Factors Affecting the Reliability and Precision of the Spectroscopic Determination of Vitamin A*

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Summary

BY means of simple statistical methods the effects of technique, laboratory equipment, solvents, temperature, and instruments on the precision and accuracy of the determination of the extinction coefficient at 328 mμ of fish liver oils are outlined.

Methyl, ethyl, propyl and butyl alcohols give successively lower E-values.

As the temperature increases, Beckman spectrophotometers tend to give lower values.

The cells used to hold the solutions to be measured need to be individually checked.

No suitable standards for spectrophotometers are yet available, but potassium nitrate, potassium chromate, and Bureau of Standard glasses are useful.

By careful observance of all the variations discussed the determination of the E-value may be made with considerable precision; the value of σ, the standard deviation, is 0.22%.

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The determination of vitamin A can be done by biological or physicochemical assay. By definition, the biological assay is presumably the more accurate since vitamin A activity is measured in biological units, but the extreme lack of precision, the cost, and the time involved have relegated it to reference use, and most business connected with vitamin A is contracted using physicochemical methods.

Most commercial firms and many government agencies such as the War Production Board and the War Food Administration accept the value of E(1%,1cm.) (328 mμ) as proportional to the vitamin A content when dealing with fish liver oils and concentrates for manufacturing purposes. The accepted "conversion factor" is 2000 so the assay depends only on the measurement of the ultraviolet absorption of a solution of the oil to be tested.

If the results obtained from an analysis are to be understood and used intelligently, it is necessary to know the precision and accuracy of the method. The terms accuracy and precision are used by various groups in different ways. At times the words are treated as synonyms; in fact, some dictionaries list them as such. Biological chemists tend to use the term accuracy where statisticians would use precision. For the purpose of this paper, accuracy is a function of the absolute or correct value whereas precision is a measure of reproducibility.

Moran (11) has described a method whereby with the use of statistics the precision of any analytical method can be measured. His use of the "C_2" factors to adjust the 10 and 25 individual observations to infinite numbers was not correct because the tables he used were designed for averages and not individual values. However, this does not detract from the value of his paper as a stimulus to analysts in pointing out the very valuable tool which modern statistical methods become in improving analytical methods and techniques. Mandel (10) describes statistical techniques and their application to problems in chemistry and suggests the use of "variance" (standard deviation squared) rather than standard deviation for "evaluation of a precision from a given series of replicate determinations."

In this study the standard deviation (σ) and "3σ control limits" were the tools used, and from the data a control chart system was instituted which has proved valuable in keeping analytical errors at a minimum.

Checking the Precision of the Method

In order to attain the highest practical precision and accuracy the procedure was broken down into its elements and each studied separately. Many replicates of the same step were made and the 3σ limits, or "limits within which any single determination may be expected to lay 997 times out of 1000" (11), were determined. If these were too wide, indicating poor precision, changes were made until the control limits were brought to acceptable values.

A check was made first on the spectrophotometers at hand, a Hilger Medium Quartz Spectrograph and two Beckman Model DU Spectrophotometers. The range in which replicate readings gave the best reproducibility was found to lie between 0.9 and 1.25 optical density units for the former and between 0.3 and 0.8 for the latter. The narrowest control limits (3σ) which could be obtained in the optimum range of the Hilger Quartz Spectrograph were ± 2.2%, whereas the Beckman instrument could be read with ± 0.22% limits. Thus the Beckman spectrophotometer was accepted as the more precise instrument.

The volumetric glassware was examined next. The flasks commonly used were Kimble 25 ml., 50 ml., and 100 ml. sizes. These were standardized gravimetrically and, surprisingly enough, none were found to have calibration lines visibly different from the found value. A statistical check on filling a 25-ml. flask to the mark showed that the 3σ limits were considerably less than ± .1%. Low actinic glassware, as described by Embree (5), is used where exposure to natural daylight occurs as the latter quickly destroys a fraction of the vitamin A and thus contributes to inaccuracy.

