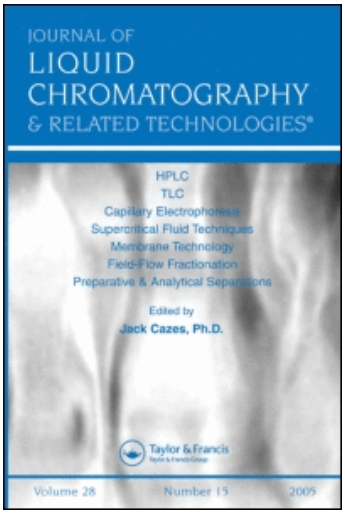

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Solvent Selection for Countercurrent Chromatography by Rapid Estimation of Partition Coefficients and Application to Polar Conjugates of p-Nitrophenol

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SOLVENT SELECTION FOR COUNTERCURRENT CHROMATOGRAPHY BY RAPID ESTIMATION OF PARTITION COEFFICIENTS AND APPLICATION TO POLAR CONJUGATES OF p-NITROPHENOL

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ABSTRACT

A rapid and moderately precise technique to measure partition coefficients of UV-absorbing solutes in solvent systems for countercurrent chromatography is described and applied to p-nitrophenol and its conjugates with glucose, sulfuric acid and glucuronic acid. It involves equilibration of one ml of each phase with solute in a narrow test tube, removal of the entire lower phase, dilution with methanol and calculation of the partition coefficient as the ratio of the absorbance values of each dilution at any suitable wavelength. The polar conjugates of p-nitrophenol can be separated by countercurrent chromatography using ethyl acetate as mobile phase and aqueous KH_2PO_4 as stationary phase.

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INTRODUCTION (1,2)

Separation of organic compounds by solvent-solvent partition has been facilitated by the recent design of numerous instruments to carry out the process of countercurrent chromatography (3,4). One of the most versatile of these instruments, the horizontal flow-through coil planet centrifuge (5,6), in which the stationary phase is retained in a helical coil by centrifugal force, is employed in the present investigation. Countercurrent chromatography is complementary to other forms of chromatography and is particularly suited for preparative separations of polar compounds and compounds which are stable over a limited range of pH, characteristics found in many natural products. The use of PTFE tubing for the chromatographic column and the absence of any solid supporting matrix for the stationary phase precludes sample loss by adsorption or surface catalysis. These features also facilitate accurate prediction of chromatographic retention based simply on a knowledge of the partition coefficient in the solvent system employed.

The attribute of the centrifugal apparatus to use as the mobile phase either phase of virtually any immiscible liquid-liquid pair allows the chromatographer to exploit a much wider range of solvent polarity and selectivity than was heretofore possible. To exploit this opportunity and to optimize solvent selection for a particular separation requires extensive appraisal of the partitioning characteristics of analytes in the available phase systems.

The methodology reported here was developed to provide a rapid technique, capable of moderate precision, for the estimation of partition coefficients of UV-absorbing compounds in the interval of 0.1 to 10, which is the range desired to achieve separation within a reasonable time. In essence, the method consists of distributing about 200 μg of compound between equal

(1 - 2 ml) volumes of each phase, separating the layers, equally diluting each layer with 5 ml of methanol, measuring the absorbance of each layer using any wavelength which provides readings in a usable range (0 - 1A), not necessarily corresponding to an absorbance maximum, and calculating the partition coefficient as the ratio of the two absorbance readings. The unique features of the procedure are the narrow diameter of the culture tube employed for equilibration and the transfer of the lower layer, which is experimentally simpler and more precise than transfer of the upper layer.

The procedure is illustrated using p-nitrophenol (PNP) and its conjugates with glucose (PNP-GS), sulfuric acid (PNP-S) and glucuronic acid (PNP-GN) in several solvent systems with 1 M phosphate buffers as the aqueous phase.

EXPERIMENTAL

Reagents (2)

p-Nitrophenol (PNP), p-nitrophenyl-β-D-glucopyranoside (PNP-GS), potassium p-nitrophenyl sulfate (PNP-S) and p-nitrophenyl-β-D-glucuronide (PNP-GN) were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade. Buffers were prepared by mixing equimolar solutions of H_3PO_4, KH_2PO_4 and K_2HPO_4 to obtain the desired pH as measured using a pH meter.

