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Toward an Efficient Method of Detecting Cocaine Metabolite in Urine

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TOWARDS AN EFFICIENT METHOD OF DETECTING COCAINE METABOLITE IN URINE

C.J. Summers
Final Paper
Research/Thesis 499
Chemistry Research Honors Project
Dr. David N. Bailey, Advisor
4/23/96

Towards an Efficient Method of Detecting Cocaine Metabolite in Urine

by


Carl John Summers

Research Honors Project in Chemistry

Illinois Wesleyan University

April 23, 1996

Signature of Author

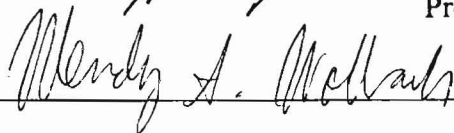
 4/23/96
Department of Chemistry

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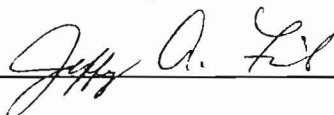


Project Advisor

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Abstract

The primary metabolite of cocaine is benzoylecgonine (BE). A desirable method for detecting cocaine use is the extraction of BE from urine into an organic phase solvent and subsequent analysis by High Performance Liquid Chromatography (HPLC). The attempt to develop such a method is hindered by the extreme water solubility of BE, making extraction into an organic phase extremely difficult. The present study attempts to use an ion-pairing agent to bind with BE and form a large, organically soluble ion-pair. The ion pairing agent used was Reinecke Salt in a 1% solution. An aqueous solution of 50 $\mu\text{g/mL}$ BE was treated with the Reinecke Salt solution and then extracted with methylene chloride. Initial analysis of the extracted ion pair was performed by Ultraviolet-Visible (UV-VIS) spectrometry. The structure of BE is pH dependent; existing as a positively charged species in acid, a zwitterion in neutral solution, and a negatively charged species in base. The pH dependence of BE results in a pH dependent ion pairing reaction; an ideal pH of 8.5 was determined for the extraction. A concentration variant peak at 255 nm was discovered using UV-VIS spectrometry, however, the peak does not exhibit linear concentration dependence and so is not currently of practical use.

Theory

Cocaine, benzoylecognine, is a well known and commonly abused recreational drug. Its fairly widespread illegal use and addictiveness result in many problems for families, businesses, and law enforcement. Due to the serious results of its abuse, simple, reliable, and efficient methods of detecting cocaine use are needed. Many drug detection methods rely on extraction of the drug or its metabolite from urine, into an organic phase which is then analyzed for the illicit drug. This method has been applied successfully in the case of nicotine, where the drug is extracted from urine into an organic phase and then detected via High Performance Liquid Chromatography (HPLC) (1). A similar technique would be desirable for use in detecting cocaine, however, an interesting difficulty is raised in the attempt to develop such a method. The problem encountered is that benzoylecognine (BE), the primary candidate for detection, is a highly water soluble species and thus is extremely difficult to extract into an organic phase.

The body metabolizes benzoylecognine into several different species, the most common of which is BE. Benzoylecognine is produced through the hydrolysis of an ester bond on cocaine (Figure 1) and accounts for 35-45% of the species excreted in urine after cocaine use (2). The remainder is secreted as either cocaine or other derivatives of cocaine such as benzoylethyl-cognine. The typical concentration range of BE in urine after intranasal use is 3-25 $\mu\text{g/mL}$ (3).

BE exists in distinct states in acidic, neutral, and basic solutions (Figure 2). The neutral state of BE is a zwitterion. Zwitterions are molecules with both a negatively and positively charged locus. The molecule thus acts as an internal salt and is correspondingly extremely water soluble. The zwitterion state of BE is amphiprotic, both able to accept and donate a proton. In acidic solution the negatively charged oxygen accepts a proton to form a molecule with one positive charge. In basic solution the positively charged nitrogen loses a proton to form a molecule with one negative charge. At all pH levels BE exists as a charged and thus polar

species. This fact results in the great degree of aqueous solubility exhibited by the molecule, thus the problem with extracting BE into organic phase for analysis.

Several different methods have been attempted to overcome this difficulty and successfully extract the BE into an organic phase. The literature includes several efforts to separate the BE by using column chromatography such as C₁₈ cartridges (4-8). These attempts have been relatively unsuccessful, time consuming, and expensive. Salting-out is a method in which the aqueous solution is saturated with a salt in an attempt to decrease the solubility of BE. This was attempted unsuccessfully by Nieuwkirk (9). So far no truly efficient and successful method of performing the extraction has been developed. This paper reports the attempt to apply the technique of ion-pairing.

The ion-pairing method involves ionic coupling of the BE molecule with an inorganic or organometallic complex which has charge centers that match up, positive-to-negative and negative-to-positive, with those of the BE molecule. This ionic bonding between the two counter ions results in a larger molecule in which the charged areas of BE are shielded by the non-polar ligands of the entire complex. This shielding results in a large non-polar "shell" surrounding the species and therefore increases solubility in organic phase solvents (10). This technique was applied successfully with nicotine, the ion-pairing agent used was sodium decane sulfonate (1). Eisman et al. have reported the results of using five different ion-pairing agents to extract cocaine into organic phase (12).

Previous work using one of the ion-pairing agents reported by Eisman et al., hexakis(thiocyanato)iron(III), to facilitate the extraction of BE was performed by Babcock (11). Adequate extraction of the ion-pair was accomplished, however this ion-pairing agent is not useful for purposes of the present research due to the non-linear absorption of the ion pair with varying concentrations of BE. The absorption detector on standard HPLC instruments operates at 255 nm. For an ion-pair to be useful as a method of detecting the quantifiable presence of BE, its absorption at 255 nm must vary linearly with concentration of BE. The ion-pair extracted by Babcock did not even exhibit a concentration dependent absorption at this wavelength.

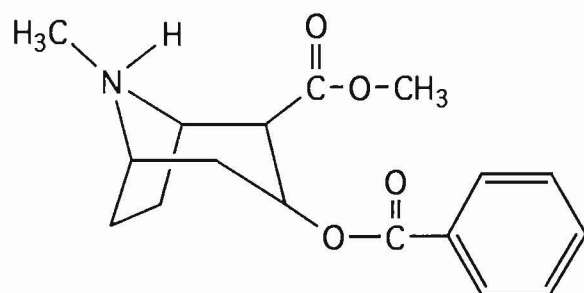
High Performance Liquid Chromatography is the preferred method of detection due to its sensitivity, the ability to separate the BE ion-pair from interfering substances in solution, and the possibility of automating the detection process (13). The use of HPLC would also allow for the possibility of a single detection process for both nicotine and cocaine use (1).

The focus of the present work is to use another of the ion-pairing agents reported by Eisman et. al., Reinecke Salt, to obtain a successful extraction of the ion-pair which absorbs linearly versus concentration at 255 nm. Reinecke Salt ($\text{NH}_4^+[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4]^-$), (Figure 3) is a synthetic reagent most commonly used to precipitate primary and secondary amines and amino acids (14).

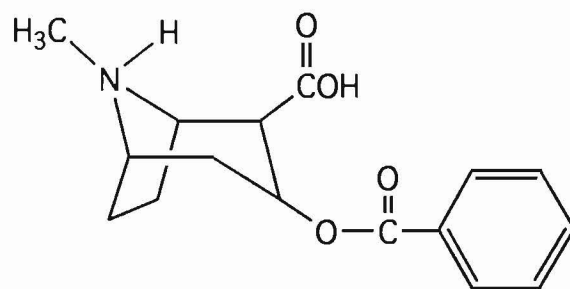
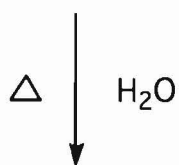
Due to the structural variation of the BE molecule at different pH levels the efficiency with which the ion-pairing agent binds with it will be pH dependent. This will make the extraction itself pH dependent and will necessitate the determination of an optimal pH for extraction. Once the optimal pH for extraction has been determined then the UV-VIS absorption spectra of the ion pair extract can be studied at 255 nm to determine if there is a peak which varies linearly with concentration. To be practically useful this peak should allow for a detection limit of approximately 0.3 $\mu\text{g/mL}$ BE in urine. This limit is desirable because the concentration of BE in urine decreases over time after intranasal cocaine use.

Figure 1:

The hydrolysis of cocaine to benzoylecognine



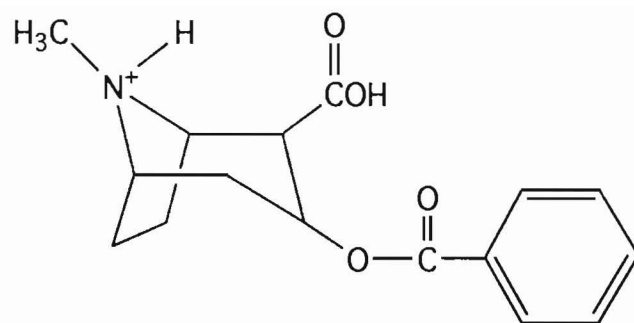
Cocaine



Benzoyl Ecognine

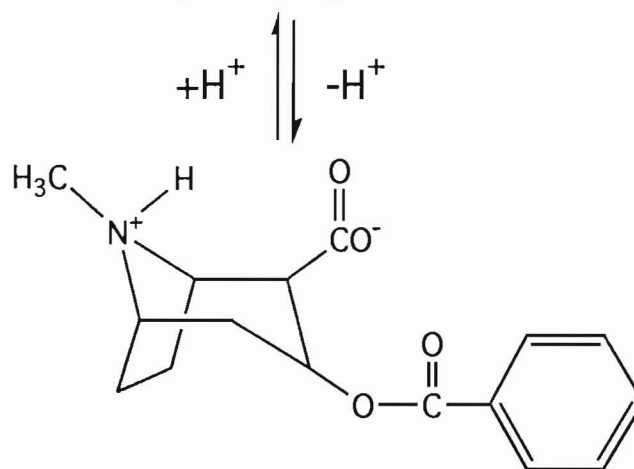
Figure 2:

