FATS, OILS AND HYDROCARBONS

Acid Value

Acid value is defined as the number of mg of potassium hydroxide required to neutralize the acids in 1 g of fatty material.

Unless otherwise directed, weigh accurately about 5 g of sample into a 500-ml Erlenmeyer flask, and add 75-100 ml of hot neutral ethanol. Agitation and further heating may be necessary to effect complete solution of the sample. For some samples, it may be necessary to use as the solvent a 1:1 mixture of neutralized diethyl ether/ethanol or petroleum spirit/ethanol. Add 0.5 ml of phenolphthalein TS and titrate immediately, while shaking, with 0.5 N KOH until the pink colour persists for at least 30 sec. (For acidity less than 2% by weight, 0.1 N KOH should be used for the titration; for acidity less than 0.2% by weight, it is necessary, in addition, to first neutralize the carbon dioxide in the reaction vessel.)

$$\text{Acid value} = (56.1 \times T \times N) / W$$

where

T is the titre (ml);
N is the normality of potassium hydroxide solution; and
W is the weight of sample (g).

Aromatic Hydrocarbons Determination

Determine by Gas Chromatography using the following conditions or equivalent that will elute n-decane before benzene:

Apparatus

Liquid phase:	Tetracyanoethylated Pentaerythritrol (TCEPE)
Length:	30 m
i.d.:	0.25 mm
Temperatures:	
Inlet:	275°
Detector:	250°
Column:	95°
Carrier gas:	N_2
Flow rate:	3 cm^3/min
Detector	Flame ionization
Split	100 – 1

Reagents

Isooctane: 99 mole percent minimum containing less than 0.05 mole percent aromatic material.

Benzene: 99.5 mole percent minimum.

Internal Standard: n-Decane and either n-undecane or n-dodecane according to the requirement of the System Suitability Test.

Reference Solution A: Prepare a standard solution containing 0.5% by weight each of the Internal Standard and of benzene in isooctane.

Reference Solution B: Prepare a standard solution containing about 0.5% by weight each of n-decane, of Internal Standard, and of benzene in isooctane.

Calibration

Select the instrument conditions necessary to give the desired sensitivity. Inject a known volume of Reference Solution A, and change the attenuation, if necessary, so that the benzene peak is measured with a chart deflection of not less than 25% or more than 95% of full scale. When choosing the attenuation, consider all unresolved peaks to represent a single compound. There may be tailing of the non-aromatic peak, but do not use any conditions that lead to a depth of the valley ahead of the benzene peak (A) less than 50% of the weight of the benzene peak (B) as depicted in Figure 1

If there is tailing of the non-aromatic material, construct a baseline by drawing a line from the bottom of the valley ahead of the benzene peak to the point of tangency after the peak (see Figure 2). Determine the areas of the benzene peak and the internal standard peak by use of an electronic integrator. Do not use integrators on peaks without a constant baseline, unless the integrator has provision for making baseline corrections with accuracy at least as good as that of manual methods.

Calculate a response factor for benzene (Rb) relative to the Internal Standard by the formula

$$A_i/W_i \times B_v/A_b,$$

in which

A_i is the area of the Internal Standard peak in arbitrary units corrected for attenuation;
W_i is the weight percent of Internal Standard in Reference Solution A;
A_b is the area of the benzene peak in arbitrary units corrected for attenuation; and
W_i is the weight percent of benzene in Reference Solution A.

System Suitability Test

Inject the same volume of Reference Solution B as in the Calibration and record the chromatogram. n-Decane must be eluted before benzene, and the ratio of A to B (Figure 1) must be at least 0.5 where A is equal to the depth of the valley between the n-decane and benzene peaks and B is equal to the height of the benzene peak.

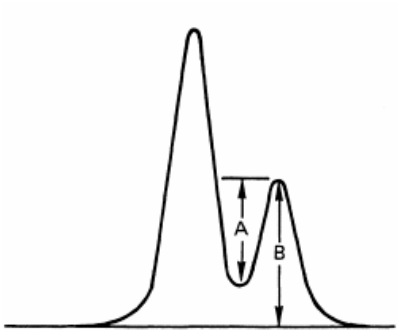


Figure 1. Illustration of A/B Ratio.

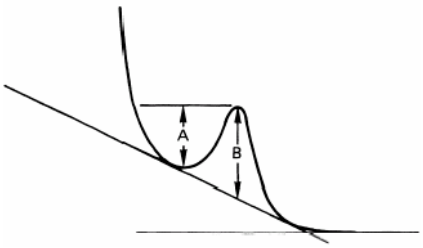


Figure 2. Illustration of A/B Ratio for a Small Component Peak on the Tail of a Large Peak.

Procedure

Place approximately 0.1 ml of Internal Standard into a tared 25-ml volumetric flask, weigh on an analytical balance, dissolve in and dilute to volume with the sample to be analyzed.

Using the exact instrumental conditions that were used in the calibration, inject the same volume of sample containing the Internal Standard. Before measuring the area of the Internal Standard and benzene peaks, change the attenuation to ensure at least 25% chart deflection.

Measure the area of the Internal Standard and benzene peaks in the same manner as was used for the calibration. Calculate the weight percent of benzene in the sample (W_B) by the formula

$$(A_b \times R_b \times W_i \times 100)/(A_i \times S),$$

in which

A_b is the area of the benzene peak corrected for attenuation;
R_b is the relative response factor for benzene;
W_i is the weight, in grams, of Internal Standard added;
A_i is the area of the Internal Standard peak corrected for attenuation; and
S is the weight, in grams of the sample taken.

Retention Times in Minutes for Selected Hydrocarbons Under the given Conditions are:

- Benzene 6.1
- Toluene 7.0
- Ethylbenzene 8.0
- p- and m-Xylenes 8.5
- o-Xylene 10.0
- n-Dodecane 6.5

Average Molecular Weight

(ASTM D 2502 See Test for Viscosity for Copyright permission)

Determine the kinematic viscosity of the sample at 37.8 and 98.9° as described in the method for Viscosity, 100°. Read the value of H that corresponds to the measured viscosity at 37.8° by the use of Table 1; linear interpolation between adjacent columns may be required. Read a viscosity -molecular weight chart for H and 98.9° viscosity (the chart is available from the American Society for Testing and Materials (ASTM)). A simplified version is shown in Figure 3 for illustration purposes only. Interpolate where necessary between adjacent lines of 98.89° viscosity. After locating the point corresponding to the value of H (ordinate) and the 98.89° viscosity (superimposed lines), read the molecular weight along the abscissa.

Kinematic viscosity, mm²/ at 37.8°	Table1 - Tabulation of H Function				
	0	0.2	0.4	0.6	0.8
2	-176	-151	-126	-104	-85
3	-67	-52	-30	-25	-13
4	-1	9	19	28	36
5	44	52	59	66	73
6	79	85	90	96	101
7	106	111	116	120	124
8	128	132	136	140	144
9	147	151	154	157	160
10	163	166	169	172	175
11	178	180	183	185	188
12	190	192	195	197	199
13	201	203	206	208	210
14	211	213	215	217	219
15	212	222	224	226	227
16	229	231	232	234	235
17	237	238	240	241	243
18	244	245	247	248	249
19	251	252	253	255	256
20	257	258	259	261	262
21	263	264	265	266	267
22	269	270	271	272	273
23	274	275	276	277	278
24	279	280	281	281	282
25	283	284	285	286	287
26	288	289	289	290	291
27	292	293	294	294	295
28	295	297	298	298	299
29	300	301	301	302	303
30	304	304	305	306	306
31	307	308	308	309	310
32	310	311	312	312	313
33	314	314	315	316	316
34	317	317	318	319	319
35	320	320	321	322	322
36	323	323	324	325	325
37	325	326	327	327	328
38	328	329	329	330	331
39	331	332	332	333	333

	H									
	0	1	2	3	4	5	6	7	8	9
40	334	336	339	341	343	345	347	349	352	354
50	355	357	359	361	363	364	366	368	369	371
60	371	374	375	377	378	380	381	382	384	385
70	386	387	388	390	391	392	393	394	395	397
80	398	399	400	401	402	403	404	405	406	407
90	409	409	410	410	411	412	413	414	415	416
100	416	417	418	419	420	420	421	422	423	423
110	424	425	423	426	427	428	428	429	430	430
120	431	432	432	433	433	434	435	435	436	437
130	437	438	438	439	439	440	441	441	442	442
140	443	443	444	444	445	446	446	447	447	448
150	448	449	449	450	450	450	451	451	452	452
160	453	453	454	454	455	455	456	456	456	457
170	457	458	458	459	459	460	460	460	461	461
180	461	462	462	463	463	463	464	464	465	465
190	465	466	466	466	467	467	468	468	468	469
	H									
	0	10	20	30	40	50	60	70	80	90
200	469	473	476	479	482	485	487	490	492	493
300	497	499	501	503	505	507	509	511	512	514
400	515	517	518	520	521	523	524	525	527	528
500	529	530	531	533	534	535	536	537	538	539
600	540	541	542	543	544	545	546	547	547	548
700	549	550	551	551	552	553	554	554	555	556
800	557	557	558	559	559	560	561	562	562	563
900	563	564	565	565	566	566	567	567	568	569
1000	569	574	578	583	587	591	594	597	600	603
2000	605	608	610	614	616	618	620	621	623	625
3000	625	626	628	629	631	632	633	634	636	637
4000	638	639	640	641	642	643	644	645	646	647
5000	648	649	650	651	652	652	653	654	655	656
6000	656	657	658	658	659	660	660	661	662	662
7000	663	664	664	665	665	666	666	667	667	668
8000	668	669	670	670	671	671	671	672	672	673
9000	673	674	674	675	675	676	676	677	677	677
10000	673	681	684	688	691	694	696	699	701	703
20000	705	707	709	711	712	715	715	717	718	719
30000	720	722	723	724	725	726	727	728	729	730
40000	731	732	732	733	734	735	736	736	737	738
50000	739	739	740	741	741	742	743	743	744	744
60000	745	746	746	747	747	748	748	749	749	750
70000	750	751	751	752	752	753	753	753	754	754
80000	755	755	756	756	756	757	757	758	758	758
90000	759	759	759	760	760	760	761	761	761	762
100000	762	762	763	763	763	764	764	764	764	765

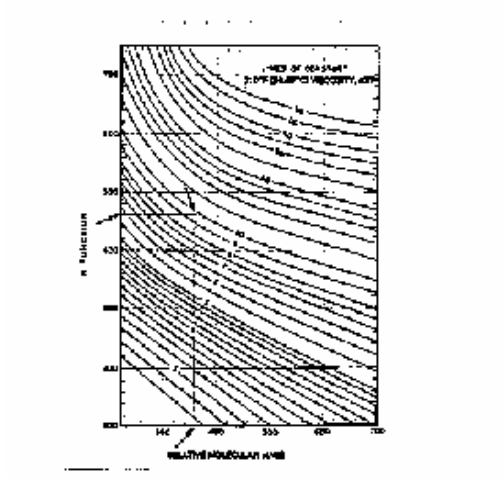


Figure 3. Lines of Constant Viscosity (mm^2/g) at 98.89°

Carbon Number at 5% Distillation Point

(ASTM D 2887 See Test for Viscosity for Copyright permission.)

"Carbon number" is number of carbon atoms in a molecule. Determine the boiling point distribution of the sample by gas chromatography using the following conditions:

The system must have the following performance characteristics:

- sensitivity: 1% dodecane must be detected with a peak height of at least 10% of full scale under the conditions prescribed below.
- stability: when operated at the required sensitivity level, the baseline drift is not more than 1% of full scale per hour
- repeatability of retention times: 6 sec for each component of the calibration mixture.
- resolution (R): determined for a solution of 1% of each of hexadecane and octadecane in n-octane is not less than three and not more than eight, using the following formula:

  $R = 2d / (W_1+W_2)$

where

d is the distance in mm between the peak maxima of hexadecane and octadecane
W1 is the peak width in mm at the baseline of hexdecane
W2 is the peak width in mm at the baseline of octadecane

Typical conditions which may be used are:

- Column: packed with: 5% SE-30
- Column temperature: 10 -350° rate: 6.5° / min.
- Carrier gas: helium
- Detector: FID; temperature: 370°
- Injection block temperature: 370°

Calibration mixture:

Prepare a mixture of hydrocarbons of known boiling points covering the range of the sample. At least one compound must have a boiling point lower than the initial boiling point of the sample.

Calibration

Cool the column to the selected starting temperature (the retention time for the initial boiling point must be at least 1 min) and inject the calibration mixture. Record the retention time of each peak maximum and the peak areas for each component. Plot the retention time of each peak versus the corresponding normal boiling point of that component in degrees Celsius to obtain a calibration curve.

Sample analysis

Using the exact conditions used in the calibration run, inject the sample. Record the area of each time segment at fixed time intervals not greater than 1% of the retention time equivalent to a boiling point of 538° obtained from the calibration curve.

Calculation

Sum the area segments to obtain the cumulative area at each time interval during the run. At the point of the chromatogram, where the baseline at the end first becomes steady, observe the cumulative area counts. Move back along the record until a cumulative area equal to 99.5% of the total at the steady point appears. Mark this point as the final boiling point. Observe the area counts at the start of the run until the point is reached, where the cumulative area count is equal to 0.5% of the total area. Mark this point as the initial boiling point of the sample. Divide the cumulative area at each interval between the initial and final boiling points by the total cumulative area and multiply by 100. This will give the cumulative percent of the sample recovered at each time interval. Tabulate the cumulative percent recovered at each interval and the retention time at the end of the interval. Using linear interpolation, if necessary, determine the retention time associated with 5% and read the corresponding boiling temperature from the calibration curve.

Congealing Range

Melt in a glass tube (25 mm in diameter and 100 mm in length, the glass being 1 mm in thickness) about 5 g of the sample by heating gently to 15-20° above the expected congealing range. By means of a perforated stopper, fasten the tube in a wide-mouthed bottle of clear glass, approximately 70 mm in diameter and 150 mm in height. Suspend a standard thermometer in the melted sample so that it will serve as a stirrer, cool if necessary, and stir the mass slowly until the mercury remains stationary for 30 sec. Discontinue stirring and allow the thermometer to hang, with the bulb in the centre of the sample, and observe the rise of the mercury column. The highest point to which it rises is the congealing temperature.

Free Fatty Acids

(Based on AOCS Method Ca 5a-40)

Unless otherwise directed in the specification monograph, weigh accurately the appropriate amount of the sample, indicated in the table below, into a 250-ml Erlenmeyer flask or other suitable container. Add 2 ml of phenolphthalein TS to the specified amount of hot alcohol, neutralize with alkali to the first faint but permanent pink colour, and then add the hot neutralized alcohol to the sample container. Titrate with the appropriate normality of sodium hydroxide, shaking vigorously, to the first permanent pink colour of the same intensity as that

of the neutralized alcohol. The colour must persist for at least 30 sec. Calculate the percentage of free fatty acids (FFA) in the sample by the formula VN/W, in which V is the volume and N is the normality, respectively, of the sodium hydroxide used, W is the weight of the sample, in g, and e is the equivalence factor given in the monograph.

FFA Range (%)	g of sample	ml of alcohol	Strength of NaOH
0.00-0.2	56.4 ± 0.2	50	0.1
0.2-1.0	28.2 ± 0.2	50	0.1
1.0-30.0	7.05 ± 0.05	75	0.25
30.0-50.0	7.05 ± 0.05	100	0.25-1.0
50.0-100	3.525 ± 0.001	100	1.0

Hydroxyl Value

(Based on AOCS Method Cd 13-60)

Note: *This method involves use of pyridine which should be used with appropriate caution.*

Hydroxyl value is defined as the number of mg of potassium hydroxide required to neutralize the amount of acetic acid capable of combining by acetylation with 1 g of sample.

Weigh accurately the appropriate amount of sample according to the expected hydroxyl value and transfer it into a 250-ml glass-stoppered Erlenmeyer flask.

Hydroxyl value	Sample weight (g)
0 to 20	10
20 to 50	5
50 to 100	3
100 to 200	2

Pipet 5.0 ml of pyridine/acetic anhydride TS into the flask. (For samples having a 0-20 hydroxyl value, add an additional 5 ml of pyridine/acetic anhydride TS to the flask.) Thoroughly mix the contents by gently swirling. Pipet 5.0 ml of pyridine/acetic anhydride TS into an empty flask for the reagent blank. (If 10.0 ml of the reagent were used for the acetylation, use a 10.0-ml blank.) Place the flasks on a steam bath, under reflux condensers, and heat for 1 h. To hydrolyze excess acetic anhydride, add sufficient water (not exceeding about 10 ml) through the condensers to the flasks. If the solution separates into two layers, add sufficient pyridine to obtain a homogeneous solution. Heat on a steam bath for 10 min with reflux condensers attached. Add 25 ml of neutralized n-butanol, about half of it through the condensers and the remainder to wash down the sides of the flasks after removal of the condensers. Add 1 ml of phenolphthalein TS and titrate to a faint pink endpoint with 0.5 N ethanolic KOH solution. To correct for free acid, mix about 10 g of the sample, accurately weighed, with 10 ml of pyridine (neutralized to phenolphthalein), add 1 ml of

phenolphthalein TS and titrate to a faint pink endpoint with 0.5 N ethanolic potassium hydroxide.

Calculate the hydroxyl value by the formula:

$$\text{Hydroxyl value} = [(B + (W_A / C) - S) \times \underline{N} \times 56.1] / W$$

where

A is ml of KOH solution required for the free acid determination;
B is ml of KOH solution required for the reagent blank;
C is the weight of sample used for the free acid determination;
S is ml of KOH solution required for titration of the acetylated sample;
W is weight of sample used for acetylation; and
N is normality of the ethanolic KOH solution.

Identification Tests for Functional Groups

Ester Hydrolysis

Reflux 1 g of sample with 15 ml of 0.5 N ethanolic potassium hydroxide for 1 h. Add 15 ml of water, acidify with dilute hydrochloric acid TS (about 6 ml). Oily drops or a white to yellowish-white solid is produced which is soluble in 5 ml of hexane.

Remove the hexane layer, extract again with 5 ml of hexane and again remove the hexane layer. Collect all the hexane extracts together. The fatty acids thus extracted may be identified by gas-liquid chromatography (see Test A). Carry out the whole of the procedure in a fume cupboard. The aqueous layer is used for Tests B through H.

Test A: Methyl esters of fatty acids

(Based on AOCS Methods Ce 1-62, Ce 1f-96, Ce 1h-05)

Apparatus

Use a suitable gas chromatograph equipped with a flame ionization detector (FID) and containing a 50-m × 0.25-mm id capillary fused silica column, or equivalent, containing a suitable highly polar stationary phase (0.20 m)film, such as CP™-Sil 88, SP-2650, SP-2340, BPX-70, or SP2560.

Note: *For accurate determination of all fatty acids present in non-ruminant animal and vegetable oils and fats, a 100m SP2560 or CP-Sil 88 column is recommended.*

Operating Conditions

The operating conditions may vary with the instrument used, but a suitable chromatogram may be obtained isothermally at temperatures between 170° and 198°, depending on the column stationary phase. Inlet temperature (injector), 250°; detector, 250°; and a suitable hydrogen or helium carrier gas flow.

Standard Solutions

Column performance is checked using a suitable mixture of fatty acid methyl esters covering the range of fatty acids under investigation Fatty acid methyl esters with a wide range of carbon numbers and double-bond configurations can be purchased. A mixture containing C12:0; 9c-18:1; 11c-18:1, 9c,12c,15c-18:3; 11c-20:1; and an Internal Standard (C21:0) using each carrier gas and column combination. Since commercial GC designs are different, to achieve optimal separation small changes in the sample size, sample concentration or oven

temperature may be required. If so, adjust the sample size, sample concentration or oven temperature until the best separation results are obtained. Baseline separation of the various components in both the standard and the sample preparations is desirable.

Sample Preparation (for fats and oils) (Based on AOCS Method Ce 2-66)

Introduce 100 to 1000 mg of the fat into a 50- or 125-mL reaction flask. Add 4 to 10 ml of 0.5 N methanolic sodium hydroxide, and add a boiling chip. Attach a condenser, and heat the mixture on a steam bath until the fat globules go into solution. This step should take 5 to 10 min. Add 5 to 12 ml of 12.5% boron fluoride–methanol reagent (this reagent contains 125 g/l of boron fluoride in methanol and is available commercially) through the condenser, and boil for 2 min. Add 2 to 5 ml of heptane through the condenser, and boil for 1 min longer. Remove from heat, remove condenser, and add about 15 ml of saturated sodium chloride solution. Stopper the flask, and shake vigorously for 15 sec. Transfer about 1 ml of the heptane solution into a test tube and add a small amount of anhydrous sodium sulfate. The dry heptane solution may then be injected directly into a gas chromatograph.

The methyl esters should be analyzed as soon as possible. They may be kept in an atmosphere of nitrogen in a screwcap vial at 2° for 24 h. For longer storage, they should be sealed in a glass ampoule, subjected first to a vacuum and then backfilled with nitrogen and stored at −20° (freezer).

Procedure

Inject an appropriate volume (1 µl) of sample into the chromatograph. If an automated system is used, follow the manufacturer's instructions; if calculations are to be done manually, proceed as follows:

Calculate the area percent of each component (CN) by the equation

$$CN = [AN/TS] \times 100,$$

in which AN is the area of the peak corresponding to component CN and TS is the total area for all detected components [$TS = \Sigma AN$].

Calculated Iodine Value (Based on AOCS Method Cd 1c-85)

Using the fatty acid composition determined above, calculate the Iodine value (IV) as follows:

- Triglycerides, iodine value = (% hexadecenoic acid × 0.950) + (% octadecenoic acid × 0.860) + (% octadecadienoic acid × 1.732) + (% octadecatrienoic acid × 2.616) + (% eicosenoic acid × 0.785) + (% docosenoic acid × 0.723)
- Free fatty acids, iodine value = (% hexadecenoic acid × 0.9976) + (% octadecenoic acid × 0.8986) + (% octadecadienoic acid × 1.810) + (% octadecatrienoic acid × 2.735) + (% eicosenoic acid × 0.8175) + (% docosenoic acid × 0.7497).

Note: *This procedure is not intended to be a rapid method, but rather gives two results from one analysis. For oils with an unsaponifiable content greater than 0.5% (e.g., fish oils), and for materials with low iodine value the calculation tends to be low. Calculated IV based on GC fatty acid composition of non-triglyceride lipid materials such as partial esters of glycerol, partial esters of sorbitol/sorbitan/isosorbide esters, partial esters of polyoxyethylene sorbitol/sorbitan/isosorbide or glycerol, etc. will provide the calculated IV of only the fatty acids used to prepare the partial esters. To obtain the actual IV of partial esters with nonfatty acid polyol diluents, the chlorinated Wijs Reagent IV method should be used. IV*

values of partial esters via the Wijs method are lower than those obtained by GC because of the dilution effect of the polyol material.

