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NEW QUANTITATIVE DETERMINATION OF 2,3-DIOXOBUTANE (DIACETYL) IN ALCOHOLIC BEVERAGES BY AN INDIRECT SPECTROFLUORIMETRIC PROCEDURE WITH USE OF 2,3-DIAMINONAPHTALENE AS REAGENT OF DERIVATIZATION

MICHÈLE MARIAUD* and PIERRE LEVILLAIN

Laboratoire de Chimie Analytique, Faculté des Sciences Pharmaceutiques, 2 Bis Boulevard Tonnellé, 37042 Tours Cedex, France

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Summary—The experimental conditions of an earlier spectrofluorimetric determination of 2,3-dioxobutane as 2,3-diaminonaphtalene derivative have been optimized. Fluorescence intensities are increased by a factor of 13 over the precedent ones. The calibration graphs are linear in the concentration range 2.5–130 μmol/l in spectrophotometry and 1.75×10^{-1}–2.5 μmol/l in fluorimetry. Limits of detection are very small: 7×10^{-1} and 2.5×10^{-2} μmol/l, respectively. Applications to alcoholic beverages are described. The new fluorimetric technique is simple and rapid since it does not require steam distillation.

In recent years, there has been increasing interest in the determination of α or β-diketones, these compounds being often the terminal stage of some chemical or biochemical degradation process. For instance, 2,3-dioxobutane (diacetyl) which is the major constituent of complex aroma of butter or some alcohols (wine or brandy) is produced by an oxydation process of acetoin, a degradation product of citric acid.

In foodstuffs, natural diacetyl contents are very weak, never greater than some ppm. Thus, they require use of very sensitive analytical procedures to be determined. All are performed after separating diacetyl by steam distillation. In alcohols, diacetyl is commonly determined after its transformation to dimethylglyoxime and subsequent reaction with Ni^{2+}[1] or Fe^{2+}.[2] These reactions must be performed in the absence of ethanol. Two distillations are required which is time consuming. Recently, a fluorimetric determination of diacetyl in butter has been described.[3] It is based on the reaction between diacetyl and 2,3-diaminonaphtalene (DAN) to yield 2,3-dimethyl[2,3-b] naphtopyrazine which possesses high absorbance and fluorescence properties. This reagent, as 2,3-diaminobenzene or 4,5-dimethoxy-1,2-diaminobenzene, was used earlier for the assay of some α diketonic acids in biological systems.[4–20]

In this paper we tested 2,3-diaminonaphtalene as reagent for diacetyl assay in alcohols in order to decrease the number of steps of earlier procedures. First, the reaction between DAN and diacetyl was optimized by spectrophotometry, directly in aqueous medium and not by fluorimetry and after extraction by cyclohexane as it was proposed earlier.[3] Then, the fluorescence of 2,3-dimethyl[2,3-b]naphtopyrazine in various medium was studied and the results allowed the sensitivity of the determination to be increased. Two applications are described: a determination of diacetyl in alcohols without any distillation and an improvement of the determination of diacetyl in butter.

EXPERIMENTAL

Reagents

All reagents, from Aldrich Chemical Company were of analytical grade. For DAN, the stock solution was prepared by dissolving 1 g of 2,3-diaminonaphtalene hydrochloride in 1 L of 0.1*M* HCl. For 2,3-dioxobutane (diacetyl), the stock solution (0.2 g/l) was prepared in water. All stock solutions were stored in the dark. Working solutions of the diketone (2×10^{-4}–1×10^{-2} g/l for spectrophotometry and 1×10^{-5}–2×10^{-4} g/l for fluorimetry) were prepared daily and left in daylight during the measurements. pH values were set between 1

*Author for correspondence.

and 8 by adding the necessary amounts of HCl and KCl[21] or citrate and phosphate[22] buffers.

Equipment

Absorption spectra were recorded on a Secomam spectrophotometer (model S1000 PC). Slits were set for a 2 nm spectral band pass and the scan speed was set at 600 nm/mm. A Secomam software package was used to obtain the derivative spectra. A 10 nm smoothing window gives the best signal to noise ratio with minimum distortion of the curves.

