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Automatic determination of 5-hydroxymethylfurfural (5-HMF) by a flow injection method

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An automated flow injection (FI) method for the determination of 5-hydroxymethylfurfural in foodstuffs without sample pre-treatment has been developed. The method is based on the Winkler reaction and makes use of only one reagent. It permits the analysis of foodstuffs or their colored extracts since it runs on reversed FI. The linear range was 5–40 ppm with a rsd at 95% of 3.6, and sample frequency 70 h^{-1}. Presence of SO_2 as interferent is bypassed adding 2% H_2O_2 previously. The procedure is applied to the analysis of spirits, wine, liquid caramel and dried plums, and agree with the values obtained with the reference method. © 1997 Elsevier Science Ltd

INTRODUCTION

5-(Hydroxymethyl)-furan-2-carbaldehyde (HMF) is a reaction product of the transformation of hexoses in an acid medium, according to a rather complex process (Ames, 1990). In many foods the control of HMF is being used as an index to detect non-microbial changes. There is a correlation between the development of strange flavors and aromas and the formation of HMF, as shown by several authors (Primo-Yúfera, 1983; Daubert *et al.*, 1990). The level of HMF in processed foods is an indication of the thermal treatment received as well as of the storage time, in such a way that the contents increase accordingly (Lo-Coco *et al.*, 1994). In other cases, the determination of HMF indicates the origin of the product; for instance, high levels in musts or juices show that they have been desulphited (Cohen *et al.*, 1994).

The determination of HMF is common in quality control laboratories. There are different methods applied to the analysis of HMF in food, all of them with a sample pretreatment by means of distillation or, more often, through extraction with organic solvents. HMF is determined in the extract by gas chromatography (Guerra Hernández *et al.*, 1988), HPLC (E.U., 1990) or spectrophotometrically by reaction of the aldehyde with thiosemicarbazide (Montilla Gómez *et al.*, 1988), according to the A.O.A.C. method (1990) or the Winkler method (Ough & Amerine, 1988). Only two of them are validated as official methods: HPLC and Winkler. The latter is the most frequently used and recommended by several international organisations such as EU and O.I.V. It is very easy and can be utilized as a routine technique. Furthermore it requires cheaper means than liquid chromatography and has a higher sample throughput. However, in the case of colored or solid samples, or those with suspended matter, a sample pretreatment is required, such as the continuous extraction with ethyl ether. Moreover, Guerra Hernández *et al.* (1988) point out some drawbacks such as the instability of the chromophore formed, the toxicity of *p*-toluidine and the use of organic solvents. Additionally, it suffers from interferences when samples contain SO_2 and other compounds (Montilla *et al.*, 1987).

The automation of analytical methods by means of flow techniques (Valcárcel & Luque de Castro, 1987) has proven to be suitable for the development of routine methods, an important increase of the precision and analysis frequency being achieved without the above mentioned disadvantages. In this sense, different FI-automated methods based on the Winkler method have been proposed. Two of them for mention are that of Salinas *et al.* (1991), applied to the analysis of honey, and that of Espinosa *et al.* (1993), which is a stop-flow method.

The aim of this work is to develop an automated method for the spectrophotometric determination of 5-HMF in food with a sufficient sensitivity and simplicity to be used routinely in quality control by the agrochemical industry.

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EXPERIMENTAL

Apparatus

A Philips SP6-550 UV/V spectrophotometer and a Hellma flow-cell (18 μl volume) is used. Detector, pump and valve are integrated and interfaced with a PC with a home-made software written in Modula-2. The FI manifold is equipped with an automatic 4-way Rheodyne 5701 valve confluences in polymethil metacrilate, a Gilson-MP3 peristaltic pump and PTFE coils (0.5 mm ID). For the official method, 250 ml decantation funnels were used.

Reagents

p-*Toluidine*
10 g of *p*-toluidine (Merck, reference 10841) were dissolved in 25 ml isopropanol (Panreac, reference 131885.14). Afterwards 10 ml of acetic acid (Merck, reference 63) are added and the volume made up to 100 ml with isopropanol.

