High-Performance Liquid Chromatographic Determination of 2-Furaldehyde and 5-Hydroxymethyl-2-furaldehyde in Fruit Juices

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Abstract

A method for the determination of 2-furaldehyde (F) and 5-hydroxymethyl-2-furaldehyde (HMF) in fruit juices by high-performance liquid chromatography (HPLC) is described. The method is based on the formation of the 2,4-dinitrophenylhydrazones of carbonyl compounds and subsequent separation of these derivatives. Derivatization is carried out by utilizing an acidic solution of 2,4-dinitrophenylhydrazine in acetonitrile. Precipitation of the derivatives of carbonyl compounds is thus avoided, and direct injection of the sample into the HPLC system is allowed. The procedure offers a high specificity and a detection limit of the order of 10^{-8} mol/L. Recoveries of 95–98% are obtained from apple juice spiked at different levels with both analytes. The reproducibility (mean of six determinations) is ± 2% for F and ± 3% for HMF.

Introduction

The occurrence and level of 2-furaldehyde (F) and 5-hydroxymethyl-2-furaldehyde (HMF) in fruit juices are indications of quality deterioration (1–5). Fruit juices undergo flavor, taste, color, and nutritional changes when stored at warm temperatures and/or for prolonged periods of time (4,6–10). Both F and HMF are formed during thermal processing or storage at improper temperatures; for this reason, both are useful indicators of temperature abuse in fruit juices (3,4,6,8,11–13). In particular, F is virtually absent in fresh juices, whereas large amounts have been found in juices stored at improper temperatures (6,9,13,14). A close relationship between flavor changes and F content has been demonstrated; for this reason, the F content is useful as an off-flavor indicator (6–9,11,13). On the other hand, HMF is the main decomposition product of glucose and fructose (5,14) and plays a role in nonenzymic browning reactions that occur in fruit juices (4,8,11,13,15). The detection and quantitative determination of these components become more and more important as aseptic processing and packaging of fruit juices assert themselves (6). Aseptic packaging allows higher temperatures during distribution and storage of the product to be adopted without microbial spoilage, but off-flavors and loss of nutritional value may develop as fruit juices are exposed to these conditions (6).

The classical methods for the quantitative determination of F and HMF in fruit juices are based on spectrophotometric measurements (7,9,11,12,14,16). These methods (*a*) are time consuming, (*b*) make use of toxic or hazardous chemicals, (*c*) require a strict control of both reaction time and temperature because the instability of the reaction product may lead to low recoveries and wide statistical variations of the results, and (*d*) are not specific (1,2,6,11,12,14,16). In recent years, high-performance liquid chromatographic (HPLC) methods have been proposed (1–6,11,13,17). These methods are less time-consuming; offer improved accuracy, sensitivity, and specificity; and utilize less hazardous reagents (4,6,11).

However, the HPLC methods proposed so far call for the injection of the sample without derivatization (1–6,11,13,17). Both sensitivity and selectivity of the method could be improved by a derivatization step in order to introduce a selective chromophore into the molecule. This type of derivatization has already been employed by us for the determination of F and HMF in other kinds of food matrices (18–21).

In this paper, a method is described that is based on the formation of the 2,4-dinitrophenylhydrazones (DNPH-ones) of carbonyl compounds. The DNPH-ones are then separated by HPLC and determined with spectrophotometric detection.

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Experimental

Standards and reagents

2-Furaldehyde (Prolabo, Paris, France) was doubly distilled at atmospheric pressure; the fraction with a boiling point of 161–163°C was collected and kept in an airtight vial at –20°C to prevent browning of the product. Both 5-hydroxymethyl-2-furaldehyde and 2,4-dinitrophenylhydrazine (DNPH) (Prolabo) were purified by successive crystallizations with HPLC-grade methanol and kept in a refrigerator at 0–4°C. Water was distilled, deionized, and then further purified with a Milli-Q system (Millipore, Milford, MA).

pH determination

pH determination was performed using an S 202 digital pH-meter (SEAC, Calenzano, Italy) supplied with an Inlab 420 electrode (Mettler-Toledo, Greifensee, Switzerland).

