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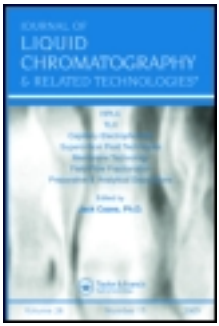

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 2-FURALDEHYDE AND 5-HYDROXYMETHYL-2-FURALDEHYDE IN PROCESSED CITRUS JUICES

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ABSTRACT

The occurrence of 2-furaldehyde (F) and 5-hydroxymethyl-2-furaldehyde (HMF) in processed citrus juices is an indication of quality deterioration. A close relationship between flavor changes and F content exists, while HMF can give rise to browning reactions. Both F and HMF are formed during heat processing or storage at improper temperatures. The detection of these compounds becomes more and more important as aseptic processing and packaging of citrus juices are becoming widespread. Aseptic packaging allows higher temperatures during distribution and storage to be employed without microbial spoilage, but off-flavors develop as citrus products are exposed to these conditions.

In this paper a method of determination 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 reversed-phase 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 determination offers a high specificity and a detection limit of the order of 10^{-8} mol/l for both analytes. Recoveries from an orange juice spiked at different levels are quantitative. Reproducibility data are presented.

INTRODUCTION

The occurrence of 2-furaldehyde (F) and 5-hydroxymethyl-2-furaldehyde (HMF) in processed citrus juices is an indication of quality deterioration[1-3]. Citrus juices undergo flavor, taste, color and nutritional changes when stored at warm temperatures and/or for prolonged periods of time[3-7]. 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 processed citrus juices[3,6,8-11]. In particular, F is virtually absent in fresh citrus juices, whereas large amounts have been found in juices stored at improper temperatures[4,6,10]. 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[4,6-8]. On the other hand, HMF is correlated with browning reactions[3,8,10-12]. The detection and quantitative determination of these components become more and more important as aseptic processing and packaging of citrus 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 citrus juices are exposed to these conditions[6].

The classical methods for the quantitative determination of these components in citrus juices are based on colorimetric measurements[1,2,4,6-9,13,14]. These methods I) are time consuming, II) make use of toxic or anyhow hazardous chemicals, III) require a strict control of both reaction time and temperature, since the instability of the colored reaction product may lead to low recoveries and wide statistical variations of the results and IV) no one of the methods is specific[1,2,6,8,9,12-14]. Gas chromatographic procedures have also been proposed for the determination of F and/or HMF not only in citrus juices, but also in other types of food matrices, in particular alcoholic beverages; they offer much higher specificity, sensitivity and speed[5,15-17]. In recent years, high performance liquid chromatographic (HPLC) methods have also been proposed[1-3,6,8,10,18]. These methods are less time consuming, offer improved accuracy, sensitivity and specificity as compared to the colorimetric procedures and utilize less hazardous reagents[3,6,8].

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

MATERIALS

Standards and Reagents

2-Furaldehyde (Prolabo) was doubly distilled and kept in a refrigerator at 0-4°C.

Both 5 hydroxymethyl-2-furaldehyde and 2,4-dinitrophenylhydrazine (Prolabo) were purified by successive crystallizations with HPLC-grade methanol and kept in a refrigerator at 0-4°C.

The Carrez clarification reagent (Carlo Erba) consisted of a 15% (w/v) solution of Carrez I (potassium ferrocianide) and of a 30% (w/v) solution of Carrez II (zinc sulfate).

Perchloric acid (70%) was obtained from Prolabo and acetonitrile (HPLC-grade) from Carlo Erba. Water was distilled, deionized and further purified with a Milli-Q system (Millipore).

2,4-Dinitrophenylhydrazine Solution

A stock reagent solution containing 2.5×10^{-3} mol/l of 2,4-dinitrophenylhydrazine (DNPH) was prepared in acetonitrile. By successive dilutions reagent solutions containing down to 2.5×10^{-6} mol/l of 2,4-dinitrophenylhydrazine were prepared.

2-Furaldehyde and 5-Hydroxymethyl-2-furaldehyde Standard Solutions

A stock standard solution containing 10^{-2} mol/l of both F and HMF was prepared in water. By successive dilutions working standard solutions containing down to 10^{-7} mol/l of both analytes were prepared.