Barbituric acid
0.375 g of barbituric acid and 1 ml of acetic acid are dissolved in ca. 20 ml of hot water. Once cool, the mixture is made up to 100 ml with water.

Standards
in all cases, a stock solution of 1000 mg litre^{-1} is prepared by dissolving 5-(hydroxymethyl)-furan-2-carbaldehyde for synthesis, (Merck, reference 820678), in distilled water. From this solution, standards of 5, 10, 20, 30 and 50 mg litre^{-1} were prepared daily by dilution with distilled water.

For the interference study, a 1000 mg SO_2 litre^{-1} stock standard solution was prepared by dissolving sodium sulphite (Panreac, reference 141698) in water, and stabilized by adding 1 ml of 30 g litre^{-1} Na_2EDTA and 1 ml of 4 M NaOH. The SO_2 content was obtained by iodometric titration according to the official method (EU, 1990). All reagents used were of analytical grade.

Reference method
We followed the method recommended by the EU (1990). Approximately 10 ml of sample or its corresponding aqueous extract is taken and placed in a 250 ml decantation funnel, then 12 ml of ethyl ether are added. The solution is stirred for three minutes and the organic phase is collected with 2 ml of distilled water. This procedure is repeated four times. Finally, we let the organic phase to evaporate and water is added up to 10 ml.

2 ml of the extract are poured into two 25 ml volumetric flasks and filtered through a Whatman-5 paper if necessary, then 5 ml of *p*-toluidine solution are added to each one. Finally, 1 ml of barbituric acid is added to one of the flasks and 1 ml of distilled water (blank) to the other one. Both are homogenized and after 2–5 minutes the absorbance is measured at 550 nm.

Sample treatment
Commercial samples of wines, apple juices and spirits (whisky and vodka) were directly analyzed without any treatment. The sample of syroup caramel was diluted 1/150 with water due to the high content in hmf. Dry plums were pitted, cut into pieces and triturated in water (1/10). Afterwards, the resulting mixture was filtered with whatman-5 filter paper and the residue washed three more times with distilled water. Finally, the extract was made up to a final volume of 50 ml.

Proposed method
A schematic diagram of the flow system is shown in Fig. 1. The reagent mixture (14.17 μl) was injected into the carrier stream (sample solution) at a flow rate of 1.3 ml min^{-1}. After mixing in the reaction coil (300 cm length, 0.5 mm ID) the coloured complex formed was spectrophotometrically monitoring at 550 nm.

Results and discussion

A study of the reagents was carried out to set up the flow method. Initially, *p*-toluidine dissolved in isopropanol was used; it reacts with the analyte in the presence of barbituric acid.

The use of reagents containing important amounts of isopropanol present some problems due to the difficulty of obtaining a homogeneous mixture between *p*-toluidine and barbituric acid. The original mixture showed a high drifting of the baseline. Moreover, the development of the reaction after injecting the sample (aqueous solution of HMF) was scarcely reproducible. The removal of isopropanol in the reagents turned out to be infeasible since it had two drawbacks. The first one was the great difficulty in dissolving substantial amounts of *p*-toluidine so that a high sensitivity could be achieved. On the other hand, isopropanol avoids the precipitation of *p*-toluidine, thus improving its stability. It is to be pointed out that the response of this reagent varies from one day to the other; therefore it must be used after stabilization for 24 h.

The use of an SBSR-type reactor ($\varnothing_i$ 0.8 mm, 30 cm length) did not solve the problems due to the difference of the refractive index. Contrary to expectations, this reactor caused the appearance of precipitates and overpressures, which produced malfunctions in the system. Also the signal was lower than that yielded by the standard reagent.