Test B: Acetic Acid

Transfer about 5 ml of the aqueous layer resulting from the hydrolysis into a dish, add excess calcium carbonate and evaporate until dry. Transfer the major part of the residue into a glass tube. Place a filter paper, moistened with Reagent for acetone (a saturated solution of o-nitrobenzaldehyde in sodium hydroxide TS, freshly prepared) on top of the tube. Heat over a micro flame. The yellow colour of the paper changes into greenish blue by reaction of the Reagent for acetone, with the calcium acetate formed.

Test C: Succinic Acid

Transfer one drop of the aqueous layer resulting from the hydrolysis and a drop of a 0.5% solution of ammonium chloride and several mg of zinc powder into a micro test tube.

The mouth of the tube is covered with a disk of filter paper moistened with a solution in benzene of 5% p-dimethylamino-benzaldehyde and 20% trichloroacetic acid. The bottom of the test tube is heated vigorously with a micro flame for about 1 min. Depending on the amount of succinic acid or succinimide, a red-violet or pink stain appears on the paper.

Test D: Fumaric Acid

Transfer 1 ml of the aqueous layer resulting from the hydrolysis with 1 ml of 2 N sodium carbonate into a test tube. Add 2 or 3 drops of 0.1 N potassium permanganate. The solution is promptly discoloured.

Test E: Tartaric Acid

Evaporate about 5 ml of the aqueous layer resulting from the hydrolysis in a porcelain dish until dry. Add 2 ml of concentrated sulfuric acid containing 0.5% of pyrogallol and heat on a steam bath. An intense violet colour is produced.

Test F: Citric Acid

To 3 ml of the aqueous layer resulting from the hydrolysis add a few drops of 1% potassium permanganate and warm until the colour has disappeared. Then add an excess of bromine TS. A white precipitate (pentabromoacetone) is formed immediately or on cooling.

Evaporate 1 ml of the aqueous layer resulting from the hydrolysis in a porcelain dish, add 1 ml of a mixture of 1 vol acetic anhydride and 5 vol of pyridine into the warm dish. A violet colour is produced. (Tartaric acid produces a green colour.)

Test G: Lactic Acid

Transfer 0.2 ml of the aqueous layer resulting from the hydrolysis and 2 ml of concentrated sulfuric acid into a test tube and place for 2 min in boiling water. Cool and add 1 or 2 drops of a 5% guaiacol solution in ethanol. A red colour is immediately produced.

If tartaric acid is present according to Test E, it must be removed as follows: transfer 3 ml of the aqueous layer resulting from the Hydrolysis and an excess of calcium hydroxide as a powder into a test tube, place in boiling water for 5 min, shaking several times, cool and filter.

Test H: Glycerol

Transfer 5 ml of the aqueous layer resulting from the hydrolysis into a test tube. Add excess calcium hydroxide as a powder, place in boiling water for 5 min, shaking several times, cool and filter.

Transfer one drop of the filtrate into a tube and add about 50 mg of potassium hydrogen sulfate. Place a filter paper, moistened with Reagent for acrolein (a 5% solution of disodium pentacyanonitrosylferrate in water and a 20% piperidine solution in water; mix the solutions 1:1 immediately before use) on the top of the tube. Heat over a micro flame. A blue coloured filter paper indicates the presence of glycerol. The colour changes to light red after addition of sodium hydroxide TS.

The test cannot be employed in the presence of ethylene glycol or lactic acid, since they decompose under the prescribed conditions yielding acetaldehyde which reacts with the reagents in the same manner as acrolein.

Iodine Value (Modified Wijs Method)

(Based on AOCS Method Cd 1d-92)

The iodine value (IV) is a measure of unsaturation and is expressed as the number of g of iodine absorbed, under the prescribed conditions, by 100 g of the test substance. For fats and oils, the Iodine Value may be calculated from the results of gas chromatographic quantification of methyl ester (see Methyl Esters of Fatty Acids, above)

Reagents

Cyclohexane
Glacial acetic acid
Wijs Solution: this reagent should be purchased commercially.
Potassium iodide TS
N sodium thiosulfate

Procedure

The appropriate weight of the sample, in g, is calculated by dividing the number 25 by the expected iodine value. Melt the sample, if necessary, and filter it through a dry filter paper. Transfer the accurately weighed quantity of sample into a clean, dry, 500-ml glass-stoppered bottle or flask containing 20 ml of glacial acetic acid/cyclohexane, 1:1, v/v, and pipet 25.0 ml of Wijs Solution into the flask. The excess of iodine should be between 50% and 60% of the quantity added, that is, between 100% and 150% of the quantity absorbed. Swirl, and let stand in the dark for 1.0 h where the iodine value is <150 and for 2.0 h where the iodine value is ≥150. Add 20 ml of potassium iodide TS and 100 ml of recently boiled and cooled water, and titrate the excess iodine with 0.1 N sodium thiosulfate, adding the titrant gradually and shaking constantly until the yellow colour of the solution almost disappears. Add starch TS, and continue the titration until the blue colour disappears entirely. Toward the end of the titration, stopper the container and shake it violently so that any iodine remaining in solution in the glacial acetic acid/cyclohexane layer may be taken up by the potassium iodide solution. Concomitantly, conduct two determinations on blanks in the same manner and at the same temperature.

Calculation

Calculate the iodine value by the formula

$$(B - S) \times 12.69N/W$$

in which

B – S is the difference between the volumes of sodium thiosulfate required for the blank and for the sample, respectively;
N is the normality of the sodium thiosulfate;
W is the weight, in g, of the sample.

1-Monoglyceride and Free Glycerol Contents

Preparation of Samples

Solid Samples in Flake Form: Mix without melting and take a portion for analysis.

Solid Samples not in Flake Form: Melt at not more than 10° above melting point, mix thoroughly and take a portion for analysis. Do not attempt to test samples which contain so much free glycerol that it separates when the sample solidifies.

Semi-solid and Liquid Samples: Liquefy by heating at not more than 10° above melting point, mix thoroughly, and take a portion for analysis. Do not attempt to test samples which contain so much free glycerol that it separates from the sample when cooled to room temperature.

Caution: *The sample must not be subjected to a temperature in excess of that required to melt it, as this may reduce the monoglyceride content if any soap is present.*

Procedure for 1-Monoglyceride

Weigh to the nearest mg duplicate samples of 1 g into a 100-ml glass-stoppered volumetric flask. Dissolve in 50 ml of chloroform. Add 25 ml of water and shake vigorously for 30-60 sec. Transfer the aqueous layer to a glass-stoppered 100-ml volumetric flask, using a glass siphon. If an emulsion forms due to the presence of soap in the sample, add 3 or 4 ml of glacial acetic acid to break the emulsion. Extract 3 more times using 25, 25 and 20 ml of distilled water. Add chloroform to the flask until the level of the chloroform coincides with the 100-ml mark. Using the glass siphon, transfer as much as possible of the aqueous layer above the chloroform layer to the flask containing the aqueous extracts. The aqueous extracts in the volumetric flask are saved for the determination of free glycerol.

Pipet 50 ml of acetic periodic acid TS into each of a series of 500-ml glass-stoppered Erlenmeyer flasks. Prepare 3 for blanks, adding 50 ml of chloroform to two and 50 ml of water to the third. The titrations of the water and chloroform blanks are used as a check (within 0.5 ml) on the chloroform. Pipet 50 ml of chloroform sample solution into one the flasks containing 50 ml of acetic periodic acid TS and shake gently to effect thorough mixing. Allow to stand for at least 30 min but not longer than 1.5 h. To each flask add 20 ml of potassium iodide TS. Mix by gentle shaking, allow to stand at least 1 min but not more than 5 min before titrating. Do not allow to stand in strong sunlight. Add 100 ml of distilled water and titrate with 0.1 N sodium thiosulfate. Use a variable speed magnetic stirrer to keep the solution thoroughly mixed. Continue the titration to the disappearance of the brown iodine colour from the aqueous layer. Add 2 ml of starch TS and continue the titration to the disappearance of iodine from the chloroform layer and the disappearance of the blue iodo-starch colour from the aqueous layer.

Calculation of 1-monoglyceride content as pure monostearate:

$$\% \text{ 1-monoglyceride} = [(B - S) \times N \times 17.927] / W$$

where

B is the sodium thiosulfate consumed in the titration of blank containing 50 ml of chloroform;
S is the sodium thiosulfate consumed in the titration of sample;
N is the exact normality of 0.1 N sodium thiosulfate; and
W is the weight of sample, represented by aliquot pipetted for test.

The 1-monoglyceride content may be calculated in terms of a monoester other than the monostearate by dividing the molecular weight of the monoglyceride by 20 and substituting this value for 17.927 in the formula above.

Procedure for free Glycerol

Add distilled water to the combined aqueous extracts from the monoglyceride determination until the volume is 100 ml and mix thoroughly. Pipet 50 ml of acetic periodic acid TS into each of a series of 500-ml glass-stoppered Erlenmeyer flasks. Pipet 50 ml of aqueous sample solution into one of the flasks containing 50 ml of acetic periodic acid TS and shake gently to effect thorough mixing. Continue as described under the procedure for monoglyceride, second paragraph commencing "Allow to stand for at least 30 min...".

Calculation of glycerol content:

$$\% \text{ free glycerol} = [(B - S) \times N \times 2.30] / W$$

where

B is the sodium thiosulfate consumed in the titration of blank containing 50 ml of water;
S is the sodium thiosulfate consumed in the titration of sample;
N is the exact normality of 0.1 N thiosulfate; and
W is the weight of sample represented by aliquot pipetted for test.

Polycyclic Aromatic Hydrocarbons

General Instructions

Because of the sensitivity of the test, the possibility of errors arising from contamination is great. It is of the greatest importance, therefore, that all glassware be scrupulously cleaned to remove all organic matter such as oil, grease, detergent residues, etc. Examine all glassware, including stoppers and stopcocks, under ultraviolet light to detect any residual fluorescent contamination. As a precautionary measure it is a recommended practice to rinse all glassware with purified isooctane immediately before use. No grease is to be used on stopcocks or joints. Great care to avoid contamination of wax samples in handling and to assure absence of any extraneous material arising from inadequate packaging is essential. Because some of the polynuclear hydrocarbons sought in this test are very susceptible to photo-oxidation, the entire procedure is to be carried out under subdued light.

Apparatus

Separatory funnels: 250-ml, 500-ml, 1,000-ml, and preferably 2,000-ml capacity, equipped with tetrafluoroethylene polymer stopcocks.

Reservoir: 500 ml capacity, equipped with a 24/40 standard taper male fitting at the bottom and a suitable balljoint at the top for connecting to the nitrogen supply. The male fitting should be equipped with glass hooks.

Chromatographic tube: 180 mm in length, inside diameter to be 15.7 ± 0.1 mm, equipped with a coarse, fritted-glass disc, a tetrafluoroethylene polymer stopcock, and a female 24/40

standard tapered fitting at the opposite end. (Overall length of the column with the female joint is 235 mm).

Disc: Tetrafluoroethylene polymer 2-inch diameter disc approximately 3/16-inch thick with a hole bored in the center to closely fit the stem of the chromatographic tube.

Heating jacket: Conical, for 500-ml separatory funnel. (Used with variable transformer heat control).

Suction flask: 250-ml or 500-ml filter flask.

Condenser: 24/40 joints, fitted with a drying tube, length optional.

Evaporation flask (optional): 250-ml or 500-ml capacity all-glass flask equipped with standard taper stopper having inlet and outlet tubes permit passage of nitrogen across the surface of the liquid to be evaporated.

Vacuum distillation assembly: All glass (for purification of dimethyl sulfoxide); 2-l distillation flask with heating mantle; Vigreaux vacuum-jacketed condenser (or equivalent) about 45 cm in length and distilling head with separable cold finger condenser. Use of tetrafluoroethylene polymer sleeves on the glass joints will prevent freezing. Do not use grease on stopcocks or joints.

Spectrophotometric cells: Fused quartz cells, optical path length in the range of 5.000 ± 0.005 cm; also for checking spectrophotometer performance only, optical path length in the range 1.000 ± 0.005 cm. With distilled water in the cells, determine any absorbance differences.

Spectrophotometer: Spectral range 250 - 400 nm with spectral slit width of 2 nm or less, under instrument operating conditions for these absorbance measurements, the spectrophotometer shall, also meet the following performance requirements:

- Absorbance repeatability, ±0.01 at 0.4 absorbance.
- Absorbance accuracy, ±0.05 at 0.4 absorbance.
- Wavelength repeatability, ±0.2 nm.
- Wavelength accuracy, ±1.0 nm.

Nitrogen cylinder: Water-pumped or equivalent purity nitrogen in cylinder equipped with regulator and valve to control flow at 5 p.s.i.g.

Reagents and materials

Organic solvents:

All solvents used throughout the procedure shall meet the specifications and tests described below. The isooctane, benzene, acetone, and methyl alcohol designated in the list following this paragraph shall pass the following test:

To the specified quantity of solvent in a 250-ml Erlenmeyer flask, add 1 ml of purified n-hexadecane and evaporate on the steam bath under a stream of nitrogen (a loose aluminium foil jacket around the flask will speed evaporation). Discontinue evaporation when not over 1 ml of residue remains. (to the residue from benzene add a 10-ml portion of purified isooctane, re-evaporate, and repeat once to insure complete removal of benzene).

Alternatively, the evaporation time can be reduced by using the optional evaporation flask. In this case the solvent and n-hexadecane are placed in the flask on the steam bath, the tube assembly is inserted, and a stream of nitrogen is fed through the inlet tube while the outlet tube is connected to a solvent trap and vacuum line in such a way as to prevent any flow-back of condensate into the flask.

Dissolve the 1 ml of hexadecane residue in isooctane and make to 25 ml volume. Determine the absorbance in the 5 cm path length cells compared to isoooctane as reference. The absorbance of the solution of the solvent residue (except for methyl alcohol) shall not exceed 0.01 per cm path length between 280 and 400 nm. For methyl alcohol this absorbance value shall be 0.00.

Isooctane (2,2,4-trimethylpentane): Use 180 ml for the test described in the preceding paragraph. Purify, if necessary, by passage through a column of activated silica gel (Grade 12 or equivalent) about 90 cm in length and 5 cm to 8 cm in diameter.

Benzene, reagent grade: Use 150 ml for the test. Purify, if necessary, by distillation or otherwise.

Acetone, reagent grade: Use 200 ml for the test. Purify, if necessary, by distillation.

Eluting mixtures:

- 10% benzene in isooctane: Pipet 50 ml of benzene into a 500-ml glass-stoppered volumetric flask and adjust to volume with isooctane, with mixing.
- 20% benzene in isooctane: Pipet 50 ml of benzene into a 250-ml glass-stoppered volumetric flask, and adjust to volume with isooctane, with mixing.
- Acetone-benzene-water mixture: Add 20 ml of water to 380 ml of acetone and 200 ml of benzene, and mix.

n-Hexadecane, 99% olefin-free: Dilute 1.0 ml of n-hexadecane to 25 ml with isooctane and determine the absorbance in a 5-cm cell compared to isooctane as reference point between 280-400 nm. The absorbance per centimeter path length shall not exceed 0.00 in this range. Purify, if necessary, by percolation through activated silica gel or by distillation.

Methyl alcohol, reagent grade: Use 10.0 ml of methyl alcohol. Purify, if necessary, by distillation.

Dimethyl sulfoxide: Pure grade, clear, water-white, m.p. 18º minimum. Dilute 120 ml of dimethyl sulfoxide with 240 ml of distilled water in a 500-ml separatory funnel, mix and allow to cool for 5-10 min. Add 40 ml of isooctane to the solution and extract by shaking the funnel vigorously for 2 min. Draw off the lower aqueous layer into a second 500 ml separatory funnel and repeat the extraction with 40 ml of isooctane. Draw off and discard the aqueous layer. Wash each of the 40 ml isooctane portions three times with 50 ml portions of distilled water. Shaking time for each wash is 1 min. Discard the aqueous layers. Filter the first isooctane portion through anhydrous sodium sulfate prewashed with isooctane (see sodium sulfate below for preparation of filter), into a 250-ml Erlenmeyer flask, or optionally into the evaporating flask. Wash the first separatory funnel with the second 40 ml isooctane portion, and pass through the sodium sulfate into the flask. Then wash the second and first separatory funnels successively with a 10 ml portion of isooctane, and pass the solvent through the sodium sulfate into the flask. Add 1 ml of n-hexadecane and evaporate the isooctane on the steam bath under nitrogen. Discontinue evaporation when not over 1 ml of residue remains. To the residue, add a 10 ml portion of isooctane and re-evaporate to 1 ml of hexadecane. Again, add 10 ml of isooctane to the residue and evaporate to 1 ml of hexadecane to insure complete removal of all volatile materials. Dissolve the 1 ml of hexadecane in isooctane and make to 25 ml volume. Determine the absorbance in 5 cm path length cells compared to isooctane as reference. The absorbance of the solution should not exceed 0.02 per cm path length in the 280-400 nm range.

Note: *Difficulty in meeting this absorbance specification may be due to organic impurities in the distilled water. Repetition of the test omitting the dimethyl sulfoxide will disclose their presence. If necessary to meet the specification, purify the water by re-distillation, passage through an ion-exchange resin, or otherwise.*

Purify, if necessary, by the following procedure: To 1,500 ml of dimethyl sulfoxide in a 2 l glass-stoppered flask, add 6.0 ml of phosphoric acid and 50 g of Norit A (decolorizing carbon, alkaline) or equivalent. Stopper the flask, and with the use of a magnetic stirrer (tetrafluoro-ethylene polymer coated bar) stir the solvent for 15 min. Filter the dimethyl sulfoxide through four thicknesses of fluted paper (18.5 cm, Schleicher & Schuell, No. 597, or equivalent). If the initial filtrate contains carbon fines, refilter through the same filter until a clear filtrate is obtained. Protect the sulfoxide from air and moisture during this operation by covering the solvent in the funnel and collection flask with a layer of isooctane. Transfer the filtrate to a 2-l separatory funnel and draw off the dimethyl sulfoxide into the 2-l distillation flask of the vacuum distillation assembly and distil at approximately 3 mm Hg pressure or less. Discard the first 200 ml fraction of the distillate and replace the distillate collection flask with a clean one. Continue the distillation until approximately 1 l of the sulfoxide has been collected.

At completion of the distillation, the reagent should be stored in glass-stoppered bottles since it is very hygroscopic and will react with some metal containers in the presence of air.

Phosphoric acid, 85% reagent grade

Sodium borohydride, 98%

Magnesium oxide (Sea Sorb 43, Food Machinery Company, Westvaco Division, distributed by chemical supply firms, or equivalent): Place 100 g of the magnesium oxide in a large beaker, add 700 ml of distilled water to make a thin slurry, and heat on a steam bath for 30 min with intermittent stirring. Stir well initially to insure that all the absorbent is completely wetted. Using a Buchner funnel and a filter paper (Schleicher & Schuell No. 597, or equivalent) of suitable diameter, filter with suction. Continue suction until water no longer drips from the funnel. Transfer the absorbent to a glass trough lined with aluminium foil (free from rolling oil). Break up the magnesia with a clean spatula and spread out the absorbent on the aluminium foil in a layer about 1-2 cm thick. Dry for 24 h at 160±1°. Pulverize the magnesia with mortar and pestle. Sieve the pulverized absorbent between 60-180 mesh. Use the magnesia retained on the 180-mesh sieve.

Celite 545: Johns-Manville Company, diatomaceous earth, or equivalent.

Magnesium oxide-Celite 545 mixture: Place the magnesium oxide (60-180 mesh) and the Celite 545 in 2 to 1 proportions, respectively, by weight in a glass-stoppered flask large enough for adequate mixing. Shake vigorously for 10 min. Transfer the mixture to a glass trough lined with aluminium foil (free from rolling oil) and spread it out on a layer about 1 to 2 cm thick. Reheat the mixture at 160±1° for 2 h, and store in a tightly closed flask.

Sodium sulfate, anhydrous, reagent grade, preferably in granular form: For each bottle of sodium sulfate reagent used, establish as follows the necessary sodium sulfate prewash to provide such filters required in the method: Place approximately 35 g of anhydrous sodium sulfate in a 30 ml coarse, fritted-glass funnel or in a 65 ml filter funnel with glass wool plug; wash with successive 15 ml portions of the indicated solvent until a 15 ml portion of the wash shows 0.00 absorbance per cm path length between 280 nm and 400 nm when tested as prescribed under Organic solvents above. Usually three portions of wash solvent are sufficient.

Procedure

Before proceeding with the analysis of a sample, determine the absorbance in a 5 cm path cell between 250 nm and 400 nm for the reagent blank by carrying out the procedure, without a wax sample, at room temperature, recording the spectra after the extraction stage and after the complete procedure as prescribed. The absorbance per cm path length following the extraction stage should not exceed 0.040 in the wavelength range from 250 to 400 nm; the absorbance per cm path length following the complete procedure should not exceed 0.070 in the wavelength range from 250 to 299 nm, inclusive, or 0.045 in the wavelength range from 300 nm to 400 nm. If in either spectrum the characteristic benzene peaks in the 250-260 nm region are present, remove the benzene by the procedure under Organic solvents, above, and record absorbance again.

Place 300 ml of dimethyl sulfoxide in a 1-l separatory funnel and add 75 ml of phosphoric acid. Mix the contents of the funnel and allow to stand for 10 min. (The reaction between the sulfoxide and the acid is exothermic. Release pressure after mixing, then keep funnel stoppered). Add 150 ml of isooctane and shake to pre-equilibrate the solvents. Draw off the individual layers and store in glass-stoppered flasks.

Place a representative 1 kg sample of wax, or if this amount is not available, the entire sample, in a beaker of a capacity about three times the volume of the sample and heat with occasional stirring on a steam bath until the wax is completely melted and homogenous. Weigh four 25 ± 0.2 g portions of the melted wax in separate 100 ml beakers. Reserve three of the portions for later replicate analyses as necessary. Pour one weighed portion immediately after re-melting (on the steam bath) into a 500 ml separatory funnel containing 100 ml of the pre-equilibrated sulfoxide-phosphoric acid mixture that has been heated in the heating jacket at a temperature just high enough to keep the wax melted. *(***Note:** *In preheating the sulfoxide-acid mixture, remove the stopper of the separatory funnel at intervals to release the pressure).*

Promptly complete the transfer of the sample to the funnel in the jacket with portions of the pre-equilibrated isooctane, warming the beaker, if necessary, and using a total volume of just 50 ml of the solvent. If the wax comes out of solution during these operations, let the stoppered funnel remain in the jacket until the wax re-dissolves. (Remove stopper from the funnel at intervals to release pressure).