Apparatus

Countercurrent chromatography was done with a horizontal flow-through coil planet centrifuge (5,6) using the planet gear drive at 400 rpm, β 0.25 and a column consisting of 5 m of 2.6 mm ID PTFE tubing wound (98 turns) on a 12.5 mm rod. Column volume was 25 ml. Solvent was delivered with a Milroyal model

HDB-1-30R pump (Laboratory Data Control, Riviera Beach, FL). Column effluent was monitored at 280 nm using either an LKB Uvicord S monitor (LKB Instruments, Inc., Rockville, MD) with a 1.8 mm cell and an LKB model 6520 recorder or a Glenco model 5480 UV monitor fitted with a 3 mm cell and a Houston-OmniScribe recorder.

Spectrophotometric measurements for partition coefficient determination were made using either a Beckman model DU or a Varian model 634 spectrophotometer with 1-cm cells.

Culture tubes were Kimble no. 45066-A, 13 x 100 mm with teflon-lined caps. Disposable Pasteur pipets should have tip diameters of approximately 1 mm and should be free of chips. A Universal Repipet® dispenser model 3010-A-U (Labindustries, Berkeley, CA) was used to dispense methanol for dilution.

Methods

Partition coefficients. Approximately 0.2 ml of a methanol solution containing approximately 1 mg/ml (7) of test compound was evaporated with an air stream in a 13 mm diam. culture tube. Exactly 1 ml (2 ml where indicated) of each of two mutually saturated solvents was added by pipet (8). The tube was closed with a teflon-lined cap and shaken gently for 10 min (9), then centrifuged briefly. A disposable Pasteur pipet attached to a 2-ml syringe-type filling device (10) was inserted with the tip touching the bottom of the culture tube and the lower layer was precisely removed and transferred to a second tube containing 5 ml of methanol or other appropriate diluent (11) previously added using a Repipet®. The pipet used for the transfer was then rinsed by inspiring and expelling a portion of the solution. The same volume of diluent was added to the upper phase. The absorbance of each solution was determined at any convenient wavelength, usually on the longer wavelength side of the absorbance maximum, chosen to provide a reading not greater than

1.0A for either solution. Minor corrections for solvent absorbance were made by subtracting absorbances of similarly diluted aliquots of mutually saturated blank solvents. The partition coefficient was calculated as the ratio of the net absorbance values.

Countercurrent chromatography. A solvent system was prepared by shaking 300 ml of ethyl acetate and 120 ml of 1 M KH_2PO_4 (pH 4.5) in a separatory funnel. After filling the column by pumping in the organic phase at a rate of 1.34 ml/min the apparatus was rotated at 400 rpm and exactly 15 ml of aqueous phase introduced by means of a loop injector. The stationary phase volume was calculated by subtracting the unretained portion of aqueous phase as measured by collecting the column effluent in a 25-ml graduated cylinder. The difference between the stationary phase volume, V_S, and the total column volume (25 ml) is the volume of mobile phase (V_m). A sample mixture was prepared by evaporating 0.5-ml aliquots of individual methanol solutions of the test compounds in a test tube and dissolving the residue in stationary aqueous phase to provide a solution containing PNP (0.25 mg), PNP-GS (0.5 mg), PNP-S (0.5 mg) and PNP-GN (0.5 mg) in 0.5 ml. An 0.5-ml aliquot was injected using a sample loop of 18 gauge (1.02 mm ID) PTFE tubing. Effluent was monitored at 280 nm with an LKB monitor using a 1.8 mm cell.

In studies on the effect of KH_2PO_4 concentration on the resolution of the glucoside and sulfuric acid ester, solvent systems were prepared by equilibrating 750 ml of ethyl acetate, presaturated with water, with 150 ml of 1 M, 0.75 M or 0.5 M KH_2PO_4. Instrument settings were the same as indicated heretofore for the mixture of four compounds. An 0.5-ml sample contained PNP (0.25 mg), PNP-GS (0.5 mg) and PNP-S (0.6 mg). Effluent was monitored at 280 nm with a Glenco monitor using a 3 mm cell.

RESULTS AND DISCUSSION

Efficacy of lower phase transfer

To test the efficacy of lower layer transfer, the partition coefficient of PNP was measured in two solvent systems in which partitioning into the upper layer was less than 0.02. In this case, virtually all of the PNP will be in the lower layer and any increase in the partition coefficient over the expected value will represent incomplete transfer of the lower layer. The expected value was determined in a separate experiment using larger phase volumes in which an aliquot was removed by pipet from each phase so as to eliminate the possibility of error by contamination of one phase with traces of the other.

