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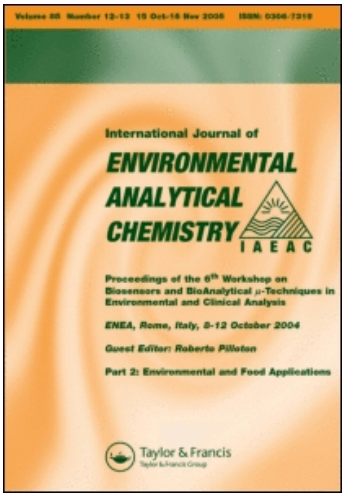

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KINETIC DETERMINATION OF DICOUMAROL ON GRAIN BY USING STOPPED-FLOW MIXING METHODOLOGY

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A simple and fast kinetic method for the direct determination of dicoumarol on grain was developed. It involves measuring the initial rate of the colour-forming reaction (λ = 475 nm) undergone by Gibbs' reagent in a basic medium (pH 9.0) in the presence of dicoumarol. Stopped-flow mixing methodology was used to obtain kinetic curves. This allowed reagent manipulation to be minimized and the method to be automated to some extent. The calibration graph was linear over the range 3–20 μg ml^{-1}. Other anticoagulant rodenticides such as bromadiolone, difacinone and warfarin are tolerated in 20-fold excess. The method was satisfactorily applied to the direct analysis of wheat and oat samples spiked with dicoumarol (mean recovery, 92.7%).

KEY WORDS: Dicoumarol, kinetics, stopped-flow, grains.

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INTRODUCTION

Dicoumarol [3,3'-methylene-bis(4-hydroxycoumarin)] which shows an anticoagulant action that has been used to control mouse and rat populations in agriculture[1-3], displays poisoning hazards for non-target animals owing to its toxicity to all mammals. Given its anticoagulant action it has also been used in medicine[4-7] but at present, because of its unpredictable response and the slow onset and long duration of its action, dicoumarol has been largely replaced by warfarin sodium.

Dicoumarol can be determined by various photometric methods[1,8,9], all of which are based on equilibrium measurements. No kinetic-based analytical methods for dicoumarol have yet been reported. Because kinetic methodologies currently offer major advantages over their equilibrium counterparts (especially in connection with the stopped-flow mixing technique), we developed a kinetic method for the determination of dicoumarol based on the behaviour of Gibbs' reagent (2,6-dichloro-p-benzoquinone-4-chlorimine) in a basic medium containing this pesticide. The proposed method was applied to grain and could be extended to other environmental problems involving different matrices. Gibbs' reagent has previously been used[2,10] together with other compounds to detect rodenticidal coumarins by thin-layer

chromatography, but never so far for the quantitative determination of dicoumarol. Even though the batch technique could be used for the kinetic determination of dicoumarol (no significant differences in the reaction rate were observed on replacing this technique with the stopped-flow mixing technique), the use of stopped-flow mixing methodology is always advisable, especially in routine analyses where it shortens the data acquisition time and minimizes manipulation of reactants, which helps to obtain to a high sample thoughput and low reagent consumption[11].

EXPERIMENTAL

Reagents

A stock solution of dicoumarol (Sigma) was prepared by dissolving 10 mg in 100 ml of 0.1 M sodium hydroxide. Working solutions of lower concentrations were made by dilution to the required volume with distilled water. A Gibbs' reagent solution was freshly prepared at a concentration of 2×10^{-2} M in ethanol dissolving 0.21 g of 2,6-dichloro-*p*-benzoquinone-4-chlorimine (Sigma) in 50 ml of ethanol. All reagents were A.R. grade chemicals.

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Apparatus

A Lambda 5 spectrophotometer (Perkin-Elmer) fitted with a stopped-flow module[12] (Quimi-Sur Instrumentation, Spain) was used for kinetic measurements. The acquisition and treatment of kinetic data was performed by a 98561AE computer equipped with a 16-bit 98640A analoge-to-digital converter (Hewlett-Packard). The spectrophotometer cell compartment was thermostated by using a Peltier electronic system (Perkin-Elmer) and the solutions in the stopped-flow module were kept at a constant temperature by means of a circulating waterbath.

