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FULL PAPER

Development an in House Validation for 5-Hydroxy-2-methyl-furfuraldehyde (HMF) Analysis in Fermented Beverages Produced from Honey, Cane Syrup and Corn Syrup by HPLC-UV

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Abstract:

A chromatographic direct method using HPLC/UV for quantification of 5-hydroxy-2-methyfurfuraldehyde (HMF) content in different fermented beverages (mead, cane syrup fermented and corn syrup fermented) was developed and in house validated using sophisticated statistical tools for the first time in this work. HMF separation was executed with isocratic elution of a mobile phase comprising water (with 0.5% formic acid) and acetonitrile (90:10, v v^{-1}), at 30 °C, flow rate of 1.0 mL min^{-1}, injection volume of 5.0 µL and detection at 285 nm. Validation study demonstrated that the developed method has good performance, presenting low limits (LOD and LOQ of 0.16 and 0.53 mg L^{-1}, respectively), good accuracy (recovery rates between 82.3 and 95.9%) and precision (RSD values between 3.87 and 8.84% and Horrat values between 0.43 and 0.76). Adequate selectivity and linearity estimates were also observed (R^2 > 99.5%). Fermented beverages from honey, cane syrup and corn syrup presented HMF contents lower than starting foods used in fermentation. Besides this, results demonstrated that fermentation conditions are a key parameter for obtaining fermented beverages with low levels of this contaminant and that the fermentation can be a strategy for mitigation of HMF in foods such honey, cane syrup and corn syrup.

Keywords: fermented beverages; HMF; fermentation process; method validation

1. Introduction

Fermented beverages with nutritional properties are part of an expanding market that aims to attend consumers who are increasingly demanding and concerned about adopting a healthy lifestyle and eager for novelties. These facts have aroused the interest of researchers and food industries in the development of new functional fermented beverages from foods such as milk, cereals and fruit juices or in the optimization on production processes of traditional beverage such as mead [1–4]. However, the technological processes of production of these new fermented beverages need to be optimized, and chemical and sensorial characterizations of the final product are essential to offer the consumer a drink with functional attributes, acceptable organoleptic characteristics and safe for consumption [4].

Mead is a fermented beverage obtained by the fermentation of a mixture of water and honey with an alcoholic content between 8.0 and 18.0% (v/v) of ethanol [5, 6]. Recently, in the literature, researchers has shown a concern in the optimization of the fermentation process in order to obtain meads with better organoleptic characteristics, in a lower time of fermentation and with a lower level of contaminants [2, 6]. Considering that, meads may present in its composition considerable quantities of sugars,

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such as glucose and fructose, low pH values, and a contaminant known as 5-hydroxy-2-methylfurfuraldehyde (HMF) is normally founding in varying quantities [2, 7, 8]. The concentration of this contaminant in the starting food and the conditions of the fermentation, maturation and storage process can strongly influence the evolution of HMF in this type of fermented beverage [1–3, 5, 6]. Therefore, the reliable HMF determination in meads is very important.

Fermented beverages from foods rich in sugars are not common in literature. To the best of our knowledge, no effort has been realized to produce fermented beverages from cane and corn syrups. Cane syrup is a food resulting from the evaporation of sugarcane juice containing high sugar contents. In addition to sugars, cane syrups may also contain compounds with antioxidant activity (polyphenols), minerals and vitamins. This food has traditionally been used for human consume and as an ingredient of confectionery, soft drinks, candies and as a substitute for fruit preserves [9,10]. This food naturally contain high levels of HMF [9], so the concentration of this contaminant in the cane syrup fermented beverages need to be monitored. On the other hand, a drink fermented from cane syrup may present compounds of nutritional interest such as polyphenols, vitamins and minerals. Corn syrup is a well-known ingredient in the food and beverage industries, having numerous applications [9]. Being the corn syrup a food obtained by thermal processing that contains high sugar contents and low pH values, it presents normally high levels of HMF [9]. The fermentation could be a strategy for the mitigation of this contaminant in corn and cane syrups and therefore, the HMF levels need to be determined in the final product of the fermentation.