DNPH solutions

A stock reagent solution containing 2.5×10^{-3} mol/L of DNPH was prepared in acetonitrile. By successive dilutions with acetonitrile, reagent solutions containing as little as 2.5×10^{-6} mol/L of DNPH were prepared.

F and HMF standard solutions

A stock standard solution containing 1.0×10^{-2} mol/L of both F and HMF was prepared in water. By successive dilutions with water, working standard solutions containing as little as 1.0×10^{-7} mol/L of both analytes were prepared.

Calibration graphs

A 5-mL volume of each working standard solution and 4 mL of a DNPH solution five times more concentrated than the working standard solution were transferred into a 10-mL glass-stoppered volumetric flask. A 0.4-mL volume of 70% perchloric acid (Prolabo) was added, and the volume was made up to the mark with an additional amount of the DNPH solution. The content of the volumetric flask was tranferred into a 25-mL beaker, and the pH was eventually adjusted to 1 with a few drops of perchloric acid. The beaker was kept on a magnetic stirrer at room temperature for at least 25 min; then 10 µL of the solution was immediately injected into the HPLC system.

Sample processing

A 5-mL volume of fruit juice was added with 4 mL of the stock DNPH solution and subjected to the same procedure described in the *Calibration graphs* section. The acetonitrile solution of the DNPH-ones of carbonyl compounds was centrifuged for 3 min at 150 g before injection into the HPLC system. A syringe attached to a Millex-LCR_{13} (Millipore) was used to remove all particles larger than 0.5 µm.

Determination of recoveries

A 2.5-mL volume of a working standard solution containing from 1.0×10^{-2} to 1.0×10^{-6} mol/L of both F and HMF was added to 10 mL of an apple juice sample. The sample obtained was processed as described in the *Sample processing* section. Each determination was carried out in triplicate; each solution was injected twice.

HPLC

A Spectra-Physics model 8700 (Carlo Erba, Milan, Italy) HPLC equipped with a Knauer model 8700 (Knauer, Berlin, Germany) variable-wavelength spectrophotometric detector and a 10-µL loop was used. A Supelcosil LC-18 (Supelco, Bellefonte, PA) stainless-steel column (250 × 4.6-mm i.d.; 5-µm particle size) was employed. Analyses were carried out isocratically at room temperature with acetonitrile–water (55:45, v/v) as the eluent at a flow rate of 1 mL/min. The spectrophotometric detector was set at 385 nm. Peak areas were determined by means of a Spectra-Physics model 4270 (Carlo Erba) integrator.

HPLC–mass spectrometry

The same HPLC system described in the preceding section was employed, with the exception that a Supelcosil LC-18 stainless-steel preparative column (250 × 10-mm i.d.; 5-µm particle size) and a 50-µL loop were used. A 100-mL volume of fruit juice was subjected to the same procedure described in the *Sample processing* section. The water–acetonitrile solution of the DNPH-ones of carbonyl compounds was reduced to a 2-mL volume in a rotary vacuum evaporator at 60°C; 10 50-µL aliquots were successively injected into the HPLC system. The fractions corresponding to the elution of the two analytes of interest were collected in two different vials and dried in a stream of nitrogen. The two solid samples obtained (about 100 µg each) were directly introduced into the mass spectrometric (MS) system. A Hewlett-Packard model 5988A (Hewlett-Packard, Palo Alto, CA) MS, equipped with a direct insertion probe source, was used; fragmentation was induced under a 70-eV electron impact.

Statistical analysis

A student *t*-test was used to determine if significant differences existed among results obtained under different experimental conditions.

Results and Discussion

Optimization of the derivatization step

The key to the determination of F and HMF in fruit juices lies in the sample preparation because the matrix contains compounds that may interfere with the analytes (1,6). For this purpose, procedures such as distillation (6,7,9,16), solvent extraction (10,11), juice filtration (2,5,17), and clarification with the Carrez solution (1,13,15) have been developed. However, preliminary distillation allows for recovery only of F, gives low and variable yields, and also collects other components (7,16). Solvent extraction is affected by incomplete recoveries and is not specific for the analytes of interest (1). Filtration is certainly the less troublesome procedure, but it does not allow the elimination of any of the possibly interfering compounds. A preliminary cleanup procedure that involves