With an aqueous lower phase, Table 1, contamination of upper phase by residual lower phase results in an absorbance reading for the upper phase no greater than 0.020 units, which represents a contamination of the upper phase with less than 2.1% of lower phase using either 1-ml or 2-ml phase volumes.

Transfer of a lower phase organic layer, Table 2, is even more precise, resulting in an excess absorbance reading of 0.002, which represents a contamination of upper phase by lower phase of well under 1%.

Reproducibility

The reproducibility of the method is demonstrated by the data in Table 3 summarizing replicate measurements of the partition coefficient of PNP between 0.2 M HOAc and EDC with relative standard deviations of 1.4% and 0.9% using 1-ml and 2-ml phase volumes respectively.

Application to polar conjugates of PNP

Partition coefficients determined for a variety of solvent systems for possible use in separation of polar conjugates of

TABLE 1

Efficacy of Lower Aqueous Phase Transfer

Sample: 100 µg p-nitrophenol
Upper Phase: heptane
Lower Phase: 50% methanol in water
Diluent and Analytical Wavelength: methanol, 312 nm

PHASE VOLUMES		ABSORBANCE OF DILUTED PHASES		$K_{U/L} = \frac{\text{UPPER-EXPECTED}}{\text{LOWER-BLANK}}$
		UPPER	LOWER	
1 ML		0.031	1.088	0.019
		0.036	1.089	0.024
		0.029	1.076	0.018
		0.034	1.145	0.021
		0.032	1.127	0.020
	EXPECTED	0.010	---	
	BLANK	0.003	0.005	
	MEAN ± SD			0.020 ± 0.002
2 ML		0.030	0.898	0.024
		0.022	0.874	0.015
		0.021	0.918	0.013
		0.023	0.863	0.016
		0.027	0.917	0.020
	EXPECTED	0.009	---	
	BLANK	0.005	0.007	
	MEAN ± SD			0.019 ± 0.004

TABLE 2

Efficacy of Lower Organic Phase Transfer

Sample: 100 μg p-nitrophenol
Upper Phase: 0.2 M HOAc
Lower Phase: 10% pentanol in ethylene dichloride
Diluent and Analytical Wavelength: methanol, 312 nm

PHASE VOLUMES		ABSORBANCE OF DILUTED PHASES		$K_{U/L} = \frac{\text{UPPER-EXPECTED}}{\text{LOWER-BLANK}}$
		UPPER	LOWER	
1 ML		0.017	1.162	-0.003
		0.017	1.108	-0.003
		0.018	1.114	-0.002
		0.022	1.122	0.002
		0.022	1.125	0.002
	EXPECTED	0.020	---	
	BLANK	0.000	0.004	
	MEAN ± SD			-0.002 ± 0.001
2 ML		0.014	1.001	-0.003
		0.015	0.987	-0.002
		0.018	1.010	0.001
		0.015	0.975	-0.002
		0.014	0.992	-0.003
	EXPECTED	0.017	---	
	BLANK	0.002	0.007	
	MEAN ± SD			-0.002 ± 0.001

TABLE 3

Reproducibility of K Determination

Sample: 100 μg p-nitrophenol
Upper Phase: 0.2 M HOAc
Lower Phase: ethylene dichloride
Diluent and Analytical Wavelength: methanol, 312 nm

$K_{UPPER/LOWER}$

	1 ML PHASES	2 ML PHASES
	0.222	0.217
	0.220	0.215
	0.226	0.214
	0.221	0.213
	0.219	0.219
MEAN ± SD	0.222 ± 0.003	0.216 ± 0.002

PNP are summarized in Table 4. Most represent single determinations using 1 ml of each phase. Solvents range in polarity from heptane to neat isopropanol. All provided 2-phase systems with 1 M phosphate buffers. Partition coefficients greater than 20 or less than 0.05 were not considered to be sufficiently accurate for intercomparison and, in any case, are beyond the desirable range for countercurrent chromatography.

