Changes in the soluble and insoluble compounds of shelf-stable orange juice in relation to non- enzymatic browning during storage

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26 **ABSTRACT**

27 For the first time in literature, this study revealed the participation of polymeric components of orange
28 juice cloud and pulp (such as proteins, arabinogalactan proteins, or protein-pectin complexes) during non-
29 enzymatic browning. In a quest to better understand the non-enzymatic browning of shelf-stable orange
30 juice during storage, the juice was fractionated into different fractions depending on the solubility in
31 water/ethanol and the obtained fractions were characterized. The results showed that brown compounds
32 formed during storage of orange juice distributed over water insoluble (pulp), ethanol insoluble (cloud), and
33 ethanol soluble (serum) fractions. In the ethanol insoluble fraction, the brown compounds are hypothesized
34 to be associated with proteins, arabinogalactan proteins and/or protein-pectin complexes of this fraction
35 without significantly changing their molecular weight distribution, monosaccharide composition, and protein
36 content. The changes in the ethanol soluble fraction including ascorbic acid degradation, acid-catalyzed
37 hydrolysis of sucrose, and formation of furfural and 5-hydroxymethylfurfural were highly correlated to the
38 browning development of the juice during storage.

39 **KEYWORDS**

40 Non-enzymatic browning; orange juice; storage; fractionations, soluble compounds, insoluble compounds

41 INTRODUCTION

42 Orange juice is one of the most popular and widely consumed juices in the world owing to its attractive
43 color, refreshing taste, and high nutritional value. Among the quality attributes of orange juice appreciated
44 by consumers, its bright orange color has been highlighted as largely influencing consumer acceptance.¹
45 Unfortunately, upon storage, an unacceptable brownish color appears which limits the product's shelf-life
46 and negatively influences consumers' purchasing behavior.² In shelf-stable orange juice, the formation of a
47 brown color during storage is mainly attributed to non-enzymatic browning (NEB).³⁻⁵ Besides orange juice,
48 NEB is also known to be a main cause of color deterioration during storage of other shelf-stable fruit juices
49 such as lemon juice, grapefruit juice, strawberry juice, mango juice, and apple juice.⁶⁻¹⁰

50 Non-enzymatic browning in orange juice has been proposed in literature to involve multiple pathways,
51 namely ascorbic acid (AA) degradation, acid-catalyzed sugar degradation, and Maillard-associated
52 reactions.^{3,11-14} Most studies have attempted to quantitatively investigate the changes in NEB precursors
53 such as AA, glucose, and fructose or the formation of NEB intermediates like furfural,
54 5-hydroxymethylfurfural (HMF) and their relationship to browning during storage.^{5,13,15,16} Nonetheless, the
55 mechanisms of NEB are rather complex and have not been fully elucidated. Although the chemical structures
56 and the reaction pathways leading to various chromophores in model system of amino acids and pentoses
57 or hexoses have been described¹⁷, this classical Maillard reaction type is reported to be of minor importance
58 in citrus juice browning (including orange juice) during storage because of the high acidity involved.¹⁵

59 Orange juice is a complex system consisting of a serum phase (e.g. organic acids, sugars), and cloud and
60 pulp (e.g. proteins, polysaccharides) which have different solubility in water and/or ethanol thereby enabling
61 the separation of orange juice into different fractions.^{18,19} Although all these fractions are simultaneously
62 present in the orange juice, only the soluble compounds present in the serum such as AA and sugars have
63 received attention so far in studies related to NEB in citrus juices.^{3,5,12} On the other hand, the contribution of
64 insoluble compounds present in the cloud and pulp to the browning has not been investigated yet.
65 Nevertheless, in other food matrices such as coffee brew, brown compounds (i.e. melanoidines) are
66 commonly found to be associated with polymers including polysaccharides and proteins.²⁰ Additionally, it
67 has also been proposed that the brown melanoidins might be formed by the crosslinking of low molecular

weight chromophores and high molecular weight colorless proteins.²¹ Therefore, we hypothesize that the brown compounds which are formed in pasteurized shelf-stable orange juice during storage might also be associated with naturally occurring polymers in the juice cloud and pulp. In addition, although the composition of orange juice cloud has been well documented in literature,^{19,22,23} its changes during storage are not studied yet. Hence, it was of interest to investigate the changes in the insoluble (cloud and pulp) and soluble (serum) compounds of orange juice in relation to NEB during storage.

The main objective of this study was to gain a better understanding of NEB of orange juice during storage through simultaneously (i) investigating the distribution of brown compounds over different fractions of orange juice obtained through (stepwise-)fractionation and (ii) characterizing the changes in insoluble and soluble compounds in relation to NEB. For these purposes, pasteurized shelf-stable orange juice was stored at an elevated temperature (42 °C) for varying times. The non-stored and stored orange juice was fractionated into water/ethanol insoluble and soluble fractions. The insoluble compounds were characterized for their molecular weight distribution, monosaccharide composition, and protein content, and changes in the soluble compounds such as AA, sugars, furfural, and HMF with storage time were quantified as well.

MATERIALS AND METHODS

Materials

A single batch of freshly produced single strength shelf-stable orange juice (Minute Maid®) filled in 60 polyethylene terephthalate (PET) bottles of 330 mL was used in this study. The juice was reconstituted from concentrate. pH and total soluble solid content (°Brix) of the orange juice were 3.9 and 11.3, respectively, which remained unchanged during storage.