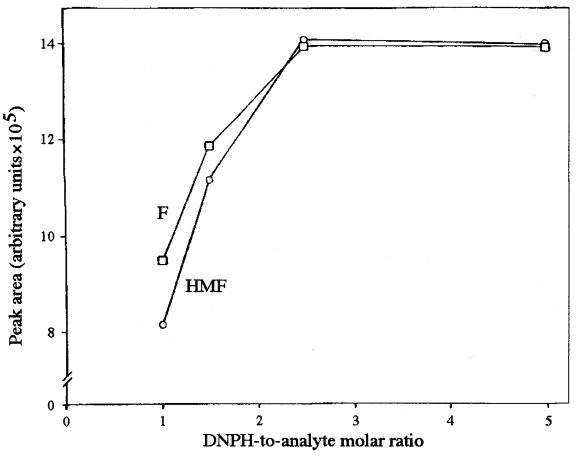

Figure 1. Conversion of F and HMF to their DNPH-ones as a function of the DNPH-to-analyte molar ratio. Medium pH, 2.5; reaction time, 30 min.

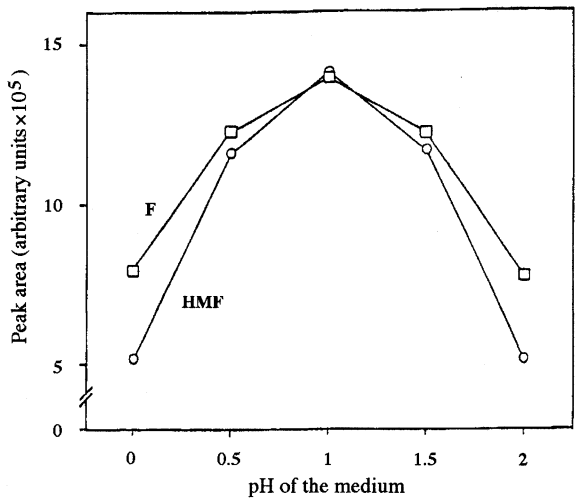

Figure 2. Conversion of F and HMF to their DNPH-ones as a function of the acidity of the medium. DNPH-to-analyte molar ratio, 2.5; reaction time, 30 min.

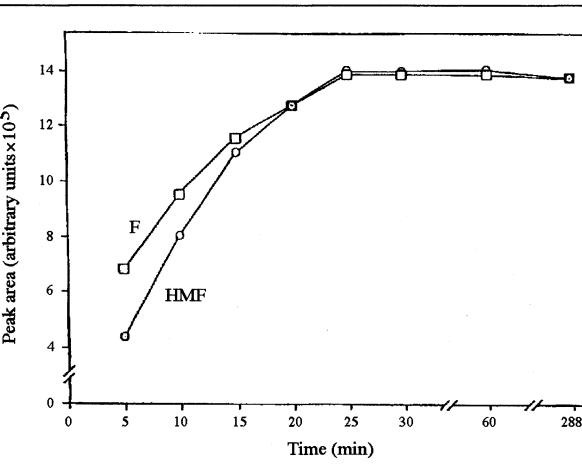

Figure 3. Conversion of F and HMF to their DNPH-ones as a function of reaction time. DNPH-to-analyte molar ratio, 2.5; medium pH, 1.

juice clarification by means of the Carrez solution allows the elimination of pulp, proteins, fats, and carotenoids (1,13,15). In our case, we have adopted a centrifugation of the sample combined with the use of a filtration cartridge before injection into the HPLC system.

The DNPH-ones are usually obtained by employing an excess of DNPH aqueous solution in the presence of hydrochloric acid. The utilization of an acetonitrile DNPH solution in the presence of perchloric acid offered the advantage of obtaining a derivative solution that could be injected directly into the HPLC system. Long and tedious steps, such as filtration and washing of the derivatives obtained and preparation of a derivative solution in a suitable solvent before the HPLC determination, may therefore be avoided. The use of perchloric acid instead of hydrochloric acid was due to its higher solubility in acetonitrile.

