

Role of Different Factors Affecting the Formation of 5-Hydroxymethyl-2-furancarboxaldehyde in Heated Grape Must

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The influence of concentration and water activity (a_w) on the formation of 5-hydroxymethyl-2-furancarboxaldehyde (HMF) in thermally treated grape must was evaluated. Must was cryoconcentrated and then heated to study the pure effect of sugar concentration. Moreover, NaCl was added to the must to lower a_w , maintaining the same sugar concentration, with the purpose of evaluating the pure effect of a_w . Finally, the influence of minimal pH changes on the formation of HMF was evaluated by means of a model solution. The results showed that a_w and sugar concentration are both determinant in the formation of HMF in grape must. Sugar concentration influences the reaction by supplying substrates; low a_w enhances the formation of HMF by changing the equilibrium in the dehydration step of the reaction.

KEYWORDS: a_w ; balsamic vinegar; cryoconcentration; heat; HMF; must

INTRODUCTION

Concentrated grape must is used by the food industry as a sweetener but, in particular, it is employed in the wine industry for addition to musts with low sugar content to improve the alcohol level of the final product. Its use is allowed by European Union (EU) regulations (1), which define concentrated rectified must as the liquid, noncaramelized, obtained by partial dehydration of the rectified must: the required chemical and physical characteristics are also specified. Concentrated grape must is also used in the production of traditional balsamic vinegars, for which the must concentration is performed by heating the mass at atmospheric pressure in open pans (2). Thermal treatments are applied to fruit juices not only to concentrate the volume but also to extend their shelf life by inactivating the enzymes and lowering the microbial contamination. In any case, the heating processes can affect the quality of such products by causing nonenzymatic browning reactions. The heat supply and the acid values of the must (the pH of must is ~ 3) determine the formation of 5-(hydroxymethyl)-2-furancarboxaldehyde (HMF) and correlated compounds: the reaction involves hexoses through an acid-catalyzed dehydration and cyclization mechanism. The first step of the reaction consists of a structural change of hexoses into an intermediate 1,2-enediolic form, which rapidly eliminates water (3). HMF is also an indicator of adulteration of food products with acid-converted invert syrups; moreover, it is commonly associated with heat damage, and it

is an index of improper storage conditions of fruit juices, whereas its level is close to zero in fresh foods (4). HMF is also a major product of the Maillard reaction; however, on the basis of previous studies (5, 6), and considering the small concentrations of free aminic groups in comparison with sugar concentrations, this pathway to HMF formation is negligible in grape must and fruit juices. Different amino acids have different reactivities in the Maillard reaction and, in particular, the concentration of reactive amino acids in grape juice is very small (7). Similarly, different sugars have different influence on the formation of HMF: fructose was found to be twice as reactive as glucose in the formation of HMF and brown pigments (3, 7). EU regulations have imposed limits on the recovery of HMF in some products; for example, concentrated musts should have a maximum of 25 mg/kg total sugars (1). HMF has not only an importance as an indicator of adulteration or heat damage but carries sanitary concern, as it has been suspected of being an initiator and promoter of colon cancer (8, 9).

Many studies have been performed on the formation of HMF in model systems (5, 7, 10, 11) and in heated and thermally concentrated fruit juices (5, 12–14). Some authors have studied the correlations between HMF levels and the time of storage of traditional balsamic vinegars (TBV) (15, 16); however, the increase of HMF level was mainly due to the concentration of the product by evaporation through the staves of the barrels. Few studies (6, 11) have considered grape must and its chemical changes during thermal concentration: the latter work considered the influence of cooking time on the formation of furfurals in must, showing how the levels of HMF, furaldehyde, and furoic acid increase with the time of heating. The extent of the browning reaction during the heating is also due to a decrease

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Table 1. Water Activity and pH Values of Cryoconcentrated Must Samples

°Brix	a_w	pH	°Brix	a_w	pH
19.0	0.914	3.11	35.5	0.889	3.03
23.0	0.906	3.07	37.0	0.884	3.03
26.5	0.903	3.07	39.0	0.880	3.00
29.5	0.897	3.05	43.0	0.865	2.98

of water content, as reported by Eichner and Karel (17) in model solution. During the must cooking both the extent of heat supply and sugar concentration rise; therefore, the contributions to the HMF formation given by each variable separately cannot be evaluated through the process. Hence, the aim of the present study is to break up a complex system into factors and to show each single net contribution; in particular, cryoconcentration of the must allowed us to study the rate of formation of HMF in must samples at different sugar concentrations with exposure to the same heat treatment. Moreover, the addition of salt to must was performed to lower the a_w in solutions with the same sugar concentration to study the rate of formation of HMF as a function of a_w .

