Kinetic–Fluorometric Determination of Malonaldehyde Based on the Hantzsch Reaction: Application to Olive Oil Analysis

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A kinetic study was completed on the formation of 1,4-disubstituted-1,4-dihydropyridine-3,5-dicarbaldehyde from malonaldehyde and methylamine, via the Hanztsch reaction. The influence of *n*-hexylaldehyde and acetaldehyde in the reaction has been studied, and it has been concluded that the presence of the aldehydes is not necessary for the reaction. The fluorescence emission of the reaction product was monitored at 470 nm, with excitation at 405 nm, and the effect of instrumental and experimental variables on the reaction was investigated. A pH of 4.0, obtained by a 0.25 M sodium acetate–acetic acid buffer solution, a 10 mM methylamine concentration, a 30% (v/v) 2-propanol content, and a temperature of 75 °C were selected for the reaction. A kinetic–fluorometric method to determine malonaldehyde has been proposed, for malonaldehyde concentrations ranging from 0.5 to 2.8 μg mL^{-1}, and a wide study of the possible interferences in the reaction has been performed. The kinetic–fluorometric method was applied to olive oil samples.

Keywords: *Malonaldehyde; Hantzsch reaction; olive oil; kinetic–fluorimetry*

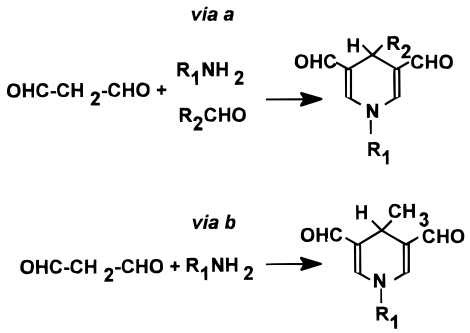
INTRODUCTION

Lipid peroxidation involves the oxidative deterioration of polyunsaturated fatty acids, and secondary decomposition products from peroxidized lipids are known to be health hazards. These facts have stimulated research in food chemistry and biochemistry related to lipid peroxidation processes (Robards et al., 1988; Sanders, 1983).

Malonaldehyde (MLD) is a well-known secondary decomposition product from peroxidized lipids of unsaturated fatty acids (Janero, 1990). Different derivatization reactions have been proposed to determine MLD by spectrophotometric and spectrofluorometric techniques. Hence, the TBA test was adopted from the mid-1950s, as a simple spectrophotometric assay for lipid peroxidation by measuring TBA-reactive MLD, in the form of a stable 1:2 MLD:TBA adduct (Patton et al., 1951).

Several modifications on the sample preparation protocols and different instrumental techniques have been tested attempting to increase the specificity of the TBA test (Ohkawa et al.,1979; Ikatsu et al., 1992; Espinosa-Mansilla et al., 1993; Draper et al., 1993). Some condensation reactions involving MLD, which are expected to give more structural selectivity than the TBA reaction, yield derivatives that are notable for their spectral properties. Hence, pyrazole derivatives from several hydrazide compounds were obtained and applied in HPLC fluorometric detection methods of MLD (Hirayama et al., 1984; Tsuruta et al., 1994). Also, 1,4-disubstituted-1,4-dihydropyridine-3,5-dicarbaldehydes are formed by reaction of MLD with primary amines. This reaction is known as the Hantzsch reaction and can take place in the presence (via a) and in the absence (via b) of monofunctional aldehydes (Scheme 1). The reaction products exhibit strong fluorescence emission (Kikugawa et al., 1987, 1984).

Scheme 1



Hantzsch reaction

An equilibrium fluorometric method, based on the Hantzsch reaction, has been reported for the determination of MLD in oxidized lipids (Kikugawa et al., 1988). The method used methylamine and acetaldehyde to obtain the heterocyclic fluorescent derivative.

In this work, the kinetics of formation of 1,4-disubstituted-1,4-dihydropyridine-3,5-dicarbaldehyde, by reaction of MLD with methylamine, are described. The kinetic effect of the presence of mono- and difunctional aldehydes, amino acids, biogenic carbonyl compounds, and unsaturated fatty acids has been studied. A kinetic–fluorometric method for the determination of MLD has been proposed and applied in olive oil samples.