The pipetting technique was also examined and the 3σ control limits were obtained for standardized "Exax" pipettes of 1, 2, 5, 10, and 25 ml. size. These were found (gravimetrically) to be ± 0.8%, ± 0.4% ± 0.4%, ± 0.25%, and ± 0.215%, respectively. Pipettes of 1 ml., which had often been used formerly, were discontinued.

The balances used for weighing the samples before dilution were the "Precision" type of torsion balance manufactured by the Roller-Smith Company.[1] Sample weights of the order of 40 mg. were conveniently weighed by dipping a tared coil of stain-

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[1]Roller-Smith Company, Bethlehem, Pennsylvania.

less steel wire into the oil to be tested and weighing the adhering oil. The 3σ limit of this practice was found to be $\pm$ .27%. Long experience with four of these balances has shown that after considerable use the accuracy falls off. Therefore it is essential to check them regularly against weights standardized by the U. S. Bureau of Standards. When the accuracy is no longer within acceptable limits the balances are relegated to less important work or are returned to the manufacturer for overhauling.

Since the over-all precision of an analytical method is a function of the individual steps, the precision of an entire procedure can be calculated by taking the square root of the sum of the squares of the individual values. In this case, it is $\pm$ .53% (assuming a value of $\pm$ .1% for filling the volumetric flasks).

The control chart, illustrated in Fig. 1, has been set up on the basis of data from 50 duplicate assays of Standard Vitamin A Capsules,[2] one of which is submitted daily for routine assay. In this chart is the combined data from two routine charts showing the use of ethanol and isopropanol as solvents. It is clear that isopropanol gives significantly lower values of E(1%,1cm.).

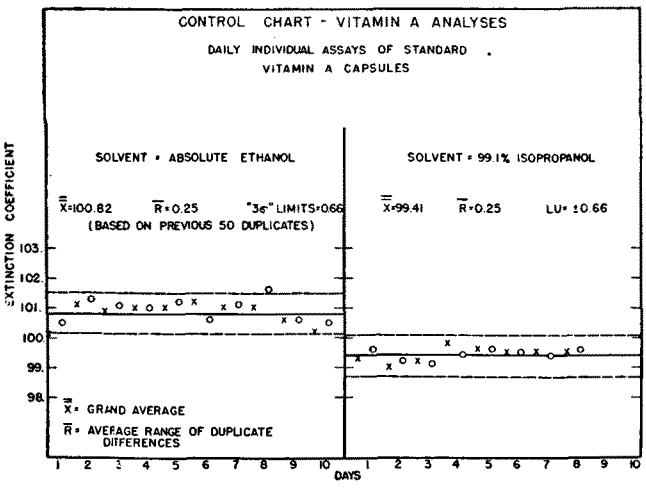

FIG. 1.

The chart shows operating limits of $\pm$ .65%, which are slightly larger than the value of $\pm$ .53% calculated above. The difference is no doubt due to the instinctive human facility of doing a slightly better job on a specific test run than during routine operations.

The value of a control chart is that it not only serves as a check on the technique of the operator but is invaluable in detecting incipient trouble in the equipment. Thus, if more than .3% of the individual values fall outside the established control limits, the operator can be sure that this effect is not "experimental error" but can be attributed to some particular cause. A gradual falling off of precision and accuracy due to failing "B" batteries in the Beckman Spectrophotometer was detected by such statistical methods. The manufacturer of this instrument[3] states that failing batteries are manifested "by failure of the needle to fluctuate completely across the scale." However, at this point the precision limits, within which the instrument gave replicate readings, were extended to $\pm$ 1.1% indicating a 5-fold drop in precision. Replacement of the batteries immediately returned the instrument to its customary precision of $\pm$ .2%. (For a complete explanation of control chart methods, the reader is referred to references 1, 15, and 12.)