MATERIALS AND METHODS

Must Concentration and Preparation of Samples. The influence of the concentration level on the formation of HMF was investigated by heating must samples previously concentrated at different °Brix levels. The must, obtained from Nero d'Avola grapes produced in Sicily (Italy), was cryoconcentrated to obtain eight final concentrations. The must was frozen in PET bottles at $-18\text{ }^\circ\text{C}$, and then it was brought again to room temperature and collected as it thawed. The must that melted first was the most concentrated (43 °Brix) because the freezing point is a colligative property of solutions, which decreases at increasing concentrations of solutes.

Once thawed, the must was adjusted to the following concentration levels, expressed as degrees Brix: 19.0, 23.0, 26.5, 29.5, 35.5, 37.0, 39.0, and 43.0. Five milliliters of each concentration was dispensed in 10 mL glass bottles.

Five milliliters of the must at 19 °Brix was put in 10 mL glass vials, and different amounts of NaCl were added to reach final concentrations of 0, 5, 10, 15, 20, and 25% (w/v) to decrease the water activity, maintaining, at the same time, similar concentrations of sugars. In the case of the highest addition of NaCl (25% w/v) the solution volume increase of 10.4% corresponded with a decrease of sugar concentration. The slight variation of sugar concentration due to the volume increase caused by NaCl addition is reported in **Table 2** and was taken into account in the expression of the results. The final water activity is reported in **Table 2**. All of the vials were hermetically sealed with silicon tops and put in a preheated oven at $95\text{ }^\circ\text{C}$ for 1 h.

A model solution at 24 °Brix (glucose/fructose = 1) was acidified with acetic acid, following this procedure: 100 mL of solution was stirred in a beaker, and drops of acetic acid were added while the pH was measured. Five aliquots of 5 mL were withdrawn at pH 3.19, 3.12, 3.04, 2.97, and 2.90 and dispensed into 10 mL glass bottles, which were hermetically sealed with silicon tops and put in a preheated oven at $95\text{ }^\circ\text{C}$ for 2 h.

At the end of each treatment, the bottles were rapidly cooled by immersion in a cold water bath prior to analysis.

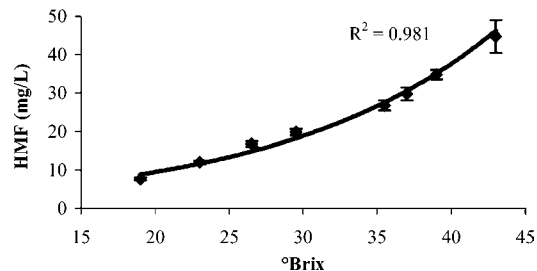
Determination of Water Activity and °Brix. Water activity was measured at room temperature ($21\text{ }^\circ\text{C}$) on 3 mL of the sample by a Hygrometer AwV C (Rotronic AG, Bassersdorf, Switzerland). The a_w values, together with the pH values measured for the must samples, are shown in **Table 1**. **Table 2** shows the a_w values of the model solution with different NaCl additions.

Degrees Brix values, which were assumed as a measure of the sugar concentration, were determined by a manual refractometer at $20\text{ }^\circ\text{C}$.

Determination of Furanic Compounds. The HPLC was a Varian 9012 liquid chromatograph equipped with a diode array detector (Varian

Table 2. Effective Sugar Concentration and Water Activity of Model Solutions Added with Different Amounts of NaCl

NaCl (% w/v)	reducing sugars (% w/v)	a_w	NaCl (% w/v)	reducing sugars (% w/v)	a_w
0	18.8	0.914	15	17.6	0.842
5	18.4	0.895	20	17.2	0.811
10	18.0	0.868	25	16.8	0.779

**Figure 1.** Formation of HMF in grape must at different levels of concentration.

330 Star), a Lichrospher 100 RP-18 ($5\text{ }\mu\text{m}$, $125\text{ mm} \times 4\text{ mm}$) column, and a $20\text{ }\mu\text{L}$ loop. Samples were used undiluted, filtered through $0.45\text{ }\mu\text{m}$ nylon filters, and injected into the HPLC. HMF was separated using $\text{H}_2\text{O}/\text{acetonitrile}$ 95:5 (v/v) as mobile phase, at a flow rate of 1 mL/min. Chromatograms were acquired from 250 to 300 nm, but HMF was monitored at 285 nm. Quantification was performed by calibration with external standard using a calibration line obtained with appropriate dilutions of commercial standard (Sigma, Milan, Italy). Water, acetonitrile, and methanol were of HPLC grade (Merck).

