cell. The precipitate should be washed with hot 0.5% perchloric acid to aid filtration.

With regular steels the sample is cooled, the spattered drops are rinsed down, and the solution is diluted to about 80 ml. A little colloidal silica, if present, does no harm.

Both tungsten and other steels are treated similarly from here on.

The sample solution is transferred to the titration cell with 0.5% perchloric acid, and 3 ml. of 85% phosphoric acid and then 0.1 *N* ferrous sulfate solution (in 0.5% perchloric acid) are added until a slight excess is shown by ferricyanide on a spot plate. Then 5.0 ml. of the ferrous solution in excess are added.

The instrument is adjusted, as previously described, to a wavelength of 525 mμ, and 2.5 ml. of freshly prepared ammonium persulfate solution (15 gram per 100 ml.) are added. After 1 minute, the solution is titrated with standard permanganate solution. If 0.1 *N* permanganate is used, 0.1-ml. increments are most convenient, while with 0.02 N permanganate 0.4-ml. portions are suitable.

Procedure for Ferrotitanium. A 0.5-gram sample containing 1 to 2 mg. of vanadium is weighed out into a 300-ml. Kjeldahl flask, and 25 ml. of water and 10 ml. each of 1 to 1 sulfuric, nitric, and hydrochloric acids are added. The sample is boiled gently over a flame until decomposition is complete, more nitric acid, hydrochloric acid, or water being added if necessary. Then the solution is boiled down twice to fumes of sulfur trioxide and cooled, 50 ml. of water are added, and the solution is filtered through medium porosity paper. The flask and precipitate are washed with 0.1 *N* sulfuric acid; finally, the filtrate is diluted to 100 ml. with water and transferred to the titration cell. The procedure used with steel is then followed.

DISCUSSION OF RESULTS

Tables I and II show that the accuracy of this method equals that of the methods used by the cooperating analysts in the analysis of the National Bureau of Standards standard samples. This procedure allows the convenient persulfate method to equal or surpass the accuracy of other existing methods for determining milligram amounts of vanadium. In addition to the determination of vanadium in steel and ferrous alloys, this general procedure should be applicable to other systems where an accurate determination of small amounts of vanadium is required.

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Determination of Small Amounts of Chromium in Human Blood, Tissues, and Urine

Colorimetric Method

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A rapid, colorimetric method for the determination of small amounts of chromium in human blood, tissues, and urine is described. The method is sensitive to 0.005 microgram of chromium per milliliter of final solution. Samples are ashed in borosilicate glass beakers, using a combination of wet- and dry-ashing methods. The blood and tissue samples are oxidized by bromine in alkaline solution. Oxidation of the urine samples is accomplished by sodium bismuthate in acid solution. Diphenylcarbazide is used as the color reagent. A clear red-violet color is obtained which can be measured colorimetrically or spectrophotometrically. Comparative standardization curves and typical analyses are given.

IN A recent industrial cancer investigation it became necessary to determine small amounts of chromium in human blood, urine, and autopsy specimens. As the number of samples to be run was large, it was necessary to develop new, sensitive, and more rapid techniques than those reported in the literature.

The best methods for estimating small amounts of chromium are colorimetric methods using diphenylcarbazide (*5*), which gives a clear red-violet color with minute amounts of chromium (*29*). Diphenylcarbazide was first used by Moulin (*23*). It has since been used extensively, and a large number of the influencing factors and interferences have been studied and reported (*5, 9, 10, 27, 29, 37, 39*). The method is nearly specific for chromium; hexavalent molybdenum is the only element giving a similar, but much less sensitive, violet color in acid solution (*29*). Mercury, copper, cadmium, silver, lead, nickel, cobalt, manganese, magnesium, zinc, vanadium, and iron also give colors under various conditions (*11, 37*). However, under the analytical conditione used for determining chromium, most of these metals either react with low sensitivity or not at all (*10, 27*).