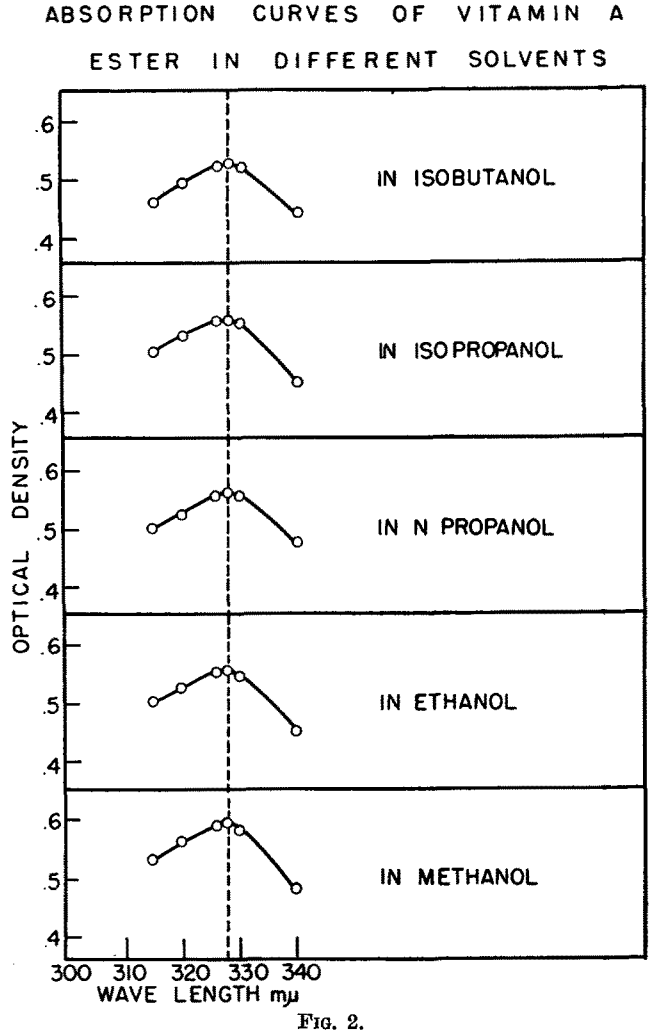

FIG. 2.

Vandenbelt (18) showed an unexpected limitation of the precision in his Beckman Spectrophotometer. In Figs. 1 and 2 of his paper he plotted extinction values against optical densities of a series of solutions whose concentrations were fixed so that the maximum optical densities varied over the whole instrument scale range. Values below .4 and above 2.0 were shown to be seriously in error.

The writers repeated this experiment using two Beckman Spectrophotometers and a sample of oil whose potency was approximately 200,000 units of vitamin A per gram. With these instruments the density could be read with accuracy down to 0.1.

Effect of Solvents on the Measurement of Optical Density

Ewing, *et al.*, (6) and Barthen and Leonard (2) stated that ethanol and isopropanol can be used interchangeably in the determination of the 328 mμ extinction coefficient of vitamin A. Before the advent of the Beckman Spectrophotometer this may have been so, but since the assay can now be run with greater precision, it is evident that solvents do affect

[2]Distilled Vitamin A Concentrate, 0.5 g. capsules, Control No. PC-3, Distillation Products, Inc., Rochester 13, N. Y.

[3]Beckman Spectrophotometer, National Technical Laboratories, S. Pasadena, California.

the value. Fig. 1, a routine control chart, shows that isopropanol gives values approximately 1.5% lower than ethanol. Other solvents were studied and the results given in Table I show that in the alcohol

TABLE I.

Solvent	Vita. A Ester Concentrate	Vita. A Alcohol	Vita. A Acetate	2-Phenyl-Azo-P-Cresol in Corn Oil
Methanol	102.1	.....	1511	27.1
Ethanol	100.8	1702	1483	27.2
Isopropanol	99.3	1706	1433	27.1
Isobutanol	97.6	.....	.....	27.2
N Propanol	99.0	.....	.....	27.1
N Butanol	98.0	.....	.....	27.0
Cyclohexane	98.4	.....	.....	27.4

Extinction coefficients at 328 mμ (325 mμ for 2-phenyl cpd.) of above compounds.

series as the molecular weight increases there is a corresponding decrease in extinction coefficient values. The values found when cyclohexane is used are also included since this solvent is used rather widely for this analysis. The table lists data which show that this effect is true only for esters of vitamin A and that vitamin A alcohol and the new "standard" proposed by Kreider (8)—2-phenyl-azo-p-cresol—do not show this effect. The data in the table are for a corn oil solution of 2-phenyl-azo-p-cresol. Table IV

TABLE IV.

