar products consumed in large amount. As for all factory-scale decoction, the rate of temperature rise after 90 °C was lowered by reducing steam supply and slight boiling was maintained for 60 min at least. However, decoction in factory employed diverse patterns of temperature rise (Figure 1), which were likely to cause different effects on the AA of vinegars. To speed up production, undecocted vinegars were heated by all steam heaters so that vinegars would reach 90 °C in 7 min as the typical decoction procedure 1 shown in Figure 1. However, when decoction needed to be slowed down, an energy-saving procedure with a preheat system would be applied as the typical decoction procedure 2 presented. In factory, not only did the temperature-rise patterns vary dramatically, but also when to add sugar into vinegar was unfixed. Different sugar addition times might alter AA of decocted vinegars since it has been reported that the addition of sugar contributes to better AA of coffee due to the higher formation of Maillard reaction products, such as melanoidins, during heat treatment

(Andueza et al., 2004; Ludwig et al., 2013; Sánchez-González et al., 2005). Both reasons mentioned above would lead to large deviation that obscured the variations induced by decoction. Therefore, to clearly reveal the effect of decoction on the AA of ZAV, decoction in laboratory under much more controllable conditions than those in factory was indispensable and proposed in the present work.

Temperature-rise procedure of laboratory-scale decoction that turned vinegars undecocted into boiled for different boiling periods is also shown in Figure 1. Standard deviations of temperature at each heating time were less than 3 °C which suggested that the decoction degree rather than any other factors would be responsible for variations in analysis results. On average, time needed to reach 90 °C was 17 min which was between those of two factory-scale decoction procedures.

The laboratory-scale decoction yielded vinegar samples boiled for 0, 30 and 60 min. Water evaporation during decoction determined an increase of the total solids content and, at least to a certain extent, resulted in the slight rise in titratable acidity and limited reduction in pH (Table 1). Decoction insignificantly increased the total solids by 3.0% of that of the initial undecocted vinegar. The mild concentration effect of decoction alone was not enough to explain the significant rises in A420 (Table 1) which was commonly used to monitor the brown colour in vinegar (Falcone & Giudici, 2008; Lei et al., 2014). With the progressive decoction, A420 increased to 1.44 times as much as that of the undecocted vinegar. Caramelisation was unlikely to

occur in decoction because it generally needs higher temperature of about 140 °C (Kroh, 1994), but brown colour development in heat-treated acid products (80-95 °C , pH ranging from 2.72 to 3.08) was attributed to the formation of melanoidins through Maillard reaction (Piva et al., 2008). So, we estimated melanoidins content in vinegar samples according to A420 as in the previous study on traditional balsamic vinegar (Falcone & Giudici, 2008), and speculated that melanoidins were accumulated during decoction.

3.2. The Effect of Decoction on Antioxidative Activity of ZAV

As occurs in other ET-based assays, DPPH radical scavenging activity is strongly influenced by the solvent and the pH of reaction (Magalhães et al., 2008). The 50% (v/v) aqueous ethanol solutions are a suitable choice for lipophilic and hydrophilic antioxidants as concluded by Stasko et al. (2007). And the pH of reaction was fixed by Tris-HCl buffer (pH 7.4) as described by Xu et al. (2007). To make results more comparable, IC₅₀ was transferred into vitamin C equivalent antioxidative capacity (VCEAC).

Both DPPH radical scavenging activity and ORAC values (Figure 2) of vinegar samples continuously increased during decoction. It confirmed the results reported by Xu et al. (2007) that DPPH radical scavenging activity of decocted vinegar was significantly higher than that of undecocted vinegar at the concentration of 0.125% (v/v). And similar increase in AA of coffee brew with the degree of heat treatment has