Storage and sampling of the orange juice

To generate samples at different storage moments in a short timeframe, an elevated temperature of 42 °C was applied for storage in the present study. This temperature was selected since previous work¹³ has confirmed that storage of shelf-stable single strength orange juice at 42 °C only accelerates the NEB process without including new reaction pathways. The orange juice was stored for 15 weeks at 42 °C in an incubator

protected from light. Sampling was done prior to the storage and at three different storage moments (5, 10, and 15 weeks). At each sampling moment, 15 orange juice bottles were randomly taken from the incubators, mixed, and uniformly divided into smaller portions. These tubes were frozen in liquid nitrogen and stored at -40 °C until use. Prior to analysis, samples were thawed in a circulating water bath at 20 °C for a standardized time and vortexed.

Color measurement

Color of the orange juice was measured using a Hunterlab ColorFlex® EZ colorimeter (D65 illuminant, 45°/ 0° geometry, and 10° observer angle, operating in a reflection mode) at room temperature. Prior to the measurement, the instrument was calibrated with a standard black and white ceramic tile. To make the measurements, a glass container was filled with 5 mL of sample, closed with a white ceramic plate and covered with a black cylinder. Color measurement was performed in triplicate with five readings for each sample. Color results were expressed as CIE $L^*a^*b^*$ value. The L^* value represents lightness, ranging from black (0) to white (100). The a^* and b^* values indicate color direction green ($-a^*$)/red ($+a^*$) and blue ($-b^*$)/ yellow ($+b^*$), respectively. These values were used to calculate the total color difference (ΔE^*) (Eq. (1)) which is a measure of the difference in color between the pasteurized stored sample and the pasteurized non-stored sample (i.e. a control sample, subscript 0 in Eq. (1)).

$$\Delta E^* = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (1)$$

Browning index measurement

Browning index (BI) of the orange juice was determined using a spectrophotometric method described by Klim & Nagy²⁴ with slight modifications. The juice was centrifuged at 1000xg, 4 °C for 15 min (J2-HS centrifuge, Beckman, Brea, CA, US) and the supernatant was collected. Subsequently, the supernatant was mixed with 95% ethanol in a ratio 1:1, and the obtained solution was placed in an ice bath for 15 min for flocculation, followed by a second centrifugation step (1000xg, 4 °C, and 15 min). The supernatant was filtered (0.45 µm, hydrophilic) and the absorbance was measured at 420 nm using a spectrophotometer.

Fractionation of the ethanol soluble and ethanol insoluble fraction of orange juice

Orange juice is comprised of serum, cloud, and pulp. While the orange juice pulp consists of the large particles ($> 2 \mu\text{m}$) that tend to settle upon storage and are pelletable under a mild centrifugation¹⁸, the orange juice cloud can be separated from the serum based on their difference in ethanol solubility. Therefore, centrifugation and ethanol precipitation (at 70% ethanol) were used to separate these three fractions of orange juice^{25–27}. Furthermore, the orange juice cloud (ethanol insoluble fraction) is rich in polymers such as pectin, proteins, celluloses, and hemicelluloses^{18,19} which may be selectively precipitated at different ethanol concentrations.²⁶ Hence, a stepwise ethanol precipitation procedure with increasing ethanol concentration was carried out to further fractionate the ethanol insoluble fraction into different sub-fractions.

Ethanol precipitation

Centrifugation and ethanol precipitation were applied to separate the serum, cloud, and pulp fractions. A schematic overview of this step is shown in **Figure 2A** in the results and discussion section. Specifically, 200 mL of the juice was mildly centrifuged (1000xg, 4 °C, and 10 min) (J2-HS centrifuge, Beckman, Brea, CA, US) to separate the large cell wall fragments (i.e. orange juice pulp) from the supernatant which contains orange juice serum and cloud. The pellet was washed with ultrapure water, centrifuged (10000xg, 4 °C, and 15 min), suspended in ultrapure water, and lyophilized. To the supernatant, absolute ethanol was added under continuous stirring until an ethanol concentration of 70% (v/v) was reached. The mixture was kept overnight in centrifuge bottles at 4 °C for precipitation. The precipitation conditions (70% ethanol and 4 °C) were chosen to maximize the amount of precipitated polymers. The mixture was centrifuged (10000xg, 4 °C, and 15 min), and the ethanol precipitated material was washed with 70% (v/v) ethanol solution, centrifuged (10000xg, 4 °C, and 15 min), dissolved in ultrapure water, and lyophilized resulting in a 70% ethanol precipitated fraction (EP). The supernatant was subjected to a solvent evaporation using a rotavapor at 40 °C to remove ethanol yielding an ethanol soluble fraction (ES). In addition, carotenoids were removed from all the fractions extracting with a solvent mixture containing hexane, ethanol, and acetone (2:1:1 (v/v/v)). After that they were lyophilized yielding three fractions, namely the pellet, the EP, and the ES which were stored in a desiccator until further use or analysis.

Stepwise ethanol precipitation

Since different types of polymers (polysaccharides and proteins) may be precipitated at different ethanol concentrations, stepwise ethanol precipitation was used to further fractionate the 70% ethanol precipitated fraction (EP) following a procedure adapted from Bekedam et al.²⁸ Readers are referred to **Figure 2B** in the results and discussion section for a visualization of this step. The lyophilized EP was dissolved overnight in ultrapure water, then absolute ethanol was slowly added until an ethanol concentration of 20% was reached. The solution was kept for precipitation and then centrifuged (10000xg, 4 °C, and 15 min) (J2-HS centrifuge, Beckman, Brea, CA, US) to separate the supernatant and the residue. The residue was washed with 70% ethanol and centrifuged (10000xg, 4 °C, and 15 min) while the supernatant was subjected to further ethanol precipitation in which the ethanol concentration was stepwise increased to 40 and 60% following the same procedure. Ethanol was removed by evaporation and the obtained fractions were lyophilized yielding EP20, EP40 and EP60 for the materials that precipitated at 20, 40 and 60% ethanol, respectively and ES60 for the soluble material at 60% ethanol. All the lyophilized fractions were stored in a desiccator until further analysis.