Fluorescence measurements were made by using a model JY3CS Jobin Yvon fluorescence spectrophotometer equipped with a xenon arc source, excitation and emission monochromators, 1 cm quartz cells and R-212 photomultiplier. The excitation and emission slits were adjusted to provide a 4 nm spectral band pass. The spectrofluorimeter response (time constant) was set at 0.2 sec and the scan speed at 40 nm/sec. The fluorescence data are given without spectral correction.

Formation of pure 2,3-dimethyl[2,3-b]naphtopyrazine hydrochloride

A sample of 80 mg of DAN was dissolved into 50 ml of methanol. To this solution were added 2 ml of 12*M* HCl, 10 ml of distilled water and 1 ml of pure 2,3-dioxobutane for diketone to be in excess, a precipitate rapidly appeared. The mixture was then evaporated under vacuum at 80°C. The resulting solid phase was dissolved anew in methanol and the solution left aside for slow crystallization. The purity of the 2,3-dimethyl [2,3-b]naphtopyrazine was checked by noting the stability of the position (nm) of the zero-crossing points of the first and of the second derivative curves of the normal spectra of solutions of this compound with the concentration.

Procedure

Derivatization and determination of diacetyl in pure solutions. In spectrophotometric procedure, place 3 ml of each working solution of diacetyl (or water) in a screw-capped test tube with Teflon lining. Measure 1.5 ml of the buffer and 0.5 ml of DAN hydrochloride stock solution. Heat at 56°C in a water bath for 1 min, then cool at room temperature. Measure the mixtures and the reagent blank between 220 and 450 nm.

In fluorimetric procedure, the diacetyl derivatization is made on 10 ml of each working solution (or water) added with 1 ml of 0.1*M* HCl and 0.5 ml of DAN hydrochloride stock solution. After heating at 56°C in a water bath during 1 min then cooling at room temperature, add 3.5 ml of cyclohexane to each tube, mix and separate organic and water phases. To 2 ml of organic phase, add 1 ml of ethanol and measure relative fluorescence intensities at 363/475 nm.

Determination of diacetyl in foodstuffs. For alcohols, operate on 10 ml of wine or brandy diluted with distilled water if necessary (concentrations must be in the interval 0.175–3 μmol/l) according to the fluorimetric method described in the preceding paragraph.

For butter, diacetyl is extracted by steam distillation according to a precedent procedure[3] but operating on only 5 g of butter. Fluorimetric determinations are made as described above, on 10 ml of distillate.

RESULTS AND DISCUSSION

Spectrophotometric study of the reaction between DAN and diacetyl

pH effects. DAN absorption spectra, much like the absorption spectra of 2,3-dimethyl[2,3-b]naphtopyrazine are pH-dependent (Figs 1(a) and (b)). DAN, whose pka are given as 0.7 ($DANH_2^{2+}/DANH^+$) and 3.4 ($DANH^+/DAN$)[23] is characterized by an hypsochromic shift of the absorption maximum located at 241 nm in alkaline medium (Fig. 1(a), curve A): 6 nm for the first protonation (Fig. 1(a), curve B), 20 nm for the second one (Fig. 1(a), curve C). These shifts are usually observed for the aromatic amines and correspond to a more or less important conjugation of nitrogen lone pair electrons with electrons of the aromatic ring when one goes from the basic form (the more important conjugation) to the monoprotonic and to the diprotonic forms (the weaker conjugation).