Type of Oil	E(1%, 1CM.) Ethanol	E(1%, 1CM.) Isopropanol
Shark Liver Oil	14.07	13.95
Shark Liver Oil	27.10	26.45
Dogfish Liver Oil	9.71	9.54
Shark Liver Oil	41.0	40.7
Shark Liver Oil	27.5	27.4
Shark Liver Oil	41.7	41.0
Shark Liver Oil	35.1	34.45
Dogfish Liver Oil	5.68	5.68
Cod Liver Oil	5.40	5.36
Cod Liver Oil	3.90	3.78

Comparison of extinction coefficients of crude fish liver oils determined in ethanol and isopropanol.

is included to illustrate that this phenomenon holds for crude fish liver oils as well as for concentrates.

Morgareidge (12) showed that vitamin A alcohol gave different extinction values when dissolved in isopropanol than in cyclohexane and explained that observation on the basis of association of a polar compound in a polar solvent. He also showed that a shift in the position of the maximum occurred since the alcohol form of vitamin A in isopropanol had a maximum absorption at 324 mμ rather than 328 mμ. Our investigation shows that this shifting did not take place when different solutions of the ester form of vitamin A were measured in the various solvents. Fig. 2 gives absorption curves determined with the Beckman Spectrophotometer to illustrate this, and in all cases, regardless of the solvents studied, the peak occurred at 327-328 mμ.

Since the three commonly used solvents—ethanol, isopropanol, or cyclohexane give relatively different results, it is now clear that to define accurately an extinction coefficient, the solvent used and the form in which the vitamin A is present (alcohol or ester) must be specified.

Effect of Temperature on Accuracy

Large fluctuations in room temperature, such as diurnal changes of temperature in summer, affect the accuracy of the determination. Variation of the temperature of an alcohol solution of vitamin A ester in the cell was studied by cooling the solution to approximately 6°C. before placing it in the cell and inserting a small copper-constantin thermocouple connected to a sensitive galvanometer (Leeds and Northrup Wall Type). The temperature was recorded as it rose to approximately 25°C. Optical density readings at 328 mμ taken at 5-minute intervals showed no significant changes over the temperature range covered. Two Beckman Spectrophotometers which were matched when kept side by side in a constant temperature room were used to examine the effect of temperature on the instruments. One instrument was moved into a regular non-airconditioned laboratory, and determinations of the optical density of solutions of vitamin A oils were made with both instruments. The procedure followed was to prepare the alcohol solution of the oil, transfer it to the reading cell, determine the optical density in one instrument, and remove the cell and holder and place them in the second instrument for the second reading. Fifteen separate determinations listed in Table II show small

TABLE II.

Sample	Instrument No. 1 (24°C.)	Instrument No. 2 (32°C.)
Vita. A Conc	.544	.541
Vita. A Conc	.513	.511
Vita. A Conc	.611	.609
Vita. A Conc	.533	.528
Vita. A Distillate	.568	.565
Crude Fish I.O	.538	.533
Ref. Fish I.O	.580	.578
Vita. A Distillate	.552	.548
Vita. A Distillate	.538	.532
Vita. A Distillate	.516	.510
Vita. A Conc	.558	.552
Vita. A Conc	.503	.498
Vita. A Conc	.484	.480
Vita. A Conc	.518	.512
Vita. A Conc	.599	.594

Optical density measurements of ethanol solutions of vit. A oils at 328 mμ on two Beckman Spectrophotometers held at different temps.

but consistent differences in the readings, the higher temperatures giving the lower values by about 0.8%.