been reported before (Anese & Nicoli, 2003; Daglia et al., 2000). DPPH radical scavenging activity of 60-min boiled vinegar rose by 63% of that of the undecocted vinegar and as for ORAC value, it was 25%. After whole 60-min boiling, the decocted vinegar, among tested samples, was nearest to the final ZAV product and had an ORAC value of 16.2 μ M of Trolox equivalents/mL which was higher than those of wine vinegars determined by the same method (Dávalos et al., 2005). Concentration effect imposed by decoction was calculated in proportion to the total solids (Table 1) of each sample and is also displayed in Figure 2. Concentration effect could only result in a rise equal to 3.0% of the DPPH radical scavenging activity or ORAC value of undecocted vinegar. Therefore, we concluded that significant improvement in DPPH radical scavenging activity and ORAC value of ZAV took place after the decoction, and the improvement could be attributed to chemical reactions rather than physical concentration during the decoction.

Moreover, satisfactory correlation between A420 (Table 1) and AA measured by DPPH radical scavenging activity assay (Pearson's $r=0.987$) and ORAC assay (Pearson's $r=0.997$) was obtained. This implied Maillard reaction was the sole or prevalent reaction in ZAV subjected to the laboratory-scale decoction (Manzocco et al., 2000), and the accumulation of melanoidins might be the main reason why the AA of ZAV rose progressively with the laboratory-scale decoction.

Cellular antioxidant activity (CAA) assay is a more biologically relevant model

to measure AA compared with chemical assays (Wolfe & Liu, 2007). Besides, measurement of CAA coupled with *in vitro* digestion provides further information close to the real physiological conditions (Faller et al., 2012). *In vitro* digestion is particularly necessary when it comes to ZAV, since foods containing phenolic compounds and melanoidins, two reported antioxidants in ZAV, tend to exert modified AA due to digestion. It has been reported that, when subjected to an *in vitro* digestion, foods containing phenolic compounds had higher CAA values than the original extract (Faller et al., 2012; Huang et al., 2014). Plus, gastrointestinal digestion is necessary to study potential bioactivity of melanoidins too, for that low molecular weight compounds released from melanoidins after digestion possessed significant AA, and was available for a rapid absorption (Rufián-Henares & Morales, 2007).

Median effective doses (EC_{50}) and CAA values of lyophilized vinegar samples are listed in Table 2. CAA values of vinegars before lyophilisation are also shown, which were calculated by multiplying those of lyophilized counterpart by lyophilized vinegar yields (Table S1). Until today, there have been no reports on the use of the CAA assay on vinegars. The lyophilisation allowed CAA values to be determined at concentrations without cytotoxicity against HepG2 cells (Table 2) as reported by previous study (Seki, 2008).

After vinegar samples boiled, CAA values continuously increased with the growing boiling time up to 60 min and 60-min boiled vinegar had a highest CAA

value among undecocted and decocted vinegars. These were comparable to the results of the two chemical assays. Actually, despite of the risk brought by Relative Standard Deviations ranging from 6.2 to 24.4%, CAA value of 60-min boiled vinegar was calculated as 1.54 times as much as that of undecocted vinegar, which, coupled with results of chemical assays, proved that the laboratory-scale decoction significantly improved the AA of ZAV. Also, the improved AA of ZAV cannot be explained by the physical concentration and the consequent limited change in pH and titratable acidity. But, unlike results from the chemical analyses, CAA value of 0-min boiled vinegar, instead of undecocted vinegar, was the lowest. While undecocted vinegar was heated to boiling point, both DPPH radical scavenging activity and ORAC value rose, but CAA value decreased. It might be explained by the hypothesis that new antioxidants were formed but as a whole the antioxidants were less absorbable.

CAA value of 60-min boiled vinegar (Table 2) was calculated as 0.36 mM QE/g, which was similar to cranberry and raspberry (Wolfe et al., 2008). It was an indication that decocted vinegar after entire 60-min boiling has strong AA, though vinegar intake is not likely to be much.