Only a few methods have been given in the literature for the determination of chromium in physiological specimens, although many methods appear for leather, which often contains large amounts of chromium introduced during the tanning process. Investigators have reported quantitative methods for the deter-

mination of chromium in human tumors (6, 7), animal tissues (e), tissue ash (26), plant ash (36), milk (35), fats (31), toxicological specimens (17), vegetation (28), animal feces (21), and sewage (10, 32).

ASHING

A majority of the investigators cited h v o reported dry-ashing methods for the physiological specimens. Usually, platinum ware and muffle furnaces nt a temperature of approximately 500" C. were used (6, 21, 36). Dry-nshing physiological specimens at low temperatures is, at best, time-consuming. Muffle space and the price of platinum would limit the use of such methods on a large scale.

Several wet–nshing procedures have been reported. The use of sulfuric and nitric acid combinations has been reported in the treatment of sewage (10, 32). Sulfuric, nitric, and perchloric acid combinations or perchloric ncid alone have been used (2, 15, 30, 35). Perchloric acid rapidly oxidizes organic matter, but it has the distinct disadvantage of volatilizing chromium as chromyl chloride whether chlorides are present or not (13, 16). Brard (2), following the wet-ashirig method of Kahane (16), used a perchloric ncid method to digest 200- to 300-gram samples of animal tissues. The escaping gases were passed through a condenser and returned to the original flask. This method rcquircs a great deal of attention and is loo cumbersome to be used for a large number of samples. The end of the digestion is difficult to judge with some samples, and the digestion products are difficult to concentrate, without loss of chromium, to the small volumes necessary for measuring minute amounts of chromium.

A considerable nniount of work has been reported on the ashing of physiological specimens for the dctcrmination of lead (18, 20, 40). A study of these methods showed that wet ashing could be accomplished in glass beakers nnd in a comparatively short time. After experimenting with several different methods, the authors adopted a combination wet- and dry-ashing procedure using borosilicate glass beakers.

OXIDATION OF CHROMIUM

The osidation of chromium in pbysiological specimens has been accomplished by means of permangnnate (.%'Tpersulfate (2, 28, 36), perchloric acid (35), hydrogen peroxide (26), sodium peroxide (10, 17), and various fusion misturcs (7,31). For the authors' purposes, oxidation in an alkaline medium was desirable, as it would afford a convenient means of removing iron, manganese, and other substances insoluble in an alkaline medium. At first, hydrogen peroside was used, but this was found to be undependable (12). The last trnccs of peroxide were difficult to decompose, and when the sample was made acid, just before the addition of the dye, the hydrogen peroxide would reduce the chromate ion (19). Oxidation with broininc in alkaline solution (12, 14, 22) worked more satisfactorily, and the excess bromine was easily removed with phenol, which reacts to form tribromophenol.

The relatively large amounts of phosphorus (as phosphate), calcium, nnd mngnrsium in the urine ash made an alkaline oxidation impractical because of the voluminous precipitate formed. A persulfate oxidntion in acid solution with silver nitrate as a catalyst was used successfully. An objection to this method was that the developed color would fade rather quickly. In the meanwhile, it was found that sodium bismuthate, ordinarily used to oxidize manganese in strongly acid solution (1, 4), would, under the proper conditions, oxidize chromium (33). The authors were unable to find any report of previous work on the use of sodium bismuthate in the quantitative determination of chromium. However, after a number of preliminary studies, the proper conditions were found by which satisfactory results were obtained.

REAGENTS

Unless otherwise specified, all reagents are of analytical quality. Standard chromate solution was made by dissolving 0.2829 gam of potassium dichromate obtained from the National Bureau of Standards in double-distilled water and making up to 1 liter. One milliliter of this solution is equivalent to 100 micrograms of chromium, solutions containing 10 and 1 microgram per milliliter are made by diluting this stock solution with double-distilled water.

Bromine–sodium bydmxide oxidizing solution, 6 ml. of saturated bromine water per 100 ml. of 1 N sodium bydroxide solution.

Phenol, 1.2% solution of redistilled phenol prepared with double-distilled water and stored in an amber bottle.

Diphenylcarbazide, 0.25% solution in 1 to 1 acetone and double-distilled wnter.