Effect of Cell Characteristics on Accuracy

Cells for spectrophotometers are usually supplied in matched sets, but it has been found necessary to check them, as the data in Table III indicate. The cells, listed with their manufacturer's numbers and arranged in "matched" pairs as received, were measured for their adherence to their stated dimensions (1.000 cm.) by the use of a Brown and Sharp micrometer caliper and a Starrett Small Hole Gauge. Lack of parallelism in one or both planes was a common occurrence so the measurement used was the average of the top and bottom midline dimensions. The variations from 1.000 cm. for each cell are given in Column 3 of Table III. The absolute absorption against air at 328 mμ of each cell filled with isopropanol is given in Column 4. The absorption and deviation from the average of an alcohol solution of 2-phenyl-azo-p-cresol are given in Columns 5 and 6. Here cell No. 2444 was arbitrarily chosen as the blank and all other cells were compared with it. Since it was one of a "matched" pair with No. 2525, the roles of the two cells were reversed to get a reading for No. 2444.

Dimensions of some cells are seriously off, as illustrated by cell No. 1 and cell No. 2452. These would give inaccurate results according to Lambert's law.

TABLE III.

Cell No.	Mean Inside Dimension (Inches)	% Deviation From .3937"	Optical Density at 328 mμ	Optical Density of 2-Phenyl-Azo-P-Cresol Solution at 328 mμ	% Deviation From Average
1435 (S)	.3941	+.11	.044	.659	+ 4.0
1441 (S)	.3942	+.13	.040	.664	— 2.4
1 (S)	.3975	+.95	.054	.672	+ 4.5
2 (S)	.3943	+.14	.049	.662	—1.05
11 (C)	.3937	0	.077	.669	0
12 (C)	.3935	—.05	.077	.668	— .15
13 (C)	.3937	0	.077	.668	— .15
14 (C)	.3939	+.06	.083	.668	— .15
2512 (C)	.3930	—.19	.094	.682	+1.90
2452 (C)	.3957	+.51	.093	.686	—2.50
5 (C)	.3930	—.19	.075	.662	—1.05
6 (C)	.3933	—.11	.083	.662	—1.05
2525 (C)	.3942	+.11	.104	.696	+4.0
2444 (C)	.3939	+.05	.083	.653	—2.4

Characteristics of cells used in Beckman Spectrophotometer. (S) = Silica. (C) = Corex.

The two "matched" pairs 2525:2444 and 1:2 are quite unmatched, as shown by their relative deviations from the average optical density. The use of such cells would greatly affect the accuracy of the determinations and cause differences between laboratories collaborating on analyses. The fact that an error in dimension is partly compensated by an opposite difference in transmission characteristics does not make the cell acceptable since this compensation will vary at different spectral ranges. Fig. 3 illustrates the transmission curves of silica and corex cells. It is seen that either type is acceptable for readings at 328 mμ.

The Hilger Spectograph vs. the Beckman Spectrophotometer

The relative merits of these two commonly used instruments as described here are based on considerable experience with both. As mentioned before, the precision with which replicate runs can be measured is approximately ten times as great with the Beckman as with the Hilger medium quartz spectrograph. (Perhaps this wide divergence could be narrowed by the use of a micro-densitometer for reading the plates obtained from the spectrograph.)

Both instruments are rugged, but they have idiosyncrasies which lead to insidious errors. The Hilger quartz spectrograph has been found to develop two such phenomena. First, the casting holding the separate parts of the photometer, collimators, and cell holders became warped, necessitating the realignment of the separate parts by the use of shims. Second, the density selector of the Spekker Photometer (3, 17) became worn to such a degree that optical density readings no longer obeyed Lambert's law, and readings, especially in the higher ranges, were seriously in error. A solution whose peak optical density was 0.6 when in a 1 cm. cell was 1.27 and 2.0+ when 2 and 3 cm. cells were used. The photometer appeared to be adjustable, but the need of special techniques for this was apparent. Adjustments were made by trial and error by inserting shims between the point of the selector and the base of the aperture frame. Alkaline potassium chromate solution for absolute standards as described by Hogness (7) was used.