Statistical Analysis. Each test was repeated twice, and single analyses were carried out in triplicate. The data shown in the figures are the average of all repetitions, whereas the error bars are the standard deviation.

RESULTS AND DISCUSSION

The HPLC analyses evidenced only HMF, identified by comparison of the retention time and the spectrum with those of pure standard. Furaldehyde was detected in traces in the sample at 43 °Brix, whereas it was not possible to detect furaldehyde or any other furanic compound among those found by other authors in TBV (16). This is probably due to the short heat treatment (1 h) compared to the common cooking times (up to 20 h) of the must for TBV production. Moreover, some compounds, such as 5-(acetoxymethyl)-2-furaldehyde, originate from microbial activity of acetic acid bacteria, which has to be excluded in the context of this study.

HMF was not detected in nonheated samples, confirming that the concentrations found are due exclusively to heat damage.

The concentration of HMF could be well correlated ($R^2 = 0.98$) with the sugar concentration level by an exponential curve, which emphasizes that the HMF level in samples at higher concentration was more than proportional to sugar concentration (**Figure 1**). This also leads to the fact that the rate of formation of HMF during the evaporation process should change with concentration, being slow at the first stages, and increasing afterward. A similar result was observed by Antonelli et al. (6) in cooked must samples; however, that result (i.e., the exponential trend) was affected by the progressive length of the heat treatment, meaning that different samples, along with increasing °Brix, had undergone different heat damage. Göğüş et al. (7) found a similar trend for HMF accumulation (plotted with boiling time) in model systems simulating grape juice, emphasizing a lag period, the length of which depends on temperature. This result is in agreement with that of Antonelli et al. (6),

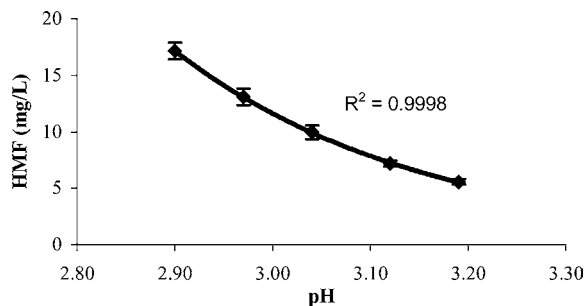


Figure 2. Influence of pH on the formation of HMF.

because in this latter study HMF content is plotted with °Brix, which was assumed as proportional to the concentration process. These studies evaluated the level of HMF in heated must at different time intervals and at different temperatures; however, such approaches do not help to explain the role that the concentration level plays. Treating cryoconcentrated samples with the same heat supply allowed us to state that HMF develops more than proportionally to °Brix. A proportional relationship would have been expected between HMF and °Brix: as sugars are the substrate of the reaction, higher concentrations should end in higher amounts of HMF recovered. However, to explain the exponential trend of formation, we hypothesized a contribution of the pH of the samples: because the degradation of hexoses is an acid-catalyzed reaction, a decrease of pH should enhance HMF production from hexoses. To validate this hypothesis, we tested five model solutions at different pH values (ranging over the same pH values as the must samples) and with the same sugar concentration to determine whether the increase in HMF had been influenced by the pH of the samples. The pH decreased slightly with the cryoconcentration of the must (from 3.11 in must at 19 °Brix to 2.98 in must at 43 °Brix), as a consequence of the concentration of the acids. This variation seemed of marginal importance, because the decrease of pH amounted to 4%. However, the results (Figure 2) demonstrated a clear role played by the minimal variations of pH on the formation of HMF. As a function of pH, the formation of HMF increased along an exponential curve ($R^2 = 0.9998$) (Figure 2), and this could justify the higher rate of formation of HMF at higher concentration levels in heated must. This explanation could, moreover, justify the trend of growth of HMF in previous studies (6). In fact, the cooking of the must for TBV production determines a decrease of pH of ~0.3 (pH decreases from 3–3.07 to 2.74–2.77), which is much more than that observed in the present research. Therefore, it is reasonable to state that in the normal conditions of must cooking, the rate of formation of HMF increases at later stages due the reduction of pH.