Procedure

A solution containing 0.5 ml of Gibbs' reagent (2×10^{-2} M in ethanol) in a final aqueous volume of 10 ml was used to fill one of the two 10-ml reservoir syringes, while the other was filled with a solution containing 2 ml of ammonium chloride-ammonia buffer (0.4 M, pH 9.0) and a dicoumarol standard solution at a final concentration between 3 and 20 μg ml^{-1} and a final volume of 10 ml. After the two 2-ml drive syringes were filled, 0.15 ml of each solution was mixed in the mixing chamber in each run. The variation of the absorbance throughout the reaction was monitored at 475 nm and displayed on a chart recorder. All measurements were carried out at 50 °C. The initial rate method was applied to the acquired absorbance values, which were processed by linear regression using the microcomputer. The reaction rate was determined in about 1 min and each standard was assayed in triplicate. The blank signal was subtracted from each reaction rate value obtained for the standards.

Determination of dicoumarol in grains

About 10 g of accurately weighed wheat or oat were sprayed with 10 ml of an aqueous dispersion containing between 20 and 40 mg ml^{-1} of dicoumarol, after which the sample was allowed to dry in the sun for 2 h and then in the shade for a day in order to remove extraneous moisture. For each determination a blank assay was carried out by spraying similar amounts of grain with 10 ml of water. The samples were weighed again in order to determine the amount of dicoumarol retained, and each sample was treated with 100 ml of 0.1 M sodium hydroxide. Aliquots of the resulting solutions were filtered and processed as described above without further treatment.

RESULTS AND DISCUSSION

Gibbs' reagent is a general reagent for phenolic compounds[13] that yields indophenol derivatives of maximum absorbance at 540–660 nm in basic media. It was formerly used as a reagent for phenols bearing no para substituent even though a number of para-substituted phenols have been reported to react with Gibbs' reagent to yield coloured products with maximum absorbances lying in similar wavelength ranges. Gibbs' reagent solutions (in 2% ethanol) do not absorb in the visible region, whereas basic solutions of this compound are unstable and slowly give rise to an absorption maximum at 475 nm (Figure 1), which is also obtained in the presence of dicoumarol, though somewhat faster. This suggests that, unlike

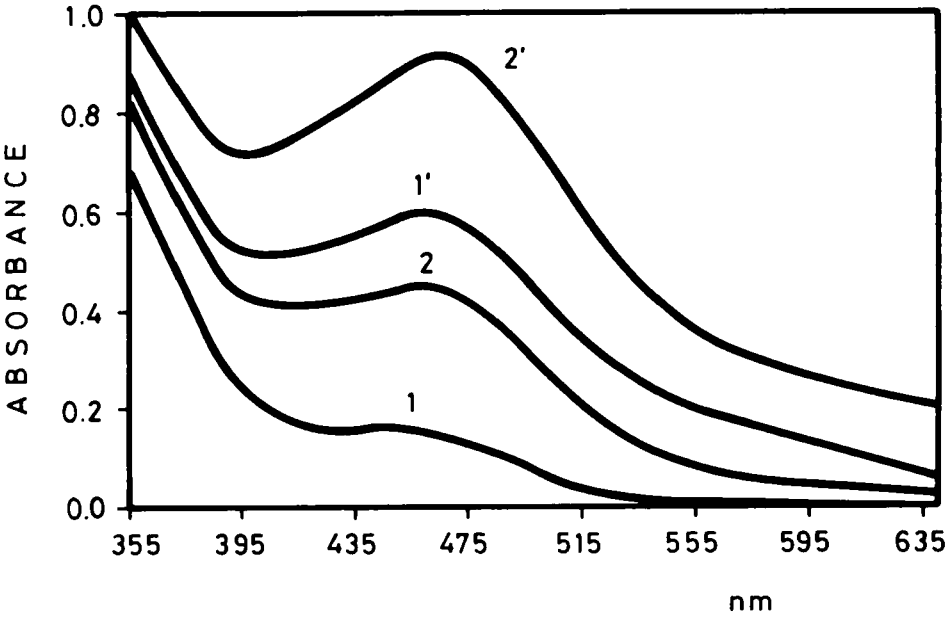

Figure 1 Absorption spectra of Gibbs' reagent in a basic medium (pH 9.0) in the absence (1 and 1') and presence (2 and 2') of dicoumarol. Temperature, 20 °C. Dicoumarol, 5 μg ml^{-1} Gibbs' reagent, 5×10^{-4} M. Time: 1 and 2, 1 min; 1' and 2', 8 min.