Furthermore, the light source used in this spectrograph leaves much to be desired. It consists of a high

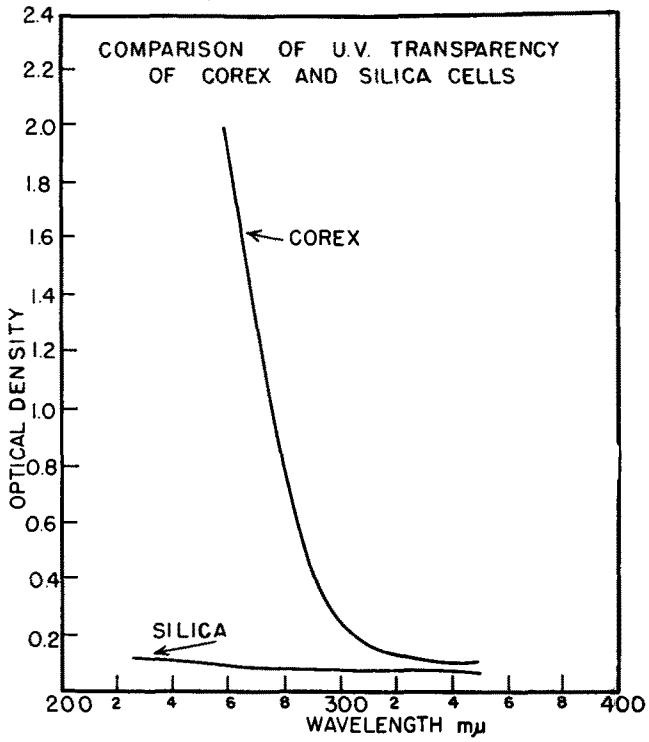

FIG. 3.

potential condensed spark between two tungsten steel electrodes. The instrument is sensitive to the position of the "point source" of light as fixed by the midpoint of the spark although Twyman (17) states that an advantage of this instrument is its lack of sensitivity to the position of the light source. However, a change of position of either electrode as little as .005 inch is detectable in the final spectrum photograph of the spark.

For a while uneven wear of the electrodes caused some concern. The trouble was at first alleviated by installing a "polarity" changing switch in the high potential leads, which changed the "polarity" of the electrodes every time the spark was switched on. Further investigation, however, showed that the phenomenon was due to unequal "capacity to ground" effect of the high potential leads connecting the condenser in the electrical circuit. Placing the condenser in a new position and using leads of equal length solved the trouble, and the "polarity" changing switch was no longer needed.

The Beckman Spectrophotometer (4), employing electronic amplification of phototube currents and a "balanced bridge" circuit, is eminently satisfactory. However, two sources of trouble have been found. First, failure of the "B" batteries supplying the plate potential to the tubes leads to lack of precision as mentioned previously. This difficulty is readily overcome by periodic replacement of the batteries. A second ailment, apparently due to trouble with the phototube, was evidenced by failure of the instrument to read zero when the cell was filled with India ink. This was a "constant" error which affected all readings made throughout the range of the instrument in inverse proportion to the scale reading. Readings of approximately 0.5 optical density were in error about 2%. To correct this, it was necessary to return the instrument to the manufacturer for overhauling.

Suggested Materials for Standardizing Spectrophotometers

The spectrum of potassium chromate in 0.05 N aqueous potassium hydroxide has been carefully measured by Hogness, Zscheile, and Sidwell (7) and for this reason has often been suggested as a spectrophotometric standard. Their values of molecular extinction coefficients of 3660 at 272.5 mμ and 4830 at 371 mμ have been corroborated various times in the literature, and the writers have used this material numerous times with good success obtaining values for the molecular extinction coefficients within 1% of the above values. The advantages of this material are that it is a stable, inorganic, crystalline compound. The disadvantages are that it must be made with freshly prepared aqueous potassium hydroxide solution and that it absorbs at the wrong wavelengths for spectrophotometers primarily engaged in vitamin A analyses.