HMF formation was inversely correlated with a_w in must with added NaCl ($R^2 = 0.99$) (Figure 3). The pure effect of water activity on the reaction was evaluated by means of NaCl additions to the must: the sugar concentration being similar, HMF formation increases proportionally with the decrease of water activity (Figure 4). In particular, the HMF concentration grew from 0.04 mg/g reducing sugars, in the sample without added NaCl, to 0.30 mg/g reducing sugars in the sample with 25% NaCl, following a linear trend. To standardize the HMF content, it was expressed per gram of reducing sugars. The sugar content was converted from °Brix into grams by means of the following equation: reducing sugars (% w/v) = °Brix + [0.2 · (°Brix) - 4]. Glucose and fructose are able to bind water, lowering water activity; therefore, sugar concentration and the corresponding reduction of water activity are different, but synergistic, factors that determine the production of HMF in

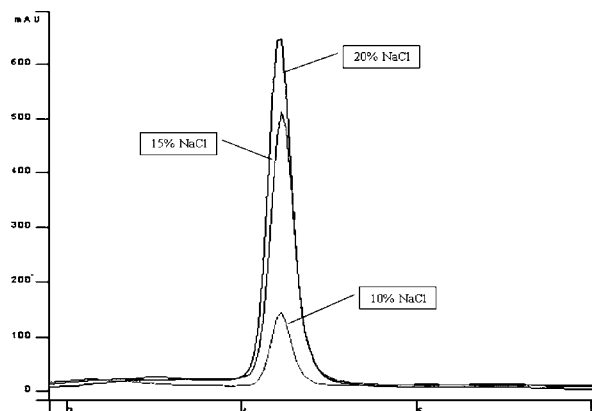


Figure 3. Effect of NaCl additions on HMF formation. Increasing peaks are, respectively, HMF in must without NaCl and HMF in samples to which 15 or 20% NaCl has been added.

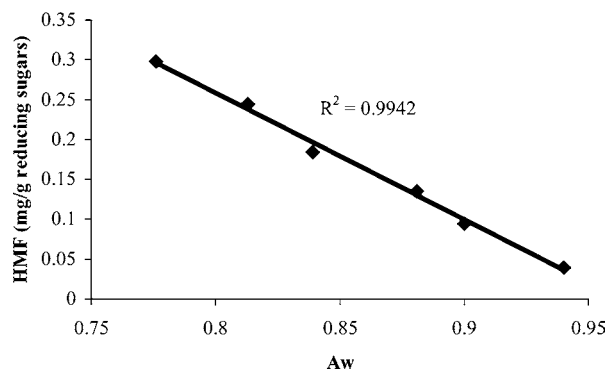


Figure 4. Influence of water activity on HMF formation in must at 19 °Brix with 0, 5, 10, 15, 20, and 25% NaCl added.

must and sugary matrices. The importance of a_w in the formation of HMF is also emphasized by the higher recovery of this furanic compound during the storage of concentrated musts (P. Giudici, private communication), when, for long periods of conservation, the level of HMF can exceed the limits fixed by law (1).

Sugar concentration and a_w exert a different role in the reaction. The former is a measure of the availability of substrate for the reaction; therefore, higher concentrations result in higher formation of HMF. On the other hand, the reason low values of a_w speed the formation of HMF could be explained with a change in the equilibrium of the acid-catalyzed dehydration and cyclization of hexoses, in particular in the dehydration steps: it is known that, after a slow enolization, the degradation of each molecule of hexose comprises proton-catalyzed β -elimination of three molecules of water from the intermediates (3). Therefore, in a system at low a_w , where many H_2O molecules are bound to sugars or other substances, the equilibrium of the reaction is shifted to the right, due to the easier elimination of water. A similar hypothesis was furnished by Eichner and Karel (17), who explained the increase of browning rate at decreasing water content with laws of mass action. This should give an explanation for the higher formation of HMF at increasing concentration. The same could be applied to the formation of furan derivatives, as the mechanism involves loss of water molecules.

The presence of HMF in heated grape musts depends on temperature, pH, sugar concentration, and the a_w of the system. Water activity plays an important role in the degradation of sugars, which could be evaluated independently from heating and sugar concentration, even if the factors act together during the concentration of grape must and fruit juices in general. The

explanation given not only highlights some mechanisms that occur during the concentration of grape musts but also helps to clarify the higher formation of HMF in concentrated musts and juices during storage and takes into consideration the chance that they could exceed the limits fixed by law.

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