Sulfuric acid solution, 25% by volume in double-distilled water. Solution must be free of reducing substances.

Nitric acid, specific gravity 1.42, redistilled.

Distilled Water. Ordinary distilled water does not contain noticeable amounts of chromium. However, there is always a possibility that the water may contain free chlorine, organic mnttcr, or otber volatile substances which may react with the chromate ion. Once the chromium is oxidized, it is necessary to use fresh double-distilled water or double-distilled water that has been well kept.

Glassware. All glassware should be rinsed with a strong ncid solution, preferably aqua regia, after it has been cleaned arid before its final rinse with distilled water. The comnion sulfuric ncid-chromic ncid cleaning solution must be rigorously avoided. Bcakcrs gradually become etched, but may be used repeatedly if properly cleaned. Beakers used for high chromium-containing samples should not be used for low chromium-containing samples.

APPARATUS

All measurements were made with a Beckman Model DU quartz spectrophotometer using 1-cm. cells. Wave length was set at 540 mμ nnd slit width nt 0.04 mm.

ANALYTICAL PROCEDURE

Blood and Tissues. Put 10 to 20 grams of weighed sample (tissues should be well washed beforehand) in a 100- or 150-ml. beaker, and add 2 to 3 ml. of concentrated sulfuric acid and 10nil. of concentrated nitric acid. Mix by rotating the beaker, cover with a watch glass, and heat cautiously on a hot plate until the tissues have gone into solution. Lung and liver tissues sometimes react violently with hot nitric acid. Blood, brain, and fatty tissues tend to foam; carefully treat these tissues with sulfuric arid until they are charred before adding the nitric acid. Gradually increase hent and let evaporate to sulfur trioxide fumes. Fume for 0.5 hour, remove from hot plate, cool slightly, add 5 ml. of concentrated nitric acid, return to hot plate, nnd again heat to fumes of sulfur trioxide. If the remaining organic matter is large, repeat the nitric ncid treatment. Remove the beaker cover, fume to dryness, and place the beaker in a muffle furnace at 550° C. for 0.5 hour. The ash should have a reddish to white color. Should black spots, or a grayishness, be seen, remove the beaker from the muffle, place on the hot plate to cool, digest with 2 ml. of nitric wid, dry, and again return to the muffle. Repeat if necessary.

If a muffle is not available, ash the tissues completely by alternately treating with nitric acid and heating with a Fisher burner to just short of redness. Beakers of 100- and 150-ml. size do not easily break upon cooling after being heated strongly, but precautions should be taken to let the beakers cool on a warm surface, such as that of a hot plate or a warm asbestos pad.

After nshing is complete, add 1 ml. of concentrated hydrochloric acid, rotate to wet all of the ash, add 2 ml. of concentrated nitric acid, cover with a watch glass, heat, and allow vapors to wash down sides of beaker for a few minutes. Remove cover and evaporate to dryness. Wash down sides of beaker with approximately 10 ml. of water, using a very fine stream of water, and again evaporate to dryness. Remove from hot plate, and add approximately 25 ml. of mater and 2 ml. of bromine–sodium hydroxide oxidizing solution. This should precipitate all the iron and make the solution definitely alkaline. If not, add 1 N sodium hydroxide solution until the sample is definitely alkaline. Cover, and boil gently for 0.5 hour with occasional stirring to ensure complete contact of the oxidizing solution. Evaporate to a volume of approximately 4 ml. and cool to room temperature. Transfer to a graduated 15-ml. centrifuge cone, carefully washing the insoluble residue adhering to the beaker three to four times with 1-ml. wash portions of double-distilled water from a wash bottle delivering a very fine stream of water. It is not necessary that all of the precipitate be washed into the centrifuge cone, but the precipitate should be well washed, and the total volume of the sam-

ple and washings must not exceed 8.5 ml. Centrifuge until firmly packed and decant into a 10-ml. volumetric flask. Small amounts of the precipitate escaping into the volumetric flask do not greatly affect the results; however, it is best to avoid this as much as possible. To the flask add 0.5 ml. of 25% sulfuric acid solution, making the solution 0.2 to 0.3 *N*. Shake, check to see that an excess of bromine (indicated by its color) is present, add 0.5 ml. of phenol solution, and shake to remove all free bromine. Add 0.5 ml. of diphenylcarbazide solution, make up to mark, shake, and take readings at a wave length of 540 mμ on a spectrophotometer or compare colorimetrically with previously prepared standards. If colors developed are too intense for reading, make proper dilutions.