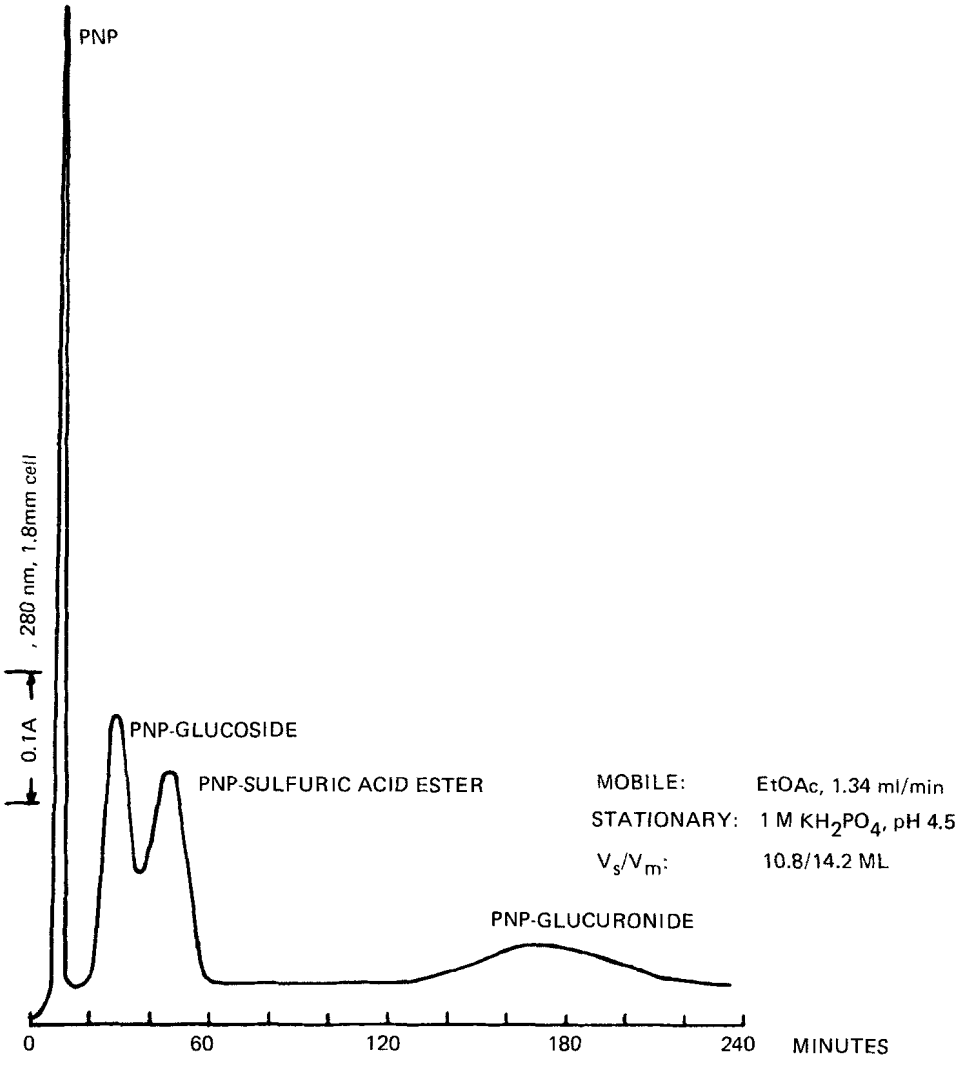
It is apparent that the four compounds could be separated using 1 M KH_2PO_4 as the stationary phase and either stepwise elution with a series of solvents or with a gradient of EtOAc or an alcohol in heptane. A separation using the isocratic system 1 M KH_2PO_4-EtOAc is shown in Fig. 1. Note that the partition coefficients, $K_{s/m}$, calculated from the chromatographic capacity factors, k′,

