Determination of Furanic Compounds in Traditional Balsamic Vinegars by Ion-Exclusion Liquid Chromatography and Diode-Array Detection

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Abstract

A method for the determination of furanic compounds in traditional balsamic vinegars is proposed. It is based on ion-exclusion chromatographic separation and diode-array detection of furans through an isocratic elution with 0.01N phosphoric acid and 16% acetonitrile. Preliminary trials on standard compounds stability in heat–acidic conditions are also performed. In all the 19 samples analyzed, 2-furoic acid, 5HMF, and furfural are found. No sample contains 4-hydroxy-2,5-dimethyl-3-(2H)-furanone (DHMF); 2-acetylfuran; or furfuryl alcohol. Three unknown compounds are also detected. The last eluting of these compounds is identified as 5-acethoxymethylfurfural, and, notwithstanding a partial hydrolysis in our chromatographic conditions, its quantitation can be carried out.

Introduction

The occurrence of furanic compounds in a wide range of foods and beverages has been reported by several authors (1–7). Furans are a class of heterocyclic compounds generated by heat–acidic treatment of monosaccharides commonly found in animal- or vegetable-derived matrices. Among them, 5-hydroxymethyl-2-furaldehyde (5HMF) and fural-2-aldehyde (F2A), coming from thermal degradation of hexoses and pentoses, respectively (8), have been extensively studied because of their influence on food color and flavor changes during storage (3,9,10). In juices, furfural is thought to be related to direct degradation of endogenous or added ascorbic acid (11), whereas 2-furoic (2FA) has been proven to be a side-product of dehydroascorbic acid degradation in aqueous solutions (12). Moreover, 4-hydroxy-2,5-dimethyl-3-(2H)-furanone (DHMF) is formed by thermal degradation of 6-dehoxysugars [i.e., rhamnose (13,14) and pentoses (15)] in the presence of aminoacids or directly from hexoses (16). It demonstrated to have a fruity, caramel-like flavor with an odor threshold of 160 mg/kg in water (14) and strong antioxidant activity (17). Furfuryl alcohol and 2-acetylfuran are other furans coming from monosaccharides degradation (18) and Maillard reactions (19). In wood-aged alcoholic beverages (i.e., wines, brandies, and whiskies), significant amounts of furfuryl alcohol were reported as a consequence of enzymatic or chemical reduction of furfural (20–22).

Traditional balsamic vinegar (TBV) is an Italian specialty produced exclusively in the provinces of Modena and Reggio Emilia (Emilia Romagna). It is obtained through alcoholic fermentation and acetic oxidation of cooked must from selected grapes. The must is previously heat concentrated up to two times. The product is aged at least 12 years in barrels of decreasing volume and made of different woods. During this period, the must concentrates and turns into tasteful sweet-sour syrup, but with low volatile acidity (i.e., low acetic acid content) and a pleasant smell. As a consequence of this unique method of production, furanic compounds should be expected in TBV. However, only a few works were devoted to this topic, whereas the largest efforts have been focused on aromatic (23–26) or acid characterization (27,28). Recently, a detailed study dealing with the content of 5HMF in vinegars from different sources (i.e., malt, sherry, wine, and apple) was reported (29). In this study, only balsamic vinegars demonstrated contents of 5HMF higher than 0.3 g/kg, reaching, in TBV, concentrations as high as 5.5 g/kg. The same authors proposed 5HMF as an indicator of the TBV age. Unfortunately, only 5HMF was quantitated, and no information is given for the content of other furans. The aim of the present work was to define a simple and rapid method for determining, besides 5HMF, the presence of several furanic compounds formed in TBV during the production process.

Experimental

Vinegars samples

Nineteen TBVs, provided by the Consorzio tra i Produttori di Aceto Balsamico Tradizionale di Reggio Emilia (Regio Emilia, Italy), were analyzed for their content in furanic compounds.

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Before analysis, each sample was diluted up to 10 times and filtered at 0.22 mm.

Standard solutions

F2A, 5HMF, DMHF, 2FA, 2-acetylfuran, and furfuryl alcohol were purchased from Aldrich Chemical (Milwaukee, WI). Standard solutions were solubilized into the mobile phase at concentration ranges shown in Table I.