Characterization of the insoluble compounds

Molecular weight distribution

The molecular weight distribution of the samples was analyzed based on the work of Shpigelman et al.²⁹ High performance size exclusion chromatography was performed on a series of three columns with exclusion limits of 8×10^4 , 4×10^6 , and 1×10^7 g mol⁻¹, respectively, in combination with a guard column. Elution took place at 35 °C with 0.1 M acetic acid in 0.1 M NaCl (pH 4.4) at 0.5 mL min⁻¹. The eluent was monitored by refractive index detection in combination with light scattering detection, and the absorbance was measured at 280 nm by diode array detection in the Agilent 1200 system. Lyophilized samples were dissolved overnight in eluent buffer and filtered (0.45 µm, hydrophilic) prior to injection (100 µL). All the samples were analyzed in duplicate.

Monosaccharide composition analysis

Monosaccharides including neutral sugars and galacturonic acid were determined using high performance anion exchange chromatography combined with pulsed amperometric detection following the

171 method described previously³⁰ with some modifications. For hydrolysis of polysaccharides to
172 monosaccharides, 1 mg of the lyophilized sample was dissolved overnight in 1 mL ultrapure water and 80 μ L
173 was dried in a test tube under a nitrogen evaporator at 45 °C. Next, 2 mL of 2M methanolic HCl was added
174 to the tube and gently mixed, then the methanolysis was carried out at 80 °C for 16 h in an oil bath. After
175 cooling to ambient temperature, the samples were dried at 30 °C under a nitrogen evaporator. Subsequently,
176 2 mL of 2M trifluoroacetic acid (TFA) was added to the samples and acid hydrolysis was done incubating the
177 samples at 121 °C for 1 h. The samples were cooled to ambient temperature and dried under a nitrogen
178 evaporator at 45 °C to remove TFA. Dried samples were dissolved in 400 μ L of ultrapure water and filtered
179 (0.45 μ m, hydrophilic) into dark brown vials with glass inserts for the analysis. The hydrolysis procedure was
180 performed in duplicate.

181 The determination of monosaccharides was performed on a HPLC (Dionex, Sunnyvale, CA, USA) equipped
182 with a CarboPacTM PA20 column, a CarboPac PA20TM guard column and an ED50 electrochemical detector.
183 The system was equilibrated for 5 min using 100 mM NaOH and for additional 5 min using 0.5 or 25 mM
184 NaOH depending on the eluent concentration used during sample elution. Samples (10 μ L) were injected and
185 eluted at a flow rate of 0.5 mL min⁻¹ with eluent concentration of 0.5 mM in order to detect fucose, rhamnose,
186 arabinose, galactose, glucose, xylose, mannose, and galacturonic acid. The analysis of the samples was
187 repeated using a 25 mM eluent concentration to obtain a better peak resolution for rhamnose and arabinose.
188 Commercial neutral sugar standards at different concentrations were used as external standards for
189 identification and quantification.

190 ***Protein analysis***

191 Total nitrogen content of the samples was determined by the Dumas method using an EA 1108 elemental
192 analyzer. Protein content in the sample was calculated from the nitrogen content using a nitrogen-to-protein
193 conversion factor of 6.25.³¹ All samples were analyzed in duplicate.

Analysis of the soluble compounds related to non-enzymatic browning

Determination of the ascorbic acid and dehydroascorbic acid content

The determination of the ascorbic acid (AA) and dehydroascorbic acid (DHAA) content was performed following a method proposed by Wibowo et al.¹³ Sample was mixed with extraction buffer (1% (w/v) m-phosphoric acid with 0.5% (w/v) oxalic acid, pH 2.0) in a ratio 1:3 (v/v). Subsequently, the mixture was centrifuged (24000xg, 4 °C, and 15 min) (J2-HS centrifuge, Beckman, Brea, CA, US). pH of the obtained supernatant was adjusted to 3.5 using 1M NaOH or 1M HCl. To determine the AA content, phosphate buffer (20 mM NaH₂PO₄ + 1 mM Na₂EDTA, pH 3.5) was added to part of the pH-adjusted supernatant in a ratio 2:1 and the mixture was filtered (0.45 µm, hydrophilic) prior to the HPLC analysis. The DHAA content was quantified indirectly after reduction to AA by adding a reducing agent (TCEP 2.5 mM tris (2-carboxyl-ethyl) phosphine in phosphate buffer, pH 3.5) to the other part of the pH-adjusted supernatant in a ratio 2:1 (v/v). The mixture was centrifuged (19900xg, 23 °C, and 15 min) (Microfuge 22R, Beckman Coulter) and filtered (0.45 µm, hydrophilic).

The analysis of AA was performed in a HPLC (Dionex, Sunnyvale, CA, USA) with an AD25 UV-Vis detection at 245 nm. A prevail C18 column (250x4.6 mm, 5 µm particle size) with a corresponding guard column was used for chromatographic separation. The mobile phase was a solution of 1 mM Na₂EDTA and 10 mM CH₃COONH₄ and an isocratic elution at 0.8 mL min⁻¹ and 25 °C was performed. The injection volume was 25 µL. Quantification was carried out using a calibration curve of AA in extraction buffer.