The derivatization step was optimized with respect to three parameters: the DNPH-to-analyte molar ratio, the acidity of the medium, and the reaction time. For this purpose, the amounts of the derivatives obtained were evaluated on two standard solutions containing 10^{-4} mol/L of F and HMF, respectively. The results obtained are shown in Figures 1–3. As may be seen, the derivatization reaction was quantitative when the reagent-to-analyte ratio was at least 2.5:1 for both analytes and the acidity of the medium, as evaluated with a pH-meter, was about 1. Under these conditions, both F and HMF were quantitatively converted into their DNPH-ones within 25 min. The derivatives obtained were stable at room temperature for at least 48 h.

The derivatization step was carried out on the same sample of apricot juice for 30, 60, 90, and 120 min to verify that no artifactual amounts of either of the analytes were produced during the preliminary sample processing under acidic conditions; each solution was injected three times. A slight increase of the amount of F was obtained at 60 min, but this was followed by a decrease at both 90 and 120 min to levels even slightly lower than those at 30 min. A slight, progressive increase in the amount of HMF was observed. However, none of the values obtained for both F and HMF were significantly different ($p < 0.05$) with respect to the amount observed at 30 min.

Table I. Statistics Relative to Calibration, Detection Limits, and Reproducibility for the Determination of F and HMF

	F	HMF
Slope	1.0014	1.0098
Intercept	10.1501	10.1781
Standard deviation of the slope	0.0010	0.0015
Standard deviation of the intercept	0.0053	0.0076
Standard error	0.0080	0.0113
Number of data points	6*	6*
Correlation coefficient	0.999985	0.999971
Detection limit (mol/L)	2.2×10^{-8}	2.4×10^{-8}
Reproducibility (%)	2†	3†

* Six determinations of each point.
† Mean of six determinations.