A standard amount of 5-acethoxymethyl-2-furaldehyde (5AMFA) was prepared from 5HMF by adding an equimolar amount of freshly distilled acetyl chloride in presence of pyridine. To the crude reaction product was added a diluted HCl solution, then the extract was washed with $NaHCO_3$ solution, and finally vacuum dried. The presence and purity (97%) of 5AMFA was confirmed by gas chromatography (GC)–mass spectrometry analysis.

Chromatographic conditions

The high-performance liquid chromatographcy (HPLC) apparatus (Jasco, Tokyo, Japan), was equipped with an isocratic pump (PU 980), a diode-array detector (MD 830), and an injection valve (Rheodyne, Cotati, CA) fitted with a 20-mL loop.

The samples were separated isocratically at 0.6 mL/min, using a Bio-Rad Aminex HPX 87H (Hercules, CA) hydrogen-form cation exchange resin-based column (300- × 7.8-mm i.d.) at 22°C. The chromatographic conditions tested were: 0.05–0.001N sulphuric acid, 0.05–0.001N phosphoric acid, and 15–25% acetonitrile (as organic modifier).

Detection

Chromatograms were acquired and processed with Borwin 5.0 software (JMBS Developments, Grenoble, France). For each run, a tridimensional chromatogram was recorded from 200 to 400 nm. Peaks were measured at 215 nm for furfuryl alcohol; at 254 nm for 2FA; and 280 nm for DHMF, 5HMF, F2A, and 2-acetylfuran.

Identification was carried out by a spiking technique and comparing retention times (t_R), retention factor (k'), and spectra against a known standard.

Method validation

Repeatability of the method was determined by calculating the relative standard deviation (RSD) of the analyte concentration of five repeated HPLC runs of a standard solution, containing each compound at the level commonly found in TBV.

Reproducibility was evaluated by injecting, in triplicate, standard solutions over a period of 20 working days. Standard samples were quantitated on five days, randomly chosen in this working period.

Linearity was obtained with five solutions derived by sequentially diluting a concentrated standard solution. The same calibration runs were used for detection limits, which were considered the concentration showing a signal thrice higher than the noise. Recoveries were calculated by spiking TBV with a known amount of each compound.

Results and Discussion

Ion-exclusion chromatography is a popular technique for the separation of organic acids and other ionizable chemical species (30,31). For neutral species (alcohols, hydrocarbons, and phenols), idrophobic interaction with stationary phase can occur, then driving to a delayed elution and peak tailing (32). By adding organic modifier (typically acetonitrile, up to 30%) in the mobile phase (30,31), this drawback can be minimized.

However, the relatively strong retention of furanic compounds can be useful for their separation from other interfering compounds, avoiding a time-consuming sample treatment. Use of ion-exclusion chromatography for furans separation by using sulfuric acid and acetonitrile, was recently reported (33,34) and applied to beverages and fruit juices.

Initially, chemical stability of standard molecules in an acidic and heated medium (i.e., chromatographic conditions) was investigated. In such conditions, partial decomposition has been reported for furfuryl alcohol (19,35) and DMHF (36). Figures 1 and 2 show the peak areas of each compound as affected by both acid and temperature variation in chromatographic conditions. For

Table I. Cromatographic Parameters, Concentration Ranges, Detection Wavelengths, and Method Validation for Standard Compounds

n°	Compound	t_R (min)	Retention factor (k')	Adsorption max (nm)	Detection wavelength (nm)	Range (mg/L)	Linearity (R^2)	LOD (mg/L)	Repeatability %RSD (n = 5)	Reproducibility %RSD* (n = 5)
1	Acetic acid	13.32	2.20	205	215	5000–1	0.999	0.25	n.d.†	n.d.
2	DMHF	17.37	2.87	287	280	125–0.1	0.999	0.06	0.20	0.24
3	Unknown	17.75	2.93	292,217	–	–	–	–	–	–
4	2-Furoic acid	19.10	3.16	253	254	100–0.1	0.999	0.03	0.42	0.29
5	5HMF	21.17	3.50	284,227	280	1500.1	0.998	0.02	0.48	1.20
6	Furfuryl alcohol	22.14	3.66	215	215	1500.1	0.989	0.06	0.30	2.04
7	Unknown	24.80	4.10	273	–	–	–	–	–	–
8	Unknown	29.70	4.91	278,222	–	–	–	–	–	–
9	Furfural	31.62	5.23	277,227	280	150–0.1	0.988	0.02	0.15	0.30
10	2-Acetylfuran	33.10	5.47	273,225	280	100–0.1	0.999	0.04	0.38	0.38