Determination of the sugar content

Sugar extraction and HPLC analysis were performed following the method of Vervoort et al.³² with slight modifications. The sugar extraction was carried out in triplicate adding 50 µL of Carrez I (15% w/v K₄[Fe(CN)₆]) and Carrez II (30% w/v ZnSO₄) to 1 mL of sample. The mixture was vortexed, kept for 30 min (room temperature), and centrifuged (19900xg, 4 °C, and 15 min) (Microfuge 22R, Beckman Coulter). The supernatant was filtered (0.45 µm, hydrophilic) and 2 µL of a 10-fold dilution of the filtrate was injected into the HPLC system. The analysis was performed using a HPLC (Agilent 1200 series, Santa Clare, CA, USA) with evaporative light scattering detection. Separation was carried out on a Prevail C18 column (250x4.6 mm, 5 µm particle size), coupled to a Prevail C18 guard cartridge (7.5x4.6 mm, 5 µm particle size) at 30 °C using

isocratic elution with 75% (v/v) acetonitrile/water at 1 mL min⁻¹. Sugar standards (fructose, glucose, and sucrose) were used for identification based on retention time, and standard curves were prepared for quantification.

Determination of furfural and HMF content

Furfural and HMF were analyzed based on the previously described method^{13,32} with some modifications. The extraction of furfural and HMF was performed in triplicate mixing 500 µL of each Carrez I reagent and Carrez II reagent with 10 mL of the sample. Subsequently, the sample was kept at room temperature for 30 min and centrifuged (24000xg, 4 °C and 15 min) (J2-HS centrifuge, Beckman, Brea, CA, US). After that, 1 mL of the supernatant was applied on a C18 SPE pre-column (Sep-PAK Water, Milford, USA), preconditioned with 2 mL methanol and 5 mL 0.5% acetic acid. Subsequently, 2 mL of ultra-pure water was used to wash the SPE column, then 4.5 mL ethyl acetate was applied on the column to elute furfural and HMF, and the resulting mixture was dried with anhydrous sodium sulphate. The eluate was filtered (0.45 µm, hydrophilic) and the volume was adjusted to 5 mL with ethyl acetate. The chromatographic analysis was carried out in a HPLC system (Agilent 1200 series, Santa Clare, CA, USA) with a UV-Vis detection. The separation was done using a Zorbax Eclipse XDB C18 column (150×4.6 mm, 5 µm particle size) coupled to a Prevail C18 guard cartridge, at 25 °C and acetonitrile/water 5/95 (v/v) as mobile phase at 1 mL min⁻¹ isocratic elution. Furfural was detected at 277 nm and HMF was detected at 285 nm. Furfural and HMF standards were used for identification and standard curves were established for quantification.

Data analysis

One-way ANOVA using JMP statistical software (JMP Pro 14.0.0, SAS Institute Inc.) was performed to determine the statistical significance of duplicate means. When the mean values of two groups (i.e. between the non-stored sample and 15 week-stored samples) were compared, a student's t-test was applied, while a post-hoc test using Tukey's honest significant difference (HSD) was used to compare the mean values of more than two groups (i.e. storage times).

245 RESULTS AND DISCUSSION

246 Color changes and browning development of orange juice during storage

247 Overall color changes of the orange juice during storage were assessed objectively using a colorimeter.
248 The total color difference (ΔE^*) was calculated based on the differences in the color parameters between
249 the non-stored and stored juice using equation (1). In addition, the development of browning was specifically
250 evaluated based on the browning index (BI) which is the measurement of the absorbance at 420 nm after
251 ethanol extraction. The changes in ΔE^* and BI of the pasteurized shelf-stable orange juice as a function of
252 storage time are described in **Figure 1A** and **Figure 1B**, respectively.

253 It is clear from the results that both ΔE^* and BI increased significantly during storage. The increase in ΔE^*
254 during storage is an indicator of the overall color changes of the juice, which can be caused by NEB reactions
255 and other reactions such as the degradation of natural pigments (i.e. carotenoids). On the other hand, the
256 rise in BI is mainly due to the formation and accumulation of brown compounds during storage. A strong
257 correlation ($r = 0.99$) was found between BI and ΔE^* verifying the fact that the formation of brown
258 compounds has an important contribution to the overall color changes of the pasteurized shelf-stable orange
259 juice during storage.

260 Fractionation of orange juice into pulp, cloud, and serum

261 After centrifugation and 70% ethanol precipitation, three different fractions namely the pellet, the
262 ethanol precipitated fraction (EP), and the ethanol soluble fraction (ES) were obtained and carotenoids were
263 removed from these fractions (**Figure 2A**). Freeze-dried, washed pellet and EP represented 267 ± 9 and
264 363 ± 14 mg per 100 mL of the non- stored orange juice, respectively, which was similar to the results of
265 these two fractions (247 ± 0 and 361 ± 17 mg per 100 mL of the juice, respectively) obtained from the 15
266 week-stored juice. This indicates that the amount of precipitated pulp and cloud did not change with storage
267 time.

268 From **Figure 2A**, it can be seen clearly that the pellet, EP, and ES fraction of the 15 week-stored orange
269 juice after removing carotenoids were brown compared to the white or yellow-like color of those from the
270 non-stored juice. This observation reveals that the brown compounds formed during storage of the orange

juice distributed over water/ethanol insoluble and ethanol soluble fractions. It has been stated that orange juice pulp and cloud are rich in cell wall polysaccharides and proteins.^{18,19,23} Hence, the brown compounds present in the pellet and EP fractions might be linked to water/ethanol insoluble polysaccharides and proteins, while in the ES fraction, the brown compounds are possibly present in a free state. However, it cannot be excluded that some of the brown compounds are long chain polymers that were also precipitated together with natural occurring polymers of the juice during the ethanol precipitation step.