EXPERIMENTAL PROCEDURES

Apparatus. Acquisition of kinetic data and fluorescent measurements were made on a SLM Aminco Bowman Series 2 luminescence instrument, equipped with a 150 W continuous xenon lamp, interfaced by a GPIB card and driver with a PC 386-microcomputer. Data acquisition was performed by the use of AB2 Software V 1.40, running under OS/2 2.0. The analysis of the kinetic data was performed by use of the Beckman Data Leader Software V 3.0. The kinetic curves were determined at λ_{ex} = 405 nm and λ_{em} = 470 nm. The

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excitation and emission spectra were recorded from samples in 10-mm quartz cells, at 75 °C, by use of a thermostatic cell holder and a Selecta Unitronic 320 OR thermostatic bath.

Reagents. All experiments were performed with analytical reagent grade chemicals, pure solvents, and ultrapure water. Malonaldehyde was prepared from 1,1,3,3-tetraethoxypropane (TET), in acid medium. To 0.01 g of TET was added 8.8 mL of HCl, and the mixture was held for 4 min; then the solution was neutralized by addition of NaOH and dilution with water to 100 mL. A 0.1 M methylamine solution was prepared by dissolving 0.675 g of reagent (Sigma) in 100 mL of ultrapure water. Solutions of methylglyoxal, glyoxal, diacetyl, fructose, alanine, DL-α-amino-*n*-butyric acid, L-proline, phenylalanine, dopamine, L-DOPA, glucose, galactose, acetone, *n*-hexylaldehyde, and acetaldehyde, were prepared by dissolution of the chemical reagents in ultrapure water. Solutions of oleic acid, linolenic acid, and linoleic acid were prepared by dissolution of the chemical reagents in 2-propanol. A 1 M buffer solution of pH 3.8 was prepared by mixing adequate amounts of sodium acetate with hydrochloric acid. Oil solution was prepared in a 100-mL volumetric flask, by mixing 5.0 mL of oil with pure hexane.

Procedures. General Procedure for the Kinetic–Fluorometric Determination of MLD. To an aliquot of 1 M MLD, equivalent to 5–28 μg, in a 10-mL volumetric flask were added 3 mL of 2-propanol, 2.5 mL of sodium acetate–acetic acid buffer solution of pH 3.8, and 1 mL of 0.1 M methylamine solution. The mixture was diluted with water to a final volume of 10 mL.

The change in the fluorescence intensity with time was recorded at λ_{ex} = 405 nm and λ_{em} = 470 nm, during 1500 s, maintaining the temperature at 75 °C. Finally, the MLD content was determined by measuring the reaction rate between 1250 and 1450 s and using the appropriate calibration graph. Each point of the calibration graph was run in triplicate. The statistical analysis of the calibration graph was performed following a linear regression model (Cuadros et al., 1993). The linearity was calculated as 1 minus the relative standard deviation of the slope of the regression line expressed in percentage (1 − RSD(*b*)%). The sensitivity was calculated as the regression standard deviation of the regression line divided by the slope of the regression line ($s_{y,x}/b$). The precision of the method was calculated, at each point of the calibration graph, as the relative standard deviation, expressed in percentage (RSD%).

Analysis of MLD in Oil Samples. A known volume of oil solution in hexane (10 mL), containing between 6 and 32 μg of MLD, was placed in an extraction flask and extracted with 10 mL of a solution containing 35% 2-propanol and 0.29 M sodium acetate–acetic acid buffer, pH 3.8. The phases were mixed and separated. Then, 8.6 mL of the aqueous phase was transferred into a 10-mL volumetric flask, and 1 mL of 0.1 M methylamine solution was added. The mixture was diluted with water to a final volume of 10 mL, and then the general kinetic procedure was followed.

RESULTS AND DISCUSSION

It has been reported that MLD, in slightly acid medium, reacts with primary amines producing highly fluorescence products. It has been also shown that monofunctional aldehydes, such as acetaldehyde and *n*-hexylaldehyde, affected the reaction between primary amines and MLD, promoting the formation of the fluorescent 1,4-dihydropyridine-3,5-dicarbaldehydes (Kikugawa et al., 1984). An equilibrium fluorometric method of determination of MLD has been reported that uses methylamine and acetaldehyde (Kikugawa et al., 1988).

In this work, a kinetic study has been carried out, with the object of investigating the influence of the presence of monofunctional aldehydes on the formation of the heterocyclic compound. In this regard, acetaldehyde and *n*-hexylaldehyde have been assayed. The reason for selecting those aldehydes is that, according to the literature, the reaction seems to take place at a greater rate and with higher yield in the presence of the aldehydes than in their absence (Kikugawa, 1986). In addition, acetaldehyde was used with methylamine, since it produces the most fluorescent heterocyclic derivative with MLD (Kikugawa et al., 1984).