Benzoylecognine in acidic, neutral, and basic solution



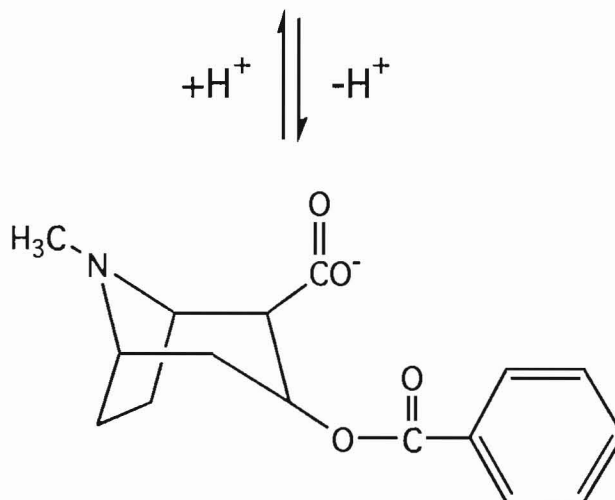
Cation

Benzoyl Ecognine in Acid



Zwitterion

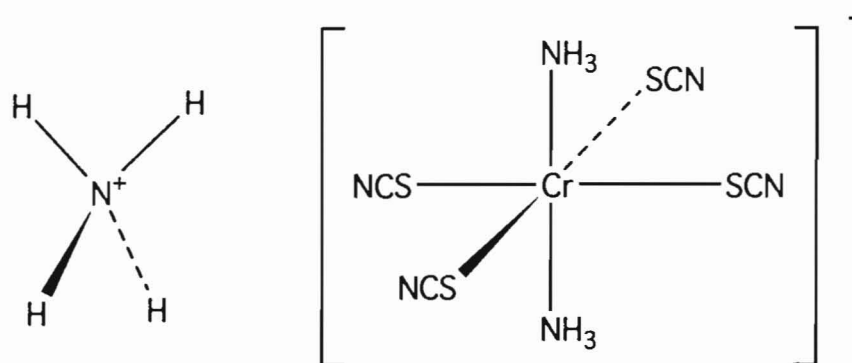
Benzoyl Ecognine in Neutral Solution



Anion

Benzoyl Ecognine in Base

Figure 3:
Reinecke Salt



Reinecke Salt

Experimental Section

Instrumentation

A Fisher Scientific Accumet Model 5 pH meter was used for all pH measurements. It was calibrated using pH = 4.00 and pH = 10.00 buffers, both from Micro Essential Laboratory (Brooklyn, NY). A Perkin-Elmer 559 Ultraviolet-Visible Spectrophotometer (UV-VIS) was used to obtain all spectra. Fisher Scientific 283 SCC 1.000 cm cells made of UV Silica (ca. 190 nm - 900 nm) were used for all UV-VIS analysis. A Waters Associates High Performance Liquid Chromatography (HPLC) system outfitted with a Fisher Recordall Series 5000 was used to obtain all chromatograms. The HPLC system included the following: an Waters Automated Gradient Controller, a Model 441 Absorbance Detector, and a Model 6000A. A μ BONDAPAK column prepacked with a C_{18} stationary phase was used in all chromatography work.

Reagents and Chemicals

1. *Preparation of Benzoyl Ecognine (BE)*

Five grams of cocaine hydrochloride from Sigma Chemical Company (St. Louis, MO) were dissolved in 750 mL of a 0.1 M/0.1 M buffer solution of Na_2CO_3 and $NaHCO_3$ certified ACS grade, Fisher Scientific (Fair Lawn, NJ). This solution was extracted with two 500 mL aliquots of purified grade diethyl ether, Fisher Scientific (Fair Lawn, NJ). The diethyl ether was evaporated in a Büchi Rotavapor. The residue was dissolved in 350 mL of distilled water and refluxed for three hours. This solution was boiled down to approximately 50 mL and then allowed to evaporate overnight in the Rotavapor. The crude BE was completely dissolved in 75 mL of hot distilled water. The hot solution was placed in several large test tubes and refrigerated overnight. The white needle-like crystals were collected, filter dried, and weighed (18.2% yield). The BE was stored in a refrigerator.

2. *Preparation of Ammonium Tetrathiocyanato Diammine Chromate (III) (Reinecke Salt)*

A 1% solution of the ion pairing agent was prepared by dissolving 10.00 grams of Acros (NJ) Reinecke Salt in one liter of distilled water.

Extraction Technique

A solution containing 10.0 mL of both the 1% Reinecke Salt and the BE solutions was added to a 125 or 250 mL separatory funnel. Three 10.0 mL aliquots of Fisher certified ACS grade methylene chloride were used in the extraction. Each aliquot was agitated with the ion-pairing/BE solution resulting in an emulsion of varying consistency and duration. The emulsion subsided with time and proper agitation of the mixture. The methylene chloride layer was then collected in a 125 mL flask.

Method Used in Determining the Effects of Varying:

1. *pH*

A solution of 10.0 mL 1% Reinecke Salt and 10.0 mL 50 µg/mL BE was prepared. The pH of the solution was adjusted using Fisher ACS reagent grade HNO₃ and NH₄OH. The solution was placed in the separatory funnel and extracted. A series of pH values varying from 0.90 to 10.52 were used.

2. *Concentration*

A series of BE solutions varying in concentration (1.0, 2.0, 3.0, 4.0, 5.0, 10.0, 20.0, and 50.0 µg/mL) were added in 10 mL aliquots to 10.0 mL of 1% Reinecke Salt solution. This solution was adjusted to the optimal pH of 8.5 (see Results and Discussion) using a minimal amount of HNO₃ and NH₄OH. The solution was then placed in the separatory funnel and extracted.

Results and Discussion

The absorption data obtained from the series of varying pH values is presented in Table 1. The absorption data indicate that a pH of 8.5 to 9.0 would be optimal for the extraction. This interpretation of the data is seen by roughly fitting a curve to the data as represented in Figure 4.

Table 1

Trial	pH of extracted solution	Absorption at 255 nm
1	1.78	0.000
2	4.04	0.043
3	6.30	0.192
4	7.75	0.289
5	9.98	0.300
6	10.44	0.125

As a result of these tests, further extractions were performed at a pH of 8.5 to optimize the absorption reading. The fact that a pH of approximately 8.5 provides the optimal extraction indicates that primarily the zwitterion species of BE is being extracted. The isoelectric point of the Zwitterion has been determined to be between a pH of 8.1 and 8.8 (13) and thus is likely the prevalent form present in the extracted ion pair.

The results of the extractions performed with varying concentrations of BE will be presented in two series in order to be more clearly understood. The first series consisted of concentrations ranging from 1 to 50 $\mu\text{g/mL}$ and the absorption measurements are reported in Table 2.

Table 2

Trial	Concentration of BE (micrograms/mL)	Absorption at 255 nm
1	1	0.035
2	3	0.098
3	10	0.125
4	20	0.165
5	50	0.188

The plot for this data is presented in Figure 5 and shows a definite concentration dependence of the measured absorption. This dependence, however, is non-linear which certainly poses a problem for the usefulness of this method. The clinical application of the detection method would involve a calibration curve which allows for quantitative determination of BE presence. A non-linear dependence provides a non-linear and thus less accurate and reliable calibration curve. Thus the ability of the method to determine the quantitative amount of BE present in analyzed solutions is hindered. This difficulty is further confounded by a problem evidenced in the second series of concentrations tested.

The second series consisted of concentrations ranging from 1 to 5 mg/mL. This series was run twice and the Absorbance values for both runs as well as the average are presented in Table 3.

Table 3

Trial	Concentration of BE (micrograms/mL)	Absorbance at 255 nm		
		First Run (a)	Second Run (b)	Average
1	1	0.091	0.112	0.098
2	2	0.143	0.158	0.151
3	3	0.145	0.152	0.149
4	4	0.144	0.161	0.153
5	5	0.138	0.158	0.148

As can be seen the absorbance values are fairly erratic and do not even exhibit concentration dependence except between 1 and 2 $\mu\text{g/mL}$. The fact that the first series showed concentration dependence would indicate that the absorption peak at 255 nm is indeed produced by either the ion pair of BE and Reinecke Salt, or pure BE. However, if this is the case it would seem that a similar concentration dependence should be shown in the series of smaller concentrations.

A possible explanation for this inconsistency is that the measurements obtained were not accurate at these small levels of concentration difference. Because the absorption level is only expected to change slightly between 1 $\mu\text{g/mL}$ increments, a fairly exact measurement must be taken to detect the true trend of absorption. In fact, inexact measurements are likely the correct

explanation of the erratic absorption levels reported in Table 3. The reason for the inaccuracy of the measurements is readily seen by consideration of the peak being measured at 255 nm on the actual UV-VIS spectra (Appendix B). The presence of the large peak at 225 nm, which is concentration independent, affects the amount of absorption detected at 255 nm in two ways. As the peak at 255 nm is merely a shoulder on that at 225 nm, the readings taken are likely to exhibit a significant amount of error because the peak being measured is not clearly defined. The other source of error is actual spreading of the absorption at 225 nm over to 255 nm resulting in error even on a quantitative digital readout. This is obviously the more significant form of error and is more difficult to deal with.

The error due to the interfering peak is likely to be large enough to make measurements over increments of a few $\mu\text{g/mL}$ completely unreliable and in fact insignificant. The effect of the peak at 225 nm is also a possible explanation for the non-linearity of the first concentration variant series. While the concentrations and thus the absorption levels of these trials are in general higher than the second series, the error in measurement at 255 nm produced by the interfering peak is large enough to cast doubt upon the reliability of these data as well.

As this concentration independent peak at 225 nm is likely the cause for inaccurate measurement of the important absorption peak at 255 nm it is obviously desirable to either remove or reduce the size of this peak. The source of this peak is not currently known and so the following is only speculation. If the peak is caused by the ion-pair itself then there is very little chance of removing it or even lessening its effect. The only option would be to consider further reacting the extracted solution to alter the ion-pair and of course this is a time consuming and probably extremely complicated further step with questionable possibility of success. If, however, the peak at 225 nm is due to another entity in solution, such as the solvent, excess of the ion pairing agent alone, the product of an unknown reaction taking place, etc., then there is a definite possibility of lessening the peak or removing its influence entirely.

Several options for solving this problem are available. The use of a different organic solvent could eliminate the peak if it is the result of methylene chloride. Adjusting of the amount

of ion-pairing agent used could reduce the size of the peak if it is the result of excess Reinecke Salt in the extracted solution. Perhaps the most likely solution to this problem is simply the application of the procedure to HPLC, the ultimate instrument on which measurements will be performed. As HPLC is a chromatographic process there is a large possibility that it would separate the ion-pair from any other entity causing the peak at 225 nm. Thus the two entities would come off of the column at different times and be detected independently of each other. If this is the case then it may be shown that the absorbance peak at 255 nm is indeed linearly dependent upon concentration. This solution was not studied but is certainly the next step in the further investigation of the ion pairing reaction between Reinecke Salt and BE.