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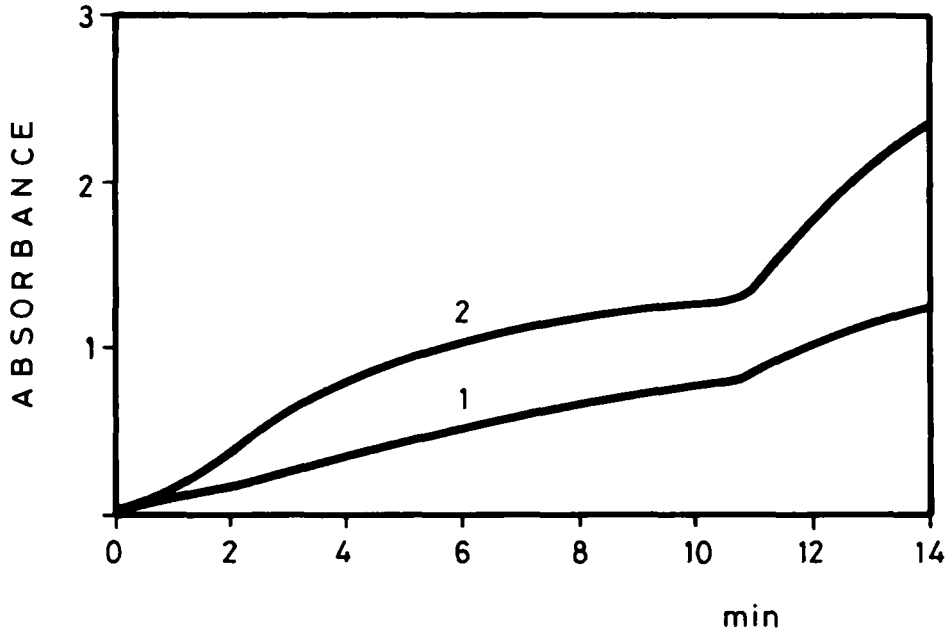

Figure 2 Kinetic curves obtained for Gibbs' reagent at pH 9.0 in the absence (curve 1) and presence (curve 2) of dicoumarol. Temperature, 20 °C. Dicoumarol, 5 μg ml^{-1} Gibbs' reagent, 5×10^{-4} M. The change in the profile of the kinetic curves at 10.75 min corresponds to the addition of a new amount of Gibbs' reagent.

other phenolic compounds, dicoumarol does not undergo a condensation reaction with Gibbs' reagent or that, if it does, the condensation product absorbs at the same wavelength. More likely, the accelerating effect is due to a catalytic effect of dicoumarol on the degradation of Gibbs' reagent. In order to clarify this point, a basic solution containing Gibbs' reagent and dicoumarol was monitored photometrically until the reaction was virtually complete. At that point, further Gibbs' reagent was added and a significant change in the slope of the kinetic curve (Figure 2, curve 1) was obtained, which indicates that dicoumarol was regenerated as equilibrium was reached and that the new addition of reagent started a new catalytic cycle. Only a small change in the slope of the kinetic curve was obtained (Figure 2, curve 2) upon the addition of further reagent when the assay was carried out in the absence of dicoumarol. However, the kinetic curves obtained in the presence of dicoumarol always showed a slightly higher absorbance at or near equilibrium than in the absence of dicoumarol. This can be attributed to the fact that the blank reaction reaches equilibrium very slowly.

Effect of variables

Variables were optimized by changing each of them in turn while keeping all others constant. All reported concentrations were the initial concentrations in the syringe (viz. twice the

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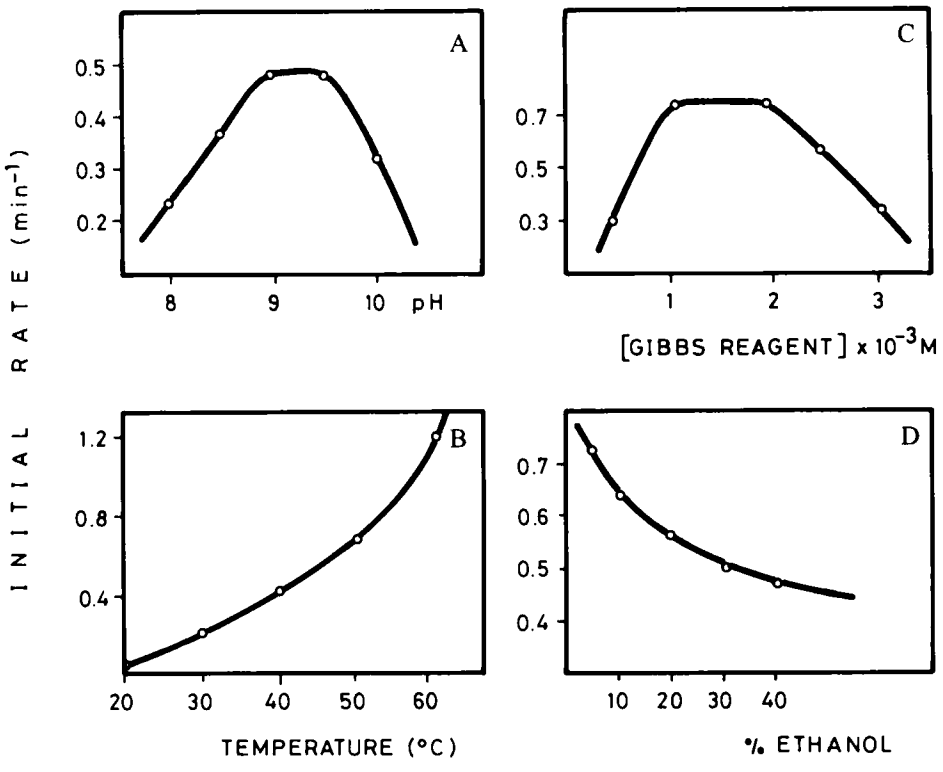