It has been intensively reported in literature that the degradation products from ascorbic acid and/or sugars may polymerize in the presence or absence of amino acids to form brown compounds during storage of shelf-stable citrus juices including orange juice.^{3,4} However, to the best of our knowledge, none of the published data reveals the participation of the endogenous polymeric components of orange juice pulp and cloud towards the juice browning or their association with the brown compounds. As the EP fraction (after removing carotenoids) is mainly composed of polymers such as polysaccharides and proteins which may have different solubility in ethanol, we intended to further fractionate this fraction using stepwise ethanol precipitation with increasing concentration of ethanol in order to verify in which fractions brown compounds are mainly present.

Further fractionation of the ethanol insoluble fraction

The ethanol precipitated fractions (EP) of the 15 week-stored and non-stored orange juice samples were sub-fractionated with stepwise ethanol precipitation in which the concentration of ethanol was increased from 20% to 40% and then to 60% (**Figure 2B**). Applying this method, four fractions were obtained, 20% ethanol precipitated material (EP20), 40% ethanol precipitated material (EP40), 60% ethanol precipitated material (EP60), and 60% ethanol soluble material (ES60).

For both the non-stored and 15 week-stored samples, most of the fractions were obtained in a significant amount (higher than 15% w/w of starting material), except for the EP60 which only accounted for around 5% w/w of the starting material (EP). Although it can be noticed that all sub-fractions of the EP of 15 week-stored juice had a brown color, the EP20 and EP40 fractions had a much darker brown color, compared to EP60 and ES60 revealing that almost all the brown compounds from the EP fraction stayed in the EP20 and EP40 fractions which were precipitated at low ethanol concentrations (20 and 40%, respectively).

Characterization of the ethanol insoluble fraction and sub-fractions

The ethanol insoluble fraction (EP) (i.e. cloud) of orange juice, which contains mainly polysaccharides and proteins^{19,22,23}, also turned brown during storage. Therefore, it was of interest to verify whether the presence of brown compounds in this fraction would change its physicochemical characteristics such as molecular weight distribution, monosaccharide composition, and protein content. The characterization was made for the EP and its sub-fractions (EP20, EP40, EP60, and ES60) of the 15 week-stored orange juice in comparison to those of the non-stored juice.

Molecular weight distribution

To investigate the effect of the presence of brown compounds to the EP fraction on the size of polymers in these fractions, the molecular weight distribution of the EP from the 15 week-stored (EP 15W) and non-stored (EP 0W) juice were compared in **Figure 3**. Based on the concentration profile (dotted lines), the EP 15W fraction, which had a brown color, consisted of two polymer populations with retention times from 35.5 min to 47.5 min and from 47.5 min to 60.0 min having a weight average molecular weight (M_w) of 2670 and 139 kDa, respectively. The materials that were eluted after 60.0 min were considered as low molecular weight population. Similarly, the EP 0W fraction, which had a white color, also contained two polymer populations with comparable retention times and M_w and had similar concentration profile. These results revealed that the presence of brown compounds in the EP 15W fraction did not influence its molecular weight distribution. Possible explanations could be that the brown compounds are small in size, or that they account for a very small portion in the EP 15W fraction. Hence, their association to the large polymers in this fraction does not significantly affect the overall polymer size.

For the sub-fractions (EP20, EP40, EP60, and ES60) obtained from the EP 15W fraction, it was observed that the EP20 and EP40 fractions which precipitated at 20 and 40% ethanol, respectively, contained the large polymer populations of the EP 15W fraction (**Figure 4**). The EP60 and ES60 fractions possessed the small polymer population with a high concentration, especially the EP60. From the results, it can be concluded that the large polymers in orange juice cloud seem to precipitate at low ethanol concentrations while higher ethanol concentrations were required to precipitate the smaller polymers. In addition, there was insignificant difference in between sub-fractions of the EP 15W and EP 0W fraction (results not shown).

Monosaccharide composition

In **Table 1**, the monosaccharide composition of the ethanol insoluble fraction (EP) and its sub-fractions (EP20, EP40, EP60, and ES60) obtained from the 15 week-stored (15W) and non-stored (0W) orange juice are given. The main monosaccharide found in the EP fraction of both the non-stored and stored samples (EP 0W and EP 15W) was galacturonic acid followed by galactose, arabinose, and glucose. As galacturonic acid is the main monomer in pectin, the abundance of this uronic acid indicates that pectin is a predominant polysaccharide in EP 0W and EP 15W. According to Klavons et al.¹⁹ pectin accounted for approximately 4.5% of the orange juice cloud. These authors found that around 60% of the cloud pectin exists as previously soluble pectin that is associated with insoluble proteins, 25-30% as calcium pectate and 15% as protopectin.¹⁹ The presence of galactose and arabinose might be related to the hydrolysis of arabinogalactan and/or the side chains of pectin. The glucose residues might be a breakdown product of non-pectic polymers such as celluloses and hemicelluloses or residues of the soluble sugar in orange juice that is trapped in the ethanol insoluble material. According to the results, no significant difference ($p < 0.05$) can be observed for the monosaccharide composition of the EP fraction from the non-stored and 15 week-stored juice. This means that the presence of brown compounds in the EP fraction does not significantly alter its monosaccharide composition.