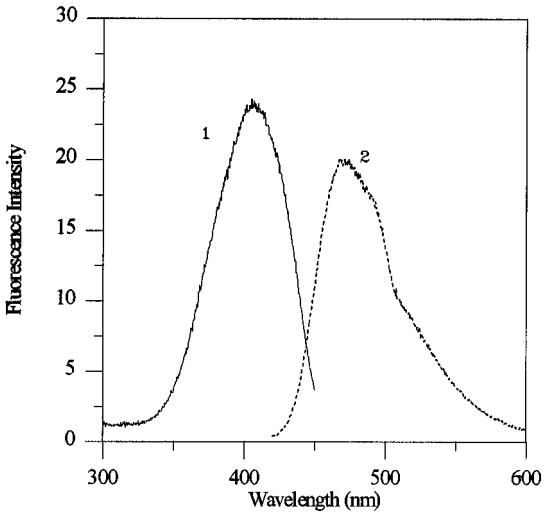

Figure 1. (1) Excitation (λ_{em} = 470 nm) (1) and emission (λ_{ex} = 405 nm) (2) spectra of the derivatized MLD product; [MLD] = 2 μg mL^{-1}, [methylamine] = 10 mM, 2-propanol percentage = 30%, T^a = 75 °C, pH = 4.0.

With the object of investigating the effect of monofunctional aldehydes in the reaction between MLD and methylamine, a comparative study of the emission maxima of the heterocyclic derivative was performed, in the absence and presence of acetaldehyde or *n*-hexylaldehyde. For the studies, a 10 mM concentration of acetaldehyde or *n*-hexylaldehyde was maintained. Excitation and emission spectra of a sample containing 2.0 μg mL^{-1} MLD in the presence of methylamine were recorded after heating at 75 °C during 25 min. These spectra are shown in Figure 1. The fluorescent derivative shows excitation and emission maxima located at 405 and 470 nm, respectively. The maximum of the emission spectra was identical for the samples containing MLD and methylamine and for the samples that also contained any of the aldehydes. This experiment allows us to conclude that, in the experimental conditions described, the presence of acetaldehyde or *n*-hexylaldehyde does not substantially affect the reaction.

The excitation was done at 405 nm, the fluorescence emission was monitored at 470 nm, and the corresponding kinetic curves were recorded. The kinetic curves obtained for both MLD–methylamine and methylamine solutions are shown in Figure 2. In the formation of the derivatized MLD, an induction period of time is observed, when the emission intensity is monitored at 470 nm. After an induction period of approximately 500 s, the emission intensity dramatically increases with heating time. The absence of fluorescence of the methylamine solution, in identical chemical conditions, is evident during the assay.

Influence of Experimental Variables. Several variables, including pH, temperature, and *n*-hexylaldehyde, methylamine, and 2-propanol concentrations could affect the kinetics of formation of derivatized MLD. The partial orders for each variable were calculated from the resulting log rate vs log concentration plots.

The influence of pH on the reaction was studied. The reaction rates were measured between 1300 and 1500

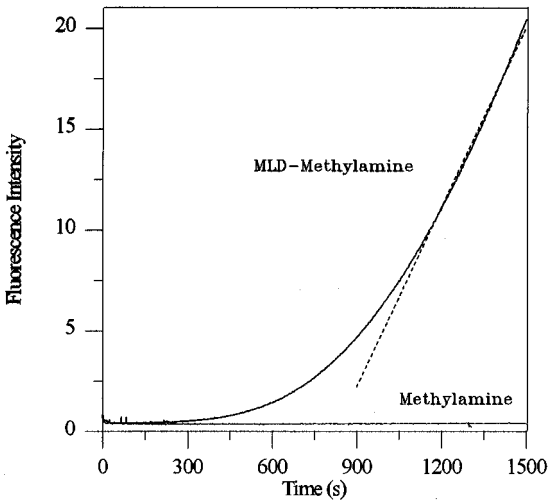

Figure 2. Kinetic curves of the derivatized MLD product and of methylamine alone; [MLD] = 2 μg mL^{-1}, [methylamine] = 10 mM, 2-propanol percentage = 30%, T^a = 75 °C, pH = 4.0.