HFM is a compound present in beverages and considered one of the parameters for the determination of quality standards if present in low concentrations [11–13]. Monakhova and Lachenmeier [12] report that the effects of HMF in humans are not completely clear up so far. High doses of this compound are not nutritionally relevant, because the HMF presents cytotoxic activity and can cause irritation to the eyes, upper respiratory system and the skin [12]. In long term studies made in rats by the National Toxicology Program (NTP), the HMF didn't show neoplastic effects in the intestinal tract [12]. However, a high manifestation of hepatocellular adenoma and liver carcinomas were diagnosed in female rats [12, 13]. These facts show a need to develop and validate analytical methods for control of the concentration of HMF in beverages for use in quality control laboratories and for standardization and identification of problems in the processing and storage of beverages in industrial level.

Recently, has been a growing concern of researchers about the quantification of HMF in mead and other fermented beverages by chromatographic methods [2, 7, 8]. However, few studies have presented figures of merit proving the reliability of the methodologies developed. Therefore, it is important to carry out validation studies to assure metrological reliability in the analysis of this contaminant in fermented beverages. As no report was found for HMF analysis in fermented beverages obtained from cane and corn syrups, the development of a new chromatographic method using reverse phase liquid chromatography with UV detection become interesting.

Chromatographic methods are the most widely used for the analysis of HMF in foods and beverages, especially the liquid chromatography with UV detection (HPLC-UV) [9, 14–18]. Although several studies are found reporting the concentration of HMF in foods and beverages by HPLC-UV, efforts for *in house* validation by different statistical techniques are still scarce [9,19,20]. *In house* validation studies well planned can facilitate the implementation of the method that has been developed in other laboratories for routine analyses, in addition to ensuring the reliability of measurements and the decision taking [19–21]. Thus, in this work an *in house* validation study of a direct chromatographic method for HMF determination in meads, corn syrup fermented beverages and cane syrup fermented beverages by HPLC-UV was carried out. Besides this, the developed method was applied to fermented beverages from honey, cane syrup and corn syrup produced in different fermentation conditions.

2. Material and Methods

2.1 Chemical and materials

5-hydroxy-2-methylfurfuraldehyde (HMF) (98%) and formic acid (95%) were acquired from

Sigma–Aldrich (São Paulo, Brazil), and acetonitrile (HPLC grade) was purchased from Tedia (Brazil). Ultrapure water (0.055 μS cm^{-1}) was produced by a simple UV Milli-Q system from Millipore (Brazil) and it was used for the solutions and mobile phase preparation.

2.2 Samples

For the method validation study fermented beverages (mead, cane syrup fermented and corn syrup fermented) obtained by utilization of the different fermentation conditions were analyzed. Initially, musts were prepared by dilution of the foods (honey, cane syrup and corn syrup) in water until concentrations in the range of 20 to 22ºBrix following pre-inoculation at 10% (v/v).

At the musts pre-inoculated was added yeast strain at a concentration of 3% (w/v). Different fermented beverages from honey, cane syrup and corn syrup were obtained variating yeast strain types (high fermentation (*Saccharomyces cerevisiae,* KOSHER GMO FREE, Munich Lallemand, lyophilized) and low fermentation (B) (*Saccharomyces carlsbergensis,* Diamond 3070 lyophilized, imported by Cooperativa Agrária Agroindustrial)), fermentation temperatures (10 °C) and 28 °C) and fermentation times (4 and 14 days). Samples were coded by letters and numbers considering the food used in fermentation (H for honey, CA for cane syrup and CO for corn syrup), yeast strain type (H for high fermentation and B for low fermentation), fermentation temperature (T1 for 10 °C and T2 for 28 °C) and fermentation time (C for 4 days and L for 14 days).

2.4 Sample and standard solutions preparation

HMF stock solutions (1000 mg L^{-1}) were prepared in ultrapure water daily and stored at 7 ºC protected from light. Standard solutions were prepared in the concentration range of 0.5 to 5.5 mg L^{-1} by dilution from the stock solution. Tests were performed previously to check the HMF range in the samples. Thus, different masses were used in the preparation of the sample solutions. For mead and cane syrup fermented beverage samples were used 1.0 g in 10 mL and for corn syrup fermented beverage sample was used 0.1 g in 100 mL. Cane syrup fermented beverage was diluted ten times and the corn syrup fermented beverage was diluted to one hundred times ensuring that HMF concentrations remained within the range concentration (0.5 to 5.5 mg L^{-1}) from analytical curve.