3.3. The Effect of Decoction on Phenolic Content

A considerable number of phenolic groups (99 µg of gallic acid equivalents/mg melanoidins) bound to ZAV melanoidins have been detected by Folin-Ciocalteu assay (Xu et al., 2007), which indicates that a proportion of phenolic compounds directly

determined in unpretreated vinegar could be attributed to the bound phenolic groups of melanoidins. So, removing melanoidins before test helps ensure that the proposed relationship between phenolic content and AA is free from the interferences from melanoidins. In the present study, before determination, high molecular weight compounds such as melanoidins were excluded by adding Carrez's solutions in order to eliminate bound phenolic groups belonging to them (Perrone et al., 2012).

Enzymatic method was employed to determine phenolic content, since it appears to have a higher specificity toward phenolic compounds and is affected by less interferences from other antioxidants than Folin-Ciocalteu assay (Stevanato, 2004). Phenolic content (Table 1) of 60-min boiled vinegar was significantly lower than that of undecocted vinegar. Besides, phenolic content tended to decrease during the laboratory-scale decoction, and if concentration effect was taken into account, this tendency was expected to be more significant.

HPLC was utilized to identify and quantify phenolic compounds in ZAV samples both undecocted and decocted. As shown in Table 3, the main kinds of phenolic compounds detected in ZAV samples closely resembled those in Taiwan black vinegar and aged oat vinegar (Chou et al., 2015; Qiu et al., 2010). Content of the individual phenolic compounds in ZAV samples varied diversely. The similar situation has been reported and ascribed to different sensitivity of phenolic compounds to heat by Adriana et al. (2005). Specifically, catechin and *p*-hydroxybenzoic were both

abundant in undecocted vinegar and significantly decreased during the decoction, which led to the decrease in total phenolic content. Nevertheless, gallic acid appeared to be more thermally stable to maintain a large amount throughout the decoction, and accounted for the largest proportion of sum content of phenolic compounds detected in 60-min boiled vinegar, which indicated gallic acid was a proper standard for the enzymatic method. Except catechin, *p*-hydroxybenzoic and gallic acid, the rest fluctuated a little and were detected to be present in very small quantities. The phenolic content (Table 3) calculated from the results of the HPLC test was lower than that determined by the enzymatic method (Table 1). This phenomenon was mainly due to the fact that results of the enzymatic method were expressed as gallic acid equivalents, while for HPLC method, sum of the individual phenolic content. And other phenolic compounds respond as much as a larger amount of gallic acid do in the enzymatic method, since that gallic acid has lower molar absorbance than other detected phenolic compounds (Ma & Cheung, 2007).

Both the enzymatic method and the HPLC assay revealed that phenolic content decreased during the decoction. This was in agreement with extensive evidences that under heat treatment, phenolic content decreases in food (Coelho et al., 2014; Katsube et al., 2009; Liu & Kitts, 2011; Perrone et al., 2012). Beside the thermal decomposition of phenolic compounds (Hečimović et al., 2011; Katsube et al., 2009; Larrauri et al., 1997), the reason for the decrease is also likely to be the incorporation of phenolic compounds into the melanoidins (Bekedam et al., 2008b; Perrone et al.,

2012), which has been proved to be a significant pathway of phenolic degradation during heat treatment (Coelho et al., 2014).

Clearly, compared with undecocted vinegar, phenolic content of decocted vinegar after 60-min boiling significantly decreased while AA measured by both chemical and cellular assay increased remarkably, thus we proposed that components other than phenolic compounds would account for the improvement of AA of ZAV induced by the laboratory-scale decoction. Considering the satisfactory correlation between A420 and AA measured by chemical assays, we hypothesized melanoidins instead of phenolic compounds were responsible for the increase of AA.

3.4. Spectroscopic Analysis and Yields of HMw

HMw samples were subjected to spectroscopic analysis to determine if further ultrafiltration or purification was needed. $K_{mix\ 420nm}$ values of HMw were all over 8.9 times as much as those of LMw (Table 4). Spectra of HMw (Figure 3) were very similar to those of melanoidins in ZAV prepared by Xu et al. (2007), and showed the characteristics of melanoidins (Clark & Sr., 1970; Hofmann, 1998), which are featureless end absorption and becoming more intense as the wavelength decreases. Hence, the performed preparation was effective and we referred the HMw samples as high-purity melanoidins.