* Five replicates within 20 days.
† n.d., not determined.

almost all the compounds, no significant variation was recorded, changing phosphoric acid from 0.05 to 0.005N (Figure 1). A decrease of 2FA peak area (up to 15%) was detected as a consequence of the acidity fall. This condition was responsible for higher dissociation of the acid. On the contrary, furfuryl alcohol benefited by weaker acidity, which probably lowered its decomposition.

Increasing elution temperature led to a fast and progressive loss of furfuryl alcohol (Figure 2). According to Choura et al. (35), condensation of furfuryl alcohol to polymeric chains can occur in heated acidic media at temperatures higher than 20°C. Therefore, this can provoke a decrease in its peak area and a rapid occlusion of the resin bed.

2-Furoic acid also tends to decrease its area with temperatures higher than 35°C, probably because of the increased dissociation degree. According to these results, the next trials were conducted at 22°C.

For method optimization, a stepwise experiment was carried out. Sulphuric and phosphoric acid at different levels were compared for their ability to separate furanic compounds with a fixed concentration of organic modifier (i.e., acetonitrile). The best combination of acid and relative concentration was then used to optimize the acetonitrile content of the mobile phase. Figure 3 shows the retention times as affected by the variation of mobile phase acidic strength. As expected, only 2FA was influenced by these changes. The weaker the acidity, the shorter its elution time was. On the other hand, 2FA's area was depressed by mobile phase low acidity (Figure 1). Because of these considerations, 0.01N phosphoric acid was chosen as the mobile phase. Various percentages of acetonitrile (14–24%) were then added to the selected solution. On increasing acetonitrile content, the t_R of furanic compounds decreased (Figure 4); and with high percentages of acetonitrile, furfural and 2-acetylfuran were unresolved. Moreover, 2FA and DMHF tend to overlap. The best elution conditions were, therefore, established with 84% 0.01N phosphoric acid–16% acetonitrile at 22°C. A typical chromatogram of standard compounds run under these conditions is shown in Figure 5. If compared with similar published methods (33,34), a comparable resolution was obtained with shorter run times (~ 33 vs. 40 min).

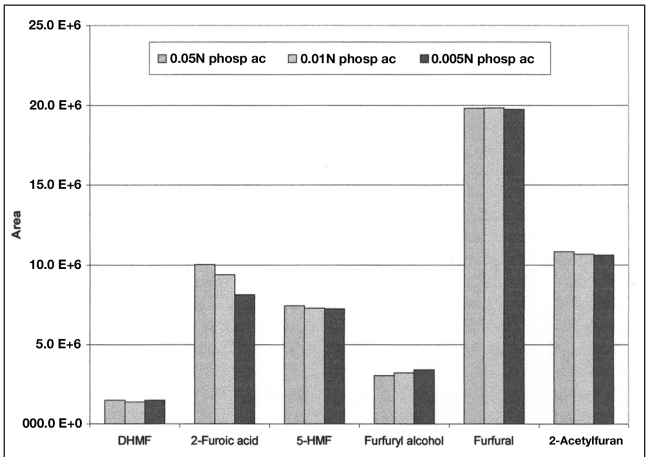

Figure 1. Peak area variations of standard compounds as affected by mobile phase acidity. Other conditions: flow, 0.6 mL/min; acetonitrile, 14%; and temperature, 22°C.