Figure 4:

Absorbance of the ion-pair at 255 nm versus pH

Chart 1

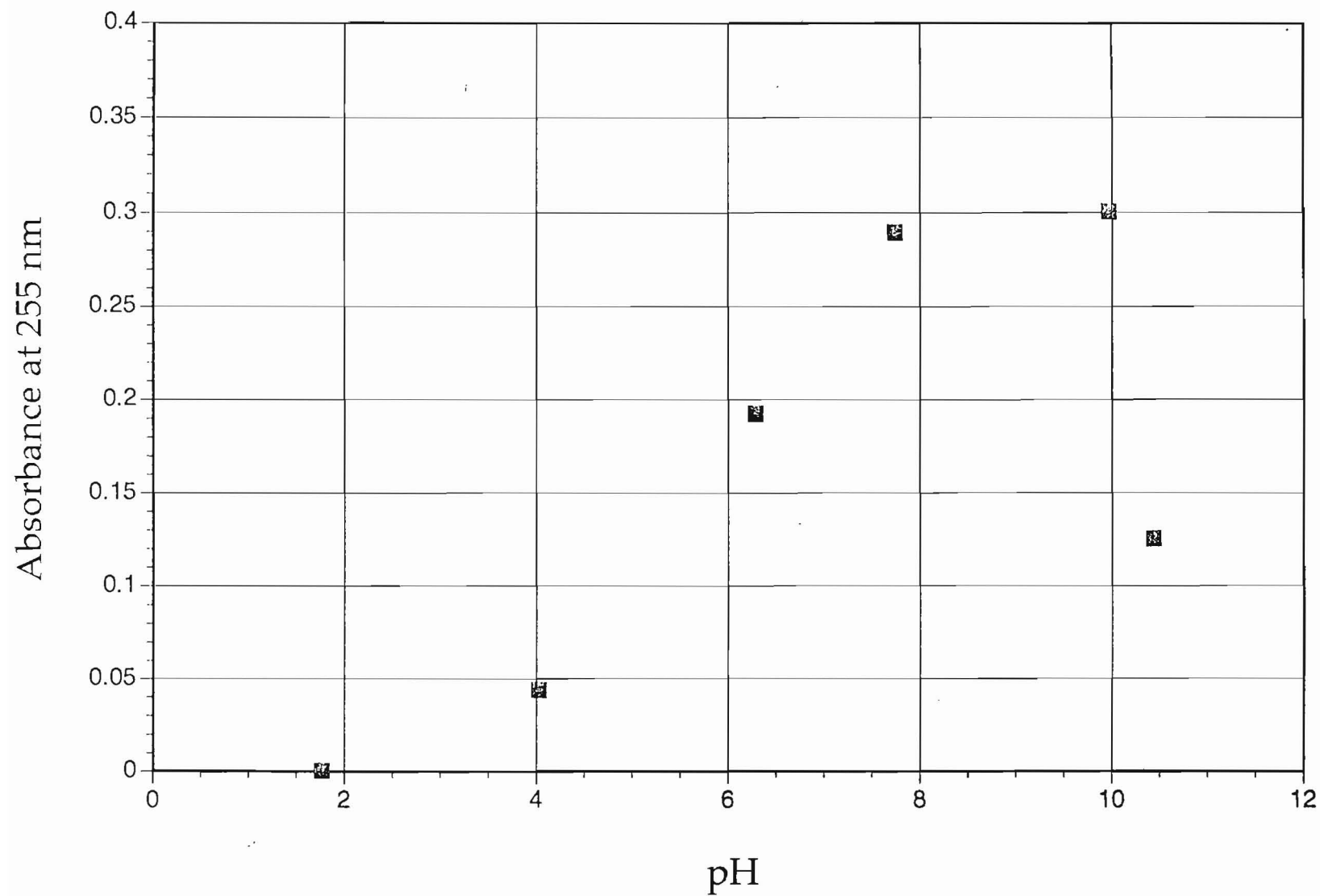
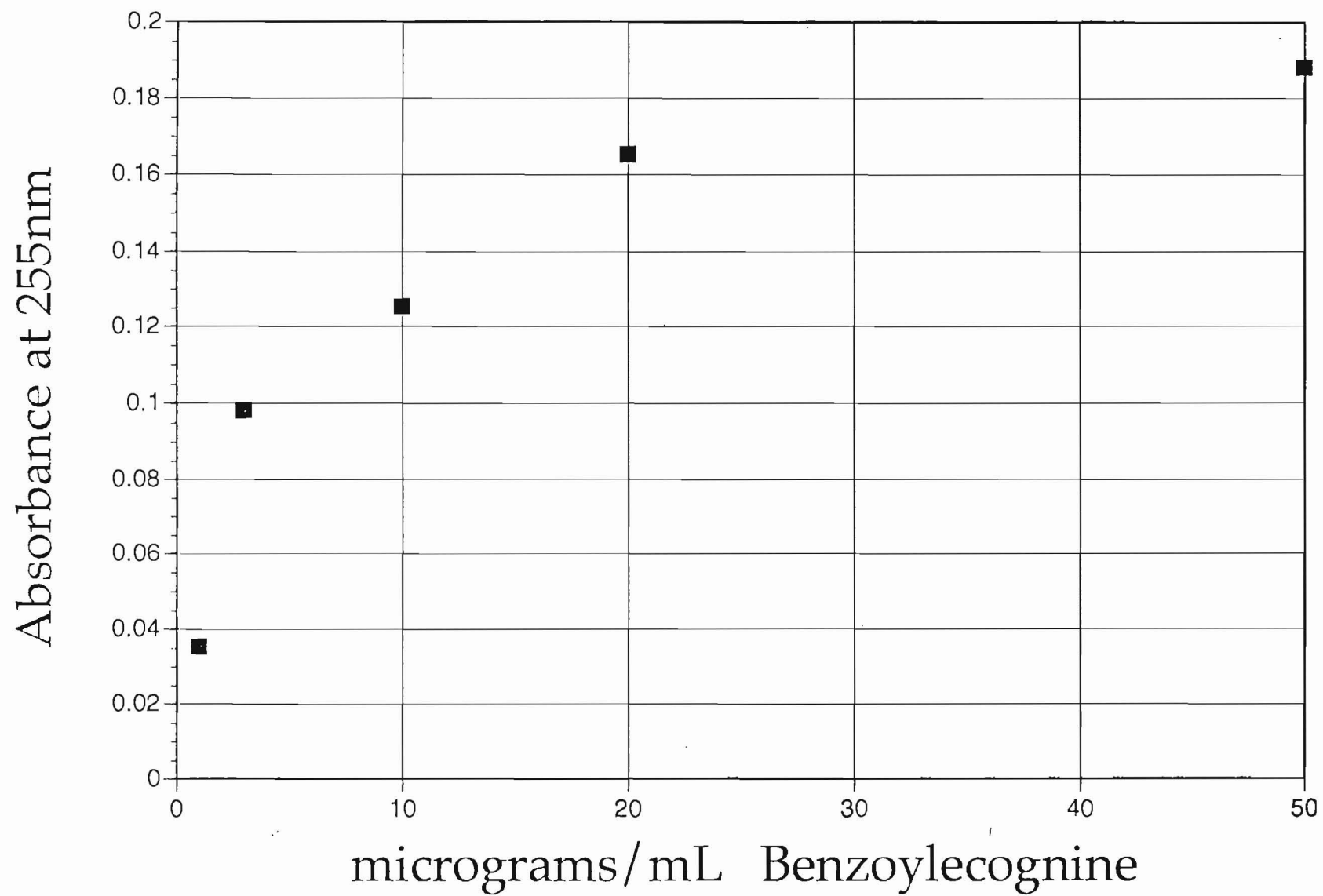


Figure 5:

**Absorbance of the ion-pair at 255 nm versus
concentration of BE**

Chart 2



References

- (1) Dunlap, C., "A Simple and Rapid Method for the Determination of Nicotine in Urine by HPLC," unpublished studies, Illinois Wesleyan University, Bloomington, IL, 1990.
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- (14) Fieser, L., Fieser, M., *Reagents for Organic Synthesis*, John Wiley & Sons, New York, 1967.

Appendix A

UV-VIS Absorption Spectra with Varying pH

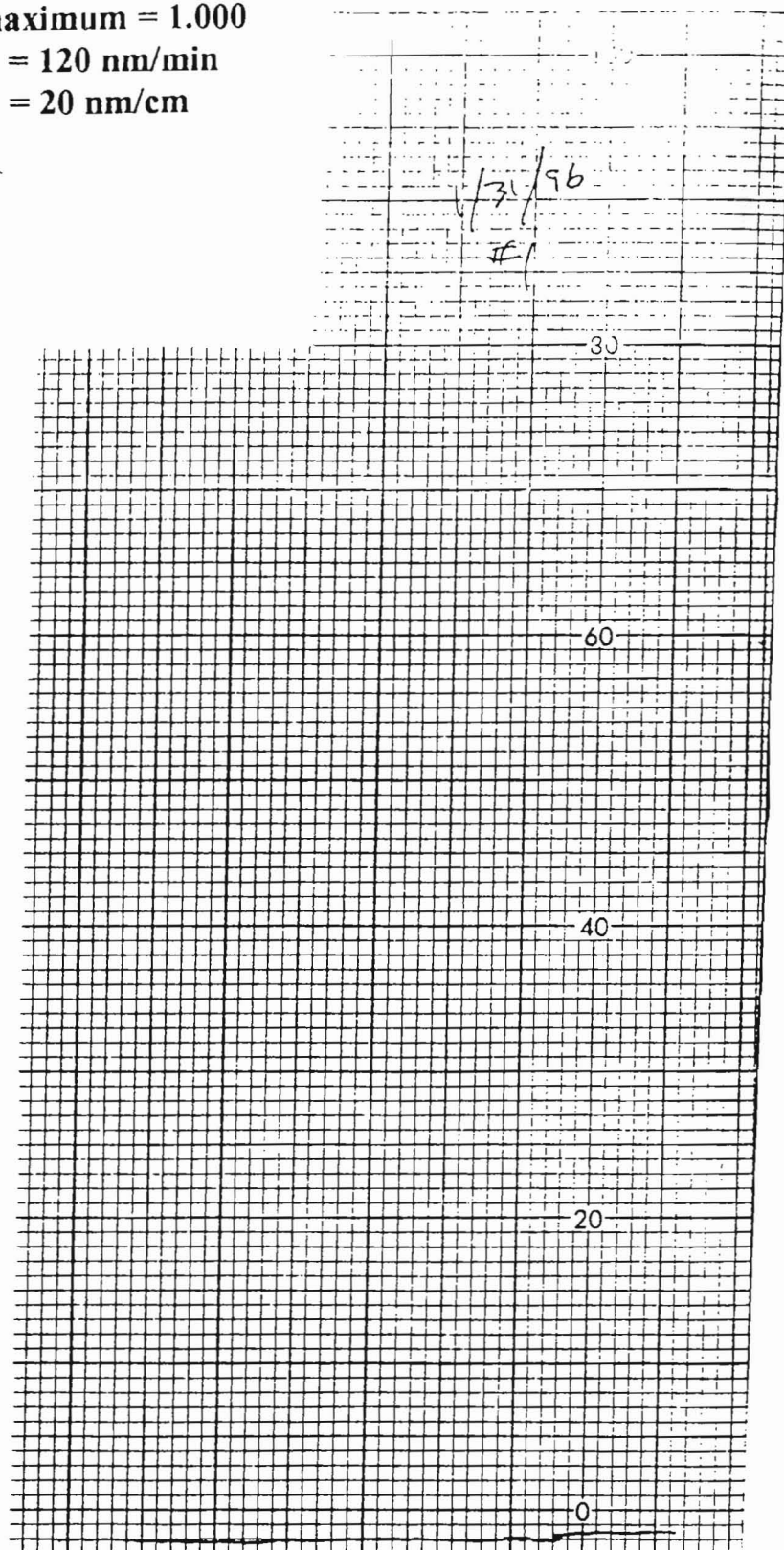
Spectrum 1.