$$K_{s/m} = K_{A/O} = k' \frac{V_m}{V_s}$$

TABLE 4

Partition Coefficients for Polar Conjugates of p-Nitrophenol

AQUEOUS PHASE, 1 MOLAR PHOSPHATE	ORGANIC PHASE	$K_{AQUEOUS/ORGANIC}$			
		PNP	PNP-GS	PNP-S	PNP-GN
pH 4.00	EtOAc			4.4	4.5
	HEPTANE	>20			
	HEPTANE + 5% EtOAc	1.3			
	HEPTANE + 10% EtOAc	0.4			
	HEPTANE + 50% EtOAc	<0.05			
	HEPTANE + 95% EtOAc		5.6	5.9	
	HEPTANE + 20% n-BuOH		>20	>20	
	ETHER	<0.05	>20	>20	
	MTBE		20	10	
KH_2PO_4	MTBE + 10% i-PrOH		3	4.3	
	MTBE + 10% n-BuOH		3	7.7	
pH 4.5	EtOAc	<0.05	2.5	5.0	14
	EtOAc + 10% i-PrOH		1.0	2.0	6.2
	EtOAc + 15% i-PrOH	0.01	0.7	1.4	4.7
	EtOAc + 20% EtOH			0.8	4.6
	EtOAc + 20% n-BuOH			0.7	3.5
	EtOAc + 20% i-PrOH			0.6	3.5
pH 5.50	n-BuOH			0.2	2.7
	HEPTANE				
	HEPTANE + 5% EtOAc	1.9			
	HEPTANE + 10% EtOAc	0.6			
	EtOAc	<0.05	2.2	3.7	
	EtOAc + 10% i-PrOH		0.8	1.1	>20
pH 7.00	EtOAc + 15% i-PrOH		0.6	0.6	>20
	EtOAc + 25% i-PrOH		0.3	0.2	12
	EtOAc + 35% i-PrOH		0.22	0.1	3.2
	EtOAc + 50% i-PrOH		0.17	<0.05	0.9
	n-BuOH		0.4	0.1	3.8
	i-PrOH			<0.05	0.2



	PNP	PNP-GS	PNP-S	PNP-GN
k' (ccc)	0	1.7	3.2	15
k (ccc)	0	2.3	4.2	20

FIGURE 1. SEPARATION OF p-NITROPHENOL AND ITS CONJUGATES WITH THE COIL PLANET CENTRIFUGE; PLANET GEAR DRIVE, 400 RPM, β 0.23; COLUMN 5 m OF 2.6 mm ID PTFE, 98 TURNS ON 12.5 mm CORE, VOL. 25 ml; SAMPLE 0.5 ml.

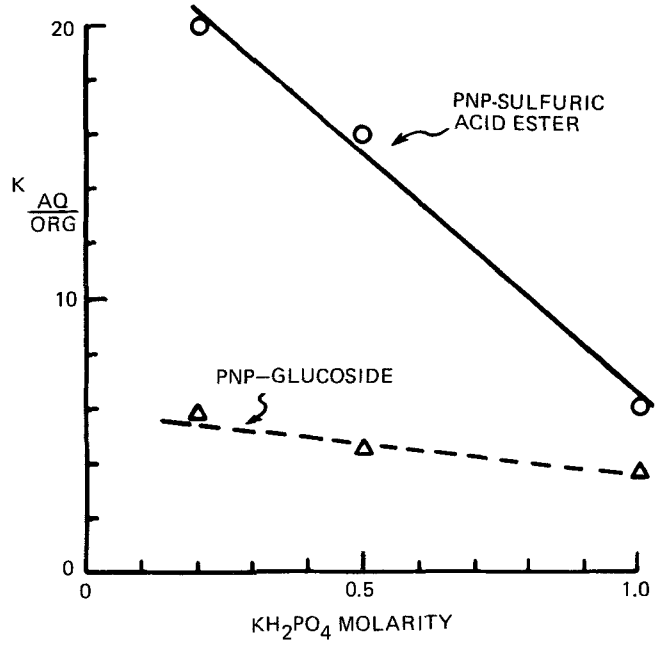


FIGURE 2. EFFECT OF BUFFER MOLARITY ON PARTITION COEFFICIENT WITH ETHYL ACETATE.

are in good agreement with those presented in Table 4. The glucoside and sulfuric acid ester are incompletely resolved.

Effect of buffer concentration

Measurement of the partition coefficients of PNP-GS and PNP-S between EtOAc and KH_2PO_4 solutions, Fig. 2, showed that $K_{A/O}$ for the ionized sulfuric acid ester is inversely related to the salt concentration, while $K_{A/O}$ for the neutral glucoside is relatively unchanged. As seen in Fig. 3, the compounds are completely resolved by countercurrent chromatography using a buffer concentration of 0.50 M.