Two aqueous solutions containing 10^{-4} mol/l of F and HMF respectively were also prepared.

METHODS

Calibration Graphs

A 5-ml volume of each working standard solution and 4 ml of a 5 times more concentrated DNPH solution were transferred into a 10-ml glass-stoppered volumetric flask. A few drops of perchloric

acid were added to pH 1 and the volume was made up to the mark with the DNPH solution. The solution was kept on a magnetic stirrer at room temperature for at least 25 min, then 10 μl of the solution were immediately injected into the HPLC system.

Sample Clarification

The procedure described by Lee et al. was adopted with some modifications[1]. 10 ml of juice were pipetted into a 50-ml beaker. 2 ml of Carrez I and 2 ml of Carrez II solution were added slowly with gentle mixing. After standing for 5 min, the mixture was filtered through a Milli-Q system, under suction, into a 25-ml volumetric flask, the filter was washed with distilled water, washings were added to the filtrate and the volume taken up to the mark with distilled water.

Preparation of the Derivatives of Carbonyl Compounds

The same procedure described under **Calibration Graphs** was applied to a 5-ml volume of clarified juice instead of working standard solution.

Determination of Recoveries

To 10 ml of a sample of orange juice was added a 2.5 ml volume of a working standard solution containing from 10^{-2} down to 10^{-6} mol/l of both F and HMF. The sample obtained was processed as described under **Sample Clarification**. A 5-ml volume of the so-obtained clarified juice was subjected to the same procedure described under **Preparation of the Derivatives of Carbonyl Compounds**. Each determination was carried out in triplicate; each solution was injected twice.

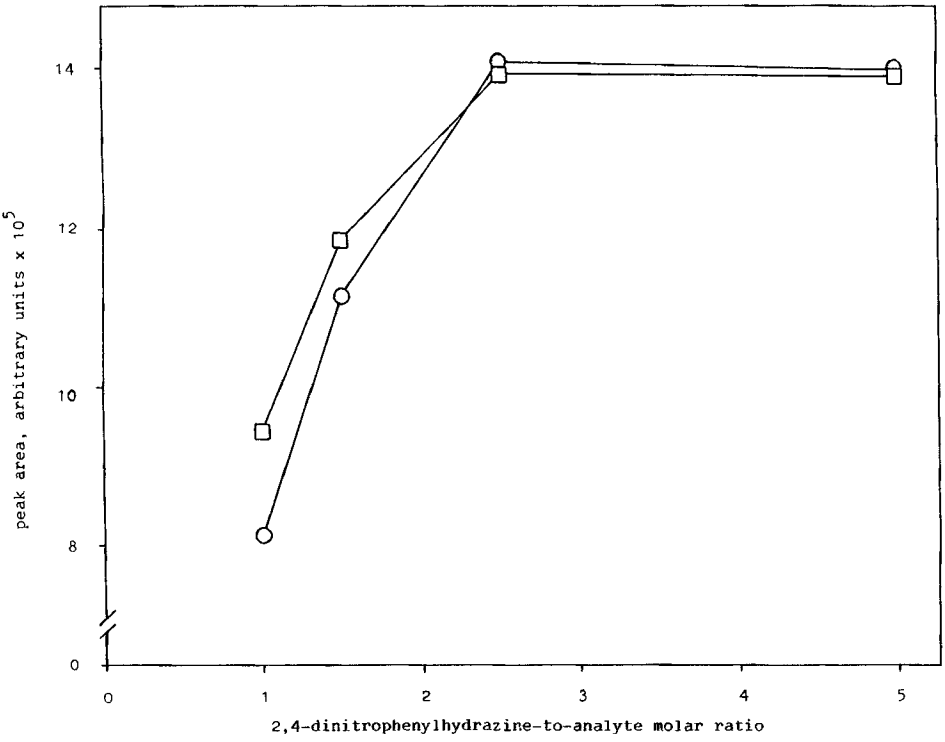

Figure 1. Conversion of 2-furaldehyde (□) and 5-hydroxymethyl-2-furaldehyde (○) to their 2,4-dinitrophenylhydrazones as a function of the 2,4-dinitrophenylhydrazine/2-furaldehyde and 2,4-dinitrophenylhydrazine/5-hydroxymethyl-2-furaldehyde molar ratios. pH of the medium = 1; reaction time = 30 min.