For 2,3-dimethyl [2,3-b]naphtopyrazine, whose absorption maxima are located at 227, 266, 350 and 363 nm (Fig. 1(b), curve A) in alkaline medium, progressive acidification induces bathochromic displacement of the absorption bands of 2, 15.5, 10 and 12 nm (Fig. 1(b), curve B). An isobestic point, located at 273 nm, characterizes an acid/base equilibrium (pka close to 2). This value is close to the ones given in the literature for molecules of the same configuration.[21]

Figure 1(a) shows that, for wavelengths higher than 350 nm, DAN absorptivity is very

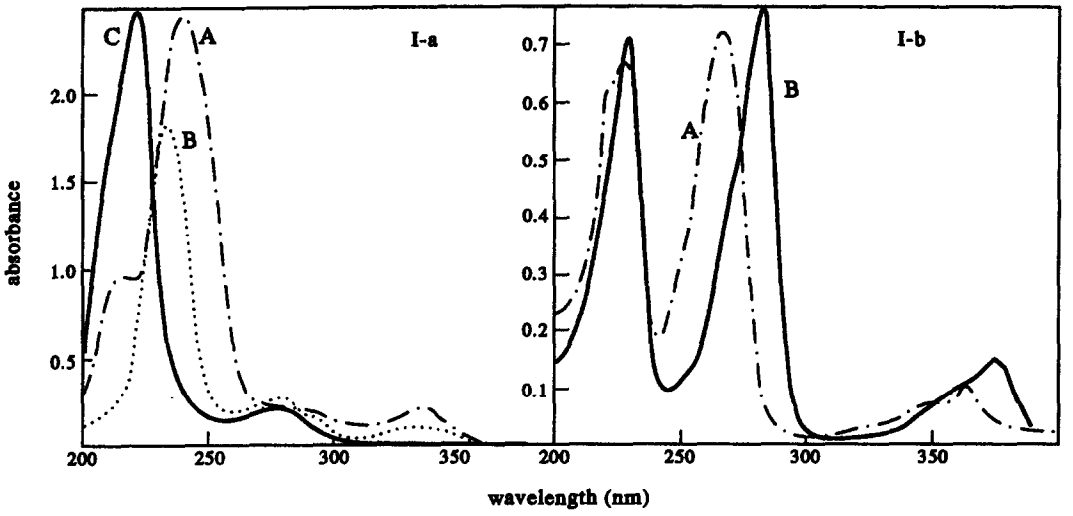

Fig. 1. Absorption spectra for (Fig. 1(a)) DAN and (Fig. 1(b)) 2,3-dimethyl [2,3-b]naphtopyrazine; pH 8 (curves A), pH 2 (curves B), 1M HCl (curve C); concentrations were 200, and 10 μmol/l for DAN and naphtopyrazine, respectively.

weak. Therefore, absorbances obtained for these wavelengths are characteristic of napthopyrazine formation. Indeed, taking into account the displacement of the position of the absorption maximum (375 nm for pH $\leqslant$ 2,363 nm for pH > 2) and of the variations of absorptivities of naphtopyrazine in function of the pH, it can be said that the reaction between DAN and diacetyl is pH-independent, the maximum yield, close to 95%, being constant between pH 1 and 8.

Temperature, heating periods and DAN concentration effects. Studies were performed at pH 2, because, at this pH, the absorptivity of naphtopyrazine at 375 nm is greater than the one obtained at 363 nm for pH values. First, the reaction is faster when temperature increases (Fig. 2). At 56°C, the maximum yield is reached in 1 min *vs* about 3 min at 37°C. A further increase in temperature does not improve the results. At 70°C, the maximum yield is smaller than 56°C. Heating for longer has no significant influence on absorbances stability either at 37°C or at 56°C. The formed compound like the DAN are therefore stable at these temperatures.

Yield in naphtopyrazine is also increased by increasing DAN concentrations. For a 2,3-dioxobutane concentration set to 40 μmol/l, yield is maximum (95%) when DAN concentration is five-fold greater than the diketone concentration and remains constant with further increasing in DAN concentration.