A continuous increase in HMw yield was with the progressive decoction, which was in accordance with the rise of A420 (Table 1), and also with previous studies

revealing the accumulation of melanoidins during heat treatment in coffee (Bekedam et al., 2008a; Del Pino-García, 2012; Vignoli et al., 2014), and in Canadian honey (Brudzynski & Miotto, 2011). Moreover, the rise in HMw yield supported the view that the increasing melanoidins was responsible for the increase in AA of ZAV when subjected to the laboratory-scale decoction.

Undecocted vinegar yielded 12.1% HMw. Unlike HMw out of green coffee (Bekedam et al., 2008a), the HMw of undecocted vinegar exhibited the highest $K_{mix\ 420nm}$ value of 0.51 and its UV-VIS spectrum (Figure 3) showed melanoidin characteristics, which indicated that the HMw of undecocted vinegar was also melanoidins instead of other macromolecules. These melanoidins of undecocted vinegar could originate from Parched Rice. Parched Rice is thick liquid produced by parching white rice into black at about 800 °C and then dissolving the black rice in boiled water to boil for over 10 min. Before leaching procedure, Parched Rice was added into vinegar substrate. Since no additive except Parched Rice is heat-treated and no procedure before decoction encompasses heat treatment that brings brown colour, we speculated the added Parched Rice could be the source of melanoidins in undecocted vinegar. Therefore, we proposed two main kinds of ZAV melanoidins: one is brought by Parched Rice and the other is newly formed during decoction.

In line with tendency of the A420 variation shown in Figure 3, $K_{mix\ 420nm}$ of HMw (Table 4) mildly decreased as the vinegar samples were decocted for longer. This was

unlikely to result from the decrease in melanoidin level of tested HMw, because the UV-VIS spectra (Figure 3) of HMw prepared from all vinegar samples present clear characteristics of melanoidins. Instead, it seemed responsible for the mild decrease in $K_{mix\ 420nm}$ of HMW that melanoidins newly formed through the decoction naturally possess lower $K_{mix\ 420nm}$ value than melanoidins brought by Parched Rice do.

3.5. The Contribution of HMw to Antioxidative Activity of ZAV

Although CAA with *in vitro* digestion provides more biologically real results compared with chemical assays, yet more variable results come along since cell response is more complex (López-Alarcón & Denicola, 2013). Thus, we employed DPPH and ORAC assay to distinguish the difference among the AA of HMw and to calculate their contributions to AA of vinegar.

Prior to determination, we investigated the presence of unbound phenolic compounds in HMw and only a free phenolic content less than 0.93% of that in corresponding vinegar sample could be found. Hence, we considered the isolation process using ultrafiltration to be efficient and it was reasonable to attribute the observed AA of HMw to the HMw themselves instead of any unbound residual from vinegars.

While undecocted vinegar was heated to boil, DPPH radical scavenging activity and ORAC value of HMw (Table 5) both declined. However, during slight boiling for up to 60 min, both rose. The opposite tendency of AA of HMw occurred during

decoction could depend on which was dominating the variation of AA, the incorporation of low molecular weight compounds in melanoidins or the thermal degradation of melanoidins. In coffee, incorporation of phenolic compounds in melanoidins occurs continuously upon heat treatment and is a significant pathway of phenolic compounds degradation (Bekedam et al., 2008a; Coelho et al., 2014), and melanoidin-bound compounds of low molecular weight account for more than 50% of the AA of melanoidins (Delgado-Andrade et al., 2005). Similar situation might appear in ZAV. Nevertheless, heat treatment also causes thermal degradation of melanoidins and a reduction in the AA of melanoidin-rich fraction (Bekedam et al., 2008a; Brudzynski & Miotto, 2011), which is favoured when high concentration of formed melanoidins exists (Brudzynski & Miotto, 2011). As for the vinegar samples analysed in the present study, since they contained formed melanoidins brought by Parched Rice, degradation of melanoidins could dominate while undecocted vinegar was heated to boil and result in the reduction in the AA of HMw. Then, the incorporation of low molecular weight compounds, especially phenolic compounds, in newly formed melanoidins prevailed and increased the AA of HMw. Further study should shed light on the structure change of melanoidins during decoction.