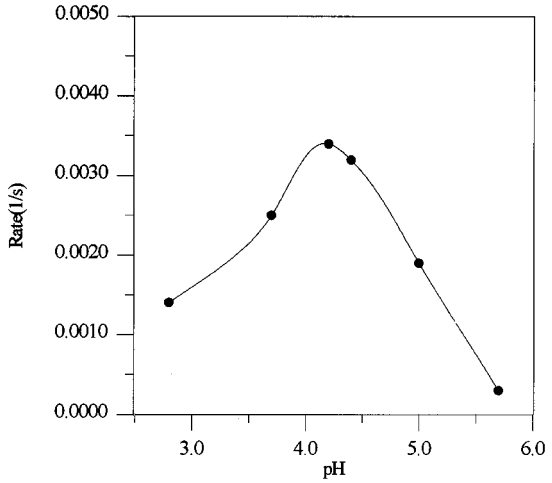

Figure 3. Influence of pH on the rate of the reaction between MLD and methylamine. [MLD] = 2 μg mL^{-1}, [methylamine] = 10 mM, 2-propanol percentage = 30%, T^a = 60 °C.

s, and the samples were maintained at 60 °C. In Figure 3, the variation of the rate of reaction with the pH is represented. The rate of the reaction increases, with an increase of pH (1/4 partial orders), up to a value of approximately 4.2 and decreases (−4/3 partial order) for higher pH values. A pH value of 4.0 was selected as optimum to obtain the greatest sensitivity.

The selected pH was obtained by addition of a 1 M sodium acetate−acetic acid buffer solution at pH 3.8. The influence of the concentration of buffer solution on the reaction was studied. The rate of reaction slightly decreases with an increment of the buffer concentration up to a value of about 0.05 M and slightly increases for values higher than this one. A buffer concentration of 0.25 M has been selected as optimum in our procedure, for control of the pH.

The effect of the temperature was examined between 54 and 84 °C. Different kinetic curves were registered, between 0 and 30 min, for samples containing 2.0 μg mL^{-1} MLD, 10 mM methylamine, 30% 2-propanol, and 0.25 M sodium acetate−acetic acid buffer solution to provide the selected pH. The rate of reaction is strongly favored with an increase in temperature up to 80 °C (Figure 4). At temperatures greater than this, the rate of reaction decreases. A temperature of 75 °C was

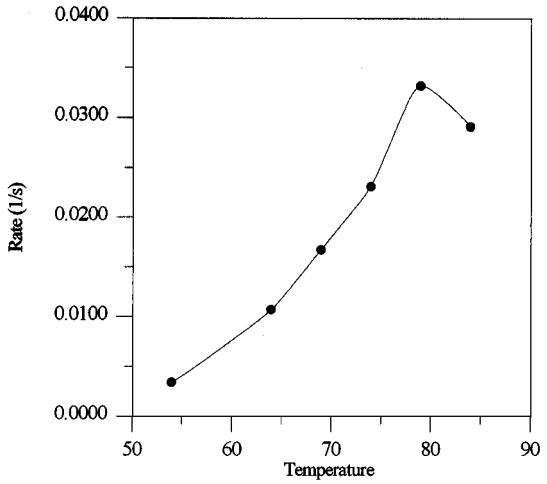

Figure 4. Influence of temperature on the rate of the reaction between MLD and methylamine; [MLD] = 2 μg mL^{-1}, [methylamine] = 10 mM, 2-propanol percentage = 30%.

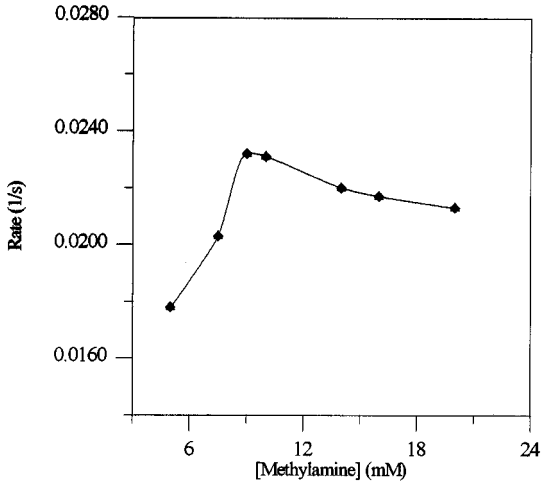

Figure 5. Influence of methylamine concentration on the rate of the reaction between MLD and methylamine; [MLD] = 2 μg mL^{-1}; 2-propanol percentage = 30%; T^a = 75 °C, pH = 4.0.