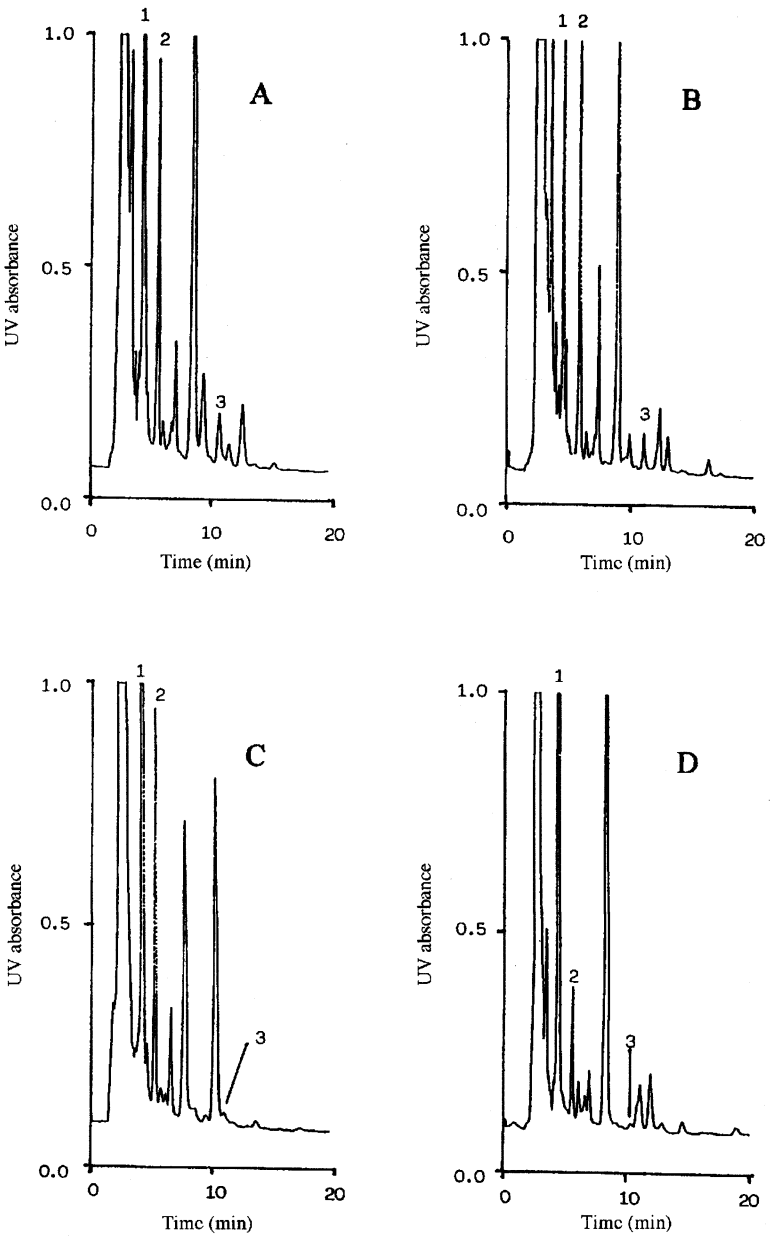

Figure 4. HPLC separation of the DNPH-ones of carbonyl compounds from commercial samples of (A) pear juice, (B) peach juice, (C) apricot juice, and (D) apple juice. For analysis conditions, see the *Experimental* section. Peaks: 1, DNPH; 2, HMF DNPH-one; 3, F DNPH-one.

Calibration

The calibration graphs were obtained by employing standard solutions of both F and HMF under optimum experimental conditions as described in the preceding section. A straight line was obtained for both analytes over the range of concentrations from 10^{-3} to 10^{-7} mol/L, which represents values typically found in real samples. The parameters of the linear regression relative to the bilogarithmic plot of the DNPH-one area versus the concentration of the analyte of interest in the sample under examination are collected in Table I.

By setting the detector wavelength at the maximum absorbance of the derivatives of both F and HMF, it was possible to determine the detection limit as $3\sigma/S$ (22), where S is the sensitivity (1.39×10^{10} for F and 1.26×10^{10} for HMF, as obtained from the calibration graphs), and σ is the peak threshold of the integrator, which was set by us at 100. The detection limits (see Table I) were much lower than those reported by other authors for the determination of F and HMF by HPLC without derivatization (1,5,6,11,17).

Specificity, recovery, and reproducibility

The method showed a high specificity because the derivatives of both F and HMF were well-separated from the other carbonyl compounds present in the sample. As an example, Figure 4 shows some typical separations obtained on commercial samples of fruit juices. The identity of the peaks was established by co-injection with the corresponding pure standards.

To verify the absence of interferences with the two analytes of interest, the fractions obtained from the chromatographic run were subjected to MS analysis. The mass spectrum of the compound corresponding to the DNPH-one of F showed the molecular ion M^+ at *m/z* 276; moreover, the mass spectrum was in excellent agreement with that of the DNPH-one of an F standard. The mass spectrum of the HPLC fraction corresponding to the derivative of HMF showed the molecular ion M^+ at *m/z* 306; again, the mass spectrum was in excellent agreement with that of the DNPH-one of an HMF standard.

Fruit juices show a highly variable composition of minor components, both in concentration and nature, several of which are carbohydrates that may form the corresponding DNPH-ones during the preliminary derivatization step. However, interferences with the two analytes of interest were observed in none of the samples examined. On the contrary, interferences may occur when the clarified sample is directly injected into the HPLC system (1,6).

DNPH must be at least 20 times more concentrated than the analytes to be determined in the analyses of real samples, as an aliquot of the reagent is employed in the derivatization of the other carbonyl compounds present. In all the samples examined so far, a 1:20 ratio was sufficient because (*a*) a large peak of the DNPH excess appeared in the chromatogram, and (*b*) area increments were not obtained for the two analytes of interest by utilizing a 1:50 analyte-to-reagent ratio.

Recoveries were determined by adding known amounts of both analytes to a sample of apple juice. The amount found with respect to the sum between the amount added and that originally present in the sample represents the recovery. The juice was selected on the basis of its low content of both F and HMF (1.87×10^{-6} and 4.85×10^{-5} mol/L, respectively), two of the lowest levels among those found in real samples. The