When the sample is in solution, remove the funnel from the jacket and shake it vigorously for 2 min. Set up three 250 ml separatory funnels with each containing 30 ml of pre-equilibrated isooctane. After separation of the liquid phases, allow to cool until the main portion of the sample-isooctane solution begins to show a precipitate. Gently swirl the funnel when precipitation first occurs on the inside surface of the funnel to accelerate this process. Carefully draw off the lower layer, filter it slowly through a thin layer of glass wool fitted loosely in a filter funnel into the first 250 ml separatory funnel, and wash in tandem with the 30 ml portions of isooctane contained in the 250 ml separatory funnels. Shaking time for each wash is 1 min. Repeat the extraction operation with two additional portions of the sulfoxide-acid mixture, replacing the funnel in the jacket after each extraction to keep the sample in solution and washing each extractive in tandem through the same three portions of isooctane.

Collect the successive extractives (300 ml total) in a separatory funnel (preferably 2-liter), containing 480 ml of distilled water, mix, and allow to cool for a few min after the last extractive has been added. Add 80 ml of isooctane to the solution and extract by shaking the funnel vigorously for 2 min. Draw off the lower aqueous layer into a second separatory funnel (preferably 2-l) and repeat the extraction with 80 ml of isooctane. Draw off and

discard the aqueous layer. Wash each of the 80 ml extractives three times with 100 ml portions of distilled water. Shaking time for each wash is 1 min. Discard the aqueous layers. Filter the first extractive through anhydrous sodium sulfate prewashed with isooctane (see Sodium Sulfate above for preparation of filter) into a 250-ml Erlenmeyer flask (or optionally into the evaporation flask). Wash the first separatory funnel with the second 80 ml isooctane extractive and pass through the sodium sulfate. Then wash the second and first separatory funnels successively with a 20 ml portion of isooctane and pass the solvent through the sodium sulfate into the flask. Add 1 ml of n-hexadecane and evaporate the isooctane on the steam bath under nitrogen. Discontinue evaporation when not over 1 ml of residue remains. To the residue, add a 10 ml portion of isooctane, re-evaporate to 1 ml of hexadecane, and repeat this operation once more.

Quantitatively transfer the residue with isooctane to a 25 ml volumetric flask, make to volume, and mix. Determine the absorbance of the solution in the 5 cm path length cells compared to isooctane as reference between 280 nm and 400 nm (take care to lose none of the solution in filling the sample cell). Correct the absorbance values for any absorbance derived from reagents as determined by carrying out the procedure without the sample. If the corrected absorbance does not exceed the limits prescribed in the Characteristics, the sample meets the ultraviolet absorbance specifications. If the corrected absorbance per cm path length exceeds the limits prescribed in the Characteristics, proceed as follows:

Quantitatively transfer the isooctane solution to a 125 ml flask equipped with 24/40 joint and evaporate the isooctane on the steam bath under a stream of nitrogen to a volume of 1 ml of hexadecane. Add 10 ml of methyl alcohol and approximately 0.3 g of sodium borohydride (Minimize exposure of the borohydride to the atmosphere. A measuring dipper may be used). Immediately fit a water-cooled condenser equipped with a 24/40 joint and with a drying tube into the flask, mix until the borohydride is dissolved, and allow to stand for 30 min at room temperature, with intermittent swirling. At the end of this period, disconnect the flask and evaporate the methyl alcohol on the steam bath under nitrogen until the sodium borohydride begins to come out of the solution. Then add 10 ml of isooctane and evaporate to a volume of about 2-3 ml. Again, add 10 ml of isooctane and concentrate to a volume of approximately 5 ml. Swirl the flask repeatedly to assure adequate washing of the sodium borohydride residues.

Fit the tetrafluoroethylene polymer disc on the upper part of the stem of the chromatographic tube, then place the tube with the disc on the suction flask and apply the vacuum (approximately 135 mm Hg). Weigh out 14 g of the 2:1 magnesium oxide-Celite 545 mixture and pour the adsorbent mixture into the chromatographic tube in approximately 3 cm layers. After the addition of each layer, level off the top of the adsorbent with a flat glass rod or metal plunger by pressing down firmly until the adsorbent is well packed. Loosen the topmost few mm of each adsorbent layer with the end of a metal rod before the addition of the next layer. Continue packing in this manner until all the 14 g of the adsorbent is added to the tube. Level off the top of the adsorbent by pressing down firmly with a flat glass rod or metal plunger to make the depth of the adsorbent bed approximately 12.5 cm in depth. Turn off the vacuum and remove the suction flask. Fit the 500 ml reservoir onto the top of the chromatographic column and pre-wet the column by passing 100 ml of isooctane through the column. Adjust the nitrogen pressure so that the rate of descent of the isooctane coming off of the column is between 2-3 ml per min. Discontinue pressure just before the last of the isooctane reaches the level of the adsorbent. (**Caution:** *Do not allow the liquid level to recede below the adsorbent level at any time*). Remove the reservoir and decant the 5 ml isooctane concentrate solution onto the column and with slight pressure again allow the liquid level to recede to barely above the adsorbent level. Rapidly complete the transfer similarly with two 5

ml portions of isooctane, swirling the flask repeatedly each time to assure adequate washing of the residue. Just before the final 5 ml wash reaches the top of the adsorbent, add 100 ml of isooctane to the reservoir and continue the percolation at the 2-3 ml per min rate. Just before the last of the isooctane reaches the adsorbent level, add 100 ml of 10% benzene in isooctane to the reservoir and continue the percolation at the aforementioned rate. Just before the solvent mixture reaches adsorbent level, add 25 ml of 20% benzene in isooctane to the reservoir and continue the percolation at 2-3 ml per min until all this solvent mixture has been removed from the column. Discard all the elution solvents collected up to this point.

Add 300 ml of the acetone-benzene-water mixture to the reservoir and percolate through the column to elute the polynuclear compounds. Collect the eluate in a clean 1-l separatory funnel. Allow the column to drain until most of the solvent mixture is removed. Wash the eluate three times with 300 ml portions of distilled water, shaking well for each wash. (The addition of small amounts of sodium chloride facilitates separation). Discard the aqueous layer after each wash. After the final separation, filter the residual benzene through anhydrous sodium sulfate prewashed with benzene (see Sodium sulfate under "Reagents and Materials" for preparation of filter) into a 250-ml Erlenmeyer flask (or optionally into the evaporation flask). Wash the separatory funnel with two additional 20 ml portions of benzene which are also filtered through the sodium sulfate. Add 1 ml of n-hexadecane and completely remove the benzene by evaporation under nitrogen, using the special procedure to eliminate benzene as previously described under Organic Solvents. Quantitatively transfer the residue with isooctane to a 25 ml volumetric flask and adjust the volume. Determine the absorbance of the solution in the 5 cm path length cells compared to isooctane as reference between 250 and 400 nm. Correct for any absorbance derived from the reagents as determined by carrying out the procedure without a wash sample. If either spectrum shows the characteristic benzene peaks in the 250 - 260 nm region, evaporate the solution to remove benzene by the procedure under Organic Solvents. Dissolve the residue, transfer quantitatively, and adjust to volume in isooctane in a 25 ml volumetric flask. Record the absorbance again. If the corrected absorbance does not exceed the limits prescribed in the Characteristics the sample meets the ultraviolet absorbance specifications.

Polyglycerol Determination in Polyglycerol Esters

Principle

Polyglycerol esters are saponified with alcoholic potassium hydroxide solution and the fatty acids removed by extraction. The polyols are converted to trimethylsilyl (TMS) derivatives and analyzed by gas liquid chromatography.

Procedure

Preparation of the polyol sample

Weigh about 0.5 g of sample and reflux with 20 ml of ethanolic potassium hydroxide solution (1 N) for 2 h. Reduce the volume of ethanol by evaporation at 45-50° in a stream of nitrogen. Add 10 ml of water and convert the soaps to free fatty acids by acidifying with concentrated hydrochloric acid. Extract the fatty acids from the aqueous phase with successive 20 ml portions of light petroleum (boiling range 40-60°). Wash the combined petroleum extracts with water (20 ml) and combine the wash with the aqueous phase.

Adjust the aqueous polyol solution to pH 7.0 with aqueous potassium hydroxide solution with the aid of a pH-meter. Evaporate to a small volume (2-3 ml) under reduced pressure and extract three times with 30 ml of boiling ethanol. Filter off any residue and evaporate the ethanol under reduced pressure to yield a viscous liquid sample of polyols.

Dissolve a 0.1 g sample of polyol in 0.5 ml of warm pyridine (previously dried over potassium hydroxide) in a 10-ml capped vial. Add 0.2 ml hexamethyl disilazane, shake, add 0.2 ml trimethylchlorosilane and shake again. Place on a warm plate (about 80°) for 3-5 min. Check that white fumes are present indicating an excess of reagent.

Gas-liquid chromatography

Any suitable gas chromatograph equipped as follows:

Stationary phase: 3% OV-1
Carrier gas: Nitrogen
Temperature of injection port: 275°
Column temperature: 90° to 330° at 4-6°/min
Detector type: FID, temperature: 350°

Inject a 2.0 µl sample of TMS derivatives of polyols. The following sequence of peaks are recorded on the resultant chromatogram :

Elution sequence of peaks	Identity	Description
1	Solvent	Out of scale
2	Glycerol	Single peak
3	Cyclic diglycerols	Single peak
4	Diglycerols	Single peak
5	Cyclic triglycerols	Single peak
6	Triglycerols	Single peak
7	Cyclic tetraglycerols	Single peak
8	Tetraglycerols	Multiple peaks
9	Pentaglycerols	Single peak
10	Hexaglycerols	Single peak
11	Heptaglycerols	Single peak
12	Octaglycerols	Single peak
13	Nonaglycerols	Barely discernible in the tail of peak 12

Calculation

Measure each peak area by a suitable method.

% di-, tri- and tetraglycerols =
(Sum of corrected areas of peaks 3 to 8 x 100) / (Sum of corrected areas of peaks 3 to 13)

% polyglycerols equal to or greater than heptaglycerol =
(Sum of corrected areas of peaks 11 to 13 x 100) / (Sum of corrected areas of peaks 3 to 13)

Polyols

Principle

The sample is hydrolysed. Fatty acids are removed by ion exchange in combination with hexane extraction. The components of the filtrate are separated by thin layer chromatography.

Procedure

Reflux 1 g of sample with 15 ml of 0.5 N ethanolic potassium hydroxide for 1 h. Add 25 g of strong cation ion exchange resin (such as Amberlite I R 120, H-form), 50 ml of hexane and 25 ml of water. Stir the mixture for about 1 h. Filter off the resin and, after allowing the layers of the filtrate to separate, take the aqueous layer for TLC.

Spot 2 to 5 µl portions of the aqueous layer onto a silica gel G plate and also 2 µl of 5% solutions of glycerol, ethylene glycol and 1,2-propylene glycol.

Develop the chromatogram using chloroform:acetone:5 N ammonia (10:80:10) as the solvent system. After development, dry the plate in a stream of air until the water and ammonia have been removed.

Spray the plate with a solution of lead acetate (1% w/v in toluene) and heat the plate for 5 min at 110°. 1,2-Diols are revealed as white spots on a brown background.

The following are examples of R_f values that may be obtained:

Polyol	Rf
Glycerol	0.35
Ethylene glycol	0.70
1,2-Propylene glycol	0.85

Propylene Glycol Dimer and Trimer Determination

Principle

Propylene glycol esters of fatty acids are saponified with alcoholic potassium hydroxide and the fatty acids are removed by extraction. The aqueous polyol fraction is analyzed by gas-liquid chromatography for di- and tripropylene glycol.

Reagents

Potassium hydroxide, ethanolic solution (56.1 g/l ethanol)
Sodium hydroxide solution (50% w/v in water)
Hydrochloric acid (1 + 1 by volume)
Hexane

Standards: propylene glycol (1,2-propanediol) dipropylene glycol (1,1-oxydi-2-propanol) tripropylene glycol triethylene glycol

Procedure

Preparation of polyols

Weigh about 50 g of sample, to the nearest 0.01 g, together with about 2 g to nearest 0.001 g, of triethylene glycol (as internal standard) into a 1-l saponification flask. Add 350 ml of ethanolic potassium hydroxide solution and reflux under an air condenser for 2 h with stirring. Transfer the contents of the flask quantitatively to an 800-ml beaker. Wash the flask and condenser with 200 ml of hot distilled water and evaporate the combined sample solution and washings to about 200 ml on a steam bath. Acidify the hot residue to pH 2 by the dropwise addition, with agitation, of hydrochloric acid (1 + 1). Transfer the hot mixture quantitatively to a 2-l separatory funnel with 200 ml of hexane and shake. Allow the layers to

separate. Transfer the lower aqueous layer to a 500-ml separatory funnel and add 200 ml of fresh hexane. Shake, then allow the layers to separate. Draw off the lower aqueous layer into a 600-ml beaker and add the hexane phase to the original 2-l separatory funnel. Wash the 500-ml separator with two further 200-ml portions of hexane and add these to the 2-l separator. Wash the original 800-ml beaker with 100 ml of water and add to the hexane solution. Mix thoroughly and allow the layers to separate. Draw off the aqueous layer into the 600-ml beaker containing the aqueous fractions. Wash the 800-ml beaker once more with 100 ml of water and add the drained aqueous layer to the combined aqueous fractions. Adjust the pH of the combined aqueous solution to pH 7.0-7.05 (using pH-meter) with sodium hydroxide solution and evaporate to about 150 ml on a steam bath. Transfer quantitatively to a 250 ml round bottom flask and concentrate further to about 50 ml by distilling through a vertical Vigreux column to prevent the loss of low boiling glycols. Decant the concentrated polyols from the precipitated salts, through a filter funnel containing Whatman No. 1 paper, into a 100-ml volumetric flask. Wash the salts and flask twice with 20 ml of water and add to the volumetric flask via the filter funnel. Dilute to the mark with water, mix well and use for the GLC polyol analysis. If salts reprecipitate in the volumetric flask refilter before sampling.

Gas-liquid chromatography

Any suitable gas chromatograph equipped with:

Stationary phase: 15% Carbowax 20 M
Carrier gas: Helium
Temperature of injection port: 290°
Column temperature: 150° to 230° at 2°/min
Detector type: FID; temperature: 250°.

Prepare a reference solution of glycols in water by weighing the glycol standards, to the nearest 0.1 mg, into a 100 ml volumetric flask as follows:

- Propylene glycol: 1 g
- Dipropylene glycol: 0.01 g
- Tripropylene glycol: 0.005 g
- Triethylene glycol: 0.01 g

Make up to the mark with water and mix.

Inject aliquots of this reference standard solution and establish the sensitivity setting to yield measurable peaks. Similarly inject the prepared sample solution.

Calculation

Measure each peak area by a suitable method, such as multiplying the peak height by the peak width at half the peak height, and calculate the % dimer and trimer in the sample as follows:

$$\% \text{ dimer} = (A_{DS} \times W_{DR}) / A_{DR} \times (W_{IS} \times A_{IR}) / (A_{IS} \times W_{IR}) \times 100 / W$$

$$\% \text{ trimer} = (A_{TS} \times W_{TR}) / A_{TR} \times (W_{IS} \times A_{IR}) / (A_{IS} \times W_{IR}) \times 100 / W$$

where

A_{DS} is the peak area of dipropylene glycol (sample solution);
A_{DR} is the peak area of dipropylene glycol (reference solution);
A_{TS} is the peak area of tripropylene glycol (sample solution);
A_{TR} is the peak area of tripropylene glycol (reference solution);
A_{IS} is the peak area of triethylene glycol (sample solution);

A_{IR} is the peak area of triethylene glycol (reference solution);
W is the weight (g) of sample of propylene glycol esters of fatty acids;
W_{DR} is the [1]weight (g) of dipropylene glycol in the reference solution;
W_{TR} is the [1]weight (g) of tripropylene glycol in the reference solution;
W_{IS} is the [1]weight (g) of triethylene glycol added to the sample solution; and
W_{IR} is the [1]weight (g) of triethylene glycol in the reference solution.

[1] Corrected for the actual content of polyol (e.g. W_{DR} is the weight of dipropylene glycol taken x % of assay).

Saponification

(AOCS Methods Tl 1a-64 and Cd 3-25)

Weigh accurately about 20 g of the sample and subject to alkaline hydrolysis by refluxing for 2 h with ethanolic potassium hydroxide TS containing a quantity of potassium hydroxide 100% in excess of the calculated amount required to saponify the sample completely. After hydrolysis, convert the ethanolic soap solution to an aqueous solution by the addition of water and evaporation of the alcohol on a steam bath. Acidify the hot aqueous soap solution with sulfuric acid to liberate the fatty acid. Extract the acid solution with 3 portions of petroleum ether to remove the fatty acid. Evaporate the petroleum ether extracts on a steam bath and dry the residue to constant weight under vacuum at 75° to recover the fatty acid. Multiply the weight of recovered fatty acid by 100/W to obtain the yield of fatty acid from a 100-g sample (where W is the exact weight of sample taken). The fatty acid can be identified by determination of the physical and chemical constants, e.g. the solidification temperature, or by gas-liquid chromatography.

Neutralize the aqueous polyol solution to pH 7 with potassium hydroxide. Evaporate the polyol solution to a moist residue on a steam bath and extract the polyol from the salts with 3 portions of hot absolute ethanol. Evaporate off the alcohol on a steam bath and dry the residue to constant weight under vacuum at 75° to yield the polyol moiety of the sample. Multiply the weight of recovered polyol by 100/W to obtain the yield of polyols from a 100-g sample (where W is the exact weight of sample taken).

Saponification Value

Definition

Saponification value is defined as the number of mg of potassium hydroxide required to neutralize the free acids and saponify the esters in 1 g of test substance.

Procedure

Melt the sample, if necessary, and filter it through a dry filter paper to remove any traces of moisture. Unless otherwise directed, weigh accurately into a 250-ml flask a sample of such size (usually about 4-5 g) that the titration of the sample solution after saponification will require between 45 and 55% of the volume of 0.5 N hydrochloric acid required for the blank. Add 50.0 ml of ethanolic potassium hydroxide TS from a pipet and allow the pipet to drain for a definite period of time. Prepare and conduct blank determinations simultaneously with the sample and similar in all respects. Connect an air condenser to each flask and boil gently but steadily, with occasional mixing, until the sample is completely saponified. (This usually requires about 1 h for normal samples). After the flasks and condensers have cooled somewhat but not sufficiently for the contents to gel, wash down the inside of the condensers with a few ml of distilled water. Disconnect the condensers, add about 1 ml of

phenolphthalein TS to reach flask, and titrate with 0.5 N hydrochloric acid until the pink colour has just disappeared.

Saponification value = [56.1 x N (A - B)] / W

Where

A is ml of HCl required for the titration of the blank;
B is ml of HCl required for the titration of the sample;
W is the weight of sample in g; and
N is normality of the HCl.

Sorbitan Ester Content

Principle

Sorbitan esters may be assayed by alkaline saponification followed by recovery of the polyol and determination of the isosorbide content by gas-liquid chromatography.

Procedure

Saponification and recovery of the polyol

Weigh accurately about 25 g of the sample into a 500-ml round-bottomed boiling flask. Add 250 ml of ethanol and a quantity of potassium hydroxide 100% in excess of the calculated amount required for saponification (approximately 7.5 g). Boil the mixture for 2 h under reflux. Transfer the saponification mixture to an 800-ml beaker. Rinse the flask with about 100 ml of water and add to the mixture. Place the beaker on a steam bath to evaporate the alcohol. Add water occasionally to replace the ethanol. When the odour of ethanol can no longer be detected, adjust the volume of the soap solution to approximately 250 ml with hot water.

Acidify the hot soap solution with stirring using sufficient 1:1 sulfuric acid to provide a 10% excess. Heat and stir the mixture until the fatty acid layer separates. Transfer the hot mixture to a 500-ml separating funnel using hot water to rinse the beaker. Cool the contents of the funnel and extract three times with 100-ml portions of petroleum ether. Combine the petroleum ether extracts in a second funnel and wash once with 100 ml of water. Combine the water wash with the aqueous phase in an 800-ml beaker.

Neutralize the polyol solution with 10% aqueous potassium hydroxide solution to pH 7 using a pH meter. Place the beaker in a steam bath and evaporate the solvent to incipient dryness. Extract the residue four times with 150-ml portions of boiling absolute ethanol. Filter the combined extracts into a 1-l suction flask through a 10-cm Buchner funnel containing a 1-3 cm bed of silicagel. Wash the funnel with absolute ethanol. Transfer the filtrate and washings to a 1,000-ml volumetric flask. Cool to room temperature and dilute to volume with ethanol. Use this as the sample solution for gas-liquid chromatography.

Gas-liquid chromatography

The experimental operating conditions for the isosorbide analyses are not critical, suitable conditions are listed below . Minor fluctuations in temperature and gas flow rate do not affect resolution or analytical results.

Stationary phase: 15% Carbowax 20 M
Carrier gas: Argon
Temperature of injection port: 295°
Column temperature: 195°

Detector type: FID; 250°

Calculation

The isosorbide content of an aliquot of the recovered polyol solution is estimated directly from a calibration curve prepared from a standard sorbitan ester or by multiplying the observed peak area by the slope of the curve (µg of isosorbide per unit area).

$$\% \text{ Sorbitan ester} = (I \times 20) / (f \times W)$$

where

I is µg of isosorbide found in the aliquot of recovered polyol solution by gas chromatography;
W is g of sorbitan ester taken for analysis; and
f is fractional isosorbide yield from standard sorbitan esters (see **Note** below).