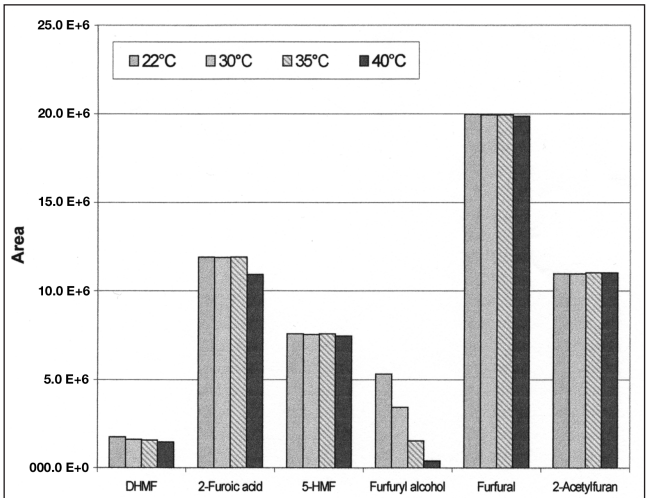

Figure 2. Peak area variations of standard compounds as affected by temperature. Other conditions: flow, 0.6 mL/min and mobile phase, 0.01N phosphoric acid (86%)–acetonitrile (14%).

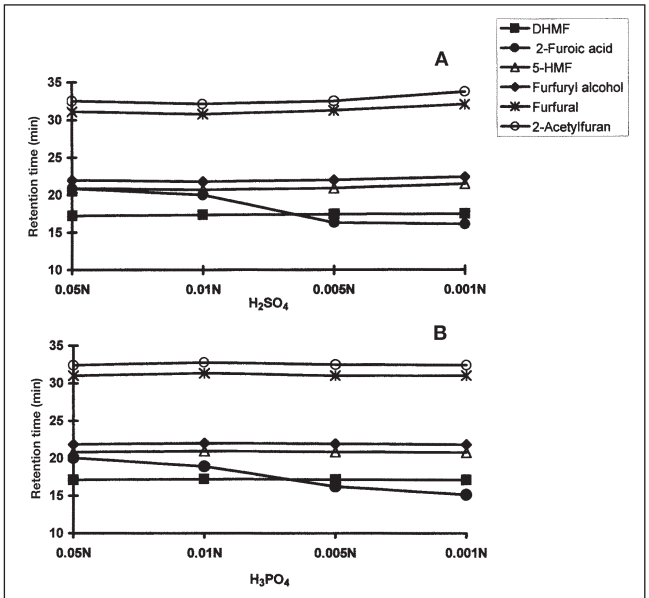

Figure 3. Effect of sulfuric acid (A) and phosphoric acid (B) content of mobile phase on retention times of standard compounds. Other chromatographic conditions: flow, 0.6 mL/min; acetonitrile 16%; and temperature, 22°C.

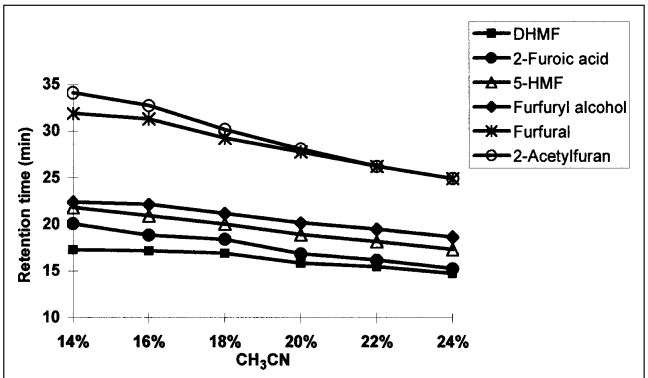

Figure 4. Effect of increasing acetonitrile content on retention times of standard compounds. Other conditions: flow, 0.6 mL/min; mobile phase, 0.01N phosphoric acid (86%); and temperature, 22°C.

Within the concentration tested, the method showed good linearity and correlation coefficients (Table I). Limits of detection were between 0.02 and 0.06 mg/L. The repeatability of the standard mixture (five replicate injections) was very good, and reproducibility, evaluated by the standard deviation (SD) of five runs randomly carried out in a 20-day working period, was satisfactory. RSD was lower than 1%, except for 5HMF and furfuryl alcohol. The latter showed the highest variability (2.04%) because of its instability in acid media. Recoveries were determined by adding known amounts of standards to an authentic sample (Table II).