High Performance Liquid Chromatography

A Spectra-Physics Model 8700 high performance liquid chromatograph, equipped with a Knauer Model 8700 variable-wavelength spectrophotometric detector and a 10-μl loop, was used. A Supelcosil LC-18 stainless-steel column (250 x 4.6 mm I.D.; film thickness 5 μm) was employed. Analyses were carried out

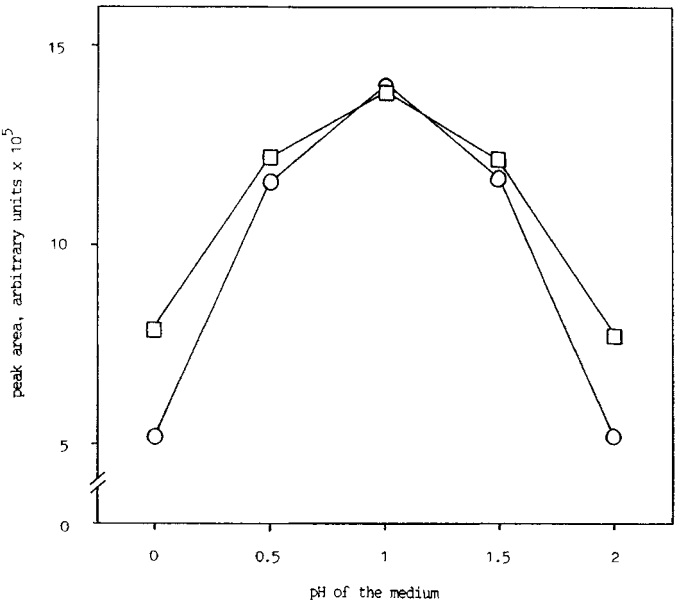

Figure 2. Conversion of 2-furaldehyde (□) and 5-hydroxymethyl-2-furaldehyde (○) to their 2,4-dinitrophenylhydrazones as a function of the acidity of the medium. 2,4-Dinitrophenylhydrazine/2-furaldehyde and 2,4-dinitrophenylhydrazine/5-hydroxymethyl-2-furaldehyde molar ratio = 2.5; reaction time = 30 min.

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 integrator.

RESULTS

The optimum experimental conditions for obtaining the DNPH-ones of carbonyl compounds present in the sample under

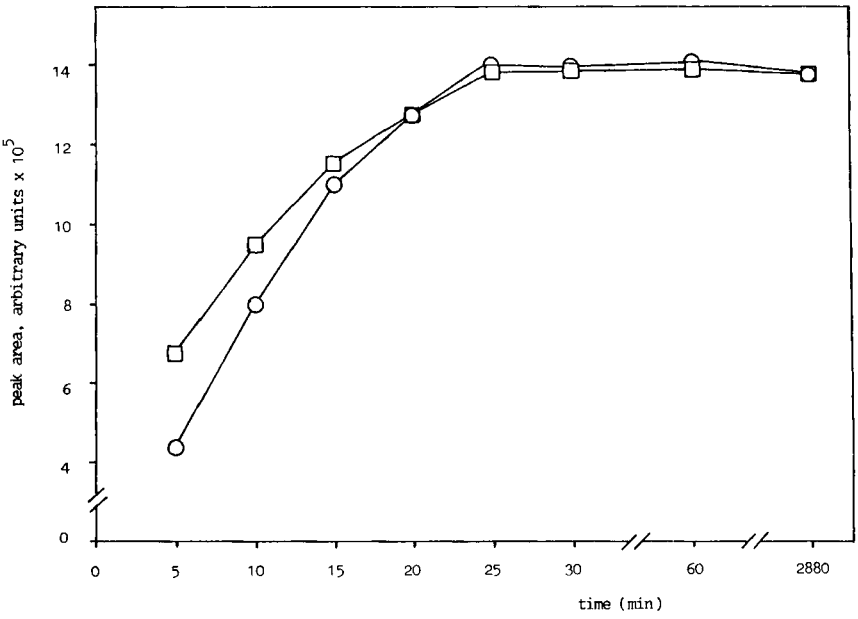

Figure 3. Conversion of 2-furaldehyde (□) and 5-hydroxymethyl-2-furaldehyde (○) to their 2,4-dinitrophenylhydrazones as a function of reaction time.
2,4-Dinitrophenylhydrazine/2-furaldehyde and 2,4-dinitrophenylhydrazine/5-hydroxymethyl-2-furaldehyde molar ratio = 2.5; pH of the medium = 1.