2.5 Chromatographic conditions

The chromatographic system consisted of a Waters 600 controller HPLC system, equipped with a photo diode array detector (2996 PDA), Empower Software. The chromatographic separation of 5-hydroxy-2-methylfurfuraldehyde was performed on a Waters column (μBondapak C18, 5.0 μm, 3.9 x 300 mm) at 30 ºC. Mobile phase consisted of water (with 0.5% formic acid) and acetonitrile in the ratio 90:10 (v/v) under isocratic conditions at a flow rate of 1.0 mL min^{-1} with an injection volume of 5.0 μL. Detection was realized at 285 nm and the total run time was 10 min. All aqueous samples solutions were filtering on 0.45 μm PTFE (Millipore - Brazil) filters before injection on chromatographic system. For analysis of HMF in the starting foods of fermentation (honey, cane syrup and corn syrup) the chromatographic conditions described by Andrade et al., (2016) were adopted with modifications in flow rate and injection volume.

2.6 Validation study

The validation study was carried out evaluating parameters as selectivity, linearity, detection limit (LOD), quantification limit (LOQ), accuracy and precision according several guides and articles from literature [19, 20, 22–24].

2.6.1 Selectivity

HMF standard solution (4.5 mg L^{-1}) and solutions from mead and fermented beverages, prepared as described in 2.3 were used to evaluate the selectivity by comparison of the retention times of HMF peak in the standard solution and beverages samples chromatograms.

2.6.2 Calibration and linearity

An analytical curve in the range of 0.5 to 5.5 mg L^{-1} was constructed from a stock solution of

HMF (1000 mg L^{-1}). This narrow concentration range was used to avoid loss of method linearity and problems in the column. Fermented beverages samples analyzed have a complex composition and this could obstruct the column and compromise its efficiency. Three standard solutions were prepared for each point of the calibration curve and these were injected in triplicate. The linearity of the chromatographic method was checked by applying a linear regression analysis and a lack-of-fit test [9,19] to data of the calibration experiment at 95% confidence level. The significance of the analytical curve coefficients (intercept and slope) by a *t*-test was tested. At the same level of confidence were built confidence and prediction intervals. All statistical analyzes were carried out using the statistical software *Minitab v. 16.2.2* [25].

2.6.3 Detection (LOD) and quantification (LOQ) limits

The limits of detection (LOD) and quantification (LOQ) were calculated based on (3 x SD)/m and (10 x SD)/m respectively, where m is the slope of the analytical curve and SD is the standard deviation on the intercept of the analytical curve [9].

2.6.4 Precision

The precision was evaluated by repeatability and the intermediate precision estimates and by Horrat values [9]. In repeatability tests, three solutions of each fermented beverage were prepared as described in 2.3 and analyzed in the same day. Relative standard deviations ((RSD (%)) were calculated from data obtained in triplicate and it were used as a repeatability estimate. The intermediate precision tests were carried out in the same way as the repeatability tests during three consecutive days. Relative standard deviations ((RSD (%)) were calculated again and they were used as intermediate precision estimates. Horrat values were also obtained, using the results of an analysis of variance (ANOVA) characteristic of a hierarchical design at 95% confidence level, as a way to verify the suitability of intermediate precision estimates for the chromatographic methodology [9].

2.6.5 Accuracy

The accuracy was assessed through recovery tests [9]. Beverage sample solutions (mead, cane syrup fermented and corn syrup fermented) were prepared as described in 2.3 and spiked in three concentration levels (1.5, 2.5 and 3.5 mg L^{-1}) with adequate volumes from stock solution of HMF (1000 mg L^{-1}). Analyses were performed in triplicate. Recovery percentages (%) were calculated as the amount of HMF recovered/amount of HMF added x 100 and used as accuracy estimates.