Urine. To the entire urine sample add enough concentrated nitric acid to equal 5% of the volume of the sample. Mix thoroughly and let sit for at least 2 hours. Take an aliquot equivalent to 50 ml. of urine, add 5 ml. of concentrated nitric acid, cover with watch glass, heat cautiously until solution has cleared, boil to near dryness, and take to complete dryness with care, as the reaction is usually very rapid at this point. Cool, add 2 to 3 ml. of concentrated nitric acid, cover with watch glass, and again evaporate to dryness. Remove cover, and place beaker in muffle at 500° C. for 20 minutes. Cool and add 2 ml. of concentrated nitric acid and 2 drops of concentrated phosphoric acid. Cover heat for a few minutes to allow acid vapors to bathe sides of beaker, remove cover, and wash sides of beaker with 10 to 15 ml. of water. Stir by rotation until nsh is completely dissolved and carefully evaporate to dryness without visible boiling. At this point the sample tends to spatter, but this can be avoided by agitating beaker just as the sample comes to dryness. Let sample bake for a few minutes and then place in the muffle for 10 minutes. Cool, add 5 ml. of water from a graduate and 1 ml. of 25% sulfuric acid. Stir until completely in solution and then add approximately 50 mg. of sodium bismuthate. Mix thoroughly by rotation, cover with a watch glass, and put on a water bath for a timed 20 minutes. Stir occasionally to ensure complete contact. Remove, cool, and wash into centrifuge cone as for blood and tissues; do not let the volume exceed 8.5 to 9.0 ml. Rinse inside of cone while making volume up to 9.5 ml. Centrifuge until the bismuthate is tightly packed and carefully decant into a 10-ml. volumetric flask. Add 0.5 ml. of diphenylcarbazide solution, make up to mark, mix, and read as for tissues. Blanks using urine from unexposed persons should be run simultaneously with each batch of samples.

STANDARDIZATION

Table I gives the optical density values obtained from duplicate samples of blood and urine which were run through the procedure as given. Ten milliliters of whole blood or 50 ml. of urine were used for each sample. The blood was obtained through the cooperation of the local American Red Cross chapter, and the urine was obtained from unexposed laboratory personnel. The chromium was added by means of a microburet.

Table I. Optical Density Values Obtained from Duplicate Blood and Urine Samples

	Chromium Added, Micrograms									
	0	0.10	0.20	0.50	0.80	1.00	2.00	5.00	8.00	10.0
Blood	0.009	0.017	0.026	0.048	0.071	0.082	0.158	0.362	0.610	0.755
	0.010	0.019	0.024	0.045	0.068	0.083	0.160	0.367	0.600	0.745
Urine	0.011	...	0.025	0.045	0.066	0.085	0.155	0.360	0 605	0.720
	0.013	...	0.026	0.047	*0.070*	0.080	0.151	0.355	0.595	0.730

Figure 1 compares the standard curves obtained from these values, after blank corrections, with the curve obtained from pure standard dichromate solution in 10 ml. of 0.2 *N* sulfuric acid solution. Recovery is relatively good and there is close agreement between the blood and urine curves. The deviations from the standard solution curve are probably due to the effects of the extraneous substances in the actual samples as well as the small amounts of chromium left in the centrifuge tubes.

Confirmation with Beer's law up to a concentration of 10 micrograms of chromium per 10 ml. of final solution (or 1 p.p.m.) is also shown. The molar extinction coefficient of the colored compound, based on the molarity of a dichromate solution, is 8.32 × 10⁴. Theoretically, this should enable the spectrophotometer to detect differences of 0.002 microgram of chromium per milliliter of solution. However, detection of differences of 0.005 microgram of chromium per milliliter of solution is the practical limit of sensitivity.