examination were evaluated. We took into account the possible influence of three variables. They are: 1) the DNPH-to-analyte molar ratio, 2) the acidity of the medium and 3) the reaction time. The results obtained are shown in Figures 1-3.

Calibration graphs were obtained for the quantitative determination of both F and HMF, and are shown in Figure 4.

A typical separation of the DNPH-ones of carbonyl compounds from a sample of orange juice is shown in Figure 5.

Recoveries were determined by adding known amounts of both analytes to a sample of orange juice. The amount found in respect

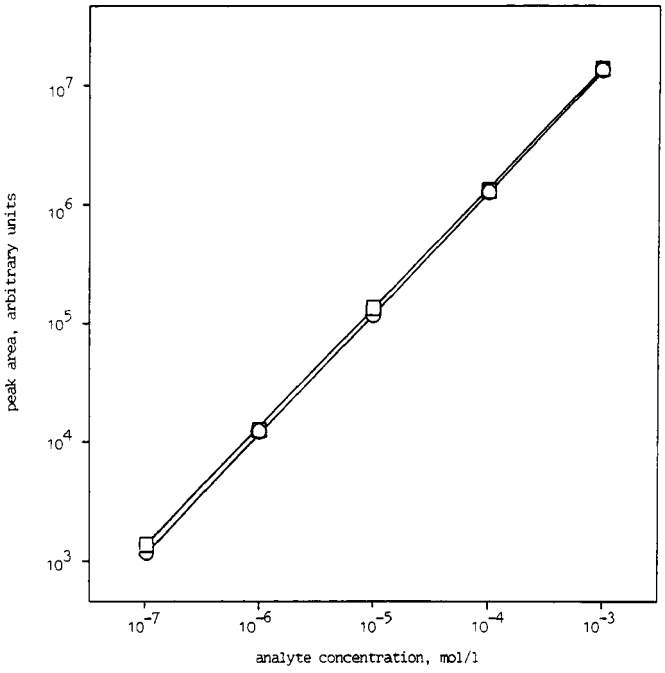

Figure 4. Calibration graph of peak area of the 2,4-dinitrophenylhydrazones of 2-furaldehyde (□) and 5-hydroxymethyl-2-furaldehyde (○) versus concentrations of the analytes. 2,4-Dinitrophenylhydrazine/2-furaldehyde and 2,4-dinitrophenylhydrazine/5-hydroxymethyl-2-furaldehyde molar ratio = 2.5; pH of the medium = 1; reaction time = 30 min.

of the sum between the amount added and that originally present in the sample represents the recovery. The results obtained are shown in Table 1.

Reproducibility was evaluated by carrying out the determination six times on the same commercial sample of orange juice over a period of 48 h; each solution was injected twice.

The procedure was applied to the determination of F and HMF in different samples of commercial citrus juices; each sample was analyzed in duplicate. The results are summarized in Table 2.