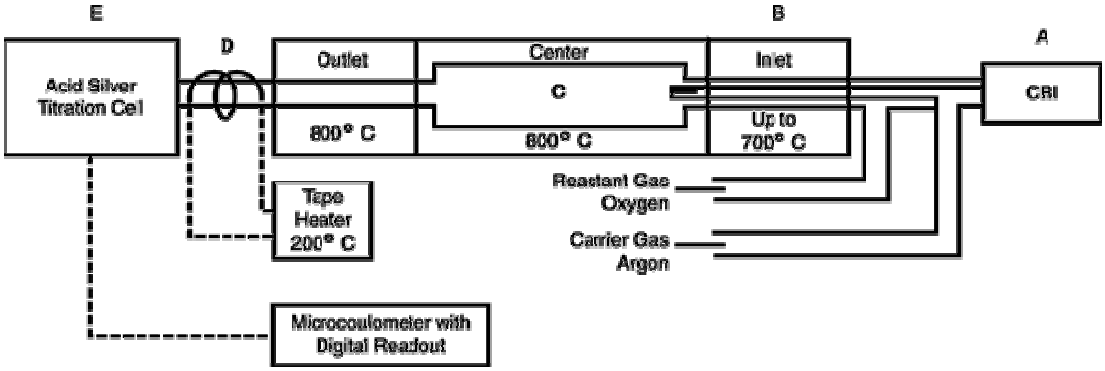
Note: *A known sample of sorbitan ester is treated as described under Saponification and recovery of the polyol above. Suitable aliquots of the solution are subjected to the gas chromatographic procedure. The fractional yield of isosorbide is calculated from the weight of sample corrected to a dry, fatty-acid-free basis. The procedure is estimated to have an accuracy of 5%.*

Sulfur

Note: *All reagents used in this test should be reagent grade: water should be of high purity, and gases must be high-purity grade.*

Apparatus

The Dohrmann Microcoulometric Titrating System (MCTS-30), or its equivalent as shown in the figure below should be used. It consists of a constant rate injector (A), a pyrolysis furnace (B), a quartz pyrolysis tube (C), a granular tin scrubber (D), a titration cell (E), and a microcoulometer with a digital readout (F).



Granular-Tin Scrubber: Place 5 g of 20/30 mesh granular reagent grade tin between quartz-wool plugs in an elongated 18/8-12/5 standard-taper adaptor which connects the pyrolysis tube and the titration cell.

Microcoulometer: Must have variable attenuation, gain control, and be capable of measuring the potential of the sensing-reference electrode pair, and comparing this potential with a bias potential, amplifying the potential difference, and applying the amplified difference to the working-auxiliary electrode pair so as to generate a titrant. Also the microcoulometer output voltage signal must be proportional to the generating current.

Pyrolysis Furnace: The sample should be pyrolyzed in an electric furnace having at least two separate and independently controlled temperature zones, the first being an inlet section that can maintain a temperature sufficient to volatilize the entire organic sample. The second zone shall be a pyrolysis section that can maintain a temperature sufficient to pyrolyze the organic matrix and oxidize all the organically bound sulfur. A third outlet temperature zone is optional.

Pyrolysis Tube: Must be fabricated from quartz and constructed in such a way that a sample, which is vaporized completely in the inlet section, is swept into the pyrolysis zone by an inert gas where it mixes with oxygen and is burned. The inlet end of the tube shall hold a septum for syringe entry of the sample and side arms for the introduction of oxygen and inert gases. The center, or pyrolysis section, should be of sufficient volume to ensure complete pyrolysis of the sample.

Sampling Syringe: A microliter syringe of 10-µl capacity capable of accurately delivering 1 to 10 µl of sample into the pyrolysis tube. Three-inch x 24-gauge needles are recommended to reach the inlet zone of the pyrolysis furnace.

Titration Cell: Must contain a sensor-reference pair of electrodes to detect changes in triiodide ion concentration, a generator anode-cathode pair of electrodes to maintain constant triiodide ion concentration, and an inlet for a gaseous sample from the pyrolysis tube. The sensor electrode shall be platinum foil and the reference electrode platinum wire in saturated triiodide half cell. The generator anode and cathode half-cell shall also be placed on a magnetic stirrer.

Preparation of Apparatus

Carefully insert the quartz pyrolysis tube into the furnace, attach the tin scrubber, and connect the reactant and carrier-gas lines. Add the Cell Electrolyte Solution (see below) to the titration cell and flush the cell several times. Maintain an electrolyte level of 3.8 cm (1.5 in.) above the platinum electrodes. Place the titration cell on a magnetic stirrer and connect the cell inlet to the tin scrubber outlet. Position the platinum foil electrodes (mounted on the movable cell head) so that the gas-inlet flow is parallel to the electrodes with the generator anode adjacent to the generator cathode. Assemble and connect the coulometer in accordance with the manufacturer's instructions. Double-wrap the adaptor containing the tin scrubber with heating tape and turn the heating tape on. Adjust the flow of the gases, the pyrolysis furnace temperature, the titration cell, and the coulometer to the desired operating conditions. Typical operating conditions are as follows:

Reagent gas flow (oxygen): 200 cm^3/min
Carrier-gas flow (Ar, He): 400 cm^3/min
Furnace temperatures
Inlet zone: 700^o (maximum)
Pyrolysis zone: 800 - 1000^o
Outlet zone: 800º (maximum)
Tin-Scrubber flow rate: 200 cm^3/min
Titration cell: Stirrer speed set to produce slight vortex
Coulometer
Bias voltage: 160 mV
Gain: 50
Constant Rate Injector: 0.25 µl/sec

The tin scrubber must be conditioned to sulfur, nitrogen, and chlorine before quantitative analysis can be achieved. A solution containing 10 mg/kg butyl sulfide, 100 mg/kg pyridine,

and 200 mg/kg chlorobenzene in isoctane has proven an effective conditioning agent. With a fresh scrubber installed and heated, two 30-µl samples of this conditioning agent injected at a flow rate of 0.5 µl/sec produces a steady increasing response, with final conditioning indicated by a constant reading from the offset during the second injection.

Reagents

- Argon or Helium, (Argon preferred) High-purity grade: two-stage regulators must be used.
- Cell Electrolyte Solution: dissolve 0.5 g of potassium iodide and 0.6 g of sodium azide in 500 ml of high-purity water, add 5 ml of glacial acetic acid and dilute to 1 L. Store in a dark bottle or in a dark place and prepare fresh at least every 3 months.
- Oxygen: high-purity grade.
- Iodine: resublimed, 20 mesh or less.
- Sulfur Standard (approximately 100 mg/kg): weigh accurately 0.1569 g of n-butyl sulfide, into a tared 500-ml volumetric flask. Dilute to the mark with isooctane and reweigh. Calculate the sulfur concentration (S), in percent, by the formula:

$$S = \frac{W_S}{W_C} \; x \; 2.192 \; x \; 105$$

where
Ws = weight of n-butyl sulfide, and
Wc = weight of the solution.

Calibration

Prepare a calibration standard (approximately 5 mg/kg) by pipetting 5 ml of Sulfur Standard into a 10-ml volumetric flask and diluting to volume with isooctane. Fill and clamp the syringe onto the constant rate injector, push the sliding carriage forward to penetrate the septum with the needle, and zero the meter in case of long-term drift in the automatic baseline zero circuitry. Switch S_1 automatically starts the stepper-motor syringe drive and initiates the analysis cycle. At 2.5 min (after setting switch S_1) set the digital meter with the scan potentiometer to correspond to the sulfur content of the known standard to the nearest 0.01 mg/kg. At the 3-min point, the number displayed on the meter stops, the plunger drive block is retracted to its original position, as preset by switch S_2 and a baseline re-equilibration period equal to the injection period must be allowed before a new sample may be injected. Repeat the Calibration step a total of at least four times.

Procedure

Rinse the syringe several times with sample: then fill it, clamp it onto the constant-rate injector push the sliding carriage forward to penetrate the septum with the needle, and zero the meter. Turn on switch S_1 to start the stepper-motor syringe drive automatically and initiate the analysis cycle. After the 3-min hold point, the number displayed on the meter corresponds to the sulfur content of the injected sample.

Viscosity, 100°

(ASTM D 445 Adapted, with permission, from the Annual Book of ASTM Standards, copyright American Society for Testing and Materials, 100 Harbor Drive, West Conshohocken, PA 19428. Copies of the complete ASTM standard may be purchased direct from ASTM)

Use a viscometer of the glass capillary type, calibrated and capable of measuring kinematic viscosity with a repeatability exceeding 0.35 % only in one case in twenty. Immerse the viscometer in a liquid bath at the temperature required for the test ± 0.1° ensuring that at no time of the measurement will any portion of the sample in the viscometer be less than 20 mm below the surface of the bath liquid or less than 20 mm above the bottom of the bath. Charge the viscometer with sample in the manner dictated by the design of the instrument. Allow the sample to remain in the bath for about 30 min. Where the design of the viscometer requires it, adjust the volume of sample to the mark. Use pressure to adjust the head level of the sample to a position in the capillary arm of the instrument about 5 mm ahead of the first mark. With the sample flowing freely, measure, in seconds (±0.2 s), the time required for the meniscus to pass from the first to the second timing mark. If the time is less than 200 s, select a viscometer with a capillary of smaller diameter and repeat the operation. Make a second measurement of the flow time. If two measurements agree within 0.2 %, use the average for calculating the kinematic viscosity. If the measurements do not agree, repeat the determination after thorough cleaning and drying the viscometer.

$$\text{Viscosity, } 100^{o} \text{ } (mm^2/s) = C \times t$$

where

C is the calibration constant of the viscometer (mm^2/s^2), and
t is the flow time (s)

FLAVOURING AGENTS

Acid Value

Dissolve about 10 g of sample, accurately weighed, in 50 ml of ethanol, previously neutralized to phenolphthalein TS with 0.1 N sodium hydroxide. Add 1 ml of phenolphthalein TS and titrate with 0.1 N sodium hydroxide until the solution remains faintly pink after shaking for 10 sec, unless otherwise directed. Calculate the Acid Value (AV) by the formula:

$$AV = (5.61 \times S) / W$$

in which

S is the number of ml of 0.1 N sodium hydroxide consumed in the titration of the sample, and W is the weight of the sample in g.

Assay

For Gas Chromatographic analysis procedures see Gas Chromatography in the Section on Analytical Techniques.

Boiling Point

See the Section on Appearance and Physical Properties, under General Methods.

Ester Determination

Transfer an accurately weighed quantity of the sample specified in the monograph into a 125-ml Erlenmeyer flask containing a few boiling stones. Add to this flask, and, simultaneously, to a similar flask for a blank test, 25.0 ml of 0.5 N ethanolic potassium hydroxide. Connect each flask to a reflux condenser, and heat the mixtures on a steam bath for exactly 1 h, unless otherwise directed in the monograph. Allow the mixtures to cool, add 10 drops of phenolphthalein TS, to each flask, and titrate the excess alkali in each flask with 0.5 N hydrochloric acid. Calculate the percentage of Ester (E) in the sample by the formula:

$$E = [(b - S)(100e)] / W$$

in which

b is the number of ml of 0.5 N hydrochloric acid consumed in the titration of the blank;
S is the number of ml of 0.5 N hydrochloric acid consumed in the titration of the sample; and
e is the equivalence factor given in the monograph, and W = the weight of the sample in mg.

Melting Point (Melting Range)

See the Section on Appearance and Physical Properties, under General Methods.

Refractive Index

See the Section on Appearance and Physical Properties, under General Methods.

Solubility in Ethanol

Unless otherwise stated in the specification, transfer a 1 ml sample into a calibrated 10-ml glass-stoppered cylinder graduated in 0.1-ml subdivisions, and add slowly, in small portions,

ethanol, the concentration and quantity of which are specified in the monograph. Maintain the temperature at 20°. A clear solution free from foreign matter should be obtained.

Solubility in General

See the Section on Appearance and Physical Properties, under General Methods.

Specific Gravity

See the Section on Appearance and Physical Properties, under General Methods.

HPLC Method for certain flavourings

3-methyl-2-oxobutanoic acid (631)
Sodium 3-methyl-2-oxobutanoate (631.1)
3-methyl-2-oxopentanoic acid (632)
Sodium 3-methyl-2-oxopentanoate (632.1)
4-methyl-2-oxopentanoic acid (633)
Sodium 4-methyl-2-oxopentanoate (633.1)
2-oxo-3-phenylpropionic acid (1478)
Sodium 2-oxo-3-phenylpropionate (1479)

Determine by HPLC using the following:

Note: All solutions should be prepared with ultra high quality (UHQ) deionized water

Apparatus:

HPLC system with a suitable pump, injector, and a data station

Column:	Stainless steel; 300 x 7.6 mm
Stationary phase:	Bio-Rad Aminex® HPX-87H or equivalent
Detector:	UV

HPLC conditions:

Column temperature:	35°
Mobile phase:	0.004 M Sulfuric acid
Flow rate:	0.6 ml/min
Injection volume:	50 µl
Detection:	210 nm
Run time:	30 min

Note: *The retention times of the compounds are as follows:*
3-methyl-2-oxobutanoic acid is 13.0 min
3-methyl-2-oxopentanoic acid is 14.8 min
4-methyl-2-oxopentanoic acid is 16.7 min
2-oxo-3-phenylpropionic acid is 24.7 min

Procedure:

Weigh about 100 mg of the sample, dissolve in a minimum amount of 0.2 M sodium hydroxide solution, and make up to 100 ml in a volumetric flask. Set up and condition the HPLC using the mobile phase. Inject the sample solution and determine the purity of the sample by the area normalization method.

Note: This method is also available from the on-line database on specifications for flavouring agents in the link to Analytical methods (Volume 4).

FOOD COLOURS

Chloride as Sodium Chloride

Note: *This determination is done in connection with Water Content (Loss on Drying) for food colours and the result is included in that calculation.*

Apparatus

- Potentiometric titration apparatus
- Silver indicator electrode
- Glass body calomel reference electrode or calomel reference electrode with potassium sulfate bridge

Reagents

- Nitric acid, 1.5 N, reagent grade
- Silver nitrate, 0.1 N, standard solution

Procedure

Accurately weigh 0.5 - 1.0 g of the colour sample (W_S), dissolve in 100 ml of water, and acidify with 5 ml of 1.5 N nitric acid. Place the silver and glass body calomel electrodes in the colour solution. If only a standard calomel reference electrode is available, connect it to the solution by means of the saturated potassium sulfate bridge. (Use of a glass body electrode as the reference electrode eliminates the need for the potassium sulfate bridge; this simplifies the apparatus considerably, and the glass body electrode is sufficiently constant to be used as a reference for this type of titration.)

Determine the chloride content of the solution by titration with the 0.1 N silver nitrate. (Each ml of 0.1 N silver nitrate is equivalent to 0.00585 g of sodium chloride.)

Calculation

Calculate the chloride content of the sample as percent sodium chloride using the following equation:

$$\% \text{ sodium chloride} = 100 \times (\text{ml titrant} \times 0.00585 \text{ g})/W_S.$$

Chloroform-Insoluble Matter

Apparatus

- Oven, 0 - 200° range
- Hot plate
- Crucible, fitted with glass fiber disk
- Vacuum flask
- Source of vacuum
- Desiccator

Reagents

- Chloroform, reagent grade

Procedure

Accurately weigh the quantity of sample indicated in each specification monograph (W_1) into a 250-ml beaker. Mix with 100 ml of chloroform (b.p. 61.1°). Stir and heat to boiling on the hot plate in a fume hood. Filter the hot solution through a weighed crucible (W_2). Transfer

the residue in the beaker to the crucible with chloroform. Wash the residue in the crucible with 10-ml portions of chloroform until the washings are colourless. Place the crucible in the oven at 100 - 150° for 3 h; cool the crucible in the desiccator. Weigh the cooled crucible (W_3).

Calculation

The percent chloroform-insoluble matter in the sample is $100 \times (W_3 - W_2) / W_1$.

Colouring Matters

Identification

Many of the colours used by food manufacturers are mixtures of colouring matters of the type described in the specification monographs, and some of the mixtures contain added diluents. A simple test to establish whether a powdered sample of colouring matter is a single colouring matter or a mixture of colouring matters is to sprinkle a very small quantity of the powder into each of two beakers, one containing water and the other containing concentrated sulfuric acid. Under these conditions, the specks of individual colouring matters can easily be seen as they dissolve; the test is surprisingly sensitive.

The positive identification of individual food colours is often quite difficult. A large number are the sodium salts of sulfonic acids, which have no precise melting point. In addition, synthesized colours usually contain subsidiary colouring matters, while colouring matters extracted from natural sources generally are mixtures of colours themselves. Identification, therefore, is best achieved by comparison of the observed properties with the properties of authentic commercial samples. The principle techniques in use are chromatography and spectrophotometry. Frequently, both are required, because the presence of subsidiary colouring matters might affect the observed spectra so that positive identification of the principal colour component cannot be made. For this reason, separation of the individual colouring matters by column, paper, or thin layer chromatography is advisable before attempting additional identification by spectrophotometry.

Subsidiary colouring matters are defined as those colouring matters that are produced during the manufacturing process in addition to the principal named colouring matter(s). Paper chromatography has been used for many years for identifying subsidiary colouring matters in water-soluble food colours. The assumption is generally made that spectrophotometric absorbances of subsidiary colouring matters are similar to that of the main colouring matter. Accordingly, standards of individual subsidiary colour matters are not required. The presence of colouring matters other than the principal and subsidiary colouring matters is usually detected on the chromatograms used to determine subsidiary colouring matters. Interpretation of the chromatograms for these colour impurities usually requires additional information.

High-performance liquid chromatography (HPLC) has been used successfully to separate, identify, and quantitate the subsidiary colouring matter contents of various food colours. Standards for individual subsidiary colouring matters are needed for this method. However, the specification limits in the monographs are, unless otherwise stated, linked to the paper chromatographic method and the conditions are provided under "Tests" in the specification.

Identification by chromatography

Paper and thin layer chromatography are often useful in identification of colouring matters and do not require expensive equipment. But it must be kept in mind that the R_f-value of a substance is generally an unreliable quantity because many factors, most of which are beyond the analyst's control, can have a major influence on the R_f-values. These factors

include: composition and age of the solvent mixture, concentration of solvent vapour in the atmosphere, quality of the chromatography paper, machine direction of commercially made paper, kind and quality of subsidiary colouring matters, concentration, pH-value of the solution, and temperature. For this reason, comparative chromatography using reference colours should always be used. By simultaneously running several colouring matters of similar concentration a number of these factors are eliminated.

Coincidence of migration distances with a single solvent system should be looked upon only as one criterion of identity and further tests should be made to confirm the finding.

The following table contains examples of the R_f-values that may be expected when 1% aqueous solutions of various colouring matters are subjected to thin layer chromatography on Silica Gel G in the ten solvent systems listed below. The compositions of the solvent systems, all of which must be freshly prepared, are:

Solvent System Number

1. iso-Propanol:ammonia (sp.gr. 0.880):water (7:2:1)
2. iso-Butanol:ethanol:water:ammonia (sp.gr. 0.880) (10:20:10:1)
3. Saturated aqueous potassium nitrate solution
4. Phenol:water (4:1, w/v)
5. Hydrochloric acid (sp.gr. 1.18):water (23:77)
6. Trisodium citrate:ammonia (sp.gr. 0.880):water (2 g:15 ml:85 ml)
7. Acetone:2-butanone :ammonia (sp.gr. 0.880):water (60:140:1:60)
8. n-Butanol:ethanol:pyridine:water (2:1:1:2)
9. iso-Propanol:ammonia (sp.gr. 0.880) (4:1)
10. n-Butanol:acetic acid (glacial):water (10:5:6)

R_f Values of Some Water-Soluble Colours (This Table does not indicate the acceptability of the listed colours for food use.)
Note: Numbers 1 to 10 refer to solvent systems (see above).

REDS	C.I. No.	INS No.	1	2	3	4	5	6	7	8	9	10
Ponceau 4R or Cochineal Red A	16255	124	0.66 (0.85)	0.75	0.88	0.03	0.95	1.00	0.60	0.90	0.11	0.52 0.00-0.57
Carmosine or Azorubine	14720	122	0.65 (0.77)	0.81	0.00-0.42	0.16	0.00 (0.00-0.32)	1.00	0.65	0.88	0.34 (0.46)	0.63 (0.11-0.70)
Amaranth	16185	123	0.62 (0.48, 0.76)	0.75 (0.83)	1.00 (0.00-1.00)	0.04 (0.16)	1.00	1.00	0.40 (0.64, 0.66)	0.90	0.10 (0.41)	0.39, 0.67
Erythrosine RS	45430	127	0.85 (0.68, 0.79)	0.91 (0.86, 0.74, 0.81)	0.00, 0.10	0.00-0.90 (0.41)	0.00	0.00-0.95	0.64, 0.66 (0.58)	0.89	0.66 (0.57, 0.43)	1.00
Red 2G	18050	--	0.68	0.80	0.37	0.12	0.00-0.71	1.00	0.64	0.90	0.36	0.68
ORANGES												
Orange G	16230	--	0.71 (0.67, 0.88)	0.80 (0.75)	0.64 (1.00, 0.35)	0.23, 0.15, 0.04	0.73	1.00	0.64 (0.62, 0.50, 0.67)	0.91	0.36 (0.32, 0.17)	0.69 (0.46, 0.82)
Orange RN	15970	--	0.83 (0.62)	0.88 (0.78)	0.00 (0.00-0.42)	0.42 (0.13)	0.13 (0.38)	0.76 (1.00)	0.68 (0.65)	0.92	0.64 (0.29)	0.82, 0.71
Sunset Yellow FCF or Orange Yellow S	15985	110	0.75 (0.68)	0.82 (0.74)	1.00 (0.00-1.00)	0.17, 0.03	1.00	1.00	0.65 (0.48)	0.90	0.34 (0.10, 0.22)	0.67 (0.46)
YELLOWS												
Tartrazine	19140	102	0.66	0.77	0.46-1.00	0.08	1.00	1.00	0.52	0.93	0.14	0.50
Yellow 2G	18965	--	0.63	0.80	0.77	0.21	0.74	1.00	0.62	0.92	0.21	0.75
Quinoline Yellow	47005	104	0.83, 0.88	0.88 (0.82)	0.00-1.00	0.65 (0.21)	0.26-1.00, 0.00-0.38	0.95 (0.35)	0.54 (0.68)	0.88	0.00-0.31, 0.64	0.11-0.75 (0.83)
Fast Yellow AB	13015	--	0.77	0.81	1.00	0.14	0.97	1.00	0.56	0.93	0.36	0.66
GREENS, BLUES, AND VIOLETS												
Green S or Acid Brilliant-Green BS or Lissamine Green	44090	142	0.44 (0.52, 0.68, 0.74)	0.61 (0.67, 0.75, 0.81, 0.84)	0.49 (0.24)	0.53 (0.05, 0.36, 1.00)	0.29 (0.43)	1.00	0.46 (0.56, 0.71)	0.75 (0.89, 0.92)	0.07	0.55
Indigo Carmine or Indigotin	73015	132	0.56 (0.70)	0.50-0.76 (0.78)	0.00 (0.05,	0.09, 0.18 (0.52)	0.92	0.94	0.66 (0.71,	0.89, 0.84	0.37 (0.00-	0.00-0.63

					0.90, 1.00)				0.73)		0.34)	
Indanthrene Blue or Solanthrene Blue RS or Anthragen Blue	69800	--	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Brilliant Blue FCF	42090	--	0.64 (0.73)	0.78	0.05	0.45 (0.68)	0.10	0.00-1.00	0.61 (0.68)	0.88	0.30 (0.49, 0.00-0.23)	0.53 (0.64)
Patent Blue V	42051	131	0.34-0.60	0.68	0.05	0.55	0.15	0.95	0.69 (0.72)	0.84 (0.92)	0.00-0.10	0.59
Violet 6B	42640	--	0.73 (0.67, 0.91)	0.80 (0.72)	0.00 (0.00-0.48)	0.62 (0.51-1.00)	0.00-0.37	0.00-1.00	0.67	0.89 (0.62)	0.37, 0.45, 0.70, 0.76	0.64 (0.71)
Methyl Violet	42535	--	0.91 (0.80)	0.56, 0.81, 0.90	0.00 (0.00-0.31)	0.79-1.00	0.00-0.80	0.00 (0.00-0.53)	0.00-0.68 (0.11, 0.28, 0.53, 1.00)	0.11 (0.90)	0.94, 0.87 (0.00-0.83)	0.75, 0.79 (0.00-0.70)
BROWNS AND BLACKS												
Brown FK	--	--	0.78, 0.71, 0.66	0.79, 0.86	1.00 (0.00-1.00)	0.69, 0.27, 0.15, 0.00	0.00-0.77	1.00	0.59, 0.64, 0.53 (0.37)	0.93	0.34 (0.26, 0.53)	0.00-0.73
Chocolate Brown FB	--	--	0.00-0.69	0.00-0.75	0.00-0.82	0.00 (0.00-0.23)	0.00-1.00	0.00-1.00	0.36, 0.51, 0.62	0.87	0.00-0.38	0.00-0.75
Chocolate Brown HT	20285	--	0.00-0.63	0.74	0.00-1.00	0.00 (0.00-0.16)	0.00-1.00	0.00-1.00	0.34, 0.43, 0.62	0.88	0.00-0.32	0.00-0.73
Black PN or Brilliant-Black BN	28440	151	0.66 (0.47)	0.75	0.00 (0.00-1.00)	0.00	1.00	1.00	0.38 (0.61)	0.85	0.05	0.00-0.43
Black 7984	27755	152	0.62	0.75	1.00	0.00	1.00	1.00	0.38 (0.61)	0.85	0.09	0.00-0.45

Key: C.I. No.: Colour Index Number; Figures in parentheses, ( ), indicate subsidiary spots of lower intensity; "0.xx-0.yy": Streak between spots.