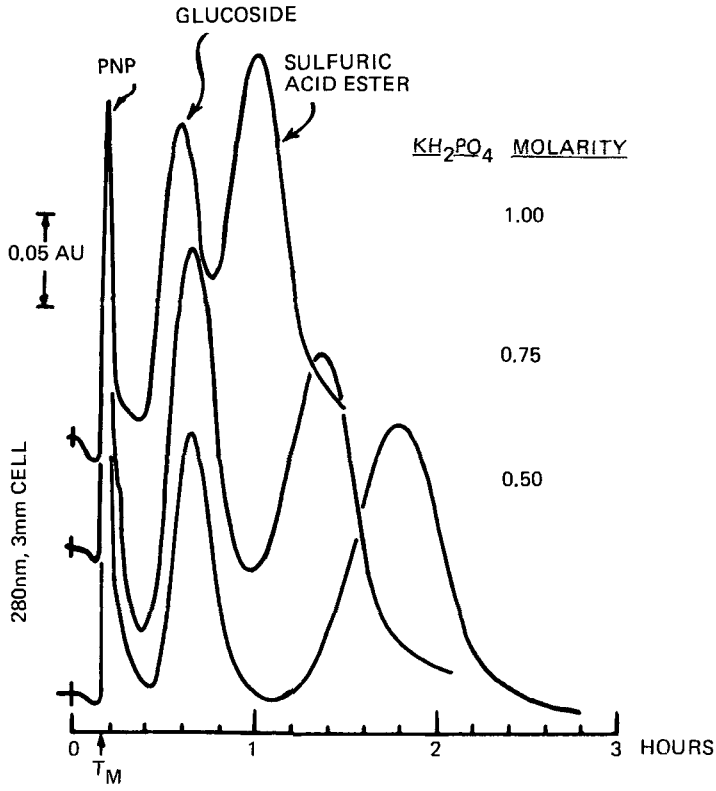


FIGURE 3. EFFECT OF KH_2PO_4 CONCENTRATION ON RESOLUTION OF PNP, PNP-GS AND PNP-S USING ETHYL ACETATE AS THE MOBILE PHASE. OTHER CONDITIONS IDENTICAL TO THOSE IN FIGURE 1.

Conclusion

The micromethod described for measuring partition coefficients is rapid, and sufficiently precise to be useful for screening solvent systems for use in countercurrent chromatography. A rapid HPLC procedure for analytical determination of PNP, PNP-S and PNP-GN in biological fluids has been reported (12). Though the countercurrent chromatographic separation reported here is slower, the capacity of the countercurrent chromatograph is greater, and the wide choice of solvents per-

mits greater flexibility in choosing conditions for separation. These features are often advantageous in isolation or preparative purification of polar substances such as drug conjugates as well as other biochemicals or natural products.

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2. Abbreviations used: PNP, p-nitrophenol; PNP-GS, p-nitrophenyl-β-D-glucopyranoside; PNP-S, potassium p-nitrophenyl sulfate; PNP-GN, p-nitrophenyl-β-D-glucuronide; PTFE, polytetrafluoroethylene; HOAc, acetic acid; EDC, 1,2-dichloroethane; EtOAc, ethyl acetate; MTBE, methyl t-butyl ether; i-PrOH, isopropanol; n-BuOH, n-butanol; $K_{U/L}$, $K_{S/m}$, $K_{A/O}$; partition coefficient expressed as concentrations in upper/lower, stationary/mobile and aqueous/organic phases respectively.

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7. A concentration of 1 mg/ml is convenient and generally appropriate, but with compounds with very high absorptivity

such as p-nitrophenol a lower concentration or smaller aliquot may be used to avoid the need to employ an analytical wavelength far removed from the absorption maximum.

8. It is convenient to use either a 1-ml tuberculin syringe fitted with a blunt needle or a positive displacement plunger pipet.

9. Vigorous hand shaking leads to emulsion formation. A wrist-action shaker or simply repeated inversion is preferable.

10. This is conveniently made from a 2-ml glass syringe having the barrel lightly coated with petroleum jelly and with cut-off 1-ml latex dropper bulb forced over the delivery end to attach the Pasteur pipet.

11. While methanol is a generally useful diluent, it precipitates salts. Although these are separated by centrifugation, an indeterminate error, not serious for screening purposes, may arise because of a volume change affecting the aqueous phase. Aqueous methanol (50%) largely avoids salt precipitation and is miscible with most organic solvents. In instances where spectral shifts may result from pH differences in dilutions of upper and lower layers it is necessary to incorporate a suitable acid, base or buffer into the diluent.

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