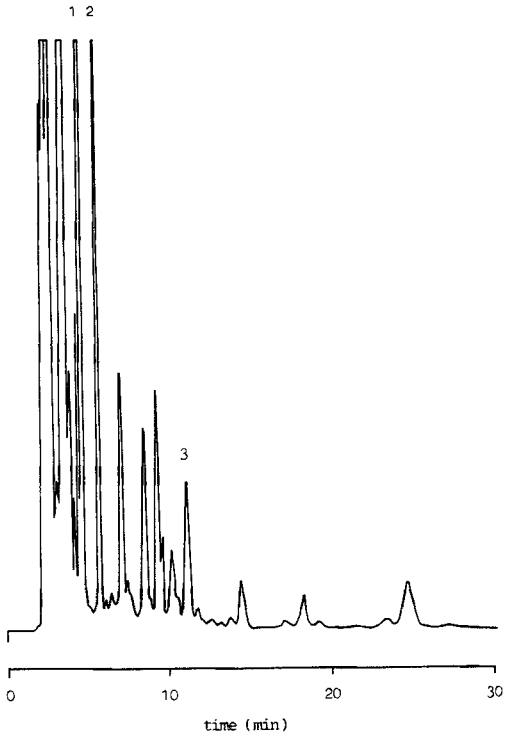

Figure 5. High performance liquid chromatographic separation of the 2,4-dinitrophenylhydrazones of carbonyl compounds from a sample of orange juice. For conditions of analysis see Methods. Peak identification: 1) 2,4-dinitrophenylhydrazine; 2) 2,4-dinitrophenylhydrazone of 5-hydroxymethyl-2-furaldehyde; 3) 2,4-dinitrophenylhydrazone of 2-furaldehyde.

DISCUSSION

Optimization of the Derivatization Step

The key to the determination of F and HMF in citrus juices lies in the sample preparation, since the matrix contains compounds that may interfere with the analytes[1,6]. For this purpose,

TABLE 1

Recoveries of 2-Furaldehyde and 5-Hydroxymethyl-2-furaldehyde Added to Orange Juice*.

Originally present	Added	Found	Recovery (%)
Concentration of 2-furaldehyde (mol/l)			
8.50×10^{-6}	1.0×10^{-7}	$(8.30 \pm 0.26) \times 10^{-6}$	96 ± 3
8.50×10^{-6}	1.0×10^{-6}	$(9.12 \pm 0.29) \times 10^{-6}$	96 ± 3
8.50×10^{-6}	1.0×10^{-5}	$(1.74 \pm 0.04) \times 10^{-5}$	94 ± 2
8.50×10^{-6}	1.0×10^{-4}	$(1.01 \pm 0.03) \times 10^{-4}$	93 ± 3
8.50×10^{-6}	1.0×10^{-3}	$(9.38 \pm 0.03) \times 10^{-4}$	93 ± 3
Concentration of 5-hydroxymethyl-2-furaldehyde (mol/l)			
5.50×10^{-5}	1.0×10^{-6}	$(5.38 \pm 0.22) \times 10^{-5}$	96 ± 4
5.50×10^{-5}	1.0×10^{-5}	$(6.11 \pm 0.20) \times 10^{-5}$	94 ± 3
5.50×10^{-5}	1.0×10^{-4}	$(1.44 \pm 0.05) \times 10^{-4}$	93 ± 3
5.50×10^{-5}	1.0×10^{-3}	$(0.99 \pm 0.04) \times 10^{-3}$	94 ± 4

*Mean of six determinations ± SD

TABLE 2

Concentrations of 2-Furaldehyde and 5-Hydroxymethyl-2-furaldehyde Found in Some Commercial Citrus Juices*

Sample	Concentration of 2-furaldehyde (mol/l)	Concentration of 5-hydroxymethyl-2-furaldehyde (mol/l)
Orange 1**	$(8.50 \pm 0.17) \times 10^{-6}$	$(5.50 \pm 0.11) \times 10^{-5}$
Orange 2	$(1.49 \pm 0.04) \times 10^{-5}$	$(1.19 \pm 0.04) \times 10^{-4}$
Orange 3	$(1.29 \pm 0.05) \times 10^{-5}$	$(1.61 \pm 0.08) \times 10^{-4}$
Grapefruit 1	$(6.51 \pm 0.19) \times 10^{-6}$	$(7.01 \pm 0.28) \times 10^{-5}$
Grapefruit 2	$(1.21 \pm 0.05) \times 10^{-5}$	$(8.51 \pm 0.26) \times 10^{-5}$
Grapefruit 3	$(1.51 \pm 0.04) \times 10^{-5}$	$(1.11 \pm 0.03) \times 10^{-4}$
Lemon 1	$(1.09 \pm 0.02) \times 10^{-5}$	$(5.79 \pm 0.13) \times 10^{-5}$
Lemon 2	$(1.11 \pm 0.03) \times 10^{-5}$	$(5.51 \pm 0.15) \times 10^{-5}$
Tangerine 1	$(1.21 \pm 0.05) \times 10^{-5}$	$(1.21 \pm 0.05) \times 10^{-4}$
Tangerine 2	$(1.29 \pm 0.06) \times 10^{-5}$	$(1.11 \pm 0.04) \times 10^{-4}$