2.7 Application in real samples

To verify the applicability of the developed method for quality control analysis of fermented beverages, the HMF contents in fermented beverages samples from honey, cane syrup and corn syrup obtained by application of different fermentative processes (yeast types (high and low fermentation), fermentation temperatures (10 °C and 28 °C) and fermentation times (4 and 14 days)) were determined. All measurements were realized in triplicate.

3. Results and Discussion

For ensure the separation of this contaminant from other components present in the beverage samples, a study to optimize the composition of the mobile phase was performed in this work. The use of mobile phases composed of water:methanol (90:10, v/v) and water:acetonitrile (90:10, v/v) are usually applied [7–9,26]. Therefore, it was chosen to work with the mobile phase composed of water and acetonitrile. This mobile phase composition already have been indicated as better for the analysis of HMF in the foods used to obtain the investigated fermented beverages (honey, cane syrup and corn syrup) [9]. Different ratios from water acidified (formic acid 0.5%) and acetonitrile (80:20 (v/v), 70:30 (v/v) and 90:10 (v/v)) (data not shown) were tested. The mobile phase composition that showed better separation of HMF from other matrix compounds was composed of water acidified with formic acid (0.5%) and acetonitrile in the proportion (90:10, v/v). Changes in flow rate between 0.8 to 1.0 mL min^{-1} were also investigated. The flow rate with the best ratio of time of analysis and separation was 1.0 mL min^{-1}. Thus, for the validation study of the methodology,

the mobile phase composition of water (with 0.5% formic acid) and acetonitrile in the proportion of 90:10 (v/v) and flow rate of 1.0 mL min^{-1} were adopted.

The validation study of chromatographic method was executed according recommendations reported in the literature [9, 14, 15, 22, 24] evaluating the parameters of selectivity, linearity, limits (LOD and LOQ), accuracy and precision by application of different statistical tools.

For evaluate the selectivity, the retention times from HMF peak in standard solution chromatograms (4.60 min) and in the sample solutions chromatograms (4.58 min for mead, 4.68 min for cane syrup fermented and 4.61 min for corn syrup fermented) were compared. Therefore, the HMF retention times from fermented beverage samples and of standard solution were very similar indicating the selectivity of chromatographic method.

For HMF quantification in fermented beverages, an analytical curve with seven concentration points ranging from 0.5 to 5.5 mg L^{-1} was prepared. For evaluate if the linear model was adequate for explain the relation between area of peak and HMF concentration, a linear regression analysis and a lack of fit test were applied to this calibration data set at 95% confidence level, and the results are presented in Table 1.

Table 1. Results of linear regression analysis and lack of fit test at 95% confidence for linearity study.

Regression [a]		**Lack of fit test** [b]	
$F_{regression}$	***p* value**	F_{lof}	***p* value**
3718.29	0.000	1.58	0.228
Linear regression coefficients ± standard deviation		$t_{observed}$ [c]	***p*-value**
Intercept	61.1 ± 226.7	0.27	0.790
Slope	4309.12 ± 70.67	60.98	0.000

[a] $F_{critical\ (0.05;\ 1,19)}$ = 4.381; [b] $F_{critical\ (0.05;\ 5,14)}$ = 2.958; [c] $t_{critical\ (0.0025,19)}$ = 2.093

These results show that the linear model adjusts well to the data, since F_{lof} value was not significant (p > 0.05). This result is confirmed by high values of F_{reg}, indicating that linear regression is very significant at the same confidence level (p = 0.000). Besides this, the significance of model parameters (intercept and slope) was evaluated by a *t*-test (Table 1). Results of *t*-test shows that the angular coefficient has a high degree of significance (p = 0.000) while for the intercept this was not observed (p > 0.05). In other words, the analytical curve passes through the origin. Thus, the equation of the linear model can be represented by $Area_{HMF} = 61 + 4309 * Concentration_{mg\ L^{-1}}$ with R^2 = 99.5%.