Figure 3 Effect of (A) pH, (B) temperature, (C) Gibbs' reagent concentration and (D) ethanol content on the initial reaction rate.

actual concentrations in the reaction mixture at time zero after mixing). Each kinetic result was the average of three measurements.

The effect of the sample pH on the reaction rate was studied by adding sodium hydroxide or hydrochloric acid to the dicoumarol solution in order to prevent alteration of the Gibbs' reagent. In each case, the pH was measured in the sample solution and in the waste, where it was the same as in the mixing chamber. In this way, a pH decrease was obtained when mixing the sample and reagent. Another pH experiment involved preparing various sample solutions in ammonium chloride-ammonia buffers of different pH (8.3–10.0). In this case, the pH of the samples was the same as in the mixing chamber. As shown in Figure 3.A, the reaction rate was independent of the pH over the range 8.8–9.6. The experiment was repeated using a borate buffer solution which, yielded lower reaction rate values. Therefore, an ammonium chloride-ammonia buffer of pH 9.0 was finally chosen to adjust the sample pH. The reaction rate was constant for buffer concentrations between 0.08 and 0.10 M; lower concentrations gave rise to slightly decreased rates.

The reaction rate increased with increasing temperatures in the range 20–60 °C (Figure 3.B). A temperature of 50 °C was selected. Figure 3.C shows the effect of the Gibbs' reagent concentration on the reaction rate. Varying the concentration from 10^{-3} M to 2×10^{-3} M had

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Table 1 Recovery of dicoumarol from spray-treated grain

Grain	*Dicoumarol (mg g^{-1})*		*Recovery (%)*
	Added	*Found**	
Wheat	15.0	13.5	90
	23.2	20.0	86
	36.4	33.3	92
Oat	14.2	15.1	106
	24.5	22.7	93
	32.1	28.8	90

*Average of three determinations.

no effect on the reaction rate. Because Gibbs' reagent was dissolved in a hydroalcoholic medium, the effect of the ethanol content in the range 5–40% (Figure 3.D) was also investigated. The reaction rate was found to decrease with an increasing ethanol content.

The initial slopes of the absorbance-time curves obtained under the above optimal experimental conditions were indicative of a first-order reaction with respect to dicoumarol.

Features of the proposed method

The absorbance-time graphs obtained for various amounts of dicoumarol were analysed by the initial-rate method. The calibration graph was linear from 3 to 20 μg ml^{-1} of dicoumarol, with a Pearson's correlation coefficient, r, of 0.990. The equation for the calibration graph was: $v = 4.6\times10^{-2}$ (ml μg^{-1} min^{-1})(dicoumarol) - 5.5×10^{-2} min^{-1}. The detection limit, as defined by IUPAC [14], was 1 μg ml^{-1}.

The relative standard deviation ($P = 0.05$, $n = 10$) obtained for 5 and 12 μg ml^{-1} dicoumarol was 4.1 and 2.0%, respectively. The selectivity of the proposed method was assessed by adding various potentially interfering anticoagulant rodenticides such as bromadiolone, diphacimone and warfarin to samples containing 5 μg ml^{-1} dicoumarol. A dicoumarol/interferent ratio of 1/20 could be tolerated in all cases. One other salient feature of the method is its speed: initial-rate measurements can be acquired within ca. 1 min.

Applications

In order to check the usefulness of the proposed kinetic method, wheat and oat samples were analysed. Table 1 lists the analytical recoveries obtained for different amounts of dicoumarol added to the grain samples. The mean recovery obtained was 92.7%.

Obviously, the proposed stopped-flow method allows the straightforward determination of dicoumarol in grain and can be readily adapted for use in routine analyses.

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