pH = 1.78

ordinate maximum = 1.000

scan speed = 120 nm/min

chart scale = 20 nm/cm



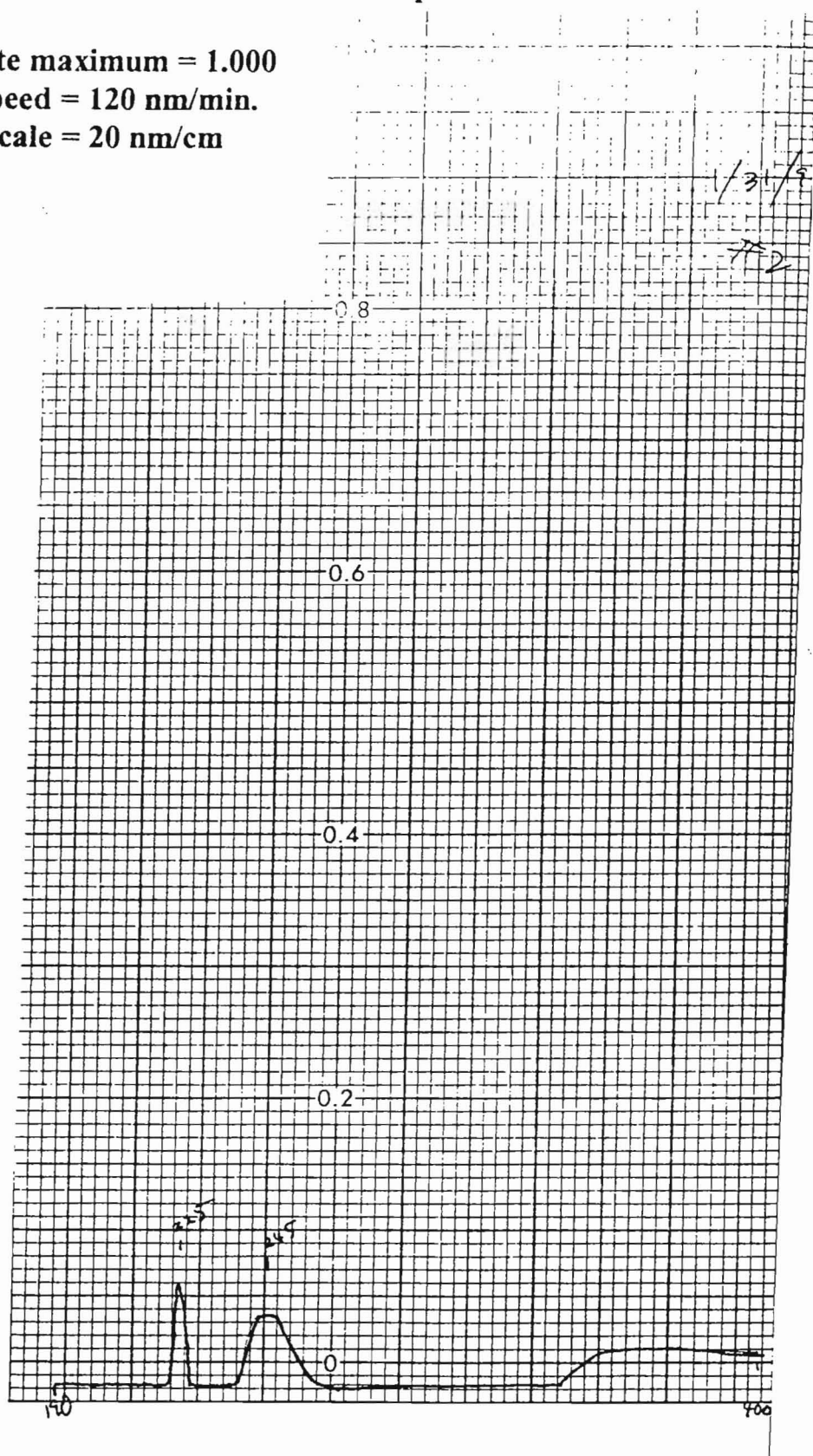
Spectrum 2.

pH = 4.04

ordinate maximum = 1.000

scan speed = 120 nm/min.

chart scale = 20 nm/cm



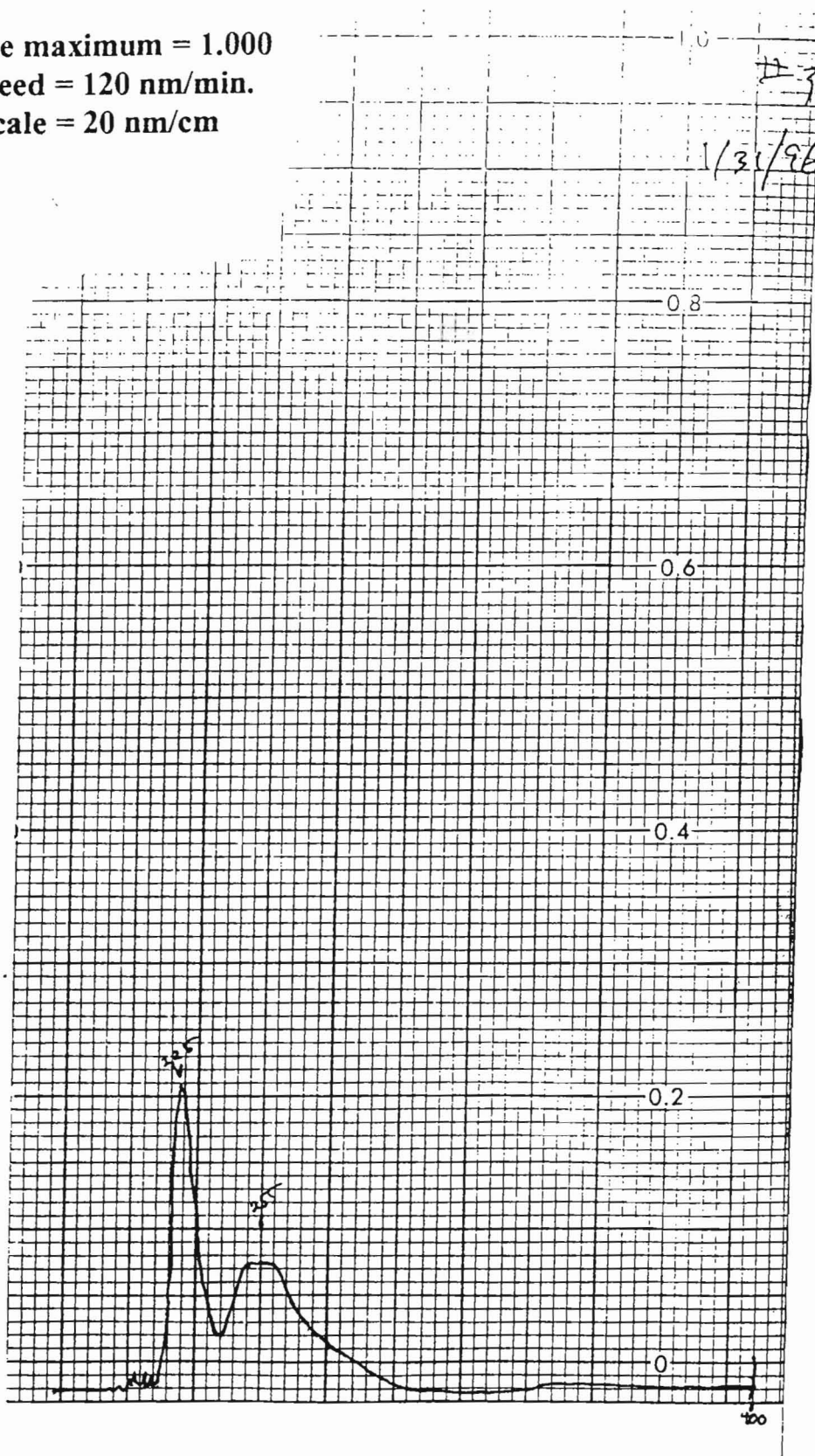
Spectrum 3.

pH = 6.30

ordinate maximum = 1.000

scan speed = 120 nm/min.

chart scale = 20 nm/cm



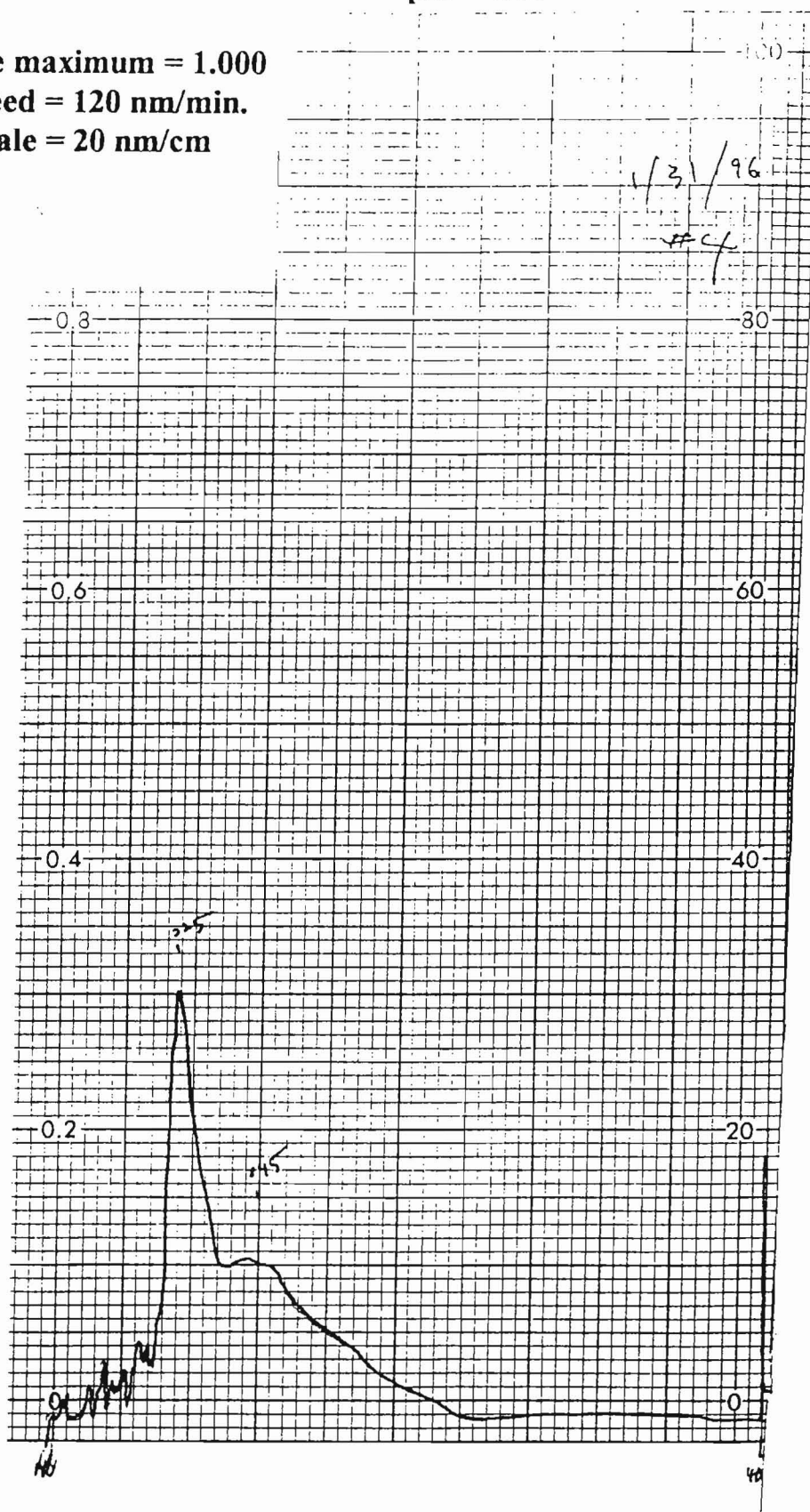
Spectrum 4.