The limits obtained in this work (LOD and LOQ) were 0.16 and 0.53 mg L^{-1}, respectively. Other researchers have found lower values for the limits in mead samples. Kahoun et al. [7] found values for detection and quantification limits of 0.05 mg L^{-1} and 0.17 mg L^{-1} and Švecová et al., (2015) showed LOD and LOQ values of 0.03 mg L^{-1} and 0.10 mg L^{-1}, respectively. A possible reason to explain why the limits in this work are higher than those reported in the literature is the fact that the limits were calculated using the method of analytical curve [23], while in the literature the limits were obtained using the relation signal/noise. Considering that the HMF concentrations in fermented beverages are determined using the analytical curve, we believe that this calculation form is more appropriate. Araujo [19] also affirms that the limits values can be different depending on calculus method used. Despite this, our limits allow the determination of HMF in low concentrations in fermented beverages demonstrating the sensitivity of methodology developed.

Repeatability and intermediate precision estimates expressed in terms of relative standard deviations (RSD (%)) were used to evaluate the precision of method (Table 2). RSD (%) values found were lower than the maximum values indicated by AOAC (2012). Thus, these precision estimates were considered adequate, since the recommended values for the concentration ranges analyzed are 7.3% and 11%, respectively, indicating that the chromatographic method

presents a good precision. Besides this, Horrat values determined for all fermented beverages (values < 1.3) confirmed the suitability of intermediate precision estimates (Table 2) [7, 9].

Table 2. Precision study results (repeatability, intermediate precision and Horrat values).

Fermented Beverages	RSD (%)		Horrat Value
	Repeatability [a]	Intermediate precision [b]	
Mead	3.87	5.15	0.43
Cane Syrup Fermented	5.37	8.84	0.96
Corn Syrup Fermented	4.62	4.72	0.76

[a] Repeatability performed in triplicate; [b] Intermediate precision performed for five consecutive days with realization of triplicates in each day.

For evaluate the accuracy of the method, recovery tests in three concentrations levels of the analytical curve (1.5, 2.5 and 3.5 mg L^{-1}) were carried out for all beverage investigated and the results are expressed as percent recovery. For the fermented beverages analyzed, recovery rates varied between 82.6 – 95.9% for mead, 82.3 – 87.4% for cane syrup fermented and 86.9 – 89.3% for corn syrup fermented in the concentration levels evaluated and these recovery rates are within the recommended limits (80 - 110%) by AOAC (2012). These results demonstrate that the chromatographic method presents a good accuracy for the analysis of HMF in these fermented beverages.

For evaluate the performance of validated chromatographic method in real samples, the HMF contents in fermented beverages obtained by different fermentative processes were determined (Table 3).

Analysis of results from Table 3 suggests that all evaluated fermented beverage samples have concentrations of HMF lower than the foods used in the fermentation (honey, cane syrup and corn syrup). Kahoun et al. [7], found similar results for mead samples. In this work, the mead samples (HHT1L, HHT2C, HBT1L and HBT2C) showed the lowest levels of HMF (0.65 to 4.08 mg L^{-1}) regardless of the fermentation conditions employed (yeast type (high and low fermentation), fermentation temperatures (10 and 28 °C) and fermentation times (4 and 14 days)) in relation to other types of fermented products analyzed. Besides this, it was observed that when the fermentation was realized at 28 °C during 4 days (HHT2C and HBT2C samples), the lowest HMF values were obtained (0.65 and 0.67 mg L^{-1}) independently of yeast type adopted (high and low fermentation) (Table 3).

Fermented beverages obtained from corn syrup, in turn, presented the highest levels (8.79 to 470.98 mg L^{-1}) of HMF. In relation to fermentation conditions adopted, a great reduction in HMF levels (8.79 and 12.10 mg L^{-1}) for this fermented beverage type (COHT2C and COBT2C samples), compared to ones determined in starting food (2,003 mg L^{-1}), was again observed when conditions of fermentation from 28 °C and 4 days were adopted (Table 3). Cane syrup fermented beverages presented similar behavior in HMF concentrations in relation to mead and corn syrup fermented samples, considering different fermentation conditions investigated (Table 3). In this way, among the conditions of the fermentation processes evaluated, it can be stated that high fermentation yeast (H) or low fermentation yeast (L), fermentation temperature of 28 °C (T2) and a fermentation time of 4 days (C), resulted in fermented beverages with lower HMF contents for all the utilized foods in the fermentation process (honey, cane syrup and corn syrup).