*Mean of six determinations ± SD
**Mean of twelve determinations ± SD

procedures such as distillation[4,6,7,14] or solvent extraction[5,8] or filtration of the juice[2,18] have been developed. However, preliminary distillation allows recovery of only F, yields are low, variable and other volatile components are collected, as well[2,6,7,14]. 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 at the same time it does not allow the elimination of any of the possibly interfering compounds. A preliminary clean-up procedure that involves juice clarification by means of the Carrez solution, already described by other Authors, allows the elimination of pulp, proteins, fats and carotenoids[1,10,12].

All the HPLC methods so far proposed for the determination of F and/or HMF in citrus juices provide for the injection of the sample without derivatization[1-3,6,8,10,18]. However, we made the clear juice undergo derivatization in order to obtain the DNPH-ones of the carbonyl compounds present. The sensitivity of the method can therefore be improved. This type of derivatization has been already employed by several Authors for the determination of F and/or HMF in other kinds of food matrices[19-21].

The DNPH-ones are usually obtained by employing an excess of DNPH aqueous solution in the presence of hydrochloric acid. Nevertheless, the utilization by us of an acetonitrile DNPH solution offers the advantage of obtaining a solution of the derivatives that may be injected directly into the HPLC system[21]. Long and tedious steps, such as filtration and washing of the derivatives obtained in aqueous solution and preparation of a derivative solution in a suitable solvent before the HPLC determination, may therefore be avoided[21]. The use of perchloric acid instead of hydrochloric acid is due to its higher solubility in acetonitrile[21].

The derivatization step was optimized by us with respect to three parameters: 1) the DNPH-to-analyte molar ratio, 2) the acidity of the medium and 3) the reaction time. For this purpose, the amounts of the derivatives obtained were evaluated on two

aqueous standard solutions containing respectively F and HMF both 10^{-4} mol/l. As can be seen in Figures 1-3, the derivatization reaction is quantitative when the reagent-to-analyte ratio is at least 2.5:1 for both analytes and the acidity of the medium, as evaluated with a pH-meter, is about 1. Under these conditions, both F and HMF are quantitatively converted into their DNPH-ones within 25 min. The derivatives obtained are stable at room temperature for at least 48 h.

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. As may be seen in Figure 4, a straight line was obtained for both analytes over a wide range of examined concentrations, which represent values typically found in real samples. By setting the detector wavelength at the maximum absorbance of the derivatives of both F and HMF, it is possible to determine the detection limit as $3\sigma/S$[22], where S is the sensitivity, which is 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 are therefore 2.2×10^{-8} mol/l for F and 2.4×10^{-8} mol/l for HMF.

Specificity, Recovery and Reproducibility

The method shows a high specificity because, under the described conditions, the derivatives of both F and HMF are well separated with respect to the other carbonyl compounds present in the sample under examination, as Figure 5 shows.

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 so far examined, a 1:20 ratio was sufficient, as I) a large peak of the DNPH excess appears in the chromatogram and II) area increments were not obtained for the two analytes of interest by utilizing a 1:50 analyte-to-reagent ratio.

Recoveries of both F and HMF were determined on a fresh orange juice. The juice was selected on the basis of its low content of both F and HMF (8.50×10^{-6} and 5.50×10^{-5} mol/l respectively), two of the lowest levels among those which we found in real samples. As Table 1 shows, recoveries for both analytes ranged from 93 to 96%. Lee et al. have already reported that no loss of both F and HMF was observed during the clarification step of citrus juices by employing the Carrez solution[1].

Reproducibility was good: the average concentration of F was 8.50×10^{-6} mol/l, with a standard deviation of 1.7×10^{-7} mol/l and a relative standard deviation of 2%. The average concentration of HMF was 5.50×10^{-5} mol/l, with a standard deviation of 1.1×10^{-6} mol/l and a relative standard deviation of 2%.

Application

As may be seen in Table 2, in all the samples analyzed the amount of HMF was one order of magnitude greater than the amount of F. The results obtained are in agreement with those already reported by other Authors on processed citrus juices.

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