pH = 7.75

ordinate maximum = 1.000

scan speed = 120 nm/min.

chart scale = 20 nm/cm



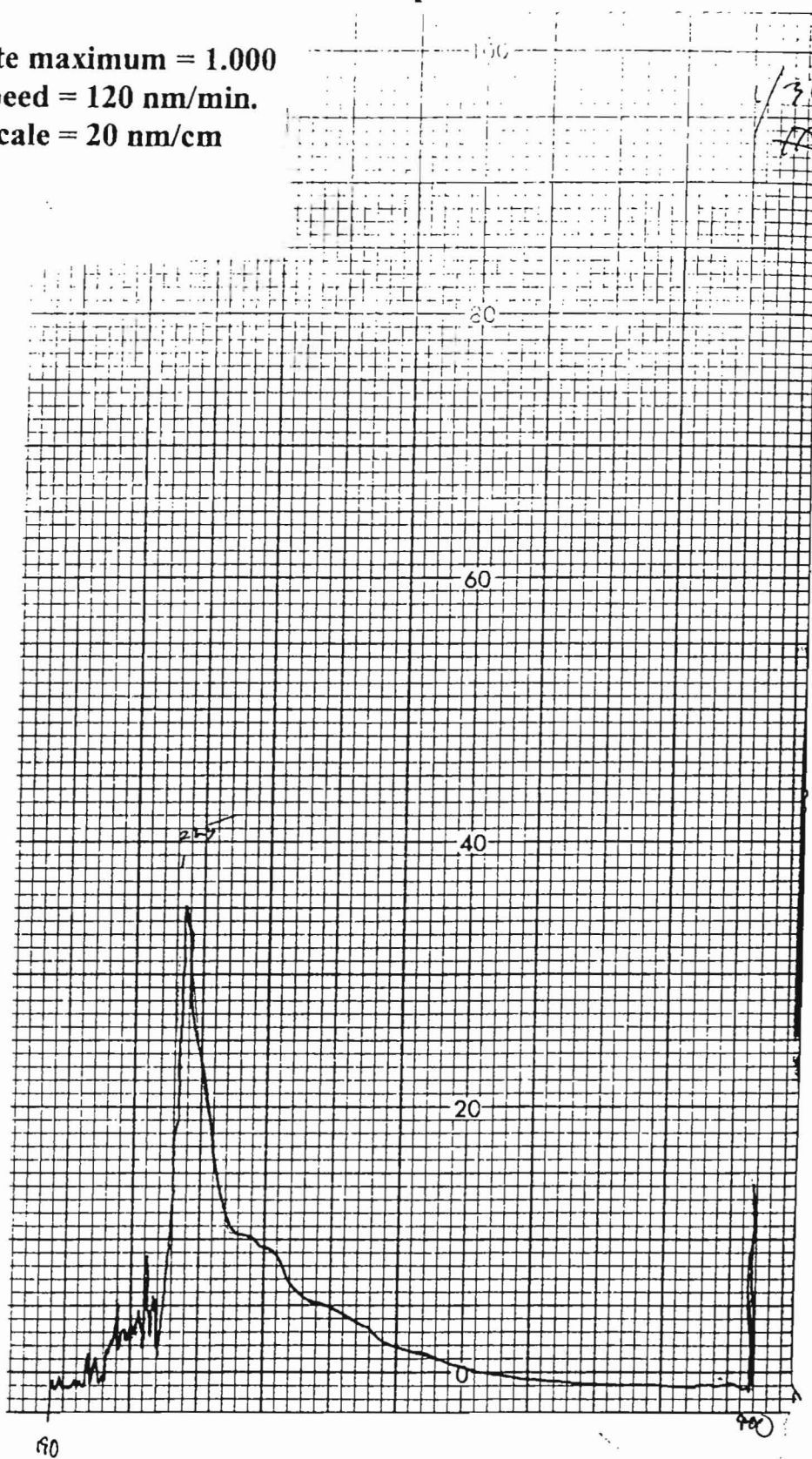
Spectrum 5.

pH = 9.98

ordinate maximum = 1.000

scan speed = 120 nm/min.

chart scale = 20 nm/cm



Spectrum 6.

pH = 10.44

ordinate maximum = 0.500

scan speed = 120 nm/min.

chart scale = 20 nm/cm



Appendix B

UV-VIS Absorption Spectra with Varying Concentration of BE

Spectrum 1.

1 $\mu\text{g/mL}$ BE

ordinate maximum = 0.500

scan speed = 120 nm/min.

chart scale = 20 nm/cm



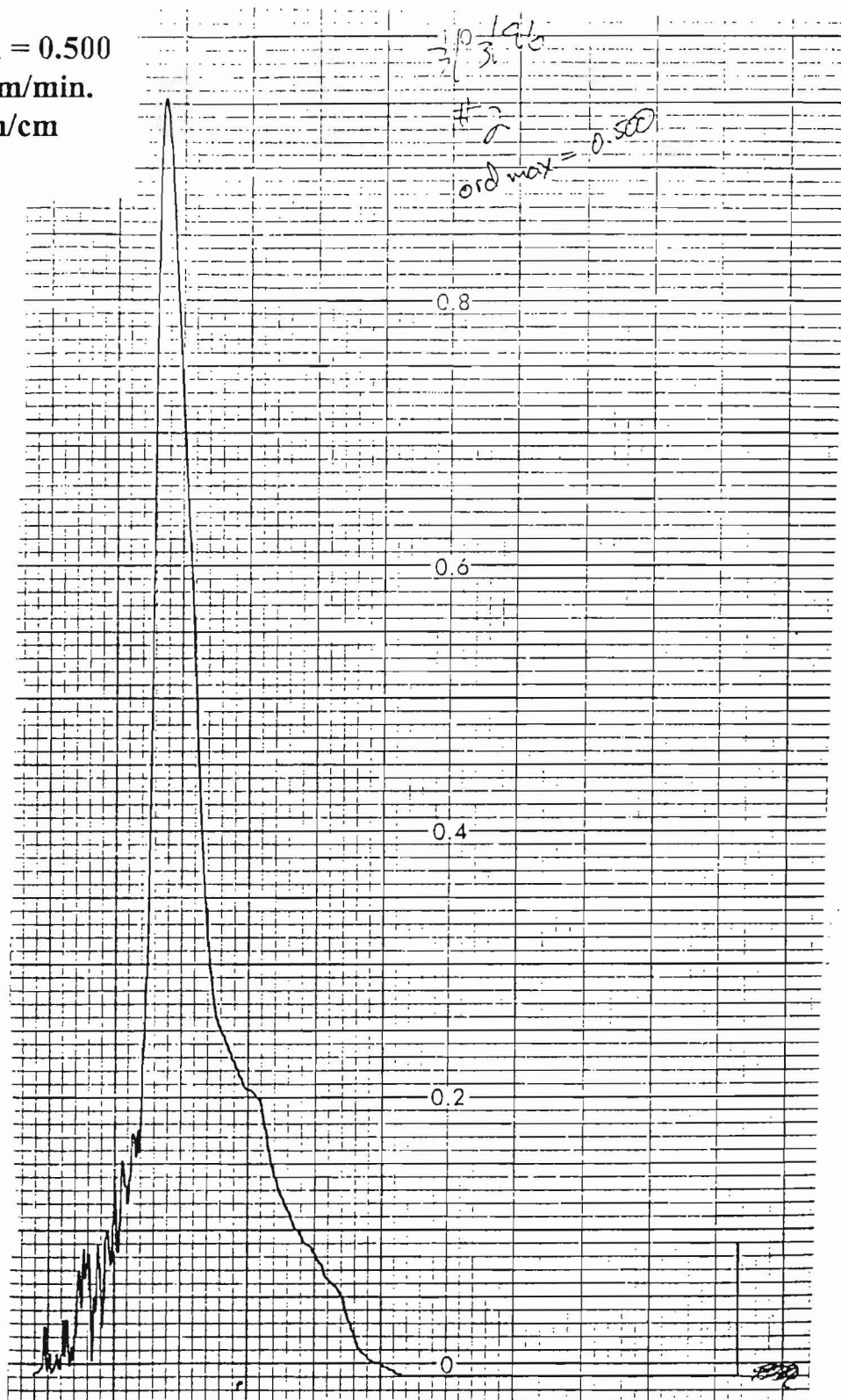
Spectra 2.

3 $\mu\text{g/mL}$

ordinate maximum = 0.500

scan speed = 120 nm/min.

chart scale = 20 nm/cm



Spectrum 3

10 $\mu\text{g/mL}$

ordinate maximum = 0.500

scan speed = 120 nm/min.