A one-reagent system was chosen as the most suitable manifold, taking into account the above mentioned problems. Several FI configurations were studied, the one shown in Fig. 1 being the easiest to use. Since most

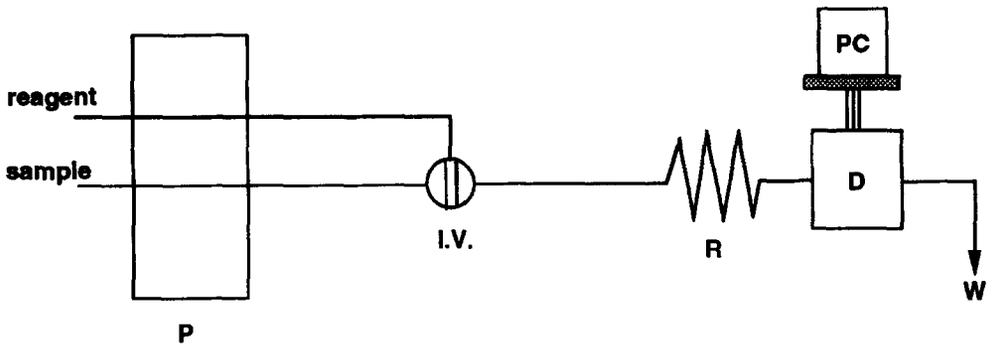

Fig. 1. Manifold used in reversed FI for the determination of 5-HMF. (P = pump; R = reactor; I.V. = automatic injection valve; D = detector; PC = computer for system control; W = waste).

foodstuffs or their extracts are colored and absorb at the monitoring wavelength (550 nm), the work is carried out using reversed FI procedure. In reversed FIA, the roles of sample and reagent are swapped over, the method being based on injection of reagent(s) into a carrier stream of sample. This has some advantages because it gives rise to an increase in sensitivity compared to direct FI procedure, partly by widening the dynamic concentration range attainable. Also, reversed FIA is of great use when very frequent analysis of abundant and inexpensive samples is required. Furthermore, it allows for the removal of the matrix effect, especially its color, apart from decreasing the fluctuations due to differences of refractive index.

Optimization of variables

The optimization of variables was carried out through the univariate method (Massart *et al.*, 1978).

Chemical variables

Different mixtures of reagent concentrations were tested to investigate the evolution of analytical signal. It was noticed that isopropanol helps in the dissolution of *p*-toluidine and increases its stability avoiding its precipitation. However, it heavily influences the FI reaction, unlike in the reference method where the homogeneization of reagents and sample extract did not offer any problem. The best experimental results were obtained with a reagent prepared by dissolving 10 g of *p*-toluidine in 25 ml of isopropanol, to which 10 ml of concentrated acetic acid were added. The mixture was made up to 100 ml with distilled water. Table 1 shows the effect of the increase in the concentration of *p*-toluidine on the analytical signal. It was found that, 10 g 100 ml^{-1} of *p*-toluidine gave the best results. Higher concentrations lower the signal, possibly due to the difference in reagent viscosity which hinders the development of the reaction. The signal increases with decreasing flow-rate (higher residence time), but the trend remains the same. Therefore, we prepared the reagent with 10 g of *p*-toluidine.

Barbituric acid solutions also precipitate with the passing of time. This can be avoided by adding 10% isopropanol. Since the addition does not prevent drifting from the mixture of this reagent with *p*-toluidine, we decided to prepare barbituric acid daily without isopropanol.

The effect of different concentrations of acetic acid in barbituric acid solution was studied using a 30 ppm HMF standard. The reproducibility increases with the addition of concentrated acetic acid and so does the solubility of the reagent. The best results were obtained by adding 1 ml of concentrated acetic acid to every 100 ml of solution. Therefore the barbituric acid solution is prepared by dissolving 0.375 g of barbituric acid in water and adding 1 ml of concentrated acetic acid; the mixture is slightly heated, and once cool its volume is made up to 100 ml with distilled water.

The effect of the presence of acetic acid was studied (Table 2) by preparing solutions of 10 g of *p*-toluidine to which 3, 7, 10 and 30 ml of concentrated acetic acid were added respectively. The final volume was made 100 ml with distilled water. The assay was carried out with a 10 ppm HMF standard and the best results were obtained with acid concentration in the order of 7% (v/v), although this is not a critical value.