Among the sub-fractions of the EP material, their monosaccharide composition did considerably vary for both the non-stored and 15 week-stored samples (**Table 1**). Generally, the sub-fraction EP20 0W/15W contained the lowest total carbohydrate content (22-23%) and possessed only about 4% of galacturonic acid while this monosaccharide accounted for more than 20% of other sub-fractions precipitated at higher ethanol concentrations (40 and 60%). This observation revealed that for both 0W and 15W samples, pectin is more predominant among the polysaccharides in EP40 and EP60 while arabinogalactan is the most important polysaccharide in EP20. Bekedam et al.²⁰ have reported that arabinogalactan might be linked to the brown compounds in coffee brew in the form of arabinogalactan proteins. This possible linkage can also be valid for the brown compounds formed in orange juice during storage based on the intensive brown color of the EP20 fraction of 15 week-stored orange juice and its considerable content of arabinogalactan. However, the presence of brown compounds did not significantly alter the monosaccharide composition of

the sub-fractions when a comparison was made between the non-stored and 15 week-stored samples. The most possible reasons could be that those monosaccharides studied are not present in the structure of the brown compounds or the brown compounds just account for a small part of the polymer material.

Protein content

The protein content of the ethanol insoluble fraction (EP) and its sub-fractions is considered as a crude protein content (**Table 1**) since no corrections were made for nitrogen from the non-protein nitrogen containing compounds. The EP 0W possessed around 29% (w/w) of proteins which was significantly higher than the protein content of the EP 15W. These results are in accordance with the observation of UV absorbance at 280 nm (**Figure 3B**) which showed a higher absorbance peak of the small polymer population for the EP 0W fraction compared to that of the EP 15W fraction. According to Brat et al.¹⁸ 50% of the orange juice cloud's alcohol insoluble residues is made of proteins, and the rest is made of cell wall materials. Since proteins constitute less than 10% of primary cell walls, most of the proteins found in the orange juice cloud are of intracellular rather than cell wall origin.¹⁸ In addition, the authors also reported that proteins were mainly responsible for the nitrogen content of orange juice cloud.

After the sub-fractionation of the EP 15W fraction through the stepwise ethanol precipitation, it can be seen that the EP20 15W fraction had the highest protein content with almost 50% w/w of the lyophilized material, while the EP60 15W had the lowest protein content (only around 5% w/w). Similar distribution of proteins was observed for the sub-fractions from the non-stored samples (0W). The intensive brown color of the high protein containing fraction (EP20 15W) could probably be due to the linkage of brown compounds to proteins or protein-pectin complexes in this fraction.²¹ A strong correlation between the melanoidin content and protein content has been reported in coffee brew and the authors have concluded that proteins are incorporated into the melanoidins.²⁸

From the results of monosaccharide composition and protein content, it seems that the material which is rich in protein tends to precipitate at a low concentration of ethanol (20%) while a higher ethanol concentration (e.g. 40%) is required to be able to precipitate the material that is rich in cell wall polysaccharides. On the other hand, the presence of the brown compounds in the ethanol insoluble material seems not to have any significant influence on its monosaccharide composition and protein content. Possibly

the ratio of brown compounds to the insoluble material was probably relatively small, so that their presence did not make any significant change in these attributes as was also observed previously for the molecular weight distribution (**Figure 4B**). The EP 20 fraction which had the highest protein content seems to possess a high amount of brown compounds as it has the most intensive brown color compared to the other fractions. Based on this observation, it can be hypothesized that the brown compounds are linked to proteins, arabinogalactan proteins, or protein-pectin complexes in this fraction.

Changes in different soluble compounds related to non-enzymatic browning

It has been proposed in literature that NEB in citrus juices including orange juice involves the degradation of several reaction precursors such as AA and sugars, and formation of reaction intermediates namely furfural and HMF.^{3,10,13,16,33} Therefore, the changes of aforementioned NEB precursors and intermediates during storage were investigated and the obtained results were summarized in **Table 2**.

Ascorbic acid and dehydroascorbic acid content

During the accelerated storage at 42 °C, the AA content decreased by 52% after 5 weeks of storage and was almost zero at the end of storage (**Table 2**). It was reported in literature that storage of commercial orange juice at room temperature for 4 months caused a loss of 29% in the AA content.³⁴ The faster drop in the AA content observed in this study could be due to the high storage temperature used.^{13,35} In addition, Gómez Ruiz and co-workers³⁶ stated that AA degradation increased with temperature and oxygen concentration. The orange juice used in this study was packed in PET bottles which have a relatively high oxygen permeability resulting in a continuous diffusion of oxygen during storage thus affecting the degradation of AA³⁷. The first-order kinetic model is the most widely used and cited model in literature describing the degradation of AA in model systems and orange juice systems.^{13,36,38,39} The changes in AA content in this work was not kinetically modelled since the data points were not sufficient.

In the presence of oxygen, AA is primarily oxidized to DHAA. The DHAA content increased significantly ($p < 0.05$) during the first 10 weeks of storage, followed by a decrease to the end of storage (**Table 2**). The increase, followed by a decrease in the DHAA content, observed in this study, is consistent with the results reported previously⁴⁰ for the changes in DHAA content of orange juice packed in materials with different

oxygen permeability. Furthermore, it could be observed that the increase in the DHAA content was much smaller than the decrease in AA content. For instance, while the AA content decreased from $234.40 \pm 3.16 \text{ mg L}^{-1}$ to $111.76 \pm 1.16 \text{ mg L}^{-1}$ after 5 weeks of storage, the DHAA increased from $11.28 \pm 1.49 \text{ mg L}^{-1}$ to $17.26 \pm 3.16 \text{ mg L}^{-1}$. There are two possible reasons for the unbalance in AA degradation and DHAA formation. First, DHAA is a very unstable compound which can be irreversibly hydrolyzed to 2, 3- diketogulonic acid. Therefore, the observed change in DHAA content is the net result of formation and degradation reactions. The second reason could be that part of the AA content might decrease through the anaerobic pathway in which DHAA is not formed. Consequently, the total vitamin C (AA plus DHAA) content (results not shown) decreased over storage time and the overall nutritional quality of orange juice is negatively affected upon storage.