Similar to the way the contribution of melanoidins to the AA of coffee (Cristina, et al., 2005; Del Pino-García et al., 2012; Rufián-Henares & Morales, 2007) and Spanish diet (Pastoriza & Rufián-Henares, 2014) was estimated, the absolute and relative contribution of HMw (Table 5) to the AA of vinegar was calculated on

grounds of the AA of HMw (Table 5), the HMw yields (Table 4) and the AA of vinegars (Figure 2). Despite the facts that the AA of HMw declined while undecocted vinegar was heated to boil, and that DPPH radical scavenging activity of HMw significantly decreased after decoction, results of DPPH and ORAC assay were in good agreement that the absolute contribution of HMw continuously rose during the laboratory-scale decoction. In addition, according to the relative contribution of HMw to AA of vinegar (Table 5), DPPH and ORAC assay both demonstrated that even though AA of vinegar rose with the progressive decoction, melanoidins still contributed significantly larger part of AA of vinegar after the decoction. These observations proved that increasing melanoidins were responsible for the increase in AA of vinegar during the laboratory-scale decoction. In fact, melanoidins accounted for 57.1% and 44.1%, according to DPPH and ORAC assay respectively, of AA of 60-min boiled vinegar, which revealed that melanoidins were main contributor to the AA of vinegar after the laboratory-scale decoction. Analogously, Maillard reaction products, mostly melanoidins, have been also reported as the principle contributor to the AA of coffee brews (Liu & Kitts, 2011), and the relative contribution of melanoidins to the AA of coffee increased after heat treatment (Smrke et al., 2013). However, it is noteworthy that only a modest contribution of melanoidins to the AA of coffee was found by other authors (Del Pino-García, 2012; Delgado-Andrade, 2005), and Vignoli et al. (2011) concluded that AA of coffee depended more upon the initial coffee composition, such as concentration of phenolic compounds and melanoidins in

the raw materials, than heat-treatment effect. Therefore, as for ZAV, the role of added Parched Rice and Raw Vinegar (RV) composition and the synergy or antagonism between melanoidins and phenolic compounds will be further studied.

4. Conclusion

Along with DPPH and ORAC assay, Cellular Antioxidant Activity (CAA) assay with *In vitro* digestion was applied for the first time to clarify the effect of decoction on the AA of ZAV in a laboratory scale. AA of ZAV increased significantly after the decoction. The present study further proved that during decoction phenolic content decreased, while melanoidins, a kind of antioxidants recently found in ZAV, were generated. Increased melanoidin content was responsible for the rise in AA of ZAV. The decoction induced remarkable rises in both the absolute and relative contribution of melanoidins to the total AA of vinegar. It suggested that production of melanoidins during decoction is main contributor to the AA of ZAV. We also proposed that ZAV melanoidins went through both degradation and formation during decoction, which calls for more elucidation of how the structure of melanoidins changes with decoction.

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Table 1

Total solids, pH, titratable acidity, A420 and phenolic contents of undecocted and decocted vinegars^a

	total solids (g/100mL)	pH	titratable acidity (% w/v)	A420 (diluted 1:25)	phenolic content ^b (mg GA/mL)
undecocted	17.4±0.1a	3.59±0.01a	5.12±0.06a	0.748±0.008d	0.426±0.005a
0-min boiled	17.6±0.4a	3.56±0.02b	5.20±0.16a	0.824±0.018c	0.416±0.010ab
30-min boiled	17.8±0.4a	3.56±0.01b	5.23±0.13a	0.940±0.021b	0.412±0.009ab
60-min boiled	17.9±0.4a	3.55±0.01b	5.31±0.14a	1.076±0.009a	0.407±0.008b

^a0-min boiled, 30-min boiled and 60-min boiled represent decocted vinegars which were heated to be slight boiling for 0, 30 and 60 min, respectively. Different letters in the same column indicate that samples are significantly different ($p < 0.05$). Data are presented as mean $\pm$ SD ($n = 3$).