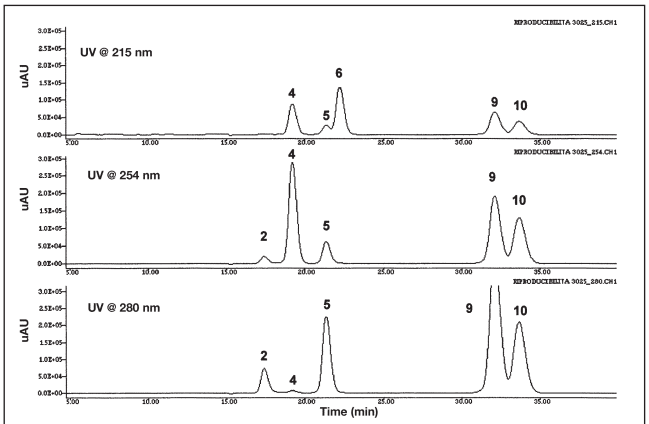

Figure 5. Chromatograms of standard solution. For peak identification, see Table I. Other conditions: flow, 0.6 mL/min; mobile phase, 0.01N phosphoric acid (86%)–ACN (14%); and temperature, 22°C.

Table II. Recoveries of Standard Addition in TBV

Compound	Content (mg/kg ± SD)	Amount added (mg/kg)	Found (mg/kg ± SD)	Recovery (% ± SD)
DMHF	ND*.	6.5	6.6 ± 0.01	101.5 ± 0.2
2-Furoic acid	1.6 ± 0.02	5.0	6.8 ± 0.03	103.0 ± 0.5
5HMF	108.0 ± 3.40	50.0	160.7 ± 2.15	101.7 ± 0.3
Furfuryl alcohol	ND	5.0	4.9 ± 0.41	98.0 ± 1.4
Furfural	1.0 ± 0.02	5.0	6.1 ± 0.01	101.7 ± 0.3
2-Acetylfuran	ND	5.0	5.2 ± 0.02	104.0 ± 0.4

* ND, not detected.

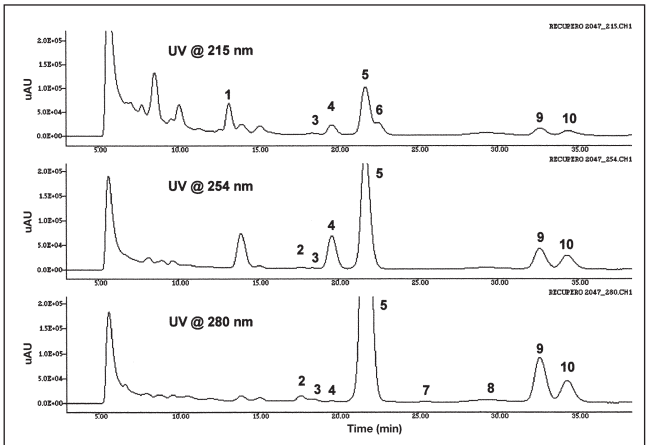

Figure 6. Chromatograms of spiked TBV. For peak identification, see Table I.

Values from 98.0% to 104.0% were obtained. Also in this case, its chemical susceptibility penalized furfuryl alcohol.

The method was applied to the 19 samples supplied by the Consorzio of TBV. Figure 6 shows a typical chromatogram of a spiked sample. Furanic compounds eluted after a group of scarcely resolved peaks, which appeared from 5 to 15 min. Sugars and acids were the main constituents of this cluster. Among them, only acetic acid was easily detectable. In Table III are the results obtained for the tested samples. The only furans detected in TBV were 5HMF, F2A, and 2FA. Among them, 5HMF was the principal compound, ranging from 2.39 to 5.36 g/kg. According to Theobald et al. (29), these contents account for TBV coming from a ten-year period of storage, at least. Significantly lower contents of F2A and 2FA were also detected in all the samples. These compounds seemed somehow related to 5-HMF; the greater the