Table from Pearson, D. (1973) J. Assoc. Public Anal., 11, 137-138. Reprinted from Environmental Carcinogens – Selected Methods of Analysis, Vol. 4 – Some Aromatic Amines and Azo Dyes in the General and Industrial Environment (IARC Publications No. 40), International Agency for Research on Cancer, Lyon, 1981. **Note:** E numbers in the original table have been replaced by the appropriate International Numbering System (INS) numbers of the Codex Alimentarius Commission.

Assessment of the colour shade should be made while the chromatograms are still moist with solvent and then again after drying. The shade should be assessed in both incident and transmitted daylight as well as under ultraviolet (UV) light, in which many colours show characteristic colour changes. Furthermore, UV light can often be used to identify the presence of colourless fluorescent impurities. If possible, use two UV emitters which yield different wave lengths; one lamp should emit around 250 nm.

Tests with acids, alkalis and other suitable reagents, in order to confirm the results, should be made. All tests may be carried out with fine capillary pipettes on each colour spot.

The following requirements should be met when identifying the colours in colouring matters by comparing to reference colours:

- equal migration distances in several solvent systems;
- equal shade in daylight and ultraviolet light; and
- equal colour changes with reagents.

Identification by spectrophotometry

Spectrophotometric methods of examination are among the most useful means of identification of colours. The UV, visible, and infrared regions of the electromagnetic spectrum are all employed.

The visible region of the spectrum is ordinarily examined as the first step in attempting to identify an unknown colour. Many colours show characteristic absorption bands in the visible region. Spectra in the UV region may also be of use and should be obtained together with the visible spectrum, if possible.

In the application of UV-visible spectrophotometry, spectra should always be obtained in more than one solvent, or if in a single solvent, under various conditions. Spectra of aqueous solutions should be obtained under neutral (buffered with ammonium acetate), acid (0.1 N hydrochloric acid), and alkaline (0.1 N sodium hydroxide) conditions.

A UV-visible absorption spectrum is ordinarily displayed as a plot of absorbance vs. wavelength. In addition to the wavelength maximum, the most characteristic and useful features of the absorption spectrum can be the "shoulders" or inflection points on the spectral curve. These features often make it possible to distinguish between two or more colouring matters that have absorption maxima at the same wavelength. Many colours can be definitively characterized by observing the extent to which the absorption maxima and other features of the absorption curve are changed by variation in pH or by other changes in the solvent.

Infrared absorption spectra offer another useful means of identification of compounds. An example of their use is in distinguishing Sunset Yellow and Orange GGN. Whereas the UV-visible absorption spectra of these colours are nearly identical, their infrared spectra are quite different in the region of the spectrum in which the sulfonic acid groups absorb strongly.

Infrared spectra of substances can be obtained using various sample preparations; the more commonly used are:

- solutions of the material in suitable solvents;
- suspensions of the material in a suitable liquid;
- potassium bromide pellets (in this technique, a small amount of the colouring matter, usually from 1 to 3 mg, is thoroughly mixed with pure, dry potassium bromide and the mixture is transferred to a suitable die and pressed into a thin pellet by exerting a pressure of 700 to 1,400 kg/cm^2).

Spectra are ordinarily displayed as % transmittance vs. wavenumber (cm^{-1}). The salient features of the spectra are the intensities of the absorption peaks, and their shapes.

Detailed discussion of the infrared absorption technique and interpretation of infrared spectra are beyond the scope of this volume. It must be pointed out, however, that some difficulties exist in the practical use of this technique for identifying colours.

The crystal structure or other physical state of the sample may affect the spectra obtained from suspensions or potassium bromide pellets. It is necessary to make certain that the unknown material has been treated in exactly the same manner as was the standard or known sample.

Care must be taken to ensure that absorption bands due to contaminants are identified. All materials to be tested should be free from water or other solvent before an infrared spectrum is obtained because water and all organic solvents absorb infrared radiation. Water-soluble colouring matters can often be prepared for analysis by dissolving samples in water, adding a little acetic acid, evaporating to apparent dryness, and then drying at about 100° to remove the residual water. Infrared spectra should be obtained of the dried solids, as well as blanks.

Other identification techniques

Sometimes chromatographic and spectrophotometric techniques will fail to provide positive identification of colouring matters. In such cases, the problem can often be solved by reducing the colouring matter or otherwise degrading it and identifying the resulting products. This technique is particularly applicable to identifying azo colours. The amine compounds resulting from the reduction can frequently be readily identified by chromatographic and spectrophotometric techniques.

Many other techniques have been applied to the identification of colouring matters. For example, many pigments have a well defined crystalline structure and can be identified by their X-ray diffraction patterns or by X-ray crystallography. Some colouring matters can be converted to crystalline derivatives and similarly identified. The descriptions of these and other available techniques are beyond the scope of this volume.

Total colouring matters content

Two general methods are used for determination of total colouring matters: ‘Colouring Matters Content by Spectrophotometry’ and ‘Colouring Matters Content by Titration with Titanous Chloride.’

When using the spectrophotometric method, the analyst should take into account the accuracy and precision of the spectrophotometer used for the analysis. All colours present in the sample that absorb in the same region as that of the main colour will contribute to the absorbance figure used to calculate the results; subsidiary colouring matters of markedly different hue will not be accounted for by this method. This method uses accepted absorptivity figures obtained from purified standard colours for calculating the total colouring matters content.

The titanous chloride reduction method assumes that isomers and subsidiary colouring matters have the same titanous chloride equivalent as the main colouring matter.

Colouring Matters Content by Spectrophotometry

Three experimental procedures are described. Procedure 1 is used for water-soluble colouring matters. Procedure 2 is used for organic solvent-soluble colouring matters, especially the synthetic carotenoids. (The solutions prepared in Procedure 2 are used in the identification tests for the carotenoids.) Procedure 3 is used for lakes.

Principle

The absorbance of a solution of the colouring matter is determined at its wavelength of maximum absorption and the total colouring matters content is calculated using a standard absorptivity value quoted in the specification monograph.

Apparatus

- UV-visible range spectrophotometer capable of accurate (± 1% or better) measurement of absorbance in the region of 350 - 700 nm with an effective slit width of 10 nm or less
- Spectrophotometer cells, 1 cm path length

Procedure 1 – Colouring matters content of water-soluble colouring matters

Accurately weigh 0.25 g (± 0.02 g) of the sample (W). Transfer to a 1-liter volumetric flask. Add freshly distilled water or the solvent prescribed in the specification monograph and swirl to dissolve. Make up to volume and mix. Dilute to a solution of suitable strength according to the details given in the specification monograph. Measure the absorbance (A) at the wavelength of maximum absorption in a 1 cm cell, using water or the prescribed solvent as the blank.

Calculation

Calculate the total colouring matters content of the sample using either of the following equations:

$$\% \text{ total colouring matters} = 100 \times (A \times 1 \text{ liter} \times F)/(a \times 1 \text{ cm} \times W)$$

or

$$\% \text{ total colouring matters} = 100 \times (A/A^{1\%}_{1cm}) \times (F/W)$$

where

A is the absorbance of the sample solution at the wavelength of maximum absorption;
$A^{1\%}_{1\ cm}$ is the specific absorbance of the standard indicated in the specification monograph;
a is the absorptivity of the standard in liter/(g·cm); and
F is the dilution factor (Volume diluted / Volume measured).

Procedure 2 – Colouring matters content of organic solvent-soluble colouring matters

Reagents

- Chloroform, reagent grade, acid free
- Cyclohexane, reagent grade

Accurately weigh 0.08 g (± 0.01 g) of the sample (W) into a 100-ml volumetric flask (V_1). Add 20 ml of chloroform and dissolve by swirling briefly. Make sure that the solution is clear. Make up to volume with cyclohexane and mix. Pipet 5.0 ml of the solution (v_1) into a second 100-ml volumetric flask (V_2) and make up to volume with cyclohexane. Pipet 5.0 ml of this diluted solution (v_2) into the final 100-ml volumetric flask (V_3) and make up to volume with cyclohexane. Measure the absorbance (A) of the twice-diluted solution at the wavelength of maximum absorption in a 1 cm cell, using cyclohexane as the blank.

Perform this procedure promptly, avoiding exposure to air insofar as possible and undertaking all operations in the absence of direct sunlight.

Calculation

Calculate the total colouring matters content of the sample using either of the following equations:

$$\% \text{ total colouring matters} = 100 \times (A \times V_1 \times V_2 \times V_3)/(a \times 10^{-3} \times v_1 \times v_2 \times W)$$

or

$$\% \text{ total colouring matters} = (A \times V_1 \times V_2 \times V_3)/(v_1 \times v_2 \times W \times A^{1\%}_{1\ cm})$$

where

A is absorbance of the sample solution at the wavelength of maximum absorption;
$A^{1\%}_{1\ cm}$ is the specific absorbance of the standard indicated in the specification monograph;
a is the absorptivity of the standard in liter/(g·cm)
V_1, V_2, and V_3 are the volumes of the three volumetric flasks (each 100 ml);
v_1 and v_2 are the volumes of the two pipets (each 5 ml);
a is absorptivity of the standard in liter/(g·cm); and
10^{-3} is the correction factor for a in ml/liter.

Procedure 3 – Colouring matters content of lakes

Reagents

- Potassium dihydrogen phosphate, reagent grade
- Sodium hydroxide, reagent grade
- Phosphoric acid, reagent grade
- Hydrochloric acid, reagent grade

Prepare pH 7 phosphate buffer as follows: Weigh 13.61 g of potassium dihydrogen phosphate into a 2000-ml beaker, dissolve in 200 ml of water, and dilute to 1,000 ml. Add about 90 ml of 1 N sodium hydroxide. Determine the pH using a pH-meter and adjust the pH to 7.0 using 0.1 N sodium hydroxide or diluted phosphoric acid.

Accurately weigh a quantity of lake which will give an absorbance approximately equal to that of the parent colour when the latter is tested according to Procedure 1, above. Transfer to a 250- ml beaker containing 10 ml hydrochloric acid previously diluted with water to approximately 50 ml. Heat with stirring to dissolve the lake, then cool to ambient temperature. Transfer to a 1-liter volumetric flask, make up to volume with pH 7 phosphate buffer, and mix. Proceed as detailed in Procedure 1, above, and in the specification monograph, using pH 7 phosphate buffer as the spectrophotometric blank.

Colouring Matters Content by Titration with Titanous Chloride

Principle

Titanous chloride (titanium trichloride, $TiCl_3$) reduces the colouring matter to yield titratable reduction products. The method assumes that isomers and subsidiary colouring matters have the same titanium trichloride equivalent as the main colouring matter.

Apparatus

- Titration apparatus (See Figures 1 and 2 below):
    - Bottle (borosilicate glass) for titrant (may be up to 5 liter volume, as needed), with 29/42 ground glass center neck (for burette), side arm for inlet gas sparge, side arm stopcock (for gas outlet), and side arm with glass stopper for refilling bottle (**Note:** *bottle may need to be custom-made*)

  - Digital burette, 25 ml – Brinkmann Digital Burette II ™ or equivalent
  - 500-ml conical flasks, sealable with No. 10 rubber stoppers
  - Overhead stirrer
  - Stopper assembly – No. 10 rubber stopper with five holes for accommodating the stirrer rod, burette delivery tip, argon source, gas outlet tubing, and 10 ml pipet
  - Glass rod stopper for the pipet inlet in the stopper assembly
  - Tubing, glass and flexible plastic, for connections
- Hot plate

Reagents

- Titanium trichloride (20% in HCl), reagent grade
- Hydrochloric acid, reagent grade
- Ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$], reagent grade
- Sulfuric acid, reagent grade
- Potassium dichromate, 0.100 N standard solution
- Ammonium thiocyanate, reagent grade
- Sodium citrate, reagent grade
- Sodium hydrogen tartrate, reagent grade
- Boiling chips
- Argon, UHP compressed gas (Carbon dioxide from a Kipp apparatus may be also be used, but is much less convenient; compressed nitrogen gas may be used provided residual oxygen is removed.)

Procedure for Colouring Matters

Note: *A water bubbler should be placed in line between the argon source and the titration apparatus.*

Preparation of 0.1 N titanium trichloride

Measure 800 ml of water for each liter of solution required into a beaker of appropriate size. On a hot plate, boil the water vigorously for 1 min, cover with a watch glass, and allow to cool to room temperature. In a fume cupboard, using graduated cylinders, add 90 ml of hydrochloric acid, stir, and add 100 ml of 20% titanium trichloride solution, for each liter of solution required. (Avoid transferring any white precipitate from the titanium trichloride reagent bottle.) Mix the solution and transfer to the titrant bottle. Attach the burette and connect the argon. Pass argon through the solution for 1-2 h with the sidearm stopcock on the bottle open to maintain ambient pressure. While maintaining a slow flow of gas, draw up titrant into the burette. Drain the burette, discard the titrant, and refill. Drain and refill the burette two more times. Stop the gas flow, close the sidearm stopcock, and store the solution for at least 72 h before use.

Figure 1

Figure 2

Apparatus for titanium chloride titrations

Standardization of 0.1 N titanium trichloride

Drain and refill the burette with 0.1 N titanium trichloride. Use within 1 h. Weigh 3.0 g (± 0.2 g) of ferrous ammonium sulfate into a 500-ml conical flask. Add 200 ml of water. Using a graduated cylinder, add 25 ml of 10 N sulfuric acid. Pipet 20 ml of standard 0.100 N potassium dichromate into the flask. Swirl to mix. Connect the flask securely to the stopper assembly (rubber stopper fitted with stirrer, gas inlet and outlet tubes, burette tip, and glass rod stopper). Gently bubble argon into the flask. Turn on the stirrer and slowly increase the speed until the solution is stirring vigorously without splashing. Wait 1 min before beginning the titration, and continue stirring throughout the procedure.

After adding 15-17 ml of 0.1 N titanium trichloride drop-wise within about 2 min, stop the flow of titrant and reduce the argon flow. Remove the solid glass rod from the stopper assembly, and pipet 10 ml of 50% ammonium thiocyanate (indicator solution) into the flask. The colour of the solution will become brownish-red. Remove the pipet, re-insert the glass rod, and restore the argon flow. Add 0.1 N titanium trichloride dropwise, with 2-3 sec pauses between drops, until a sharp colour change from brownish-red to light green is observed. The endpoint (20-21 ml) is reached when the solution returns to the original light green colour and remains that colour for 20 sec. Stop the argon flow and gradually turn off the stirrer. Record the volume (V) of 0.1 N titanium trichloride used to the nearest 0.05 ml. Perform the titration procedure in triplicate.

Determine the indicator blank by repeating the above procedure without the 0.100 N potassium dichromate. The blank determination should require less than 0.5 ml of 0.1 N titanium trichloride. Record the volume used to the nearest 0.05 ml.

For each titration, the concentration of the titanium trichloride solution is $(N \times 20)/(V - V_B)$,

where

N is the concentration of the standard potassium dichromate solution;
20 is the aliquot (ml) of the potassium dichromate solution;
V is the volume (ml) of titanium trichloride solution required to titrate the aliquot of standard potassium dichromate solution; and
V_B is the volume (ml) of titanium trichloride solution used to determine the blank.

Calculate the concentration of the standard titanium trichloride solution by averaging the three titration results. (Restandardize the solution weekly by performing one titration of 0.100 N potassium dichromate and determining one indicator blank.)

Determination of total colouring matters content of sample

Accurately weigh the quantity of sample indicated in each specification monograph (W_S, in mg) into a 500-ml conical flask. Add 10 g of sodium citrate or 15 g of sodium hydrogen tartrate, as specified in each monograph, a few boiling chips, and 150 ml of water. Wash down the walls of the flask with water, cover with a watch glass, and gently swirl to dissolve. In a fume hood, heat the solution to boiling on a hot plate. Boil vigorously for at least 10 sec to remove dissolved oxygen. (Avoid sample decomposition by boiling the solution for no more than 2 min.) Using gloves, remove the flask from the hot plate. Within 2-4 min of removing the flask from the hot plate, remove the watch glass, and connect the flask securely to the stopper assembly (flask might still be hot). Gently bubble argon into the flask. Turn on the stirrer and slowly increase the speed until the solution is stirring vigorously without splashing. Wait 1 min before beginning the titration, and continue stirring throughout the procedure. The colour will act as its own indicator unless otherwise stated in the appropriate monograph.

Rapidly add standardized 0.1 N titanium trichloride dropwise until the colour of the solution begins to change, then stop for 15-20 sec. Continue adding the titrant dropwise, with 1-2 sec pauses between drops. When the solution is close to the final colour, stop again for 20 sec. Continue adding 0.1 N titrant dropwise, with 5-10 sec pauses between drops, until the final colour is observed. The endpoint is reached when the final colour is stable for 20 sec. Stop the argon flow and gradually turn off the stirrer. Record the volume of titrant used to the nearest 0.05 ml

Calculation

The percent total colouring matters content of the sample is $100 \times (V \times F \times N) / (W_S)$,

where

V is the ml of standardized titanium trichloride solution required;
F is D/(1.00 ml × 0.1 meq/ml), where D is the weight (mg) of colouring matters equivalent to 1.00 ml of 0.1 N titanium trichloride, quoted in the specification monograph); and
N is the concentration of standardized titanium trichloride solution (in meq/ml).

Procedure for Lakes

Add 150 ml of water to a 500-ml conical flask and dissolve in it the buffer compound specified in the monograph for the parent colour. Accurately weigh a quantity of lake equivalent to 35-40 ml of 0.1 N titanium trichloride and transfer it to the flask. Add a few boiling chips, wash down the walls of the flask with water, and cover with a watch glass. In a fume hood, heat the mixture to boiling or until the lake has completely dissolved. Using gloves, remove the flask from the hot plate. Titrate with standardized 0.1 N titanium

trichloride in the manner described under Determination of total colouring matters content of sample, above.

Subsidiary Colouring Matter Content

Principle

In this method, the subsidiary colouring matters are separated from the main colouring matter by ascending paper chromatography and are extracted separately from the paper. The absorbance of each extract is measured at its wavelength of maximum absorbance by visible spectrophotometry.

Because it is impractical to identify each subsidiary colouring matter and because the subsidiary colouring matters are usually minor components of food colours, the method assumes that the specific absorbance of each subsidiary colouring matter is the same as that of the total colouring matters. The subsidiary colouring matters content is calculated by adding together the absorbances of the extracts in conjunction with the total colouring matters content of the sample.

Apparatus

Chromatography tank and ancillary equipment (Figures 3 and 4 or equivalent) comprising:

- Glass tank (A) and cover (B)
- Supporting frame (C) for the chromatography paper
- Solvent tray (D)
- Secondary frame (E) for supporting "drapes" of the filter paper
- Whatman No. 1 chromatography grade paper or equivalent, 20 cm x 20 cm sheets
- Microsyringe, capable of delivering 0.1 ml with a tolerance of ± 0.002 ml
- Visible range spectrophotometer
- Spectrophotometer cells, closed, 40 mm path length
- Test tubes
- Filter paper, 9 cm, coarse porosity

Chromatography solvents (all reagent grade)

- Water:ammonia (sp.gr. 0.880):trisodium citrate (95 ml:5 ml:2 g)
- n-Butanol:water:ethanol:ammonia (sp.gr. 0.880) (600:264:135:6)
- 2-Butanone:acetone:water (7:3:3)
- 2-Butanone:acetone:water:ammonia (sp.gr. 0.880) (700:300:300:2)
- 2-Butanone:acetone:water:ammonia (sp.gr. 0.880) (700:160:300:2)
- n-Butanol:glacial acetic acid:water (4:1:5)

Shake for 2 min, allow layers to separate. Use the upper layer as the chromatography solvent.