selected as close to the optimum. Higher temperatures were not selected to avoid possible losses of sample by evaporation. The influence of methylamine concentration was studied in the range between 5 and 20 mM. The rate of reaction increases with the concentration of methylamine up to 10 mM and slightly decreases for values higher than 10 mM (Figure 5). A concentration of 10 mM was selected as optimum. A plot of the logarithm of the rate against the inverse of the absolute temperature allowed us to calculate an activation energy of 25.3 kcal/mol (Figure 6).

The influence of methylamine concentration was studied in the range between 5 and 20 mM. The rate of reaction increases with the concentration of methylamine up to 7.5 mM (1/2 partial order) and then slightly decreases for values higher than 7.5 mM (0 partial order) (Figure 5). A concentration of 10 mM was selected as optimum.

The influence of the *n*-hexylaldehyde concentration was studied in the range between 0.5 and 14 mM. A 0 partial order for *n*-hexylaldehyde was determined for the entire range of concentrations assayed. The presence of *n*-hexylaldehyde is not necessary for the development of the reaction.

Influence of the 2-Propanol Percentage. Although both methylamine and MLD are soluble in

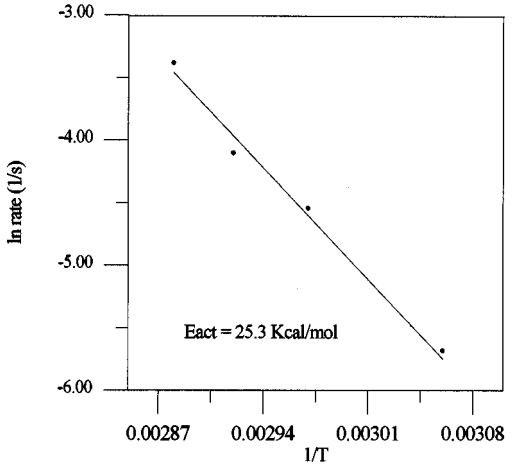

Figure 6. Logarithmic plots of rate vs inverse of the absolute temperature.

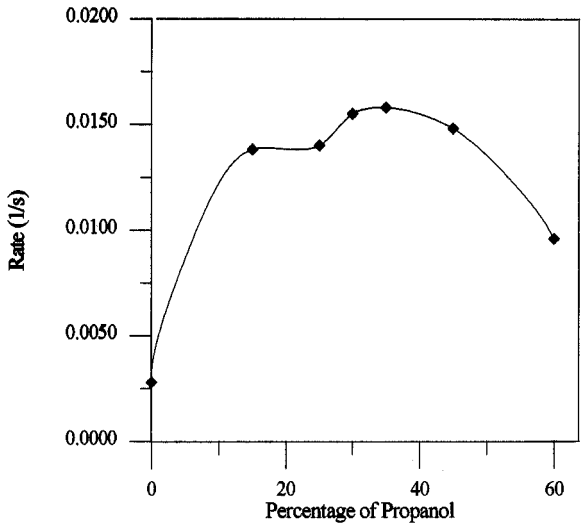

Figure 7. Influence of propanol concentration on the rate of the derivatization reaction.

water, the effect of the 2-propanol concentration on the rate of reaction was investigated, to avoid solubility problems with the application of the method to oil samples. For different samples of MLD, in the presence of methylamine, the percentage of 2-propanol in the medium was varied between 0% and 60% (v/v). Different kinetic curves were registered, and the rates of reaction were calculated. The reaction rate first increases with the content of 2-propanol in the medium up to 30% but decreases for 2-propanol percentages higher than 45%. A 30% (v/v) 2-propanol–water mixture has been selected as optimum for the procedure (Figure 7).