Table III. Content (mg/kg) of Furanic Compounds in Authentic Samples

Sample *n*°	2-Furoic acid	5-HMF	Furfural	5-AMFA
1	41.9	2754	14.8	31.2
2	41.5	2805	23.1	ND*
3	43.1	2566	33.8	ND
4	31.3	3809	65.7	ND
5	25.4	3307	27.3	ND
6	46.9	2387	4.9	47.7
7	38.6	2413	36.9	ND
8	35.4	3166	11.8	89.3
9	51.8	3462	46.1	ND
10	94.6	4039	61.7	188.1
11	50.7	5356	41.9	84.3
12	24.1	3832	46.9	ND
13	44.3	3107	18.0	67.7
14	39.2	3243	36.5	ND
15	53.6	3487	17.3	84.8
16	49.9	3817	64.5	130.0
17	35.3	3204	34.3	ND
18	35.9	3056	35.0	ND
19	13.3	3069	65.0	ND

* ND, not detected.

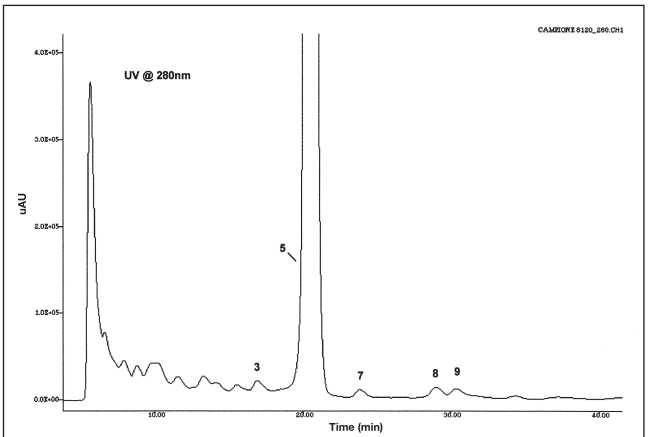

Figure 7. Chromatogram of authentic TBV sample showing the unknown peaks 3, 7, and 8.

5-HMF content, the higher the sum of F2A and 2FA found in vinegars. However, regression analyses performed on the data set showed no significant correlation among the different analytes. The substances were generated during the concentration step. Then, annual additions of cooked must have modified the original composition. In addition, during aging, a progressive concentration of the product led to an acidity increase that reached a final pH of slightly higher than 2. The lack of other furanic compounds and their uncorrelation may have been justified by both their instability and long aging time. The preparative dilution operated on the analyzed samples may have been a further cause. Thus, undiluted samples were also randomly injected, but DMHF, 2-acetylfuran, and furfuryl alcohol were not detected.

In addition to identified compounds, 3 unknown peaks were also detected (Figure 7 and Table I) and revealed at 280 nm. Compound 3 eluted just after DMHF, and its furan-like spectrum (TableI) was similar to that reported for 5-methylfurfural by Kermasha et al. (3). Compound 7 showed a spectrum similar to another unknown compound in wine and beer reported earlier by Yuan et al. (33).

The last unknown compound (*n*° 8 in Table I), was identified as 5-acethoxymethyl-2-furaldehyde on the basis of its UV spectrum and by spiking authentic samples with the standard compound. This molecule could be derived by the esterification of 5HMF with acetic acid. It was proposed by Giacco et al. (37) as a possible marker for TBV older than six years. These authors, using a GC method, found concentrations up to 20 mg/L. In our samples, 5-acethoxymethyl-2-furaldehyde ranged from 0 to 188.1 mg/kg, values that, even though we experienced an oncolumn partial hydrolysis during the calibration curve build up, are higher than that previously reported. This discrepancy was probably because of the different chromatographic technique we used and, at the moment, this substance does not seem to be an efficient quality marker for TBV. On the other hand, GC is probably a more suitable tool for the quantitation of a small amount of 5AMFA. Further studies need to confirm this hypothesis and identify the remaining unknown compounds.

Conclusion

Separation and quantitation of furanic compounds in TBVs were successfully performed by this improved HPLC method. In spite of the matrix complexity, no sample pretreatment was required, apart from dilution and filtration. In actual samples, 5HMF, furfural, and 2FA were detected. To the best of our knowledge,this is the first time that the latter acid is found in TBV. Unknown compounds were also found in almost all the samples. One of these compounds was identified as 5-acethoxymethylfurfural, a furan previously reported in TBV. With the proposed method, the quantitation of acetic acid is also possible.

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