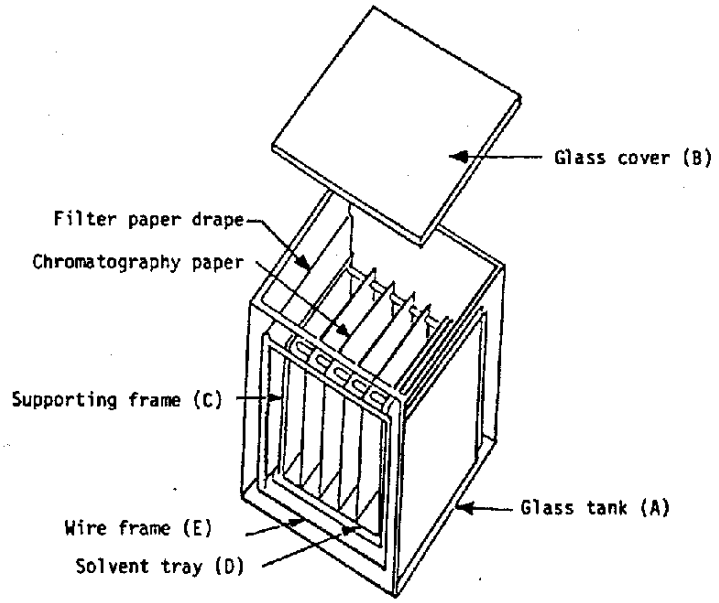


Figure 3. Assembly of Chromatography Apparatus

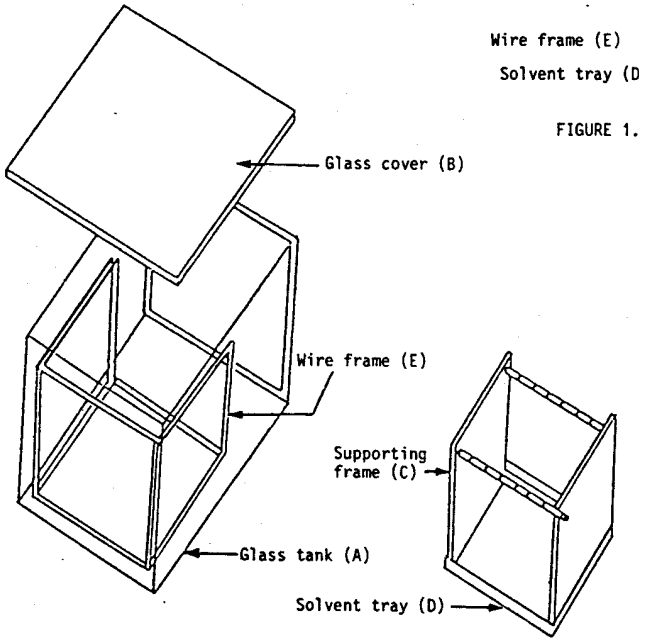


Figure 4. Components of Chromatography Apparatus

Other reagents

- Acetone, reagent grade
- Sodium hydrogen carbonate, reagent grade

Procedure

Not less than 2 h before carrying out the determination, arrange the filter-paper drapes in the glass tank and pour over the drapes and into the bottom of the tank sufficient chromatography

solvent to cover the bottom of the tank to a depth of approximately 1 cm. Place the solvent tray in position and fit the cover to the tank.

Prepare a 1.0% aqueous solution of the sample. Mark out a sheet of chromatography paper as shown in Figure 5. Apply 0.10 ml of the sample solution as uniformly as possible within the confines of the 18 cm x 7 mm rectangle, holding the nozzle of the microsyringe steadily in contact with the paper. Allow the paper to dry at room temperature for 1 - 2 h or at 50° in a drying cabinet for 5 min, followed by 15 min at room temperature. Mount the dried sheet, together with a plain sheet to act as a blank on the supporting frame. (If required, several dried sheets may be developed simultaneously.)

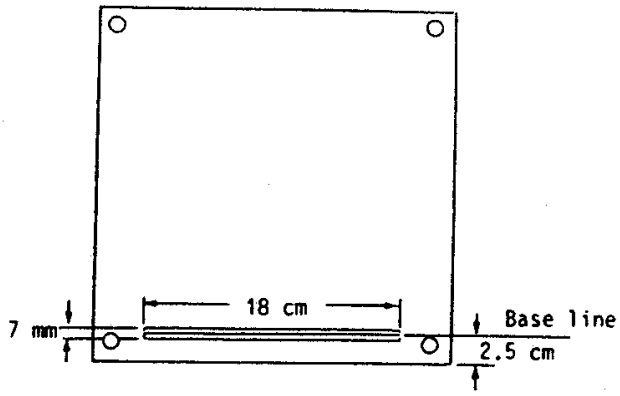


Figure 5. Method of Marking Chromatography Paper

Pour sufficient chromatography solvent into the solvent tray to bring the surface of the solvent about 1 cm below the base line of the chromatography sheets. The volume necessary will depend on the dimensions of the apparatus and should be predetermined. Put the supporting frame into position and replace the cover. Allow the solvent front to ascend the distance above the base line noted in the specification monograph, then remove the supporting frame and transfer it to a drying cabinet at 50-60° for 10-15 min. Remove the sheets from the frame.

Cut each subsidiary band from each chromatogram sheet as a strip, and cut an equivalent strip from the corresponding position of the plain sheet. Place each strip, subdivided into a suitable number of approximately equal portions, in a separate test tube. Add 5.0 ml of water:acetone (1:1 by vol) to each test tube, swirl for 2 - 3 min, add 15.0 ml of 0.05 N sodium hydrogen carbonate solution, and shake the tube to ensure mixing. Filter the coloured extracts and blanks through 9-cm coarse porosity filter papers into clean test tubes and determine the absorbances of the coloured extracts at their wavelengths of maximum absorbance, using 40-mm closed cells, against a filtered mixture of 5.0 ml of water:acetone (1:1 by vol) and 15.0 ml of the 0.05 N sodium hydrogen carbonate solution. Measure the absorbances of the extracts of the blank strips at the wavelengths at which those of the corresponding coloured extracts were measured and correct the absorbances of the coloured extracts with the blank values.

Prepare a standard solution from the 1.0% sample solution, corresponding to L/100% where L is the subsidiary colouring matters limit given in the specification monograph. Apply 0.10 ml of this solution to a sheet of chromatography paper by the technique outlined above, run a chromatogram and a blank, and dry at 50-60° for 10-15 min. Cut the band from the sheet as a

strip and cut an equivalent strip from the blank sheet. Proceed as detailed previously and determine the total absorbance (A_s) of the standard corrected for the blank.

Calculation

Calculate the percent subsidiary colouring matters in the sample using the following equation:

$$\% \text{ Subsidiary Colouring matters} = 100 \times L \times D \times (A_a + A_b + A_c \ldots A_n) / A_s$$

where

L is the limit for subsidiary colouring matters given in the specification monograph;
D is the total colouring matters content of the sample;
$A_a + A_b + A_c \ldots A_n$ is the sum of the absorbances of the subsidiary colouring matters corrected for the blank values; and
A_s is the absorbance of the standard solution;

Ether-extractable Matter

Method I

Apparatus

- Upward displacement type liquid/liquid extractor with sintered glass distributor, 500 ml working capacity with a piece of bright copper wire suspended through the condenser
- Distillation flasks: 250 and 500 ml
- Small coils of copper wire (0.5 g) for placing in distillation flasks
- Oven, 0 to 200° range
- Desiccator

Reagents

- Aluminium oxide, powdered, chromatography grade
- Ferrous sulfate, reagent grade
- Ammonium thiocyanate, reagent grade
- Titanium trichloride, 0.1 N, standard solution
- Sodium hydroxide, 2 N and 0.1 N, reagent grade
- Hydrochloric acid, 3 N and 0.1 N, reagent grade
- Ethyl ether or isopropyl ether, freshly distilled or stabilized

Ether purification

Immediately before use, freshly distilled ether should be passed through a 30 cm column of aluminium oxide in order to remove peroxides and inhibitors. Test to ensure the absence of peroxides, as follows:

Prepare a colourless solution of ferrous thiocyanate by mixing equal volumes of 0.1 N solutions of ferrous sulfate and ammonium thiocyanate. Carefully discharge any red colouration, due to ferric ions, with titanium trichloride. To 50 ml of this solution add 10 ml of ether and shake the mixture vigorously for 2-3 min. No red colour should develop.

Procedure

Alkaline ether extract. Weigh accurately about 5.0 g of the colouring matter sample (W_S). (For colouring matters with solubilities of less than 5 g/150 ml, use the lower weights

prescribed in the specification monograph under TESTS). Dissolve the sample in 150 ml of water, add 2.5 ml of 2 N sodium hydroxide and transfer the solution to a 500-ml distillation flask; dilute with water to approximately 200 ml. Add 200 ml of ether to the distillation flask and extract for 2 h with a reflux rate of about 15 ml/min. Reserve the colour solution. Transfer the ether extract to a separatory funnel and wash the ether extract with two 25-ml portions of 0.1 N sodium hydroxide and then with water. Transfer to a tared 150-ml distillation flask (W_1) containing a clean copper coil and distil off the ether in portions, reducing the volume to about 5 ml.

Acid ether extract. To the colour solution reserved above, add 5 ml of 3 N hydrochloric acid, mix and extract with a further quantity of the ether as above. Wash the ether extract with two 25-ml portions of 0.1 N hydrochloric acid and then with water. Transfer in portions to the flask containing the evaporated alkaline extract and carefully evaporate all the ether. Complete the drying in an oven at 85° for 20 min, then allow the flask to cool in a desiccator for 30 min and weigh. Repeat the drying and cooling until constant weight is obtained (W_2).

Calculation

The percent ether-extractable matter is $100 \times (W_2\text{-}W_1)/W_S$.

Method II

Apparatus

- Soxhlet extractor - Suspend a piece of bright copper wire through the condenser. Place a small coil of copper wire (0.5 g) in the distillation flask.

Reagent

- Ethyl ether or isopropyl ether, freshly distilled or stabilized

Ether purification

Purify the ether and test to ensure the absence of peroxides as directed in Method I.

Procedure

Weigh accurately about 2 g of the colouring matter sample (W_S). Transfer to the Soxhlet thimble and extract with 150 ml ether for 5 h. Concentrate the ether extract on a steam bath to about 5 ml. Dry the residue in a tared evaporating dish (W_1) on a water bath and then dry at 105° until a constant weight is obtained (W_2).

Calculation

The percent ether-extractable matter is $100 \times (W_2\text{-}W_1)/W_S$.

Hydrochloric acid-insoluble Matter in Lakes

Apparatus

- Oven, 0 - 200° range
- Sintered glass crucible, No. 4
- Desiccator

Reagents

- Hydrochloric acid, concentrated
- Hydrochloric acid, 0.5% v/v

Procedure

Accurately weigh approximately 5 g of the lake (W_S) into a 500 ml beaker. Add 250 ml water and 60 ml concentrated hydrochloric acid. Boil until all the colour and alumina have dissolved. Filter through a tared sintered glass crucible (W_1). Wash the crucible with hot 0.5% hydrochloric acid until the washings are colourless. Dry the crucible at 135°to constant weight (W_2). Cool in a desiccator before weighing.

Calculation

The percent hydrochloric acid-insoluble matter is $100 \times (W_2\text{-}W_1)/W_S$.

Leuco Base in Sulfonated Triarylmethane Colours

Principle

Air is blown through an aqueous solution containing the colouring matter, copper(II) chloride, and dimethylformamide and the solution is analyzed spectrophotometrically. Under these conditions the leuco base is oxidized to the corresponding colouring matters and the increase in absorbance is equivalent to the amount of leuco base originally present.

Apparatus

- Visible range spectrophotometer
- Spectrophotometer cells, 1 cm path lengths (flow-through cells optional)

Reagents

- Copper (II) chloride [$CuCl_2 \cdot 2H_2O$], reagent grade
- Dimethylformamide (DMF), reagent grade

Procedure

Note: *The entire procedure should be completed as quickly as possible.*

Solution A: Weigh 10.0 g of copper (II) chloride and dissolve in 200 ml of DMF. Transfer to a 1-liter volumetric flask and make up to the mark with DMF.

Solution B: Accurately weigh the quantity of sample indicated in the specification monograph (W, in mg). Dissolve in approximately 100 ml water, transfer quantitatively to a 1-liter volumetric flask and make up to the mark with water.

Solution a: Pipet 50 ml DMF into a 250-ml volumetric flask. Cover with Parafilm™ (or equivalent covering) and place in the dark.

Solution b: Accurately pipet 10 ml of Solution B into a 250-ml volumetric flask. Add 50 ml DMF. Cover with Parafilm™ (or equivalent covering) and place in the dark.

Solution c: Pipet 50 ml of Solution A into a 250-ml volumetric flask. Bubble air through the solution for 30 min in the following manner: Insert a 5-ml pipette into flexible tubing attached to a bench air flow source. Slowly, turn on the air, insert the pipette into the solution in the flask and adjust the air flow to a rapid, but controlled, rate. After 30 min remove the pipette from the solution and rinse the sides of the pipette into the flask with water from a wash bottle. Then, turn off the air flow.

Solutions d_1 and d_2 (duplicates): Accurately pipet 10 ml of Solution B into each of two 250-ml volumetric flasks. Add 50 ml of Solution A to each flask. Bubble air through the solutions for 30 min, using the method given for preparation of Solution c. After stopping the air flow, dilute solutions a- d_2 in the five flasks nearly to volume with water and place the flasks in a

water bath until they have cooled to room temperature, as heat is evolved when DMF and water are mixed. Do not leave them for longer than necessary; 5-10 min is normally enough. Dilute to volume with water. Immediately measure the absorbances of the solutions by spectrophotometry.

Spectrophotometric Determination

According to the table below, generate the absorbance curves for solutions a, b, c, d_1, and d_2 between 700 and 500 nm, using solutions a and c as blanks. Rinse cells thoroughly with each sample solution between measurements. When using the flow-through cells, use 3 separate rinses of at least 40 ml of each sample solution to be measured.

Curve	Blank	Solution	Comments
1	a	a	Set zero at 700 nm, run curve; record absorbance at wavelength of maximum absorption for colouring matter standard
2	a	b	Run curve without readjusting zero setting; record absorbance at wavelength of maximum absorption
3	c	c	Set zero at 700 nm; record absorbance at wavelength of maximum absorption for colouring matter standard
4a	c	d_1	Run curve without readjusting zero setting; record absorbance at wavelength of maximum absorption
4b	c	d_2	Run curve without readjusting zero setting; record absorbance at wavelength of maximum absorption

Calculations

Calculate the percent leuco base in the sample using the following equation:

$$\% \text{ leuco base} = 100 \times [(A_4 - A_3) - (A_2 - A_1)] \times 1 \text{ liter} \times F/(a \times b \times W \times R)$$

where

$A_1 \ldots A_4$ are the recorded absorbances of curves 1, 2, 3, and 4, respectively;
F is 250 ml/10 ml (dilution factor);
a is the absorptivity of 100% colouring matters in liter/ (mg·cm);
b is the cell path length (1 cm); and
R is the formula weight of colouring matter/formula weight of leuco base (given in the specification monograph).

Organic Compounds other than Colouring Matters

General Note

For the separation and determination of organic compounds other than colouring matters (i.e., uncoloured impurities, such as uncombined intermediate starting materials), high-performance liquid chromatography (HPLC) has several advantages over other chromatographic techniques, viz. improved separations, speed (it can be automated) and accuracy. When determining named organic compounds, standards of each compound likely to be encountered are needed before any particular colour can be analyzed.

HPLC methods are generally outlined rather than described in detail. Column packing materials, capillary columns, and type and sensitivity of detectors should be chosen for optimum separation and quantitation of impurities currently listed in food colour specifications, as well as other impurities.

The alternative (traditional) method to HPLC is column chromatography (described further below), which involves collecting the eluate in fractions, using ultraviolet spectrophotometry to identify the compounds in each fraction, and calculating their concentrations.

Determination by High Performance Liquid Chromatography

Principle

The organic compounds other than colouring matters are separated by HPLC using gradient elution and are quantitatively determined by comparison of their peak areas against those obtained from standards. The conditions prescribed must be treated as guidelines and minor modifications might be needed to achieve the separations. Deviations from the prescribed conditions, such as a different column length, other types of column packing and solvent system, and the use of paired ion procedures, can result in elution characteristics different from those for the conditions given here, such as order of elution and resolution.

Apparatus

- High-performance liquid chromatograph capable of gradient elution with
  - controller/integrator
  - pump(s), flow rate 1 ml/min
  - auto-sampler with a 20 µl injector
  - detector, UV-visible absorption
  - printer/plotter
- Chromatography column, C-18 on silica gel, 5 µm particle size, 250 × 4.6 mm.
- Guard column, C-18 on silica gel, 5 µm particle size, 15 × 4.6 mm

Reagents

- Methanol, HPLC grade
- Ammonium acetate, HPLC grade
- Reference standards as required

Instrument Parameters

- Injection volume: 20 µl.
- Eluents:
  - A: 0.2 N ammonium acetate;
  - B: methanol
- Gradient:
  - 0.0 (sample injection)
  - 0 to 35 min – 0 to 40% B (analysis)
  - 35 to 41 min – 100% B (wash)
  - 41.1 to 55 min – 100 to 0% B (return to initial gradient composition and equilibrate column)
- Flow rate: 1.0 ml per min
- Temperature: Ambient
- Pump pressure: minimum 300 psi, maximum 4000 psi
- Detector wavelengths: as required
- Integration: peak area

Procedure

Prepare 0.5% (w/w) colouring matter sample solutions in 0.02 M ammonium acetate. Prepare calibration solutions from standards of impurities named in the specification monograph.

Analyze, following the instructions given for the HPLC chromatograph and detector.

Determination by Column Chromatography

Apparatus

- Chromatography column (see Figure 6)
- UV range spectrophotometer
- Spectrophotometer cells, 1 cm path length
- Reference standards, as required

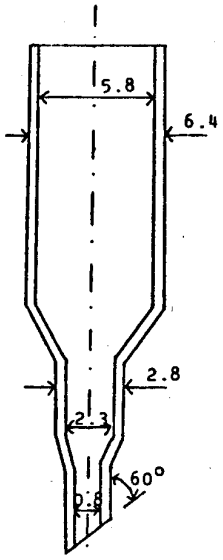


Figure 6. Chromatography Column (dimensions in cm)

Reagents

- Powdered cellulose, Whatman, or equivalent low iron cellulose
- Ammonium sulfate, reagent grade, very low in iron

Column Preparation

Prepare a 25% ammonium sulfate solution for use as the eluent. Prepare a slurry of powdered cellulose in the 25% ammonium sulfate solution, using about 75 g of cellulose to 500 ml of liquid. Place a small disk of stainless steel gauze in the constriction above the tip of the chromatography column. Pour a sufficient volume of the slurry into the column so that the height of the packing is about 5 cm from the top of the column. Tap the column occasionally to ensure efficient packing. Wash the column with 200 ml of the eluent.

Test the column by passing 200 ml of 25% ammonium sulfate solution through it and measuring the UV absorption of the solution by spectrophotometry. The absorption must be sufficiently low to avoid interference with the intended analysis.

Procedure

Weigh 0.200 g of the colouring matter sample (W) into a 150-ml beaker and dissolve in 20 ml of water. Add approximately 5 g of powdered cellulose. Add 50 g of ammonium sulfate to salt out the colour. Transfer the mixture to the chromatography column, rinse the beaker with

the 25% ammonium sulfate solution, and add the washings to the column. Allow the column to drain until flow ceases, or nearly so.

Add 25% ammonium sulfate solution to the column at a rate equal to the flow rate through the column. Collect the effluent in 100-ml fractions. Continue until twelve fractions have been collected. Reserve the column and contents until the last fraction has been examined by spectrophotometry.

Mix each fraction well, and obtain the UV absorption spectrum of each solution from 220 to 400 nm, using the eluent as the blank. If the UV spectrum of the twelfth fraction shows the presence of any compound, continue collecting fractions until the compound is eluted.

Absorptivities of the organic compounds, such as intermediate starting materials, collected in the separate fractions and expected to be present in the colouring matters are used to calculate the percent organic compounds other than colouring matters in the sample and can be found in the specification monograph of the food colour.

Calculation

Calculate the percent organic compounds other than colouring matters in the sample using the following equation:

$$\% \text{ organic compounds} = 100 \times (A \times 0.100 \text{ liter})/(a \times 1 \text{ cm} \times W)$$

where

A is the total absorbance of eluted fractions corrected for absorbance of blank;
0.100 liter is the volume of one fraction; and
a is the absorptivity in liter/(g·cm).

Note: *Usually only one compound is encountered in each eluted fraction. When more than one compound is present in significant quantities in any fraction, the spectrophotometric data will so indicate. In such cases, the amounts of the various compounds must be determined by the procedure customarily used for the spectrophotometric analysis of mixtures of absorbing materials.*

Some samples contain small amounts of various materials, particularly inorganic salts, which contribute "background absorption". Correction for this is made as follows: Determine the amount of background absorption of the fraction collected from the column immediately before and of the fraction immediately following those fractions in which the organic compounds are encountered. Subtract one-half of the sum of these two absorbances from the observed absorbance of the fractions containing the organic compounds. The remainder is taken as the absorbance due to inorganic salts.

Sulfate as Sodium Sulfate

Note: *This determination is done in connection with Water Content (Loss on Drying) for food colours and the result is included in that calculation.*

Reagents

- Sodium chloride, reagent grade, sulfate-free
- Hydrochloric acid, reagent grade
- Barium chloride, reagent grade

Procedure

Weigh 5.0 g of the colouring matter sample, transfer it to a 250-ml conical flask and dissolve in about 100 ml of water by heating on a water bath. Add 35 g of sulfate-free sodium chloride, stopper the flask, and swirl at frequent intervals for 1 h. Cool the flask, transfer the contents with saturated sodium chloride solution to a 250-ml volumetric flask, allow the solution to cool further to 20°, and dilute to volume. Shake the flask, and filter the solution through a dry filter paper. Pipet 100 ml of the filtrate into a 500-ml beaker, dilute to 300 ml with water and acidify with hydrochloric acid, adding 1 ml in excess. Heat the solution to boiling, and add an excess of 0.25 N barium chloride solution, drop by drop, with stirring. Allow the mixture to stand on a hot plate for 4 h, or leave it overnight at room temperature. Heat the mixture to about 80° and allow the precipitate to settle. Filter off the precipitated barium sulfate, wash with hot water, and ignite at a dull red heat in a tared crucible until a constant weight is obtained. Carry out a blank determination using the above procedure and correct the weight of barium sulfate found.

Calculation

Calculate the sulfate content of the sample as percent sodium sulfate:

% sodium sulfate =
100 × (2.5 × corrected weight of barium sulfate found × 0.6086)/Weight of sample.