Fluorimetric study. In aqueous medium, residual DAN concentration is very high *vs* naphtopyrazine concentration. As it was shown earlier,[3] its fluorescence interferes in the emission spectrum of naphtopyrazine and makes impossible the direct determination of this later compound to be performed. In spite of the small difference between pka values of DAN and naphtopyrazine, the extraction of naphtopyrazine by an organic solvent can be taken into account, operating at pH value about 2. In a previous publication,[3] the extraction was performed by employing cyclohexane and the organic phase was submitted to fluorimetric measurement. Although the proposed technique is more sensitive than the spectrophotometric one, its own sensitivity is relatively low.

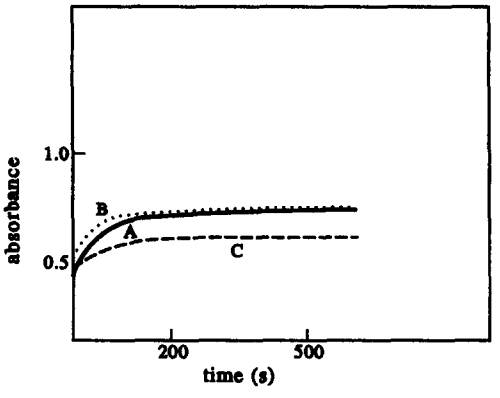

Fig. 2. Effects of temperature and heating periods on 2,3-dimethyl[2,3-b]naphtopyrazine formation; (A) 37°C, (B) 56°C and (C) 70°C; pH was 2 and concentrations were 60, and 500 μmol/l for 2,3-dioxobutane and DAN, respectively; absorbances were made at 375 nm.

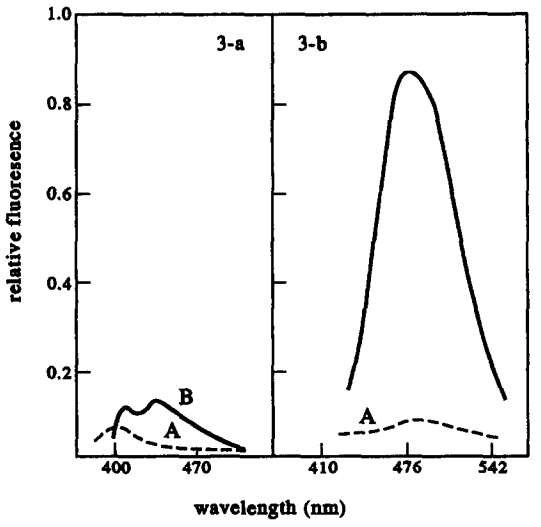

Fig. 3. Fluorescence spectrum of (curves A) DAN and (curves B) 2,3-dimethyl[2,3-b]naphtopyrazine in cyclohexane (Fig. 3(a)) or in 33% (v/v) ethanol/cyclohexane (Fig. 3(b)) $\lambda_{exc} = 363$ nm.

Fluorescence of naphtophyrazine can be strongly increased by adding an hydroxylic solvent to the organic phase (Fig. 3). The enhance is coupled with a displacement of the maximum emission wavelength which comes from 431 nm in earlier procedures [3] to 475 nm in our technique (Fig. 3). The emission maximum of DAN is displaced to the same wavelength (475 nm), but its intensity is not changed. Two factors had to be studied: the nature and the percentage of added solvant.

Fluorescence intensities are maximum when low molecular weight hydroxylic solvents are used. They increased by a factor 10 when a 33% percentage of isopropanol is added, by a factor of 13 for the same percentage of ethanol. Methanol cannot be used, because it is not miscible with cyclohexane.

Fluorescence intensity is also enhanced with increasing percentage in hydroxylic solvent. For instance, with isopropanol, fluorescence is increased by a factor 2.5 when the percentage of hydroxylic solvent comes from 7 to 93%. But, the addition of the hydroxylic solvent to cyclohexane decreases the concentration of 2,3-dimethyl[2,3-b]naphtopyrazine in the organic mixture. So, it is necessary to extract naphtopyrazine with the smallest volume of cyclohexane. In our experimental conditions, the volume of cyclohexane cannot be lower than 3.5 ml and a 33% ethanol was added before measurements.