chart scale = 20 nm/cm



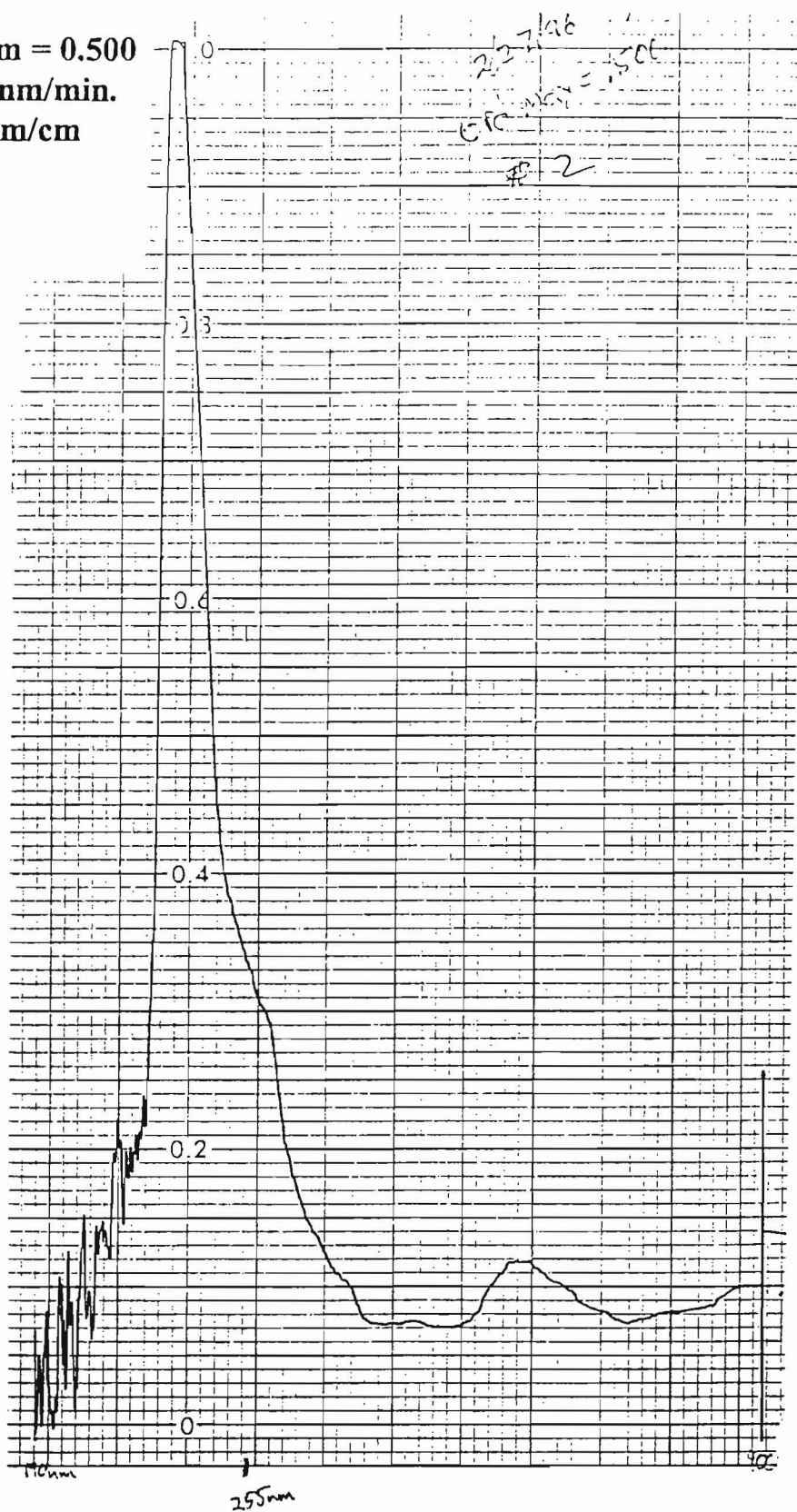
Spectrum 4

20 $\mu\text{g/mL}$

ordinate maximum = 0.500

scan speed = 120 nm/min.

chart scale = 20 nm/cm



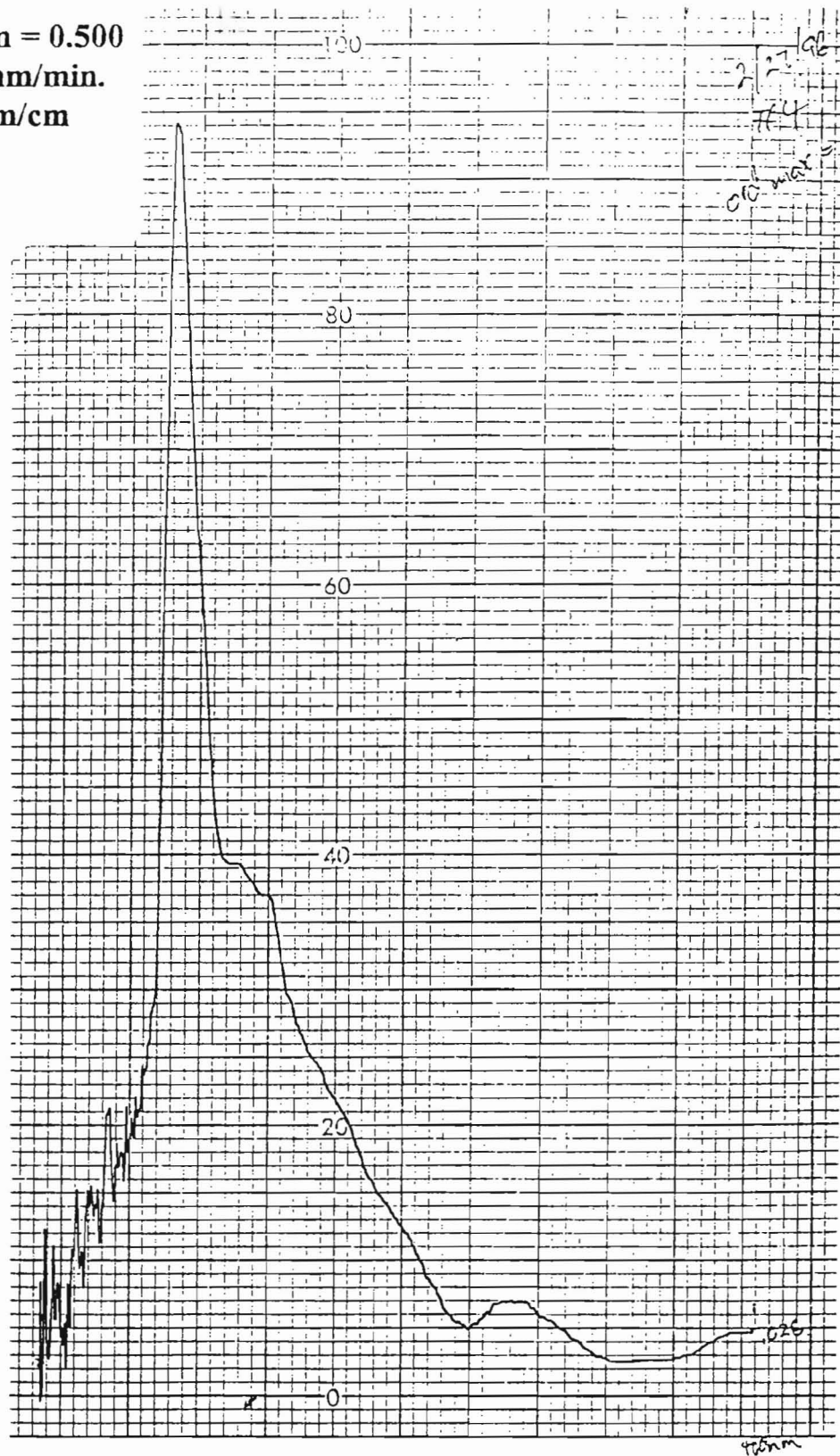
Spectrum 5

50 $\mu\text{g/mL}$

ordinate maximum = 0.500

scan speed = 120 nm/min.

chart scale = 20 nm/cm



Spectra 1a & 1b

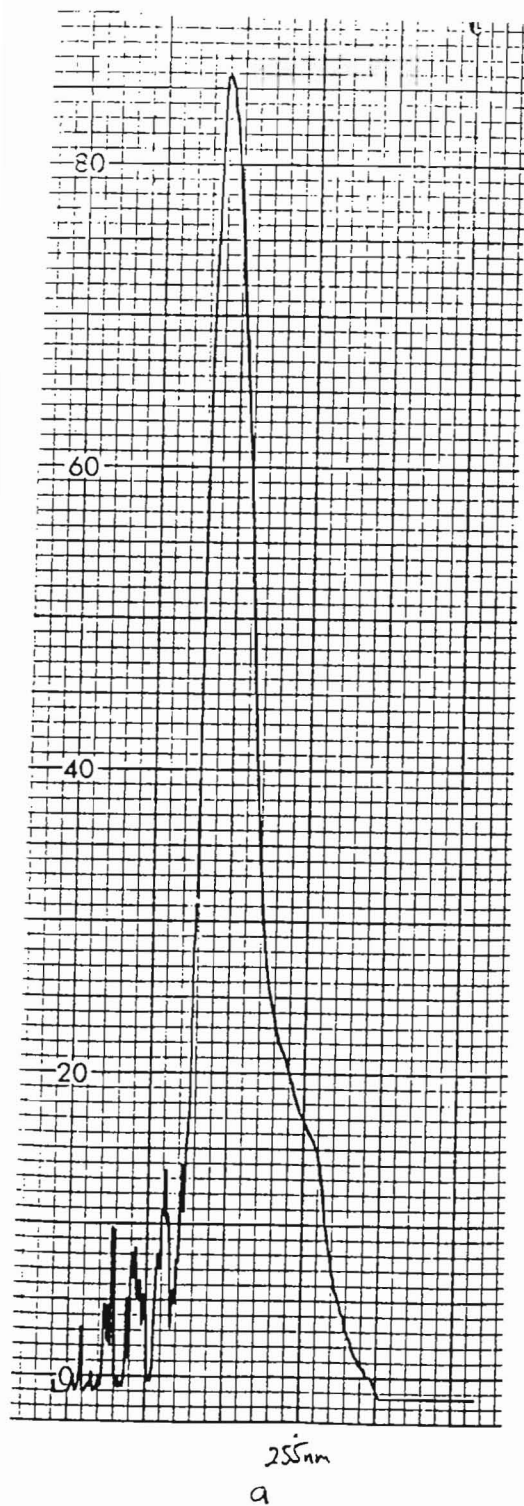
ordinate maximum = 0.500

scan speed a = 120 nm/min.