Potassium nitrate has been suggested by Morton (13), Ley and Volbert (9), as well as Vandenbelt, *et al.* (18). Each have reported values of 0.698 for E(1%,1cm.) (302 mμ). These results agree well with the value of 0.699 found by the authors. Unfortunately, this material has the same disadvantages as potassium chromate.

Salicylaldehyde and anthraquinone have also been suggested by Morton (13), but no accurate spectrophotometric data are given. The values found for the extinction coefficient E(1%,1cm.) of ethanol solutions of the compounds as received from Eastman Kodak Company were 380 and 234, respectively. Both materials absorb at 325 mμ but have obvious disadvantages, the former being a liquid and the latter being almost insoluble in common spectrographic solvents.

Taylor (16) and recently Kreider (8) have suggested 2-phenyl-azo-p-cresol. This material absorbs at 325 mμ with a value of 900 for E(1%,1cm.), as reported by Kreider and corroborated by the writers. It is reported to be very stable, is a crystalline material, and absorbs in the correct spectral range for vitamin A analysis. Kreider suggested the use of a corn oil solution for standardizing work, and the data given in Table I are for such a solution kindly supplied by him.

The use of any of these materials as adulterants for vitamin A may be readily detected by determining the vitamin A potency by the antimony trichloride method of Carr and Price (13) which is less precise but more specific than the spectrophotometric procedure.

Finally, for convenient, exact, and constant values a section of a glass filter, cut to fit in the instrument cell at right angles with the path of the light beam, makes an excellent standard for routine checking of a spectrophotometer. Unfortunately, no material of this nature has been found with an optical density peak in the 328 mμ region. Available filter glasses which have a transmission minimum in the blue and near ultraviolet are some times not stable, so that it is necessary to choose a filter in the higher ranges of the visible spectrum. Such a filter was obtained from the U. S. Bureau of Standards, along with its spectral characteristics as measured by them. This is used periodically to check the wavelength and absorption calibrations of the instruments.

The wavelength calibration of the Beckman instrument is also routinely checked by placing a mercury vapor lamp (General Electric, A-H-4, 400 watts) in the place normally occupied by the instrument lamp housing and using the mercury lines at 365.0, 404.65, 435.83, and 546.07 mμ for reference.

Contamination of Spectrographic Solvents

Solvents used for spectrographic purposes usually require more care in handling and dispensing than those used for reagent purposes. Impurities such as benzene or acetone are often met in the alcohols. Even traces are serious if spectral measurements are made between 200 and 300 mμ.

Whenever a cell of pure solvent is compared with a cell of the same solvent plus some solute, care must be exercised to see that the "blank" solvent is exactly the same as the "unknown." The blank must be obtained from the same bottle as that used for the unknown solution. The familiar siphon dispenser with a pinch clamp on a section of rubber tubing is a sure source of trouble since sufficient material may be extracted from the rubber by the solvent to affect its transmission characteristics. As this extraction proceeds very slowly, the longer a given unit of solvent is in contact with the rubber the more material will be dissolved. A "blank" prepared the first thing in the morning will be seriously different from one made after a few hundred mls. have been withdrawn. A simple experiment was performed to show the effect of rubber. Redistilled isopropanol had an optical density against air of 0.04 in a 1 cm. silica cell. Thirty seconds of contact with a new piece of best quality red rubber tubing raised the absorption to .07. When the piece of tubing (3" of ⅛" bore heavy wall) was left in a beaker with 50 ml. of the solvent for three hours the solvent was colored a faint red and had an optical density of 0.7 at 328 mμ. Thus, if a siphon dispenser is to be used, it should have a glass stopcock ground to operate without lubricant.

Acknowledgment

The authors wish to express their appreciation for the valuable help and suggestions given by Dr. Norris Embree.

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