Table II. Recoveries of F and HMF Added to an Apple Juice Sample

Originally present	Added	Found*	Recovery (%)*
Concentration of HMF (mol/L)			
4.85×10^{-5}	1.0×10^{-6}	$(4.70 \pm 0.14) \times 10^{-5}$	95 ± 3
4.85×10^{-5}	1.0×10^{-5}	$(5.61 \pm 0.11) \times 10^{-5}$	96 ± 2
4.85×10^{-5}	1.0×10^{-4}	$(1.44 \pm 0.03) \times 10^{-4}$	97 ± 2
4.85×10^{-5}	1.0×10^{-3}	$(1.02 \pm 0.02) \times 10^{-3}$	97 ± 2
Concentration of F (mol/L)			
1.87×10^{-6}	1.0×10^{-7}	$(1.89 \pm 0.06) \times 10^{-7}$	96 ± 3
1.87×10^{-6}	1.0×10^{-6}	$(2.72 \pm 0.05) \times 10^{-6}$	95 ± 2
1.87×10^{-6}	1.0×10^{-5}	$(1.14 \pm 0.02) \times 10^{-5}$	96 ± 2
1.87×10^{-6}	1.0×10^{-4}	$(0.99 \pm 0.02) \times 10^{-4}$	97 ± 2
1.87×10^{-6}	1.0×10^{-3}	$(9.81 \pm 0.19) \times 10^{-3}$	98 ± 2

* Mean of six determinations plus standard deviation.

Table III. Concentrations of F and HMF Found in Some Commercial Fruit Juices*

Sample	HMF concentration (mol/L)	F concentration (mol/L)
Apricot 1	$(6.66 \pm 0.19) \times 10^{-5}$	$(4.82 \pm 0.14) \times 10^{-6}$
Apricot 2	$(7.18 \pm 0.21) \times 10^{-5}$	$(4.87 \pm 0.09) \times 10^{-6}$
Apricot 3	$(9.19 \pm 0.36) \times 10^{-5}$	$(5.35 \pm 0.16) \times 10^{-6}$
Pear 1	$(7.74 \pm 0.15) \times 10^{-5}$	$(6.41 \pm 0.25) \times 10^{-6}$
Pear 2	$(9.16 \pm 0.18) \times 10^{-5}$	$(6.94 \pm 0.13) \times 10^{-6}$
Pear 3	$(9.01 \pm 0.27) \times 10^{-5}$	$(7.85 \pm 0.29) \times 10^{-6}$
Peach 1	$(9.82 \pm 0.29) \times 10^{-5}$	$(7.43 \pm 0.14) \times 10^{-6}$
Peach 2	$(8.57 \pm 0.17) \times 10^{-5}$	$(7.16 \pm 0.21) \times 10^{-6}$
Peach 3	$(9.08 \pm 0.36) \times 10^{-5}$	$(7.09 \pm 0.28) \times 10^{-6}$
Apple 1	$(4.85 \pm 0.19) \times 10^{-5}$	$(1.87 \pm 0.04) \times 10^{-6}$
Apple 2	$(5.42 \pm 0.16) \times 10^{-5}$	$(2.07 \pm 0.08) \times 10^{-6}$
Apple 3	$(4.65 \pm 0.13) \times 10^{-5}$	$(2.15 \pm 0.06) \times 10^{-6}$

* Mean of six determinations plus standard deviation.

results obtained are shown in Table II. Recoveries for both analytes ranged from 95 to 98%.

Reproducibility is shown in Table I and was evaluated by carrying out the determination six times on the same sample of peach juice over a period of 48 h; each solution was injected twice. The average concentration of F was 7.43×10^{-6} mol/L with a standard deviation of 1.4×10^{-7} mol/L; the average concentration of HMF was 9.82×10^{-5} mol/L with a standard deviation of 2.9×10^{-6} mol/L.

Application

The procedure was applied to the determination of F and HMF in different commercial samples of fruit juices; each sample was analyzed in duplicate. The results are summarized in Table III. As may be seen, in all the samples analyzed, the amount of HMF (range, 10^{-4}–10^{-5} mol/L) was one order of magnitude greater than the amount of F (range, 10^{-5}–10^{-6} mol/L). No apparent sign of column performance deterioration, such as tailing or the appearance of spurious peaks, was observed after six months of continuous use.

Conclusion

Both F and HMF were determined in all samples analyzed by employing the method described, whereas F was often not detected in previous HPLC reports based on direct injection of the juice after filtration or clarification. In fact, the detection limit was much lower than that reported by other authors who analyzed samples without derivatization. The method shows a high specificity; centrifugation of the juice combined with the use of a syringe attached to a filtration device was adequate to eliminate possible interferences. Recoveries for both analytes were practically quantitative over a wide range of examined concentrations, and reproducibility was very good.

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Manuscript accepted July 17, 1997.