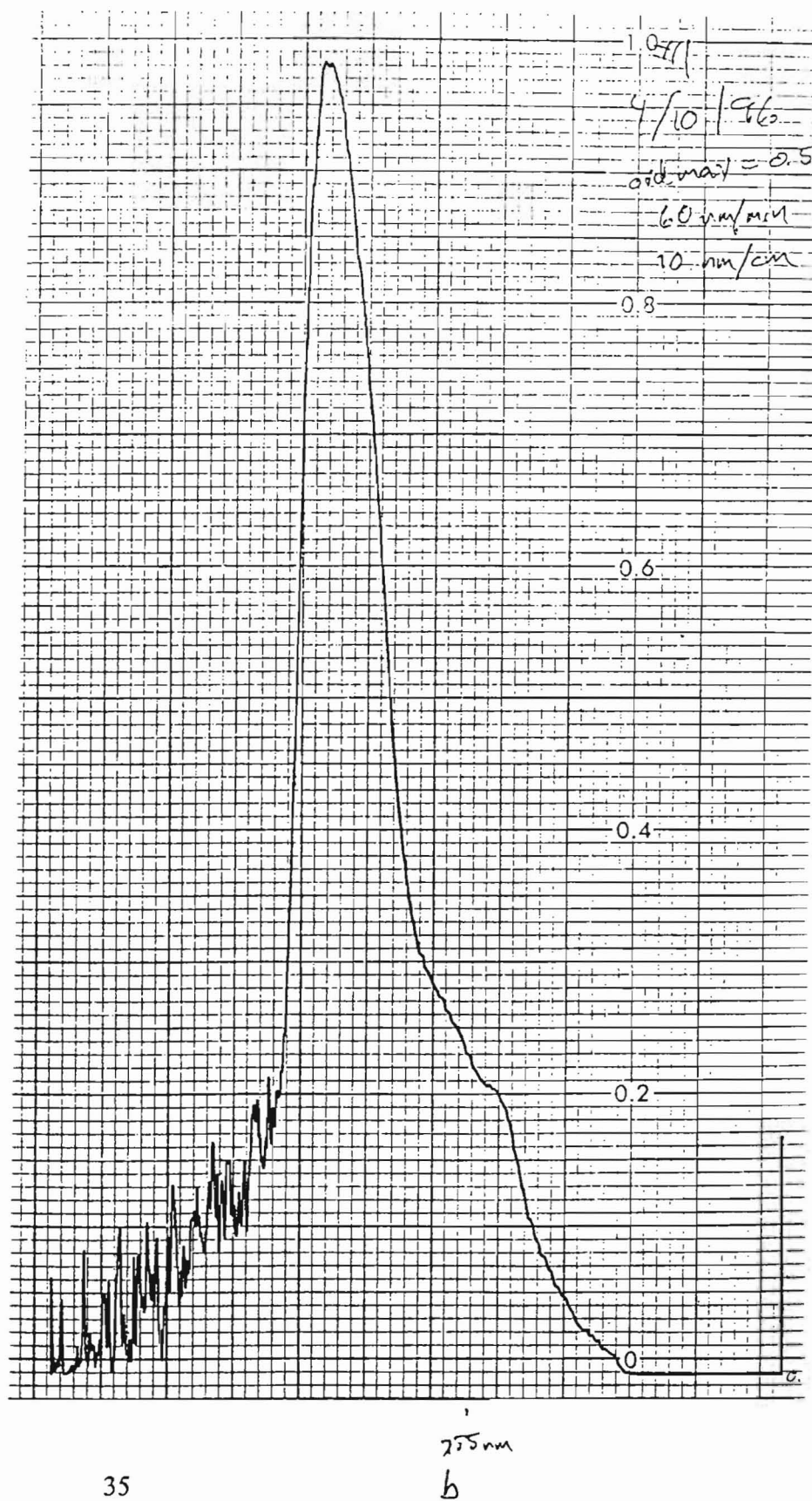
b = 60 nm/min.

chart scale a = 20 nm/cm

b = 10 nm/cm



1 $\mu\text{g/mL}$



Spectra 2a & 2b

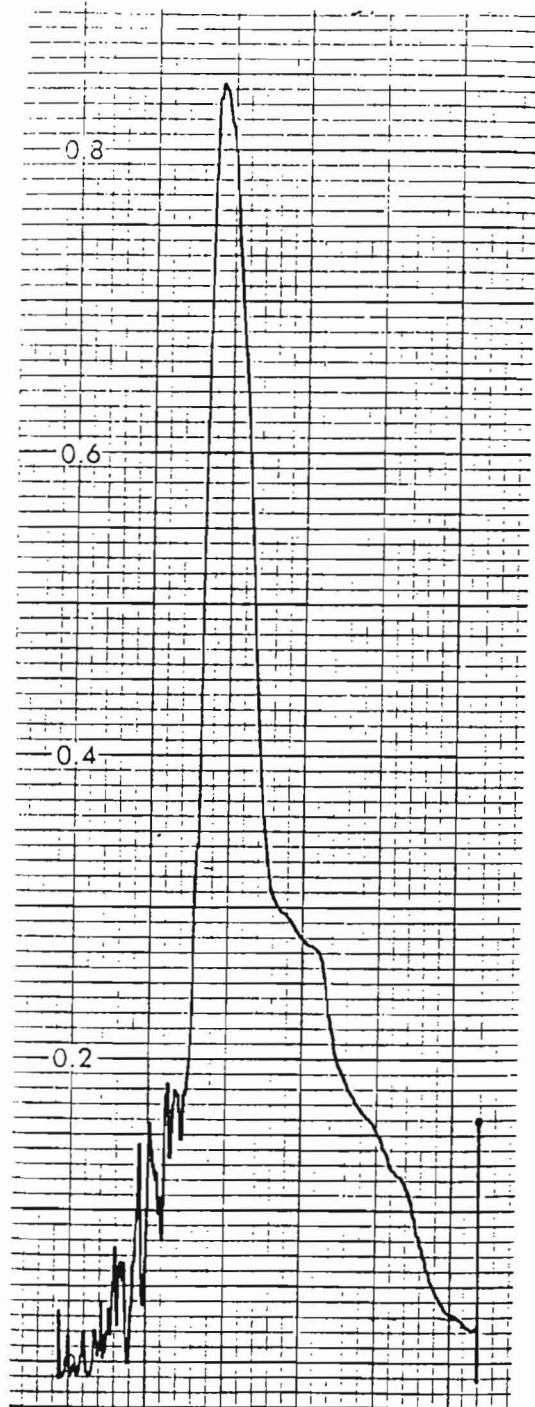
ordinate maximum = 0.500

scan speed a = 120 nm/min.

b = 60 nm/min.