Unsulfonated Primary Aromatic Amines

Principle

Unsulfonated primary aromatic amines are extracted into toluene from an alkaline solution of the sample, re-extracted into acid, and then determined spectrophotometrically after diazotization and coupling. They are expressed as aniline unless they are known to be some other amine.

Note: *This method is not sufficiently sensitive for determining aniline at low mg/kg levels or below.*

Apparatus

- Visible range spectrophotometer
- Spectrophotometer cells, 40 mm path length

Reagents

- Toluene, reagent grade
- Hydrochloric acid, 1 N, reagent grade
- Hydrochloric acid, 3 N, reagent grade
- Potassium bromide, 50% solution, reagent grade
- Sodium carbonate solution, 2 N, reagent grade
- Sodium hydroxide, 1 N, reagent grade
- Sodium hydroxide, 0.1 N, reagent grade
- R salt (2-naphthol-3,6-disulfonic acid, disodium salt) solution, 0.05 N, reagent grade
- Sodium nitrite solution, 0.5 N, reagent grade
- Aniline, reagent grade

Procedure

Preparation of a Standard Aniline Solution

Weigh 0.100 g of redistilled aniline into a small beaker and transfer to a 100-ml volumetric flask, rinsing the beaker several times with water. Add 30 ml of 3 N hydrochloric acid and dilute to the mark with water at room temperature. Dilute 10.0 ml of this solution to 100 ml with water and mix well; 1 ml of this solution is equivalent to 0.0001 g of aniline. Prepare the standard aniline solution freshly when required.

Preparation of a Calibration Graph

Measure the following volumes of the standard aniline solution into a series of 100-ml volumetric flasks: 5 ml, 10 ml, 15 ml, 20 ml, and 25 ml.

Dilute to 100 ml with 1 N hydrochloric acid and mix well. Pipet 10 ml of each solution into clean, dry test tubes; cool them for 10 min by immersion in a beaker of ice water. To each tube add 1 ml of the potassium bromide solution and 0.05 ml of the sodium nitrite solution. Mix and allow the tubes to stand for 10 min in the ice water bath while the aniline is diazotized. Into each of five 25-ml volumetric flasks, measure 1 ml of the R salt solution and 10 ml of the sodium carbonate solution. Pour each diazotized aniline solution into a separate flask containing R salt solution and sodium carbonate solution; rinse each test tube with a few drops of water. Dilute to the mark with water, stopper the flasks, mix the contents well and allow them to stand for 15 min in the dark.

Measure the absorbance of each coupled solution at 510 nm using 40 mm cells. As a reference solution, use a mixture of 10.0 ml of N hydrochloric acid, 10.0 ml of the sodium carbonate solution, and 2.0 ml of the R salt solution, diluted to 25.0 ml with water. Plot a graph relating absorbance to weight of aniline in each 100 ml of aniline solution.

Preparation and Evaluation of a Test Solution

Weigh, to the nearest 0.01 g, about 2.0 g of the colouring matter sample (W) into a separatory funnel containing 100 ml of water, rinse down the sides of the funnel with a further 50 ml of water, swirling to dissolve the sample, and add 5 ml of 1 N sodium hydroxide. Extract with two 50-ml portions of toluene and wash the combined toluene extracts with 10-ml portions of 0.1 N sodium hydroxide to remove traces of colour. Extract the washed toluene with three 10-ml portions of 3 N hydrochloric acid and dilute the combined extract to 100 ml with water. Mix well. Call this Solution T.

Pipet 10.0 ml of Solution T into a clean, dry test tube, cool for 10 min by immersion in a beaker of ice/water, add 1 ml of the potassium bromide solution and proceed as described above for the preparation of the calibration graph, starting with the addition of 0.05 ml of the sodium nitrite solution.

Measure the absorbance of the coupled test solution at 510 nm using a 40 mm cell. Use a reference solution prepared from 10.0 ml of Solution T, 10 ml of the sodium carbonate solution, and 2.0 ml of the R salt solution diluted to 25.0 ml with water.

From the calibration graph, read the weight of aniline (W_A) corresponding to the observed absorbance of the test solution.

Calculation

$$\% \text{ unsulfonated primary aromatic amine (as aniline)} = 100 \times W_A / W$$

Water Content (Loss on Drying)

Note: *See the methods to determine Chloride as Sodium Chloride and Sulfate as Sodium Sulfate. Specifications for food colours include the results of those tests as part of the calculation of Loss on Drying.*

Colouring materials containing -SO_3Na or -COONa groups are usually hygroscopic and any water retained from their manufacture (or subsequently absorbed from the atmosphere) is generally present in the form of a hydrate. When such colouring matters are dried at 135° the loss in weight can generally be equated to the total water content, but this is not always the case. For example, Erythrosine and Ponceau 4R each retain one molecule of water of crystallization at 135° and it is normal practice to take this into account when totalling the amounts of main components present in a sample.

Apparatus

- Oven, 0 - 200° range
- Weighing bottle, 50 mm in diameter and 30 mm high, with ground glass stopper

Procedure

Weigh 2.0 - 3.0 g of the sample (W_1) in a tared weighing bottle plus stopper. Heat the unstoppered bottle in the oven at the temperature prescribed in the specification monograph (±5°), until a constant weight is obtained. Cool the crucible and residue in a desiccator before each weighing.

Calculation

$$\text{Loss on drying of the sample (\%)} = 100 \times (1 - W_2/W_1)$$

where

W_2 is the weight of the dried sample. (See **Note** above)

Water-insoluble Matter

Apparatus

- Oven, 0 - 200° range
- Porcelain filtering crucible
- Glass microfiber filter disc, Whatman type GF/C, compliant with BS 1752
- Desiccator

Procedure

Weigh 4.5 - 5.5 g of the sample (W_S) into a 250 ml beaker. Add about 200 ml of hot water (80-90°), stir to dissolve, and allow the solution to cool to room temperature. Filter the solution through a tared porcelain crucible and filter disc and wash with cold water until the washings are colourless. Dry the crucible and residue at 135° until a constant weight is obtained. Cool the crucible and residue in a desiccator before weighing.

Calculation

$$\text{Water-insoluble matter in the sample (\%)} = 100 \times W_R/W_S,$$

where

W_R is the weight of the residue.

Water-soluble Chlorides and Sulfates in Aluminium Lakes

Reagents

- Nitric acid, 1.5 N, reagent grade
- Hydrochloric acid, reagent grade

Procedure

Accurately weigh 10 g of the sample into a 400 ml beaker. Add 250 ml of water. Stir to wet the sample and then stir occasionally during a period of 30 min. Filter.

Measure 50 ml of the filtrate, add 50 ml water and acidify with 5 ml of 1.5 N nitric acid. Determine the chloride content by the potentiometric method used for soluble colours (see Chloride as Sodium Chloride determination).

Measure 50 ml of the filtrate, dilute to 300 ml with water and acidify with hydrochloric acid, adding 1 ml in excess. Heat the solution to boiling and add an excess of 0.25 N barium chloride, drop by drop, with stirring. Complete the analysis by digesting, filtering, and igniting the precipitate as described in the method used for the determination of sulfate in soluble colours (see Sulfate as Sodium Sulfate determination).

ASSAY METHODS

Alginates Assay

(Carbon Dioxide Determination by Decarboxylation)

Apparatus

The apparatus required is shown in Figure 1 below. It consists essentially of a soda lime column, A, a mercury valve, B, connected through a side arm, C, to a reaction flask, D, by means of a rubber connection. Flask D is a 100-ml round-bottom, long-neck boiling flask, resting in a suitable heating mantle, E. The reaction flask is provided with a reflux condenser, F, to which is fitted a delivery tube, G, of 40-ml capacity, having a stopcock, H. On the reflux condenser is mounted a trap, I, containing 25 g of 20-mesh zinc or tin. The trap I should be connected with an absorption tower, J. The absorption tower consists of a 45-cm tube fitted with a medium-porosity fritted glass disk sealed to the inner part above the side arm and having a delivery tube sealed to it extending down to the end of the tube. A trap, consisting of a bulb of approximately 100-ml capacity, is blown above the fritted disk and the outer portion of a ground spherical joint is sealed on above the bulb. A 250-ml conical flask K, is connected to the bottom of the absorption tower. The top of the tower is connected to a soda lime tower, L, which is connected to a suitable pump to provide vacuum and air supply, the choice of which is made by a 3-way stopcock, M. The volume of air or vacuum is controlled by a capillary-tube regulator or needle valve, N. All joints are size 35/25, ground spherical type.

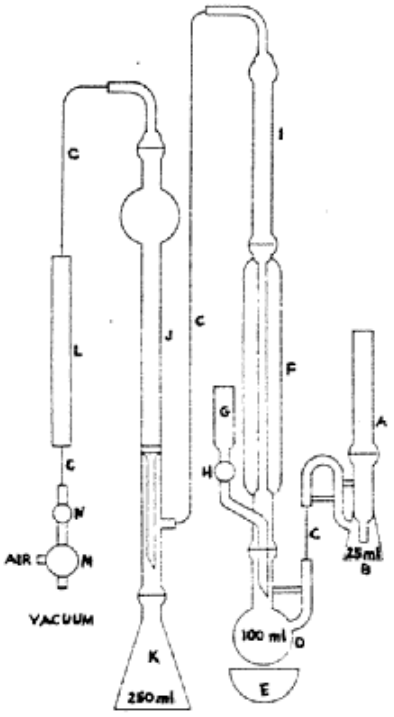


Figure 1. Apparatus for Carbon Dioxide determination by Decarboxylation

Procedure

Weigh to the nearest 0.1 mg, 250 mg of the sample, previously dried in vacuum for 4 h at 60°. Transfer into the reaction flask, D, add 25 ml of 0.1 N hydrochloric acid, insert several boiling chips, and connect the flask to the reflux condenser, F, using syrupy phosphoric acid as a lubricant.

Note: *Stopcock grease may be used for the other connections.*

Check the system for air leaks by forcing mercury up into the inner tube of the mercury valve, B, to a height of about 5 cm. Turn off the pressure using the stopcock, M. If the mercury level does not fall appreciably after 1 to 2 min, the apparatus may be considered to be free from leaks. Draw carbon dioxide-free air through the apparatus at a rate of 3,000 to 6,000 ml per h. Raise the heating mantle, E, to the flask, heat the sample to boiling, and boil gently for 2 min. Turn off and lower the mantle, and allow the sample to cool for 15 min. Charge the delivery tube, G, with 23 ml of concentrated hydrochloric acid. Disconnect the absorption tower, L, rapidly transfer 25.0 ml of 0.25 N sodium hydroxide into the tower, add 5 drops of n-butanol, and again connect the absorption tower. Draw carbon dioxide-free air through the apparatus at the rate of about 2,000 ml per h, add the hydrochloric acid to the reaction flask through the delivery tube, raise the heating mantle, and heat the reaction mixture to boiling.

After 2 h, discontinue the current of air and heating. Force the sodium hydroxide solution down into the flask, K, using gentle air pressure, and then rinse down the absorption tower with three 15-ml portions of water, forcing each washing into the flask with air pressure. Remove the flask, and add to it 10 ml of a 10% solution of barium chloride ($BaCl_2 \cdot 2H_2O$). Stopper the flask, shake gently for about 2 min, add phenolphthalein TS, and titrate with 0.1 N hydrochloric acid. Perform a blank determination.

Each ml of 0.25 N sodium hydroxide consumed is equivalent to 5.5 mg of carbon dioxide (CO_2).

Cellulose Derivatives Assay

(Ethoxyl and Methoxyl Group Determination)

Apparatus

The apparatus used for the ethoxyl and methoxyl determination is shown in Figure 2. The boiling flask A, is fitted with a capillary side-arm, B, for the introduction of carbon dioxide and is connected to a column, C, which serves to separate aqueous hydriodic acid from the more volatile ethyl or methyl iodide. The volatile iodide passes through an aqueous red phosphorus suspension in a scrubber trap, D, and is finally absorbed in the bromine acetic acid solution in an absorption tube, F. The carbon dioxide is introduced from a device arranged to minimize pressure fluctuations and connected to the apparatus by a small capillary containing a small cotton plug.

Procedure

Prepare the apparatus by placing in trap D, through the funnel K or tube F and the connecting side-arm, a volume sufficient to make trap D half-full of a suspension of about 60 mg of red phosphorus in 100 ml of water. Rinse the tube F and the side-arm with water into trap D. Dry carefully the absorption tube F and pour down the funnel K 7 ml of bromine acetic acid TS. Weigh 0.05 g of the sample, to the nearest 0.1 mg, in a tared gelatin capsule, and place it in the boiling flask along with a few glass beads or pieces of porous plate. Add 6 ml of

hydriodic acid TS and attach the flask to the condenser, using a few drops of the acid to seal the junction. Bubble carbon dioxide through the apparatus at the rate of about 2 bubbles per sec. Place the boiling flask in an oil bath heated to 150°, and continue the reaction for 40 min. Drain the contents of the absorption tube F into a 500 ml conical flask containing 10 ml of a 1 in 4 solution of sodium acetate. Rinse tube F with water, adding the rinsings to the flask, and finally dilute with water to about 125 ml. Add formic acid, dropwise, with swirling, until the reddish-brown colour of the bromine is discharged, then add 3 additional drops. A total of 12 to 15 drops are usually required. Let stand for 3 min, and add 15 ml of dilute sulfuric acid TS and 3 g of potassium iodide, and titrate immediately with 0.1 N sodium thiosulfate, using starch TS as indicator near the endpoint. Perform a blank determination, including also a gelatin capsule and make any necessary correction.

Each ml of 0.1 N sodium thiosulfate is equivalent to 0.517 mg of ($-OCH_3$) or 0.751 mg of ($-OC_2H_5$).

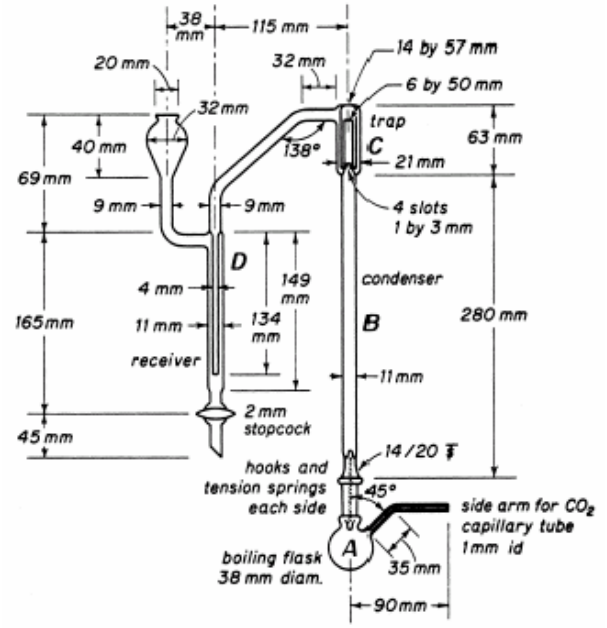


Figure 2. Apparatus for determination of ethoxyl and methoxyl groups

Polysorbates Assay

(Oxyethylene Group Determination)

Caution: *Use a safety shield and conduct the distillation in a hood.*

Principle

The oxyethylene groups are converted to ethylene and ethyl iodide which can be determined by titration. By utilizing a conversion factor determined on a reference sample, it is possible to compute the polyoxyethylene ester content.

Apparatus

An arrangement of apparatus for the analysis is shown in Figure 3. It consists in part of the reaction flasks (A), condenser, trap (B), and first absorption tube (C) of a Clark alkoxyl apparatus. These are followed by an absorption tube (D) made from a section of a spiral from a Widmer distillation column and a standard-taper (24/40) gas inlet adapter. Dimensions of

the apparatus not readily determined from the diagram are as follows: carbon dioxide inlet, capillary, 1-mm inside diameter; flask A, 28-mm diameter, 12/18 standard-taper joint; condenser, 9-mm inside diameter; inlet to trap B, 2-mm inside diameter tube; inlet to trap C, 7/15 standard-taper joint, 2-mm inside diameter tube; trap C, 14-mm inside diameter; trap D, inner tube, 8-mm outside diameter, 2-mm opening at bottom of spiral; spiral, 1.75-mm rod, 23 turns, 8.5 rise per turn; trap D, outer tube, approximately 12.5-mm inside diameter, with side-arm 7 cm from top of spiral; side-arm, 3.5-mm inside diameter, 2 mm opening at bottom. The stopcock is lubricated with silicone grease. The absorption tubes may be conveniently suspended by a series of properly spaced sheet-metal clips attached to a stick clamped at an angle of about 60°.

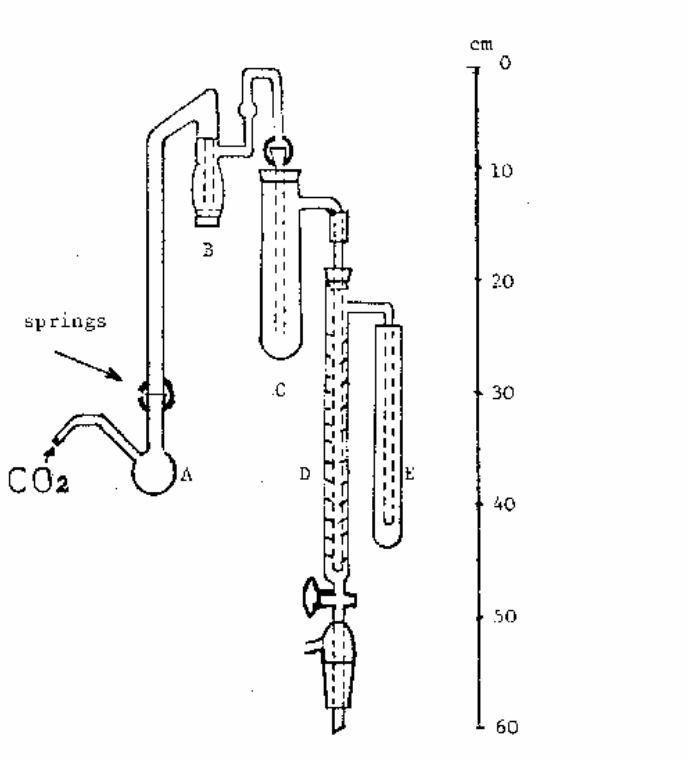


Figure 3. Apparatus for determination of oxyethylene groups

Procedure

Fill trap B with a suspension of a small amount of red phosphorus in enough water to cover the inlet tube. Pipet 10 ml of acid silver nitrate TS into tube C, pipet 15 ml of bromine-bromide TS into tube D, and place 10 ml of a 10% potassium iodide solution in trap E. Place about 0.05 g of the sample, accurately weighed, in the reaction flask A, together with a Hengar boiling granule and 10 ml of hydriodic acid TS. Connect the flask to the apparatus, pass a slow stream of carbon dioxide through (about 1 bubble per sec), and heat the flask slowly in an oil bath to 140-145°.

Maintain the flask at this temperature for at least 40 min, until there is no longer any cloudy reflux in the condenser above the reaction flask, and until the supernatant liquid in the silver nitrate trap C has clarified almost completely. Five min before the completion of the reaction, heat the silver nitrate trap C to 50-60° in a hot water bath to drive out any dissolved olefin.

On completion of the decomposition, disconnect tubes D and C cautiously in that order. Then disconnect the carbon dioxide source and remove the oil bath from flask A. Connect the spiral absorption tube, D, by its lower adapter to a 500-ml iodine-titration flask containing 10 ml of 10% potassium iodide solution and 150 ml of water. Remove the potassium iodide tube, E, and rinse the side-arm into it. Allow the bromine solution to run into the titration flask through the stopcock and rinse the tube and spiral with a few ml of water. Add the contents of the potassium iodide tube to the titration flask, stopper and allow to stand 5 min. Add 5 ml of dilute sulfuric acid TS and titrate at once with 0.05 N sodium thiosulfate, using 2 ml of starch TS as indicator.

Rinse the contents of the silver nitrate trap C into a flask, dilute to 150 ml with water, heat to boiling, cool to room temperature, and titrate with 0.05 N ammonium thiocyanate, using 3 ml of ferric ammonium sulfate TS as indicator.

Perform a blank determination omitting the sample.

Calculation

The volumes of sodium thiosulfate solution (S ml) of normality N and ammonium thiocyanate solution (S' ml) of normality N' used to titrate the contents of the bromine and silver nitrate traps are subtracted from the corresponding blank titrations (B and B' ml, respectively) and the following calculations made:

$$\% C_2H_4O = [(B - S) \times N \times 2.2] / \text{wt. of sample in g}$$

$$\% C_2H_4O = [(B' - S') \times N' \times 4.4] / \text{wt. of sample in g}$$

The sum of the values obtained from these calculations represents the total oxyethylene content of the sample. The % of polyoxyethylene ester can be estimated from the ratio of the % of oxyethylene in the unknown sample to that in a reference sample of known purity.

MEDIA, REAGENTS AND SOLUTIONS

MEDIA

The following media are used in the Microbiological Analyses Section of this volume and are referenced in that Section. The listing is alphabetical.

Baird-Parker Medium

Tryptone: 10 g
Beef extract: 5 g
Yeast extract: 1 g
Sodium pyruvate: 10 g
Glycine: 12 g
Lithium chloride $6H_2O$: 5 g
Agar: 20 g
Distilled water: 1 litre

Autoclave 15 min at 121°. Final pH is 7.0 ± 0.2. If desired for immediate use, maintain melted medium at 48-50° before adding enrichment. Otherwise, store solidified medium at 4 ± 1° up to one month. Melt medium before use.

Bismuth Sulfite Agar (Wilson and Blair)

Polypeptone (or peptone): 10 g
Beef extract: 5 g
Dextrose: 5 g
Na_2HPO_4 (anhydrous): 4 g
$FeSO_4$ (anhydrous): 0.3 g
Bismuth sulfite (indicator): 8 g
Brilliant green: 0.025 g
Agar: 20 g
Distilled water: 1 litre

Mix thoroughly and heat with agitation. Boil about 1 min to obtain uniform suspension. (Precipitate will not dissolve.) Cool to 45-50°. Suspend precipitate by gentle agitation, and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Let plates dry about 2 h with lids partially removed; then close plates. Final pH, 7.7 ± 0.2. Do not autoclave. Prepare plates on day before streaking and store in dark. Selectivity decreases in 48 h. Store plates in dark.