Table 1. Influence of the concentration of *p*-toluidine on the analytical signal (10 ppm of HMF, flow-rate 1.3 ml min^{-1})

Concentration *p*-toluidine	Absorbance*
5 g 100 ml^{-1}	0.046
10 g 100 ml^{-1}	0.166
13 g 100 ml^{-1}	0.170
20 g 100 ml^{-1}	0.101

*Arbitrary units

Table 2. Effect of the variation of acetic acid concentration on the analytical signal (10 ppm de HMF, flow-rate 1.3 ml min^{-1})

Concentration AcH (%, v/v)	Absorbance*
3	0.120
7	0.236
10	0.155
30	0.122

*Arbitrary units

Optimization of the mixture of reagents

With a single-channel scheme (Fig. 1) different mixtures were assayed using several rates of *p*-toluidine and barbituric acid. Their composition is shown in Table 3. It can be noticed that, from the point of view of sensitivity, the best mixtures are 20/10 and 30/10 of *p*-toluidine/barbituric acid, although the former is preferable since it has a wider working range. However, this reagent also precipitates with the passing of the time. In order to avoid this, the effect of the addition of different amounts of isopropanol (0, 2, 5 and 10 ml in 100 ml of mixture) was studied. The reagent to which 5 ml of isopropanol had been added did not precipitate and showed a satisfactory sensitivity. Therefore, 100 ml of the optimized reagent contained 2 g of *p*-toluidine, 8.3 ml of isopropanol, 2.1 ml of concentrated acetic acid and 40 mg of barbituric acid.

Fi variables

Sample volumes between 14.17 to 200 μl were assayed; it was noticed that volumes over 20 μl did not increase the signal and gave rise to double peaks since the reaction with the reagent plug was not complete. The best signals were obtained with a decreasing loop; therefore a minimum value-corresponding to the valve's inner volume (14.17 μl) was chosen.

Different flow-rates between 0.4 and 2.5 ml min^{-1} were tested. The best results were obtained with 1.3 ml min^{-1}, which is a compromise between sensitivity and sampling frequency. Lower values ($\sim$0.5 ml min^{-1}) are only suitable for samples containing less than 5 ppm. Consequently, the flow-rate should be adjusted to the required sensitivity. As for the reactor length, values in the range 10–500 cm were assayed. The signal increased with increasing length up to a maximum value of 300 cm.

The monitoring wavelength is not critical in this method, since the reaction product shows a wide absorption band with a maximum at 550 nm.

Features of the method

The calibration graph was run with HMF aqueous standard from 0 to 40 ppm. Under optimal conditions, the linear regression equation is: C(ppm) = 0.6 + 113 Abs, $r = 0.9989$. The precision of the method, studied on eleven different samples of a HMF standard (15 ppm) injected in triplicate, was 3.6% (RSD at 95% confidence level). The detection limit, defined as three times the standard deviation of the baseline noise, was 1.40 ppm. The sampling frequency afforded 70 h^{-1}.

Table 3. Performance of the different reagent ratios in the FI procedure

Standard HMF (ppm)	*p*-Toluidine (ml)/barbituric acid (ml)*				
	10/10	10/20	15/10	20/10	30/10
blank (water)	0.241	0.184	0.290	0.179	0.1
5	0.320	0.206	0.348	0.287	0.289
10	0.346	0.223	0.343	0.462	0.485
20	0.852	0.330	0.676	1.340	saturated
30	—	0.396	saturated	saturated	
50	—	0.550	—		

*Results as absorbance values.

Application to food samples

The proposed method was applied to the analysis of HMF in different samples whose content had previously been determined by the Winkler official method (1955). The results obtained are given in Table 4. The greatest differences are in those foodstuffs with high contents in HMF, where the sample ought to be highly diluted. The Signs Tests-Paired Samples (Statgraphics 5.0) applied to the results obtained with both methods showed no statistical differences. It can be noticed that the effect of the color is not important, due to the fact that by using reversed FI the absorption of each sample is taken to zero. This is a great advantage of this method against others that remove the color interference by stop-flow (Espinosa Mansilla *et al.*, 1993) with the subsequent decrease in sampling frequency.

However, the method cannot be applied to samples with high color intensity and low concentration of HMF, such as, for instance, red wines. Other samples like natural honey, usually with low contents in HMF, cannot be analyzed since they should be diluted prior to handling with the FI system.