^bPhenolic content was measured by peroxidase-catalysed enzymatic method after removing high molecular weight materials including melanoidins. (Perrone et al., 2012) GA: gallic acid.

Table 2

Cellular antioxidant activity (CAA) of vinegar samples with digestion^a

	EC ₅₀ (mg/mL) ^b	CAA(μM QE/g) ^c	cytotoxicity(mg/mL) ^d	CAA(μmol QE/mL) ^e
undecocted	3.76±0.87ab	1.33±0.24bc	>6.67	0.24±0.06bc
0-min boiled	4.45±0.97a	1.11±0.23c	>6.67	0.20±0.05c
30-min boiled	3.01±0.48bc	1.62±0.16ab	>13.33	0.29±0.05ab
60-min boiled	2.44±0.16c	1.97±0.07a	>13.33	0.36±0.02a

^a Vinegars were lyophilized to allow CAA values to be determined at concentrations without cytotoxicity against HepG2 cells as reported by the previous study (Seki, 2008). Lyophilized vinegar samples were subjected to *in vitro* digestion and subsequent CAA assay. Different letters in the same column indicate that samples are significantly different ($p < 0.05$). Data are presented as mean $\pm$ SD ($n = 3$).

^b EC₅₀: median effective doses of lyophilized vinegar samples.

^c QE: quercetin equivalents. CAA values are expressed as μmol of QE/g of lyophilized vinegar sample.

^d Cytotoxic concentrations of lyophilized vinegar samples.

^e CAA values of vinegars before lyophilisation were calculated by multiplying CAA values of lyophilized counterpart by lyophilized vinegar yields (Table S1) and expressed as μmol of QE/mL of vinegar before lyophilisation.

Table 3Phenolic contents determined by HPLC^{ab}

	Undecocted	0-min boiled	30-min boiled	60-min boiled
gallic acid	30.67±0.89b	29.64±1.92b	38.50±2.63a	27.39±2.24b
protocatechuic acid	2.37±0.21b	1.96±0.21b	3.46±.23a	3.37±0.34a
catechin	23.08±3.25a	12.71±1.10b	6.62±0.58c	1.00±0.37d
chlorogenic acid	8.06±0.31a	6.78±0.35b	6.57±0.37b	5.74±0.31c
vanillic acid	2.92±0.13a	1.20±0.14c	2.21±0.11b	1.40±0.09c
<i>p</i> -hydroxybenzoic acid	43.62±2.78a	29.65±1.38b	18.98±0.98c	5.67±0.20d
ferulic acid	3.29±0.10c	4.45±0.23b	3.39±0.39c	6.15±0.37a
rutin	ND ^c	ND	ND	6.21±0.83
total content	114.05±6.62a	86.42±5.13b	79.76±5.27b	56.95±4.47c

^aDifferent letters in the same row indicate that samples are significantly different ($p < 0.05$). Phenolic contents were determined by HPLC after removing high molecular weight materials including melanoidins. (Perrone et al., 2012) Data are expressed as mean $\pm$ SD ($n = 3$).

^bPhenolic contents are expressed as mg phenolic compounds/L of vinegar sample.

^cNot detected.