Calibration Curves and Analytical Parameters. Under the conditions selected, the fluorescence–time signals, at λ_{ex} = 405 nm and λ_{em} = 470 nm, were recorded between 0 and 1500 s, for solutions containing different amounts of MLD. By application of the least-squares method, different calibration equations of reaction rate vs [MLD] were analyzed. Different interval times for the measurements, comprised between 1000 and 1500 s, were used. The most favorable statistical parameters were obtained in the range between 1250 and 1450 s. In Figure 8a, the kinetic curves obtained are represented. Each kinetic curve was recorded in triplicate. The calibration graph is not linear but concave for MLD concentrations lower than 0.5 μg mL^{-1} (Figure 8b). The calibration graph is linear for MLD

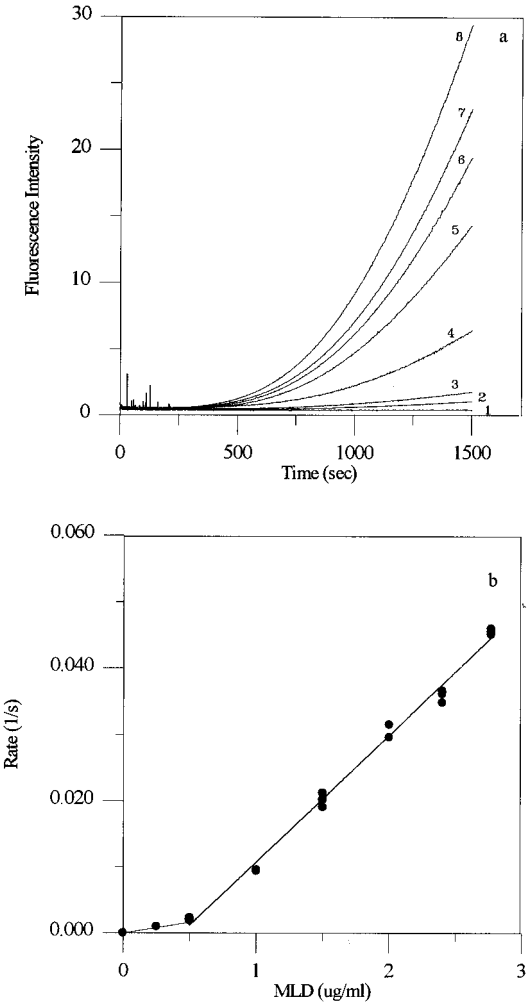

Figure 8. (a) Kinetic curves of the calibration graph for the determination of MLD and (b) calibration graph of MLD; [methylamine] = 10 mM, 2-propanol percentage = 30%, T^a = 75 °C, pH = 4.0. [MLD]: (1) 0, (2) 0.25, (3) 0.5, (4) 1.0, (5) 1.5, (6) 2.0, (7) 2.4, and (8) 2.8 μg mL^{-1}.

Table 1. Analytical Characteristics for the Kinetic–Fluorometric Determination of MLD[a]

equation ($y = a + bx$)		$y = (-0.0084 \pm 0.0008) + (0.0191 \pm 0.0004)x$
correlation coefficient (r)		0.9965
linearity (1 − RSD(b)%)		97.89%
sensitivity ($s_{y,x}/b$)		0.070 μg mL^{-1}
precision (RSD%)	0.5 μg mL^{-1}	9.12
	1.0 μg mL^{-1}	4.97
	1.5 μg mL^{-1}	2.94
	2.0 μg mL^{-1}	2.16
	2.4 μg mL^{-1}	1.97
	2.8 μg mL^{-1}	1.76

[a] a, intercerpt on the y axis; b, slope; $s_{y,x}$, regression standard deviation of (1 − RSD(b)%); RSD, relative standard deviation.

concentrations higher than 0.5 μg mL^{-1}. The nonlinear relationship for low MLD contents is similar to the behavior of the reaction of methylamine with MLD in the presence of acetaldehyde (Kikugawa et al., 1988). Statistical parameters of the linear portion of the calibration graph and analytical characteristics of the determination of MLD with the proposed method, such as linearity, sensitivity, and precision, have been calculated following a linear regression model (Cuadros et al., 1993), and the results are shown in Table 1.