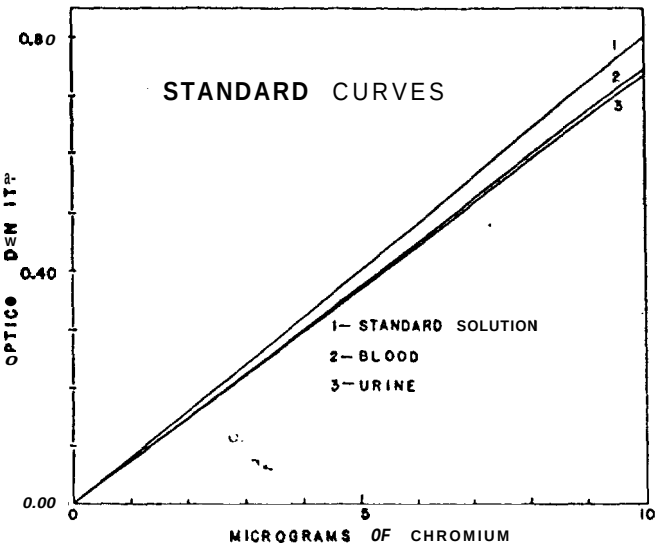

Figure 1. Comparison of Standard Solution Curve with Curves Obtained from Blood nnd Urine Standards

Table II shows the percentage of chromium recovered from approximately 10 grams of met tissue taken from autopsy specimens obtained from persons known to be nonchromate workers. The specimens were furuished through the cooperation of the Department of Pathology, The Ohio State University. Three sections, as uniform as possible, were cut from each specimen, and to each section 0, 1, or 2 micrograms of chromium were added. The sections were then analyzed as above. The original chromium in the sections to which chromium was added was calculated from the analysis of the section to which no chromium was added. This assumed a uniform distribution of the original chromium throughout the autopsy specimen, but such an assumption is not necessarily valid. However, the sections were fairly uniform, nnd the recoveries consistently fell within 10% of the amount added.

Trivalent chromium, as the nitrate, was also run through the procedures with blood and urine samples. Inasmuch as there were no notable differences in the results, the potassium dichromate salt was used as a standard because of its higher purity.

Figure 2 compares the optical density versus the time curve of a standard dichromate solution with the curves obtained from urine and blood standards. As can be seen, the urine samples fade rather rapidly. This is due, in part, to the greater acidity of the final solution combined with the effects of small traces of sodium bismuthate which are carried over with the decantate. Attempts to eliminate this fading with sodium azide *(9)* were unsuccessful. However, good results can be obtained by taking readings within 5 minutes after the diphenylcarbazide has been mixed with the sample. Blood samples should be read within 20 minutes after mixing.

DISCUSSION

More than 1000 experimental samples were run in the development of these procedures. Although the recommended sample sizes give optimum results, the size of the samples sctuslly analyzed varied from 0.1 up to 30 grams of blood or tissue and from 10 to 100 ml. of urine. Blood samples were collected with special platinum needles and evacuated-type vials. Urine samples were collected in specially prepared glass jars.

The amounts of chromium found varied from undetectable amounts up to 3 micrograms per 10 grams of blood, 450 micrograms per 10 gram of tissue, and 35 micrograms per 100 ml. of urine. The usual amounts encountered, however, were in the neighborhood of 0.5, 1, and 5 micrograms, respectively.

Among the tissues and organs analyzed were lung, kidney, liver, spleen, brain, heart, stomach, adrenal glands, lymph nodes, pancreas, thyroid, large and small intestines, muscle, fecal matter, malignant tumors, and small pieces of bone and cartilage. Tissues which contain large amounts of calcium and phosphorus, such as bone and cartilage, form a voluminous precipitate which interferes with the oxidation of the chromium. The authors were able to get some satisfactory results by extending the period of boiling with the bromine–sodium hydroxide solution; however, the method is not recommended for such tissues without further study.