Sugar content

During storage, the sucrose content significantly decreased ($p < 0.05$) while the glucose and fructose content increased (**Table 2**). After 15 weeks of storage at 42°C , approximately 82% of sucrose was hydrolyzed while the glucose and fructose content increased more than double the initial level. The observed results in sugar changes in the current work are in agreement with the literature data with the main mechanism being the hydrolysis of sucrose to glucose and fructose under acidic conditions.^{13,33} However, it can be noticed that the increase of glucose and fructose content did not coincide with the decrease of sucrose content. It is possible that glucose and fructose were also formed from the hydrolysis of other carbohydrates like orange juice fibers (e.g. pectin, hemicelluloses) during storage. The reducing sugars, including fructose and glucose, can undergo acid-catalyzed decomposition to form reactive intermediates (e.g. HMF and/or 3- deoxyglucosone), which are important in NEB of citrus juices and orange juice.^{5,41}

Furfural and 5-hydroxymethylfurfural content

The results (**Table 2**) show that both furfural and HMF were absent in the pasteurized shelf-stable orange juice prior to storage and their concentration increased significantly with storage time. After 15 weeks storage at 42°C , the concentration of furfural was 2.21 mg L^{-1} while that of HMF was 25.91 mg L^{-1} . It is

430 interesting to note that there was higher formation of HMF than furfural during storage, which is consistent
431 with the literature reported data for orange juice¹³ and for grapefruit juice.¹⁰

432 It is well established that furfural is primarily generated from AA via an anaerobic degradation
433 pathway^{10,42,43} while HMF is a breakdown product during the dehydration of glucose and fructose.^{10,41} The
434 formation of furfural and HMF in citrus juices is generally agreed to be related to the darkening of the juices
435 during storage.^{3,10} While furfural is a known reactive compound, HMF has been regarded as a by-product
436 which only accumulates because of its low reactivity in browning reactions.¹⁰

437 The changes in AA and sugar content and the formation of furfural and HMF are consistent with the
438 results of the browning development (BI). This is in agreement with literature which has demonstrated that
439 AA and sugars are precursors of the NEB reactions and their degradation causes the formation of furfural
440 and HMF which are considered as NEB reaction intermediates.³ In conclusion, the changes in soluble
441 compounds observed in this study confirm the documented literature on NEB, while the participation of
442 polymeric components (e.g. proteins, arabinogalactan proteins, protein-pectin complexes) of orange juice
443 cloud and pulp during juice browning is demonstrated for the first time in literature.

444 ABBREVIATIONS USED

445 AA ascorbic acid

446 AIJN Association of the Industry of Juices and Nectars from fruits and vegetables of the European Union

447 BI browning index

448 DHAA dehydroascorbic acid

449 EP material precipitated in 70% ethanol solution

450 EP20 material precipitated in 20% ethanol solution

451 EP40 material precipitated in 40% ethanol solution

452 EP60 material precipitated in 60% ethanol solution

453 ES material soluble in 70% ethanol solution

454 ES60 material soluble in 60% ethanol solution

455 Fuc fucose

456 Fru fructose

457	GalA	galacturonic acid
458	Gal	galactose
459	Glu	glucose
460	HMF	5-hydroxymethylfurfural
461	HPLC	high-performance liquid chromatography
462	Man	mannose
463	M _w	weight average molecular weight
464	NEB	non-enzymatic browning
465	PET	polyethylene terephthalate
466	PPO	polyphenol oxidase
467	Rha	rhamnose
468	Suc	sucrose
469	W	week(s)
470	Xyl	xylose

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FIGURE CAPTIONS

Figure 1. Changes in (A) total color difference (ΔE^*) and (B) browning index (BI) of orange juice during storage at 42 °C.

Figure 2. Schematic overview of (A) ethanol precipitation of the non-stored orange juice and 15 week-stored orange juice at 42 °C and (B) stepwise ethanol precipitation of the total ethanol precipitated materials.

Figure 3. Size exclusion profile of the total ethanol precipitated material (EP) from the non-stored (OW) and 15 week- stored (15W) orange juice at 42 °C: (A) Log molar mass distribution (solid line) of EP OW (thin) and EP 15W (thick) and concentration of EP OW (.....) and EP 15W (.-.-.) as a function of elution time (min).

608 (B) Light scattering signal at 92° angle (solid line) of EP 0W (thin) and EP 15W (thick) and UV absorbance
609 chromatogram at 280 nm of EP 0W (dotted lines) and EP 15W (dot-dash-dot).

610 **Figure 4.** Size exclusion profile of the total ethanol precipitated material (EP) and its sub-fractions from the
611 15 week- stored orange juice at 42 °C: (A) Log molar mass distribution (solid line) and concentration (dashed
612 line) as a function of elution time (min). (B) Light scattering signal at 92° angle (solid line) and UV absorbance
613 chromatogram at 280 nm (dashed line). EP - blue, EP20 - red, EP40 - black, EP60 - yellow, ES60 - green.

Table 1. Average monosaccharide composition and crude protein content of the ethanol precipitated material and their sub-fractions from the non-stored and 15 week-stored orange juice at 42 °C expressed in g/100g lyophilized sample.