Table 1. Characteristics of spectrophotometric and fluorimetric methods

Method Wavelengths (*nm*)	Spectrophotometry 375	Fluorimetry 365/475
Slope/A* μmol/l	6.9×10^{-3}	1.20
Intercept/A*	0.07	0.06
Dynamic range/μmol/l	2.5–130	0.175–2.5
Limit of detection/μmol/l	7×10^{-1}	2.5×10^{-2}
RSD%†	1.2	1.5

*Absorbance of fluorescence intensity.

†Relative standard deviations ($n = 10$). Concentrations were 10 and 1 μmol/l in spectrophotometry and fluorimetry, respectively.

QUANTITATIVE STUDY

Study on pure solutions of diacetyl

Linear calibration graphs were obtained by spectrophotometry as well as with fluorescence procedures (Table 1). With this latter method, a very high sensitivity, represented by the slope of the calibration line, is obtained and the limit of detection[24] is very small (2.5×10^{-2} μmol/l). To check the precision of the methods, replicated samples ($n = 10$) containing 10 μmol/l in spectrophotometry or 1 μmol/l in fluorimetry were measured. The relative standard deviations[24] obtained are good since they do not vary by more than 2%.

Determination of diacetyl in foodstuffs

For alcohols, the fluorimetric technique was applied directly on the alcoholic sample or on the distillate obtained after one steam distillation according to the first part of earlier techniques.[1] Table 2 shows that the results obtained by the two procedures agree well. Those obtained directly on the alcoholic samples are better than the other ones, about 1% higher.

Satisfactory recoveries were also obtained

Table 2. Recovery of diacetyl in alcohols spiked, or not spiked, with known amounts of diacetyl

		With distillation		Without distillation	
	ppm added	*ppm** found	% recovery	*ppm** found	% recovery
Brandy/1	0	3.23		3.26	
	1	4.14	97.8	4.24	99.5
	2	5.12	98.0	5.20	98.9
	3	6.11	98.1	6.20	99.1
Wine/1	0	0.87		0.89	
	0.25	1.10	98.1	1.13	99.3
	0.50	1.34	97.9	1.37	98.6
	1.00	1.84	98.5	1.88	99.6
Wine/2	0	0.96		1.03	
	0.25	1.19	98.4	1.27	99.3
	0.50	1.43	98.1	1.51	98.9
	1.00	1.91	97.5	2.00	98.9

*Each value is the mean of two replicates.

Table 3. Recovery of diacetyl in butter sample spiked or not spiked with known amounts of diacetyl

		Technique[3] without ethanol (363/431 *nm*)		With ethanol (363/475 *nm*)	
	ppm added	*ppm** found	% recovery	*ppm** found	% recovery
Butter/1	0	0.46		0.47	
	0.12	0.57	98.5	0.585	99.1
	0.25	0.70	98.2	0.72	99.5
Butter/2	0	0.35		0.37	
	0.12	0.46	98.3	0.48	98.5
	0.25	0.59	98.3	0.615	99.0

*Each value is the mean of two replicates.

when several samples spiked or not spiked with known amounts of pure diacetyl were analyzed (Table 2). Our method is rapid since it is made directly on the samples and does not require any distillation.

Diacetyl was determined in butter by our procedure, *i.e.* adding ethanol to cyclohexane extracts. Two kinds of sample were also used: spiked or not spiked with pure diacetyl. The results (Table 3) agree well with those obtained on the same samples by employing the earlier cited method[3] and show nearly quantitative recoveries of added diacetyl. The best recoveries are obtained with our technique (about 1% higher) and the increase in sensitivity permits to decrease the weight of the used butter sample by a factor of four.

CONCLUSION

The experimental conditions of an earlier determination of diacetyl[3] have been optimized. Adding ethanol to cyclohexane extracts allows the sensitivity of the assay to be increased by a factor 13. The new proposed method is proved to be very efficient for the direct determination of diacetyl in alcohols. It does not require steam distillation, and hence is less time consuming.

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