By preparing a batch of samples in the afternoon of one day, letting them digest on a sand bath or a slightly warm hot plate overnight, and finishing the batch the next day, a single laboratory technician can run fifteen to twenty samples a day. Substances, such as selenium and ashing nids *(58)* which assist in the decomposition of organic matter, were not tried because of the possibility of interference and/or contaminntion.

Most difficulties can be traced to improperly or incompletely ashed samples. Chromium is not easily volatilized under the conditions outlined, and low results are usually due to incomplete oxidation of either the organic matter or the chromium rather then to a loss of chromium.

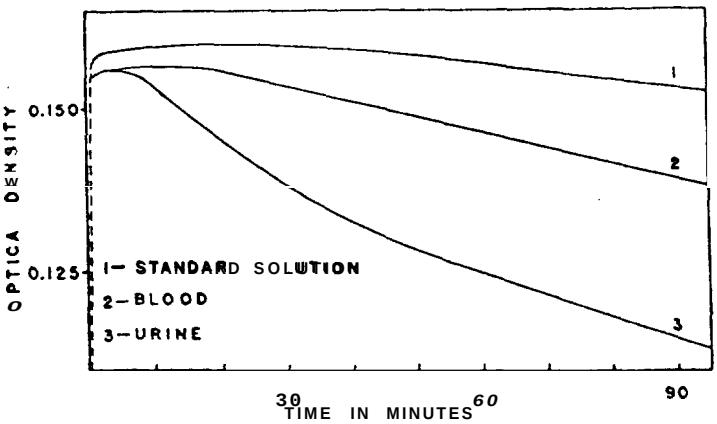

Figure 2. Comparative Fading Rates of 2 Micrograms of Chromium in 10 Ml. of Final Solution

After the samples have been nshed, the hydrochloric and nitric acid treatment completely dissolves the blood ash, the ash of most tissues, and any chromium oxides that may hsve been formed. The nitric acid, which is in excess, removes most of the chlorine and chlorides, while the subsequent evaporation with water removes most of the free acid. The phosphoric acid which is added to the urine samples compensates, in part, for variations in urine content and aids in making the ash soluble.

The sodium bismuthate osidation of the urine samples should be carefully controlled. The method as given is somewhat flexible, but excessive variations in time, temperature, or acidity may cause either high results due to side reactions or incomplete oxidation of the chromium. Chlorides and fluorides must be absent, and the amount of sodium bismuthate added to each sample should be approximately uniform. This may be accomplished by means of a small scoop made of glass tubing.

Centrifuging the excess sodium bismuthate out of the sample should also be done with some care. If a centrifuge is not available, or if slightly greater accuracy is desired, the samples may be filtered directly into the 10-ml. volumetric flasks, using approved microtechniques (84, *25*).

Should the chromium-diphenylcnrbezide color be too intense for reading, the contents of the 10-ml. flask may be rinsed into a larger volumetric flask, and the reading taken after proper adjustments for acidity and diphenylcarbazide concentration. In doing this, the loss of accuracy is not very great. If greater accuracy is desired, the blood and tissue samples with their centrifugates may be returned to their originnl beakers, reashed, and reprocessed using larger volumetric flasks or an aliquot of the samples for color development. Urine samples should be rerun entirely using smaller samples.

Table II. Chromium Recovery from Unexposed Autopsy Specimens

Tissue	Sample Weight Grams	Cr. Added γ	Cr Found γ	Cr in Tissue γ	Added Cr Recovered %
Lung	10.0	0	1.30	1.30	...
	11.1	1.00	2.40	1.46.	95
	10.2	2.00	3.15	1.33[a]	91
Liver	9.0	0	0.05	0.05	...
	9.9	1.00	1.10	0.06[a]	104
	7.6	2.00	1.95	0.04.	96
Spleen	9.6	0	0.16	0.18	...
	10.9	1.00	1.10	0.17.	93
	8.7	2.00	2.05	0.15.	96
Kidney	9.4	0	0.80	0.80	...
	10.1	1.00	1.90	0.86[a]	104
	10.7	2.00	2.90	0.91.	99

[a] Calculated.