Table 4

$K_{\text{mix } 420\text{nm}}$ and HMw yields^a

	$K_{\text{mix } 420\text{nm}}$ of HMw (L cm ⁻¹ g ⁻¹)	$K_{\text{mix } 420\text{nm}}$ of LMw (L cm ⁻¹ g ⁻¹)	HMw yields ^b (%, w/w)
Undecocted	0.51±0.01a	0.030±0.001c	12.1±0.9c
0-min boiled	0.49±0.01b	0.030±0.002c	16.8±1.5b
30-min boiled	0.46±0.01c	0.041±0.002b	19.0±2.4b
60-min boiled	0.44±0.01d	0.049±0.003a	32.6±3.1a

^aHMw: high molecular weight materials. LMw: low molecular weight materials. The specific extinction coefficient (K_{mix}) was introduced to quantify melanoidin level according to Bekedam et al., (2006). Different letters in the same column indicate that samples are significantly different ($p < 0.05$). Data are presented as mean ± SD ($n = 3$).

$K_{\text{mix } 420\text{nm}}$ values of HMw were all over 8.9 times as much as those of LMw.

^bOn the basis of total solids of the vinegar sample.

Table 5

antioxidative activity (AA) of HMw and their contributions to AA of vinegar
according to DPPH and ORAC assay^a

	antioxidative activity of HMw		absolute contribution ^b		relative contribution ^c	
	DPPH	ORAC	DPPH	ORAC	DPPH	ORAC
	(mg Vc/g)	(μ mol Trolox/g)	(mg Vc/mL)	(μ mol Trolox/mL)	(%)	(%)
Undecocted	26.63 $\pm$ 0.79a	94.2 $\pm$ 6.9c	0.56 $\pm$ 0.03c	1.98 $\pm$ 0.08d	37.1 $\pm$ 2.4b	15.2 $\pm$ 0.8c
0-min boiled	20.60 $\pm$ 1.01c	83.2 $\pm$ 4.6bc	0.61 $\pm$ 0.04c	2.47 $\pm$ 0.13c	36.3 $\pm$ 1.7b	17.9 $\pm$ 1.7c
30-min boiled	23.37 $\pm$ 1.30b	102.4 $\pm$ 6.2b	0.79 $\pm$ 0.08b	3.45 $\pm$ 0.31b	41.0 $\pm$ 3.1b	23.5 $\pm$ 2.0b
60-min boiled	24.14 $\pm$ 0.95b	122.9 $\pm$ 10.5a	1.41 $\pm$ 0.10a	7.15 $\pm$ 0.17a	57.1 $\pm$ 3.7a	44.1 $\pm$ 3.2a

^aDifferent letters in the same column indicate that samples are significantly different

($p < 0.05$). Data are presented as mean $\pm$ SD ($n = 3$). HMw: high molecular weight materials. Vc: Vitamin C.

^bAbsolute contribution is the AA resulted from melanoidins expressed as mg of Vc equivalents or μ M of Trolox equivalents/mL of vinegar sample.

^cRelative contribution is the ratio of the AA resulted from melanoidins to the AA of the corresponding vinegar sample.

Figure caption

Figure 1 Temperature-rise procedures of laboratory-scale decoction (n=3, SD< 3 °C) and of two typical decoction procedures in factory. The vinegar subjected to typical factory procedure 1 is heated by all steam heaters, therefore its temperature rises quickly. In contrast, typical factory procedure 2 only employs a proportion of steam heaters but coupled with a preheat system. Hence the temperature-rise procedure is slow but starts at a higher temperature. The temperature-rise rate of procedure in lab is between those of typical factory procedure 1 and 2. As for all decoction, the rate of temperature rise after 90 °C is lowered and slight boiling was maintained for 60 min at least.

Figure 2 DPPH scavenging activity (A) and ORAC (B) of vinegar samples. Vertical bars with different letters indicate significant differences ($p < 0.05$). Theoretical concentration effect imposed by decoction was calculated in proportion to the total solids (Table 1) of each sample and is represented by white bars. Vc: Vitamin C.

Figure 3 UV–VIS spectra of high molecular weight materials (HMw) prepared from vinegar samples. All spectra are very similar to those of melanoidins in ZAV prepared

by Xu et al. (2007), and show the characteristic of melanoidins (Clark & Sr., 1970; Hofmann, 1998), which are featureless end absorption and becoming more intense as the wavelength decreased.

Figure 1

Figure 2

Figure 2

Figure 3