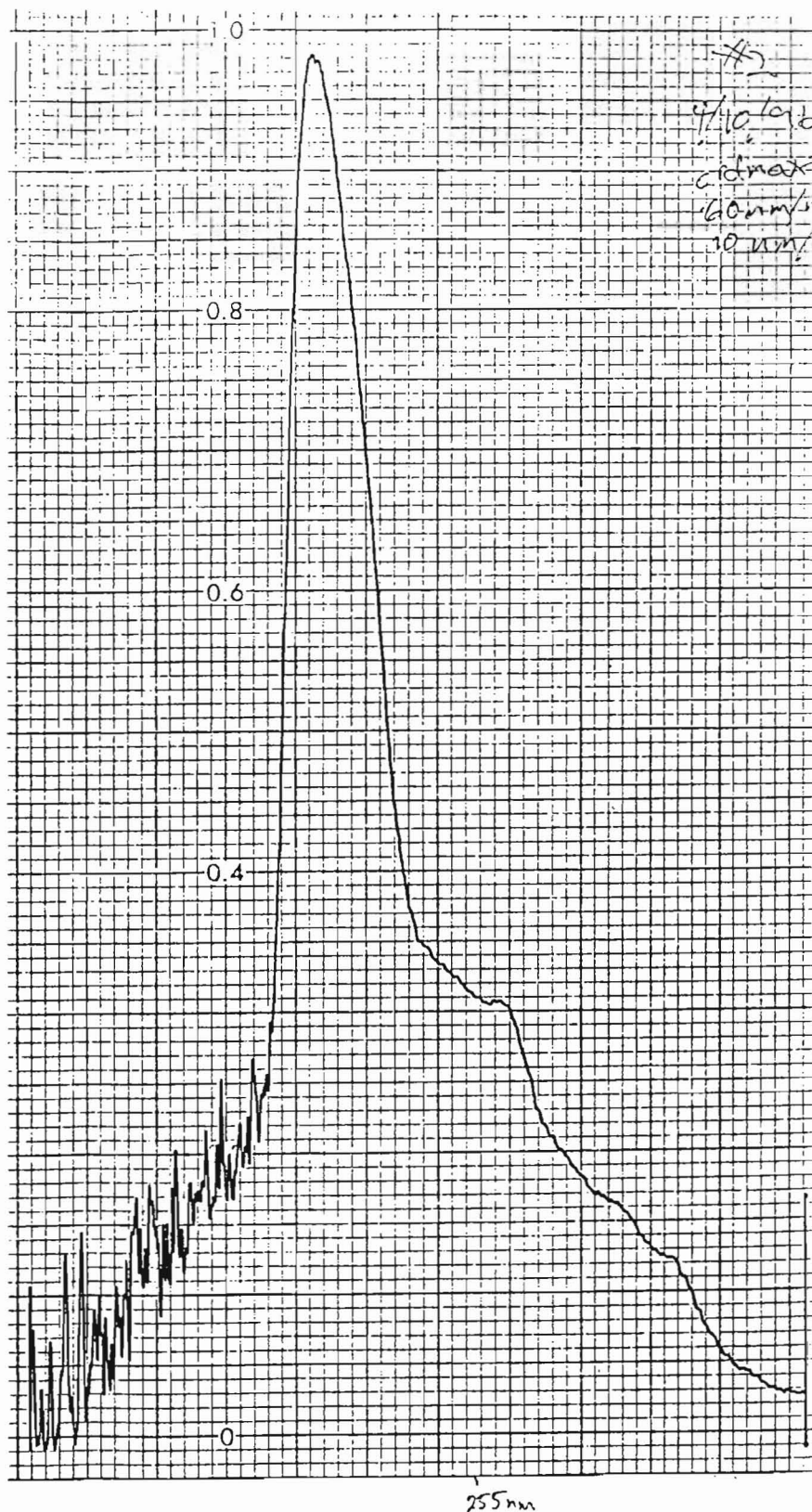
chart scale a = 20 nm/cm

b = 10 nm/cm



a

2 $\mu\text{g/mL}$



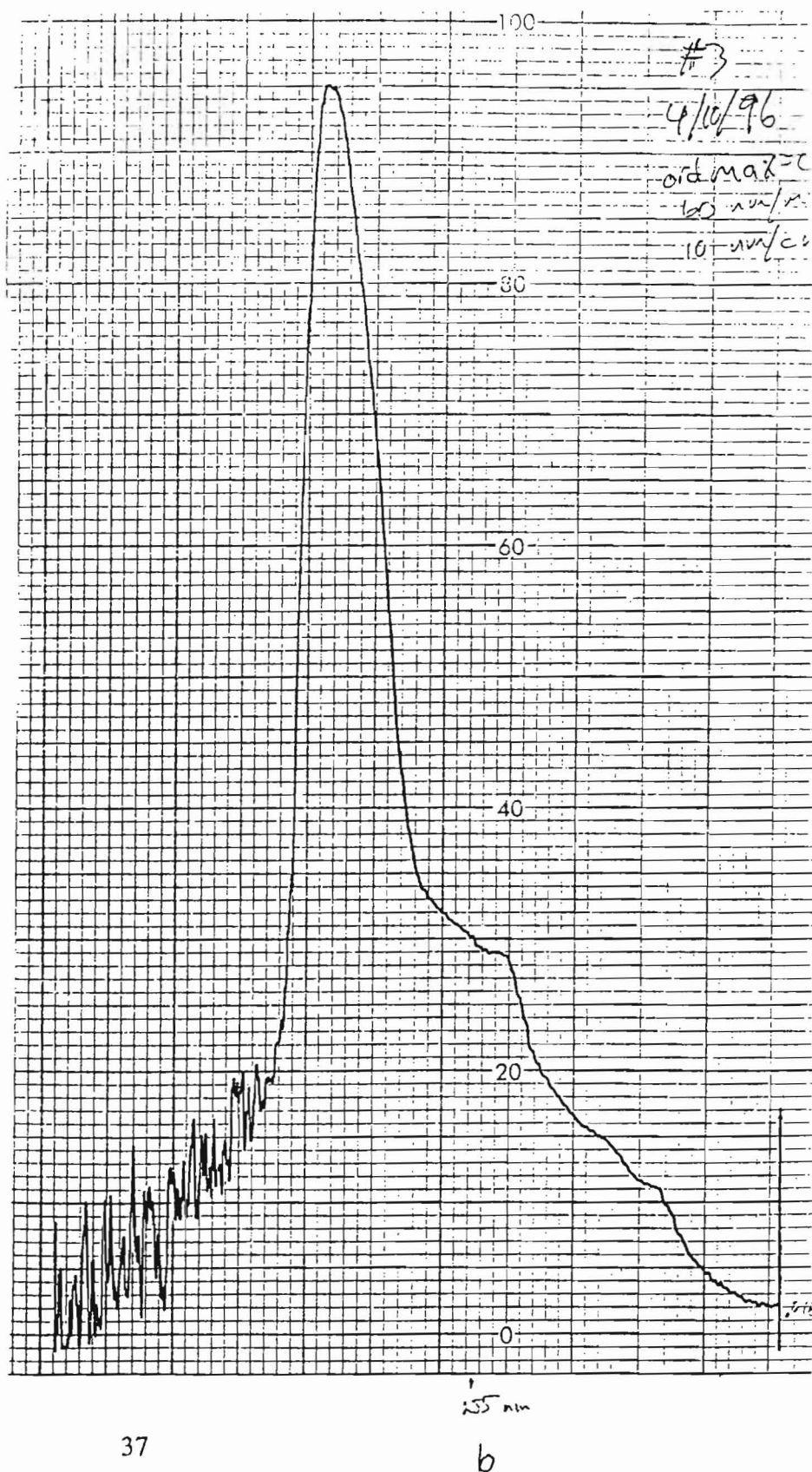
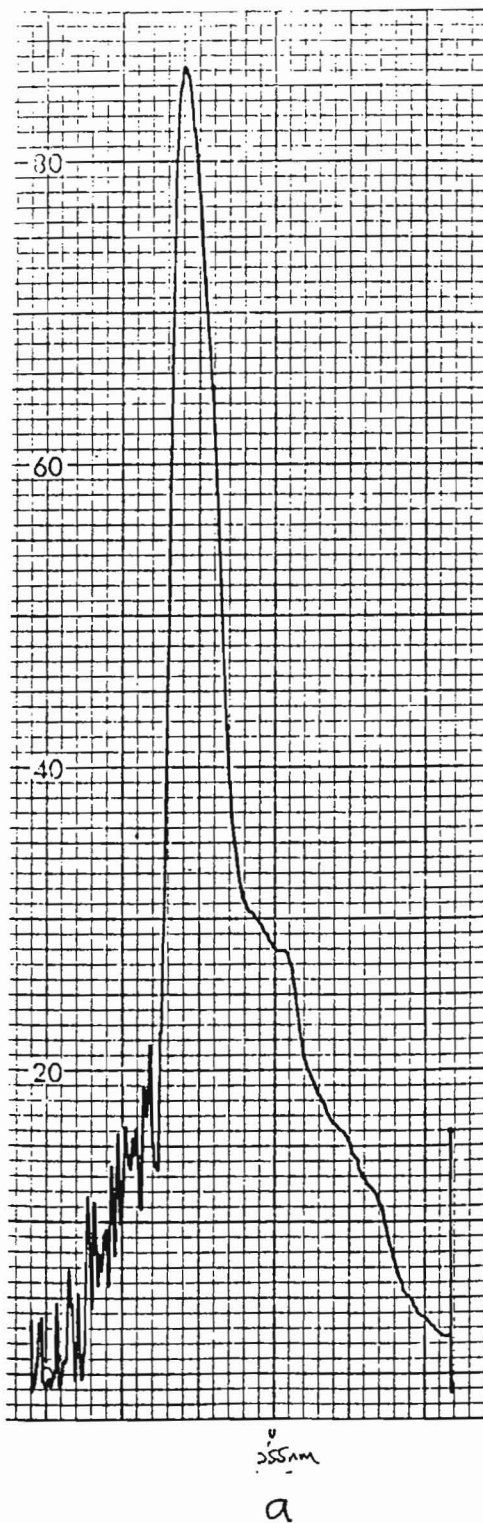
255 nm

b

Spectra 3a & 3b

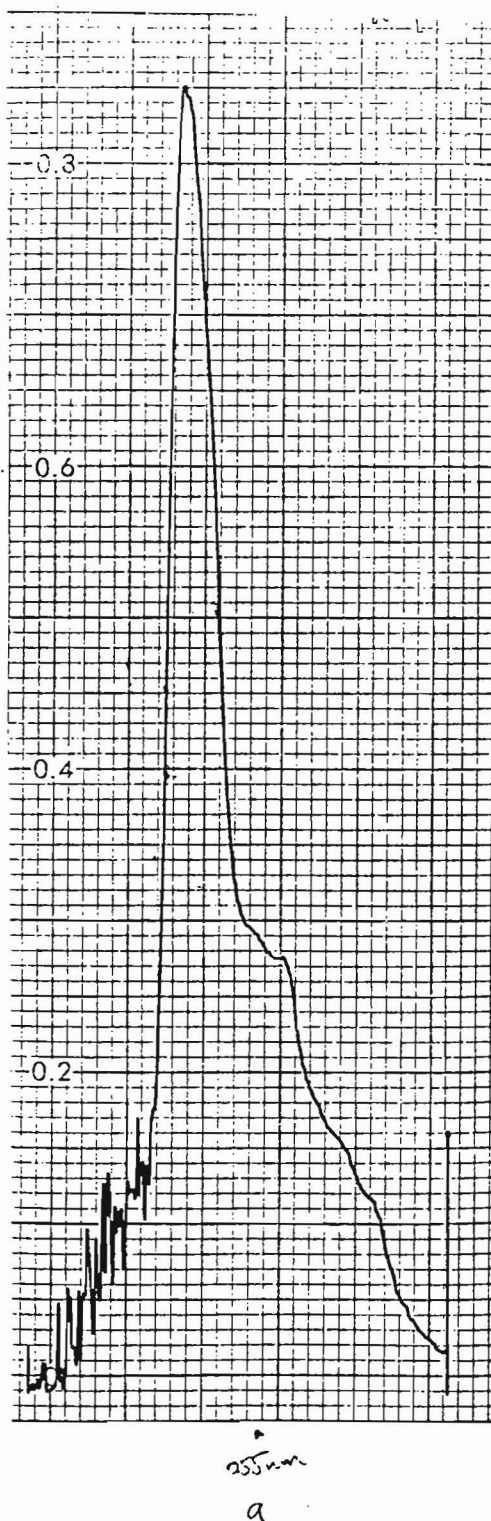
3 $\mu\text{g/mL}$

ordinate maximum = 0.500
 scan speed a = 120 nm/min.
 b = 60 nm/min.
 chart scale a = 20 nm/cm
 b = 10 nm/cm

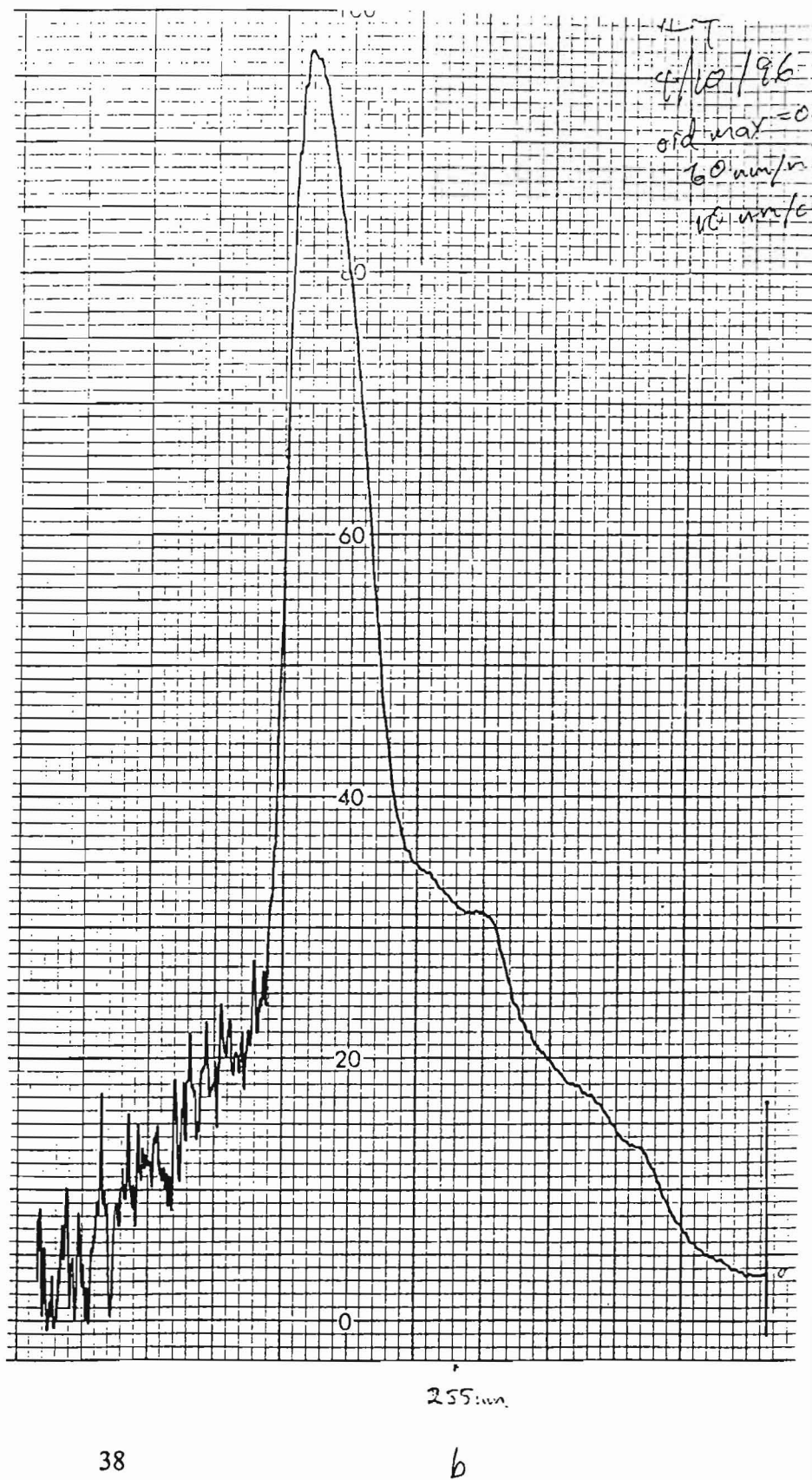


Spectra 4a & 4b

ordinate maximum = 0.500
 scan speed a = 120 nm/min.
 b = 60 nm/min.
 chart scale a = 20 nm/cm
 b = 10 nm/cm



4 $\mu\text{g/mL}$



Spectra 5a & 5b

5 $\mu\text{g/mL}$

ordinate maximum = 0.500

scan speed a = 120 nm/min.

b = 60 nm/min.

chart scale a = 20 nm/cm

b = 10 nm/cm