A 0.25% solution of diphenylcarbazide io alcohol may be used in place of the recommended acetone solution. In either case the solution becomes brown upon standing. The brown color seems to have no noticeable effect, and with good grades of acetone the coloring effect is slow. Ege and Silverman (8) have recently reported the use of phthalic anhydride as a stabilizer for the diphenylcarbazide solution; nevertheless, the authors made their solution fresh daily.

INTERFERENCES

The diphenylcarbazide method is almost specific for chromium. Ferric iron, which gives a yellow color with diphenylcarbazide, is eliminated in the method for blood and tissues. In the method for urine, what iron may be present will give little if any interfercnce because of its low sensitivity. Manganese, as permanganate, reacts with loa sensitivity to give a faintly yellow color. Very large amounts of permanganate should be reduced Kith a few granules of sodium nzide (9) before the dye is added. The interference of vanadium is a more serious possibility, because it gives a stronger yellow color than does iron. If the ratio of vanadium to chromium does not exceed 10 to 1, nearly correct results for chromium can be obtained by allowing the solution to stand for about 10 minutes after the addition of the reagent, as the vanndium-diphenylcarbazide color fades fairly rapidly. In amounts greater than this, the vanadium may be removed by treating with 8-hydroxyquinoline and extracting with chloroform (*29*).

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Partition Chromatography of Synthetic Anthocyanidin Mixtures

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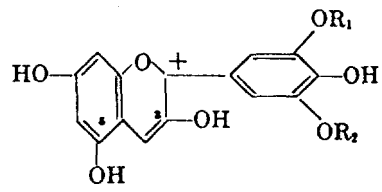
A method has been developed for the quantitative separation and analysis of small amounts of mixtures of the synthetic anthocyanidins, malvidin, petunidin, and delphinidin. Separation is accomplished by partition chromatography on columns of silicic acid with 10% phosphoric acid as the immobile aqueous phase and a mixture of phenol and toluene as the nonaqueous phase. R values are reported for the anthocyanidins. The anthocyanidin solutions after separation obey Beer's law. The concentrations of these solutions are determined spectrophotometrically.

DURING the course of an investigation into the nature of the colored substances of wine and grapes, it was necessary to consider existing procedures for the separation and quantitative analysis of small amounts of various mistures of anthocyanins and anthocyanidins. It has been shown by previous workers (*1*, 7, *10*, *15*, *17*, *25*) that the skins of some colored grapes contain anthocyanins which are mono- or diglucosides of the anthocyanidins, malvidin, petunidin, and delphinidin.



represents malvidin when $R_1 = R_2 = CH_3$, petunidin when $R_1 = CH_3$ and $R_2 = H$, and delphinidin when $R_1 = R_2 = H$. The naturally occurring glucosides of these anthocyanidins usually have glucose residues attached to the 3 or to the 3 and 5 positions. These substances are usually isolated as chlorides or picrates. In grapes or wine, the anion may be some organic ion such as tartrate. During the process of the transformation of grapes into aged wine, part of this anthocyanin mixture is probably hydrolyzed to anthocyanidins and a gradual demethylation probably occurs, so that the concentration of delphinidin or its glucosides is increased at the expense of malvidin and petunidin or their glucosides (IO).

Mixtures of anthocyanins have been separated by Karrer and his co-workers. A mixture of the picrates of the monoglucosides of malvidin, petunidin, and delphinidin obtained from the fruit of the bilberry was partly resolved by 31 recrystallizations (*16*). A similar mixture, "althaein," obtained from the flowers of the black mallow, was partly resolved by adsorption chromatography on a column of calcium sulfate (*IS*,*14*). Adsorption chromatography of red wines has also been reported (*11*, *19*), and although separate colored bands have been obtained on columns of adsorbants, no attempts at the identification of the pigments in these bands were mentioned, On the basis of these publics-