Kinetic Equation. In the optimized conditions the kinetic equation can be applied as follows: for [MLD] ≥ 0.5 μg mL^{-1} → $v = k$[MLD][H$^+$]^a[methylamine]^b[n-

Table 2. Recovery Values in the Determination of MLD in the Presence of Several Foreign Species

foreign species	interference/ MLD ratio	recovery (%)
methylglyoxal	10	46.5
	5	66.9
glyoxal	5	109.3
	2	100.0
diacetyl	10	106.9
	5	104.1
	2	99.4
n-hexylaldehyde	5	96.2
	2	97.5
acetaldehyde	250	100.0
	100	100.0
	10	99.4
fructose	100	97.2
	10	96.5
	5	97.2
acetone	50	98.2
	10	100.0
alanine	1:5	98.2
DL-α-amino-*n*-butyric acid	100	97.0
	10	105.5
	5	103.7
L-proline	100	100.6
	10	100.6
phenylalanine	100	96.3
	10	100.6
glycocole	100	109.6
	1	104.5
dopamine	10	106.4
	2	98.2
L-DOPA	10	94.8
	5	97.6
glucose	50	92.5
	10	96.4
galactose	10	91.1
	5	103.9
oleic acid	100	103.8
	10	94.8
linoleic acid	100	105.2
	2	98.8
linolenic acid	100	95.9
	1	95.9

hexylaldehyde][c], being (a) for $[H^+] \geq 6.30 \times 10^{-5}$ M → $v = k'[\text{MLD}][H^+]^{1/4}$ and (b) for $[H^+] \leq 6.3 \times 10^{-5}$ M → $v = k''[\text{MLD}][H^+]^{-4/3}$, where k' and k'' are the conditional rate constants of the condensation reaction; *b* and *c* exhibit 0 partial order value in both cases.

Influence of Foreign Species. A number of species that are known to affect the reaction of MLD with methylamine have been described in the literature. This is an important problem in the application of the proposed method in real samples. Dialdehydes (glyoxal, methylglyoxal, diacetyl), monofunctional aldehydes (acetaldehyde, *n*-hexyaldehyde), biogenic carbonyl compounds (D-glucose, D-galactose, D-fructose), unsaturated fatty acids (oleic acid, linoleic acid, linolenic acid), and amino acids and similar compounds (glycocol, phenylalanine, L-DOPA, dopamine, alanine, DL-α-amino-*n*-butyric acid, L-proline) have been described as important interferent species (Kikugawa, 1986; Kikugawa et al., 1988). The kinetic behavior of MLD with methylamine, in the presence of these compounds, was studied under the optimum conditions previously established. To investigate the degree of interference of the abovementioned species, in the reaction of MLD with methylamine, the following experiments were carried out. A 1.5 μg mL^{-1} concentration of MLD was maintained constant, in the presence of the interfering species, over a range of concentrations between 1.5 and 150 μg mL^{-1} (1:1−1:100 MLD:interference (m/m) ratio). The kinetic curves at 470 nm emission and 405 nm excitation wavelengths were obtained. In Table 2, the recovery values for MLD in the presence of the different compounds assayed are summarized. Most of the species were tolerated at a 10:1 (interference:analyte (m:m)) ratio, at least. Methylglyoxal interferes in the determination. The presence of several monofunctional aldehydes used in the Hantzsch reaction (Kikugawa et al., 1984) did not increase the reaction rate in our experimental conditions.

Table 3. Results Obtained by the Application of the Proposed Kinetic−Fluorometric Method in Olive Oil Samples

added MLD (μg mL^{-1})	found MLD (μg mL^{-1})	standard deviation[a]	recovery (%)
1.00	1.02	0.02	102
2.00	1.87	0.11	93.5
2.62	2.46	0.06	93.9

[a] For $n = 3$.

Determination of Malonaldehyde in Olive Oil Samples. The proposed kinetic method was applied to the determination of MLD in olive oil samples. A dilution of oil of 1:20 was necessary to carry out the method. The oil solution was spiked with different amounts of MLD to test the procedure. The recovery ratios, of known amounts of MLD added to the oil, were obtained by using calibration graphs (reaction rate vs concentration). Table 3 shows the assay results, as a percentage of recovery, from the average of three determinations of three different samples. The recoveries agree well enough with the added amount, and the precision is quite satisfactory.

CONCLUSIONS

Similar spectral characteristics and kinetic data are obtained in the presence and absence of *n*-hexylaldehyde or acetaldehyde. This indicates that the formation of 1,4-disubstituted-1,4-dihydropyridine-3,5-dicarbaldehyde derivatives from MLD in the presence of a primary amine, such as methylamine, occurs by means of via b Hantzsch reaction. In consequence, only the dialdehyde and the primary amine are implicated in the reaction in our experimental conditions. A very selective fluorometric−kinetic method is proposed, allowing the determination of MLD in concentrations ranging between 0.5 and 2.8 μg mL^{-1}, and adequate recovery values were obtained when the proposed method was applied to olive oil samples.

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