Finally, the analyte recovery was tested by making two standard addition (5.3 and 15.8 ppm) to one grape juice sample containing 1.4 ppm of HMF. The recovery was 99.7% and 101% for addition level 1 and 2, respectively.

Interferences

SO_2 occurs in many foodstuffs, either as a preservative or as an anti-browning agent (Iyengar & McEviliy, 1992). It generally interferes in all types of reactions. In our case, free SO_2 also affects the determination of HMF and other furanic aldehydes (O.I.V., 1979). When its content exceeds 10 ppm, it should be removed prior to the determination. For this purpose, the effect of high SO_2 levels was studied by taking several samples of apple juice to which 5, 10 and 20 ppm of HMF and

Table 4. Comparison of the results obtained by FI method and reference method*

Sample	FI (ppm)	Official (ppm)
Scotch whisky 1	9.7 ± 0.3	9.9 ± 0.2
Scotch whisky 2	12.2 ± 0.4	10.1 ± 0.1
Russian vodka	n.d.	n.d.
Dry white wine	3.3 ± 0.1	3.3 ± 0.5
Apple juice	3.7 ± 0.4	3.6 ± 0.1
Grape juice	1.8 ± 0.2	3.4 ± 0.5
Liquid caramel	2528 ± 34	2307 ± 67
Dried plums	65.1 ± 0.3	61.6 ± 0.2

*$n = 6$ determinations per sample (95% confidence level)
n.d. undetected.

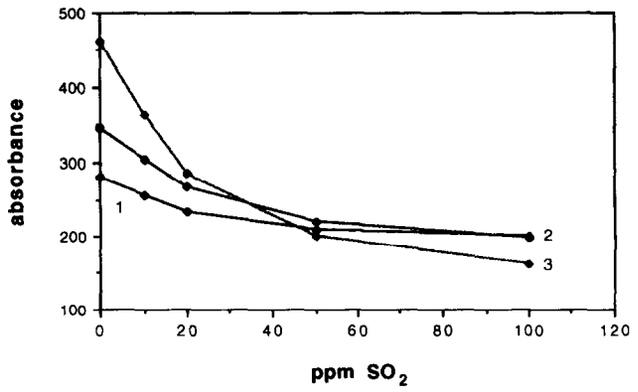

Fig. 2. Interference effect of SO_2 on the analytical signal of 5-HMF. 1–5 ppm SO_2; 2–10 ppm SO_2; 3–15 ppm SO_2.

Table 5. Removal of the interference of SO_2 through the addition of 2% H_2O_2. Sample, grape juice with 10 ppm of HMF added[a]

SO_2 added (ppm)	Absorbance[b]
0	0.295
4	0.290
10	0.287
16	0.281
20	0.285
48	0.287
100	0.283

[a]Absorbance of sample without SO_2, 0.301
[b]$n = 5$

increasing amounts of SO_2 (4,10,16,20,48 and 100 ppm) had been added. The signal decreased with increasing concentration of SO_2 up to a value of 48 ppm, over which the signal was independent from the concentration tested (Fig. 2).

To remove these interferences the effect of H_2O_2 was tested on different amounts (0.5–10 ml). It was noticed that the addition of 1 ml of 2% H_2O_2 to 100 ml of sample after 2-hour stabilization avoids the interference of SO_2 by oxidation to sulfate. Meanwhile we verified that the treatment of the samples with 2% H_2O_2 did not affect the determination of HMF. The results shown in Table 5 indicate that it is possible to easily remove the effect of SO_2 through addition of 2% hydrogen peroxide.

Conclusions

The automatic FI method based on the use of a sole reagent permits the routine analysis of HMF in different foodstuffs with a high sampling frequency. Against the reference method this one has the advantage that it does not require a previous extraction of the sample with organic solvents; additionally, if the concentration of HMF exceeds 5 ppm, colored samples can be directly analyzed, which is also an important advantage. Finally, the interference of SO_2—usual in food—can be removed by a pretreatment with H_2O_2. The results obtained with real samples correlate well with those provided by the reference method. The recovery assays carried out on real samples at two levels have also been satisfactory.

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