The reduction of HMF concentration during fermentation realized with yeast from *Saccharomyces cerevisiae* type has been reported by some authors in the literature [27, 28]. According to Liu et al. [27], yeasts reduces the aldehyde group of the furan ring of the HMF in an alcohol identified as 2,5-bis-hydroxymethylfuran. The accumulation of this compound may be lesser toxic to yeast culture than HMF, which explains the high yield of fermentation and the reduction in the levels of this contaminant. Akıllıoglu et al. [28] monitored the biotransformation of HMF in the respective alcohol in pilsner beers by chromatography and it was observed that the

transformation of the contaminant into its respective alcohol is faster in medium with higher sugar content.

Table 3. HMF concentrations expressed as averages ± standard deviations (n=3) from honey, cane syrup, corn syrup, mead, cane syrup fermented and corn syrup fermented samples from Brazil.

Foods before Fermentation	HMF contents (mg L^{-1})**	Fermented Samples*	HMF contents in the fermented beverages (mg L^{-1})
Honey (H)	2.37 ± 0.21	HHT1L	4.08 ± 0.97
		HHT2C	0.65 ± 0.06
		HBT1L	1.80 ± 0.54
		HBT2C	0.67 ± 0.02
Cane Syrup (CA)	138.4 ± 22.1	CAHT1L	31.52 ± 4.63
		CAHT2C	2.26 ± 1.04
		CABT1L	10.65 ± 5.08
		CABT2C	5.37 ± 4.64
Corn Syrup (CO)	2,003 ± 319	COHT1L	470.98 ± 68.84
		COHT2C	8.79 ± 3.40
		COBT1L	301.95 ± 98.72
		COBT2C	12.10 ± 0.97

*H = high fermentation yeast, B= low fermentation yeast, T1 = fermentation temperature equals to 10 °C, T2 = temperature fermentation equals to 28°C, L = fermentation time equals to 14 days and C = fermentation time equals to 4 days; ** HMF contents in starting foods (honey, cane syrups and corn syrups) were determined according methodology described by Andrade et al. [9].

Iglesias et al. [5], evaluating strategies for mead fermentation, suggests that for *S. cerevisiae* temperatures between 20 and 30 °C promote greater efficiency of the fermentative process regarding the conversion of sugars into alcohol. According to these authors, the use of fermentation temperatures lower than 15 °C decreases the fermentation performance, requiring longer fermentation times. This behavior of yeast during fermentation at different temperatures may explain the differences in HMF concentration in the fermented beverages of each food investigated in this work. To date, it was not found in the literature studies relating the rate of conversion of HMF to another compound during fermentation at different temperatures for the fermented beverages analyzed in this study.

Therefore, it was observed that these fermentative conditions (fermentation temperature of 28 °C and fermentation time of 4 days regardless yeast type (high and low fermentation)) would be the most appropriate to produce beverages with low levels of HMF. In view of this, it can be point out that the developed method presented a good robustness for the analysis of HMF in different fermented beverages, demonstrating the possibility of determination in a wide range of concentrations. Another aspect to be highlighting is that the optimization of the fermentation process is a key factor to obtain fermented beverages with good quality and safe for the consumption, especially when the foods used in the fermentation contain high levels of HMF.

4. Conclusions

A direct method for determination of HMF in different fermented beverages was developed and *in house* validated by application of different statistical tools. As optimized conditions of chromatographic method were adopted mobile phase composition of water (with 0.5 % formic acid) and acetonitrile in the rate 90:10 (v/v) and flow rate of 1.0 mL min^{-1}. Validation results demonstrated that the methodology developed presents good selectivity, good linearity and low limits. Besides this, good precision, good accuracy and the possibility of determination in an ample range of concentrations were also observed.

The concentration of HMF in fermented beverages obtained from cane syrup and corn syrup was determined by the first time in this work. Results indicated that these fermented beverages types can be alternatives to existent products, considering that the foods used in the fermentative process presents characteristics

interesting such as the presence of phytochemicals and minerals (antioxidants compounds in cane syrup) or low cost and facility of acquisition (corn syrup). Variation in HMF levels in the different fermented beverages obtained from the same food indicate that fermentative process is a key parameter for production of beverages with low concentration of this contaminant and, therefore, of good quality and safe for consumption.

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