Sample	Fuc*	Rha*	Ara*	Gal*	Glu*	Xyl*	Man*	GalA*	Crude protein*
EP 0W	0.18 ^A	1.60 ^A	6.13 ^A	9.13 ^A	3.58 ^A	0.24 ^A	0.26 ^A	11.62 ^A	29.23 ^B
EP20 0W	0.15 ^{Aa}	3.17 ^{Ab}	4.05 ^{Aa}	6.88 ^{Aa}	4.53 ^{Ab}	0.18 ^{Aa}	0.22 ^{Aa}	4.18 ^{Aa}	49.59 ^{Ad}
EP40 0W	0.24 ^{Ab}	0.99 ^{Ba}	4.75 ^{Aa}	7.82 ^{Aa}	1.75 ^{Aa}	0.20 ^{Aa}	0.20 ^{Aa}	22.34 ^{Ab}	25.78 ^{Bc}
EP60 0W	0.50 ^{Bc}	1.34 ^{Aa}	13.62 ^{Ac}	23.01 ^{Bc}	2.35 ^{Aa}	0.73 ^{Aa}	0.19 ^{Aa}	21.98 ^{Ab}	4.88 ^{Aa}
ES60 0W	0.09 ^{Ba}	1.07 ^{Aa}	8.19 ^{Bb}	10.33 ^{Aa}	10.46 ^{Ac}	0.64 ^{Aa}	1.21 ^{Aa}	6.71 ^{Ba}	9.95 ^{Ab}
EP 15W	0.19 ^A	1.76 ^A	6.29 ^A	10.11 ^A	5.69 ^A	0.54 ^A	0.25 ^A	15.06 ^A	25.61 ^A
EP20 15W	0.17 ^{Aa}	3.27 ^{Ab}	3.84 ^{Aa}	7.25 ^{Aa}	4.17 ^{Ab}	0.16 ^{Aa}	0.25 ^{Aa}	4.35 ^{Aa}	48.22 ^{Ac}
EP40 15W	0.25 ^{Ab}	0.76 ^{Aa}	4.22 ^{Aa}	7.70 ^{Aa}	3.36 ^{Bb}	0.20 ^{Aa}	0.18 ^{Aa}	28.86 ^{Ba}	19.69 ^{Ab}
EP60 15W	0.27 ^{Ac}	1.25 ^{Aa}	12.66 ^{Ac}	20.54 ^{Ac}	0.95 ^{Aa}	0.39 ^{Aa}	0.10 ^{Aa}	22.48 ^{Ab}	6.97 ^{Aa}
ES60 15W	0.05 ^{Aa}	0.77 ^{Aa}	7.23 ^{Ab}	10.05 ^{Ab}	11.61 ^{Ac}	0.91 ^{Ab}	0.97 ^{Ab}	4.70 ^{Aa}	10.08 ^{Aa}

* Different capital letters (in superscript) indicate statistical difference ($p < 0.05$) between the non-stored sample and 15 week-stored sample, while small letters indicate statistical differences among the sub-fractions of the non-stored sample or 15 week-stored sample.

Table 2. Changes in different attributes related to browning of shelf-stable orange juice during storage at 42 °C.

Storage time (weeks)	AA (mg L ⁻¹)*	DHAA (mg L ⁻¹)*	Suc (g L ⁻¹)*	Glu (g L ⁻¹)*	Fru (g L ⁻¹)*	Furfural (mg L ⁻¹)*	HMF (mg L ⁻¹)*
0	234.40 ^d	11.28 ^a	39.90 ^d	17.93 ^a	21.49 ^a	0.00 ^a	0.00 ^a
5	111.76 ^c	17.26 ^b	21.00 ^c	27.25 ^b	32.43 ^b	0.99 ^b	3.61 ^b
10	42.88 ^b	24.85 ^c	13.41 ^b	35.05 ^c	40.64 ^c	1.50 ^c	10.62 ^c
15	0.55 ^a	8.14 ^a	7.34 ^a	39.93 ^d	45.86 ^d	2.21 ^d	25.91 ^d

* For each attribute listed in the same column, different letters (in superscript) indicate statistical difference ($p < 0.05$) among storage moment.

Figure 1. Changes in (A) total color difference (ΔE^*) and (B) browning index (BI) of orange juice during storage at 42 °C.

Figure 2. Schematic overview of (A) ethanol precipitation of the non-stored orange juice and 15 week-stored orange juice at 42 °C and (B) stepwise ethanol precipitation of the total ethanol precipitated materials.

Figure 3. Size exclusion profile of the total ethanol precipitated material (EP) from the non-stored (0W) and 15 week- stored (15W) orange juice at 42 °C: (A) Log molar mass distribution (solid line) of EP 0W (thin) and EP 15W (thick) and concentration of EP 0W (.....) and EP 15W (. . . .) as a function of elution time (min). (B) Light scattering signal at 92° angle (solid line) of EP 0W (thin) and EP 15W (thick) and UV absorbance chromatogram at 280 nm of EP 0W (dotted lines) and EP 15W (dot-dash-dot).

Figure 4. Size exclusion profile of the total ethanol precipitated material (EP) and its sub-fractions from the 15 week- stored orange juice at 42 °C: (A) Log molar mass distribution (solid line) and concentration (dashed line) as a function of elution time (min). (B) Light scattering signal at 92° angle (solid line) and UV absorbance chromatogram at 280 nm (dashed line). EP - blue, EP20 - red, EP40 - black, EP60 - gray, ES60 - purple.

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