Brain Heart Infusion (BHI) Broth and Agar

Medium 1

Calf brain infusion: 200 g
Beef heart infusion: 250 g
Proteose peptone or gelysate: 10 g
NaC1: 5 g
$Na_2HPO_4 \cdot 12\ H_2O$: 2.5 g
Dextrose: 2 g
Distilled water: 1 litre

Dissolve ingredients in distilled water with gentle heat. Dispense broth into bottles or tubes for storage. Autoclave 15 min at 121°. Final pH, 7.4 ± 0.2.

Medium 2

Brain heart infusion : 6 g
Peptic digest of animal tissue: 6 g
NaCl: 5 g
Dextrose: 3 g
Pancreatic digest of gelatine: 14.5 g
Na_2HPO_4: 2.5 g
Distilled water: 1 litre

Suspend ingredients of *Medium 2* in distilled water and boil for 1 min to completely dissolve.

For both *Medium 1* and *Medium 2*, dispense broth into bottles or tubes for storage. Autoclave 15 min at 121°. Final pH, 7.4 ± 0.2. Commercially available BHI is acceptable.

To prepare brain heart infusion agar, add 15 g agar to 1 litre BHI broth. Heat to dissolve agar before dispensing into bottles or flasks. Autoclave 15 min at 121°.

Brilliant Green Lactose Bile Broth

Peptone: 10 g
Lactose: 10 g
Oxgall: 20 g
Brilliant green: 0.0133 g
Distilled water: 1 litre

Dissolve peptone and lactose in 500 ml distilled water. Add 20 g dehydrated oxgall dissolved in 200 ml distilled water. The pH of this solution should be 7.0-7.5. Mix and add water to make 975 ml. Adjust pH to 7.4. Add 13.3 ml 0.1% aqueous brilliant green in distilled water. Add distilled water to make 1 litre. Dispense into fermentation tubes, making certain that fluid level covers inverted vials. Autoclave 15 min at 121°. Final pH, 7.2 ± 0.1.

Buffered Peptone Water (BPW)

Peptone: 10g
NaCl: 5 g
Na_2HPO_4: 3.5 g
KH_2PO_4: 1.5 g
Distilled water: 1 litre

Autoclave at 121° for 15 min. Final pH 7.2 ± 0.2.

Dichloran 18% Glycerol (DG18) Agar

Glucose: 10 g
Bacteriological peptone: 5 g
KH_2PO_4: 1 g
$MgSO_4 \cdot 7H_2O$: 0.5 g
Dichloran (0.2% in ethanol, w/v): 1 ml
Chloramphenicol: 0.1 g
Agar: 15 g
Distilled water: 1 litre

Mix above items and steam to dissolve agar, then bring volume to 1000 ml with distilled water. Add 220 g glycerol and sterilize by autoclaving at 121° for 15 min. The final pH should be 5.6 and the final a_w, 0.955.

This medium is used as a general purpose mould enumeration medium and is preferred when the a_w of the analyzed food is 0.95 or lower. The low water activity of this medium reduces interference by bacteria and fast-growing fungi. When both yeasts and moulds must be enumerated, DRBC agar should be used (see next media).

Dichloran Rose Bengal Chloramphenicol (DRBC) Agar

Glucose: 10 g
Bacteriological peptone: 5 g
KH_2PO_4: 1 g
$MgSO_4 \cdot 7H_2O$: 0.5 g
Rose bengal (5% soln., w/v): 0.5 ml
Dichloran (2,6-dichloro-4-nitroaniline) solution (0.2%(w/v) in ethanol): 1 ml
Chloramphenicol: 0.1 g
Agar: 15 g
Distilled water: 1 litre

Final pH should be 5.6. Mix ingredients, heat to dissolve agar and sterilize by autoclaving at 121° for 15 min. Temper to 45 ± 1° in a water bath and pour plates.

EC Broth

Trypticase or tryptose: 20 g
Bile salts No. 3: 1.5 g
Lactose: 5 g
K_2HPO_4: 4 g
KH_2PO_4: 1.5 g
NaC1: 5 g
Distilled water: 1 litre

Distribute 8 ml portions to 16 x 150 mm test tubes containing inverted 10 x 75 mm fermentation tubes. Autoclave 15 min at 121°. Final pH 6.9 ± 0.2.

Hektoen Enteric (HE) Agar

Peptone: 12 g
Sodium thiosulfate: 5 g
Yeast extract: 3 g
Ferric ammonium citrate: 1.5 g
Bile salts: 9 g
Bromthymol blue: 0.064 g
Lactose: 12 g
Acid fuchsin: 0.1 g
Sucrose: 12 g
Agar: 13.5 g
Salicin: 2 g
Distilled water: 1 litre
NaC1: 5 g

Heat to boiling with frequent agitation to dissolve. Boil no longer than 1 min. Do not overheat. Cool in water bath. Pour 20 ml portions into sterile 15 x 100 mm petri dishes. Let dry 2 h with lids partially removed. Final pH, 7.6 ± 0.2. Do not store more than 1 day.

Koser's Citrate Broth

$NaNH_4HPO_4 \cdot 4H_2O$: 1.5 g
K_2HPO_4: 1 g
$MgSO_4 \cdot 7H_2O$: 0.2 g
Sodium citrate$\cdot 2H_2O$: 3 g
Distilled water: 1 litre

Dispense into screw-cap tubes as desired. Autoclave 15 min at 121°. Final pH, 6.2 ± 0.2. This formulation is listed in *Official Methods of Analysis* of the AOAC and *Standard Methods for the Examination of Water and Wastewater* of the APHA. It differs from the composition of commercially available dehydrated media. The latter have been found to be satisfactory.

Lactose Broth

Beef extract: 3 g
Peptone: 5 g
Lactose: 5 g
Distilled water: 1 litre

- For *E. coli*: Dissolve ingredients and dispense 10 ml portions into 20 x 150 mm tubes containing inverted 10 x 75 mm fermentation vials. Autoclave 15 min at 121°. Final pH 6.9 ± 0.2.
- For *Salmonella*: Dispense 225 ml portions into 500 ml Erlenmeyer flasks. After autoclaving 15 min at 121° and just before use, aseptically adjust volume to 225 ml. Final pH, 6.9 ± 0.2.

Lauryl Tryptose (LST) Broth

Tryptose or trypticase: 20 g
Lactose: 5 g
K_2HPO_4: 2.75 g
KH_2PO_4: 2.75 g
NaC1: 5 g
Sodium lauryl sulfate: 0.1 g
Distilled water: 1 litre

Dispense 10 ml portions to 20 x 150 mm tubes containing inverted 10 x 75 mm fermentation tubes. Autoclave 15 min at 121°. Final pH, 6.8 ± 0.2.

Levine's Eosin-Methylene Blue (L-EMB) Agar

Peptone: 10 g
Lactose: 10 g
K_2HPO_4: 2 g
Agar: 15 g
Eosin Y: 0.4 g
Methylene blue: 0.065 g
Distilled water: 1 litre

Boil to dissolve peptone, phosphate, and agar in 1 liter of water. Add water to make original volume. Dispense in 100 or 200 ml portions and autoclave 15 min at not over 121°. Final pH, 7.1 ± 0.2.

Before use, melt, and to each 100 ml portion add:

a. 5 ml sterile 20% lactose solution;

b. 2 ml aqueous 2% eosin Y solution; and

c. 4.3 ml 0.15% aqueous methylene blue solution.

When using complete dehydrated product, boil to dissolve all ingredients in 1 liter water. Dispense in 100 or 200 ml portions and autoclave 15 min at 121°. Final pH, 7.1 ± 0.2.

Lysine Decarboxylase Broth (Falkow) (for Salmonella)

Gelysate or peptone: 5 g
Yeast extract: 3 g
Glucose: 1 g
L-Lysine: 5 g
Bromcresol purple: 0.02 g
Distilled water: 1 litre

Heat until dissolved. Dispense 5 ml portions into 16 x 125 mm screw-cap tubes. Autoclave loosely capped tubes 15 min at 121°. Screw the caps on tightly for storage and after inoculation. Final pH, 6.8 ± 0.2 .

Lysine Iron Agar (Edwards and Fife)

Gelysate or peptone: 5 g
Yeast extract: 3 g
Dextrose: 1 g
L-Lysine hydrochloride: 10 g
Ferric ammonium citrate: 0.5 g
Sodium thiosulfate (anhydrous): 0.04 g
Bromcresol purple: 0.02 g
Agar: 15 g
Distilled water: 1 litre

Heat to dissolve ingredients. Dispense 4 ml portions into 13 x 100 mm screw-cap tubes. Autoclave 12 min at 121°. Let solidify in slanted position to form 4 cm butts and 2.5 cm slants. Final pH, 6.7 ± 0.2.

MacConkey Agar

Proteose peptone or polypeptone: 3 g
Peptone or gelysate: 17 g
Lactose: 10 g
Bile salts No. 3 (or bile salts mixture): 1.5 g
NaC1: 5 g
Neutral red: 0.03 g
Crystal violet: 0.001 g
Agar: 13.5 g
Distilled water: 1 litre

Suspend ingredients and heat with agitation to dissolve. Boil 1-2 min. Autoclave 15 min at 121°, cool to 45-50°, and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Dry at room temperature with lids closed. Do not use wet plates. Final pH, 7.1 ± 0.2.

Malonate Broth

Yeast extract: 1 g
$(NH_4)_2SO_4$: 2 g
K_2HPO_4: 0.6 g
KH_2PO_4: 0.4 g
NaC1: 2 g
Sodium malonate: 3 g
Dextrose: 0.25 g
Bromthymol blue: 0.025 g
Distilled water: 1 litre

Dissolve by heating, if necessary. Dispense 3 ml portions into 13 x 100 mm test tubes. Autoclave 15 min at 121°. Final pH, 6.7 ± 0.2.

Malt Agar

Malt extract, powdered: 20 g
Agar: 20 g
Distilled water: 1 litre

Mix ingredients, steam to dissolve agar and sterilize for 15 min at 121°. Temper medium to 45° and pour plates under aseptic conditions.

Malt Extract Agar

Malt extract: 30 g
Agar: 20 g
Distilled water: 1 litre

Boil to dissolve ingredients. Autoclave 15 min at 121°. Dispense 20-25 ml into sterile 15 x 100 mm petri dishes. Final pH, 5.5 ± 0.2.

MR-VP Broth

Medium 1

Buffered peptone-water powder: 7 g
Glucose: 5 g
K_2HPO_4: 5 g
Distilled water: 1 litre

Dissolve ingredients in 800 ml water with gentle heat. Filter, cool to 20°, and dilute to 1 litre. Autoclave 12-15 min at 121°. Final pH, 6.9 ± 0.2.

Medium 2

Pancreatic digest of casein: 3.5 g
Peptic digest of animal tissue: 3.5 g
Dextrose: 5 g
Potassium phosphate: 5 g
Distilled water: 1 litre

Dissolve ingredients in water with gentle heat if necessary. Dispense 10 ml into 16 x 150 mm test tubes and autoclave 15 min at 118-121°. Final pH, 6.9 ± 0.2.

Medium 3

Peptone: 5 g
Glucose: 5 g
Phosphate buffer: 5 g
Distilled water: 1 litre

Dissolve ingredients in water. Dispense 10 ml into 16 x 150 mm test tubes and autoclave 15 min at 121°. Final pH, 7.5 ± 0.2.

For Salmonella: Dispense 10 ml into 16 x 150 mm test tubes, and autoclave 12-15 min at 121°.

Motility Test Medium (semisolid)

Beef extract: 3 g
Peptone or gelysate: 10 g
NaCl: 5 g
Agar: 4 g
Distilled water: 1 litre

Heat with agitation and boil 1-2 min to dissolve agar. Dispense 20 ml portions into 20 x 150 mm screw-cap tubes, replacing caps loosely. Autoclave 15 min at 121°. Cool to 45° after autoclaving. Tighten caps, and refrigerate at 5-8°. To use, re-melt in boiling water or flowing steam, and cool to 45°. Aseptically dispense 20 ml portions into sterile 15 x 100 mm petri plates. Cover plates and let solidify. Use same day as prepared. Final pH, 7.4 ± 0.2.

Nutrient Broth

Beef extract: 3 g
Peptone: 5 g
Distilled water: 1 litre

Heat to dissolve. Dispense 10 m1 portions into tubes or 225 ml portions into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°. Final pH, 6.8 ± 0.2.

Phenol Red Carbohydrate Broth

Trypticase or proteose peptone No. 3: 10 g
NaC1: 5 g
Beef extract (optional): 1 g
Phenol red (7.2 ml of 0.25% phenol red solution): 0.018 g
Distilled water: 1 litre
Carbohydrate*

*Dissolve either 5 g dulcitol, 10 g lactose, or 10 g sucrose (as specified in the *Salmonella* test) in this basal broth. Dispense 2.5 ml portions into 13 x 100 mm test tubes containing inverted 6 x 50 mm fermentation tubes. Autoclave 10 min at 118°. Final pH, 7.4 ± 0.2. Alternatively, dissolve ingredients, omitting carbohydrate, in 800 ml distilled water with heat and occasional agitation. Dispense 2.0 ml portions into 13 x 100 mm test tubes containing inverted fermentation tubes. Autoclave 15 min at 118° and let cool. Dissolve carbohydrate in 200 ml distilled water and sterilize by passing solution through bacteria-retaining filter. Aseptically add 0.5 ml sterile filtrate to each

tube of sterilized broth after cooling to less than 45°. Shake gently to mix. Final pH, 7.4 ± 0.2.

For viable yeasts and moulds: Dispense 20-25 ml portions into sterile 15 x 100 mm petri dishes.

Plate Count Agar (Standard Methods)

Tryptone: 5 g
Yeast extract: 2.5 g
Dextrose: 1 g
Agar: 15 g
Distilled water: 1 litre

Heat to dissolve ingredients. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°. Final pH 7.0 ± 0.2.

Potassium Cyanide (KCN) Broth

Potassium cyanide: 0.075 g
Proteose peptone No. 3 or polypeptone: 3 g
NaC1: 5 g
KH_2PO_4: 0.225 g
Na_2HPO_4: 5.64 g
Distilled water: 1 litre

Dissolve above ingredients **except potassium cyanide** and autoclave 15 min at 121°. Cool and refrigerate at 5-8°. Final pH, 7.6 ± 0.2. Prepare KCN stock solution by dissolving 0.5 g KCN in 100 ml sterile distilled water cooled to 5-8°C. **Using bulb pipetter**, add 15 ml cold KCN stock solution to 1 litre cold, sterile base. **DO NOT PIPET BY MOUTH. Handle with gloves.**

Mix and aseptically dispense 1.0-1.5 ml portions to 13 x 100 mm sterile tubes. Using aseptic technique, stopper tubes with No. 2 corks impregnated with paraffin. Prepare corks by boiling in paraffin about 5 min. Place corks in tubes so that paraffin does not flow into broth but forms a seal between rim of tubes and cork. Store tubes at 5-8° **no longer than 2 weeks before use**.

Potato Dextrose Agar

Potato infusion: 200 ml
Dextrose: 20 g
Agar: 20 g
Distilled water: 1 litre

To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 litre distilled water for 30 min. Filter through cheesecloth, saving effluent, which is potato infusion. Mix in other ingredients and boil to dissolve. Autoclave 15 min at 121°. Dispense 20-25 ml portions into sterile 15 x 100 mm petri dishes. Final pH, 5.6 ± 0.2. Medium should not be re-melted more than once.

For potato dextrose salt agar, prepare potato dextrose agar, as above, and add 75 g NaC1 per litre.

Purple Carbohydrate Broth

Proteose peptone No. 3 : 10 g
Beef extract (optional): 1 g
NaC1: 5 g
Bromcresol purple: 0.02 g
Distilled water: 1 litre

Prepare as for phenol red carbohydrate broth (M109). Final pH, 6.8 ± 0.2.

Rappaport-Vassiliadis Medium

Tryptone: 5 g
NaCl: 8 g
KH_2PO_4: 1.6 g
Distilled water: 1 litre

Magnesium chloride solution

$MgCl_2 \cdot 6H_2O$: 400 g
Distilled water: 1 litre

Malachite green oxalate solution

Malachite green oxalate: 0.4 g
Distilled water: 100 ml

To prepare the complete medium, combine 1000 ml broth base, 100 ml *magnesium chloride solution*, and 10 ml *malachite green oxalate solution* (total volume of complete medium is 1110 ml). Broth base must be prepared on same day that components are combined to make complete medium. Magnesium chloride solution may be stored in dark bottle at room temperature up to 1 year. To prepare solution, dissolve entire contents of $MgCl_2 \cdot 6H_2O$ from newly opened container according to formula, because this salt is very hygroscopic. Malachite green oxalate solution may be stored in dark bottle at room temperature up to 6 months. Merck analytically pure malachite green oxalate is recommended because other brands may not be equally effective. Dispense 10 ml volumes of complete medium into 16 x 150 mm test tubes. Autoclave 15 min at 115°. Final pH, 5.5 ± 0.2. Store in refrigerator and **use within 1 month**.

This medium must be made from its individual ingredients. Use of commercially available dehydrated media is not recommended. Users of this medium should be aware that there are formulations and incubation temperatures for this medium other than those recommended in this volume.

Selenite Cystine Broth

Medium 1

Tryptone or polypeptone: 5 g
Lactose: 4 g
Sodium selenite ($NaHSeO_3$): 4 g
Na_2HPO_4: 10 g
L-Cystine: 0.01 g
Distilled water: 1 litre

Heat to boiling to dissolve. Dispense 10 ml portions into sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. Do not autoclave. Final pH, 7.0 ± 0.2. The medium is not sterile. Use same day as prepared.

Medium 2 (North-Bartram modification)

Polypeptone: 5 g
Lactose: 4 g
Sodium selenite ($NaHSeO_3$): 4 g
Na_2HPO_4: 5.5 g
KH_2PO_4: 4.5 g
L-Cystine: 0.01 g
Distilled water : 1 litre

Heat with agitation to dissolve. Dispense 10 ml portions to sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. Do not autoclave. Use same day as prepared.

Simmons Citrate Agar

Sodium citrate·$2H_2O$: 2 g
NaC1: 5 g
K_2HPO_4: 1 g
$NH_4H_2PO_4$: 1 g
$MgSO_4$: 0.2 g
Bromthymol blue: 0.08 g
Agar: 15 g
Distilled water: 1 litre

Heat gently with occasional agitation. Boil 1-2 min until agar dissolves. Fill 13 x 100 or 16 x 150 mm screw-cap tubes 1/3 full. Autoclave 15 min at 121°. Before medium solidifies, incline tubes to obtain 4-5 cm slants and 2-3 cm butts. Final pH, 6.8 ± 0.2.

Tetrathionate Broth

Polypeptone: 5 g
Bile salts: 1 g
Calcium carbonate: 10 g
Sodium thiosulfate·$5H_2O$: 30 g
Distilled water: 1 litre

Suspend ingredients in 1 litre distilled water, mix, and heat to boiling. (Precipitate will not dissolve completely.) Do not autoclave. Cool to less than 45°. Store at 5-8°. Final pH, 8.4 ± 0.2.

Toluidine Blue – DNA Agar

Deoxyribonucleic acid (DNA): 0.3 g
Agar: 10 g
Calcium chloride (anhydrous): 1.1 mg
NaCl: 10 g
Toluidine blue O: 83 mg
Tris(hydroxymethyl)aminomethane: 6.1 g
Distilled water: 1 litre

Dissolve the Tris(hydroxymethyl)aminomethane in 1 litre distilled water. Adjust the pH to 9.0. Add the remaining ingredients except the toluidine blue O and heat to boiling to dissolve.

Dissolve toluidine blue O in the medium. Dispense to rubber-stopped flasks. Sterilization is not necessary if used immediately. The sterile medium is stable at room temperature for 4 months and is satisfactory after several melting cycles.

Triple Sugar Iron (TSI) Agar

Medium 1		Medium 2	
Polypeptone	20 g	Beef extract	3 g
NaC1	5 g	Yeast extract	3 g
Lactose	10 g	Peptone	15 g
Sucrose	10 g	Proteose peptone	5 g
Glucose	1 g	Glucose	1 g
$Fe(NH_4)_2(SO_4)\cdot 6H_2O$	0.2 g	Lactose	10 g
$Na_2S_2O_3$	0.2 g	Sucrose	10 g
Phenol red	0.025 g	$FeSO_4$	0.2 g
Agar	13 g	NaC1	5 g
Distilled water	1 litre	$Na_2S_2O_3$	0.3 g
		Phenol red	0.024 g
		Agar	12 g
		Distilled water	1 litre

These two media are interchangeable for general use. For use with *V. parahaemolyticus*, add 25 g NaC1 per litre to either formula.

Suspend ingredients of Medium 1 in distilled water, mix thoroughly, and heat with occasional agitation. Boil about 1 min to dissolve ingredients. Fill 16 x 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave Medium 1 for 15 min at 118°. Prepare Medium 2 in the same manner as Medium 1, except autoclave 15 min at 121°. Before the media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt. Final pH, 7.3 ± 0.2 for Medium 1 and 7.4 ± 0.2 for Medium 2.

Trypticase (Tryptic) Soy Agar

Trypticase peptone: 15 g
Phytone peptone: 5 g
NaCl: 5 g
Agar: 15 g
Distilled water: 1 litre

Heat with agitation to dissolve agar. Boil 1 min. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2.

Trypticase (Tryptic) Soy Broth

Trypticase peptone: 17 g
Phytone peptone: 3 g
NaCl: 5 g

K_2HPO_4: 2.5 g
Glucose: 2.5 g
Distilled water: 1 litre

Heat with gentle agitation to dissolve. Dispense 225 ml into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2. For trypticase soy broth without glucose, prepare as above, but omit 2.5 g glucose.

Trypticase (Tryptic) Soy Broth containing 10% NaCl and 1% Na pyruvate

Trypticase or tryptose (pancreatic digest of casein): 17 g
Phytone peptone (pancreatic or enzymatic digest of casein): 3 g
NaCl: 100 g
K_2HPO_4: 2.5 g
Dextrose: 2.5 g
Na pyruvate: 10 g
Distilled water: 1 litre

Adjust to pH7.3. Heat gently if necessary. Dispense 10 ml into 16 x 150 mm tubes. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2. Store up to one month at 4 ± 1°.

Trypticase (Tryptic) Soy Broth with ferrous sulphate

Trypticase peptone: 17 g
Phytone peptone: 3 g
NaCl: 5 g
K_2HPO_4: 2.5 g
Glucose: 2.5 g
Ferrous sulphate: 35 mg
Distilled water: 1 litre

Heat with gentle agitation to dissolve. Dispense 225 ml into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2.

Trypticase Soy-Tryptose Broth

Trypticase soy broth (commercial, dehydrated): 15 g
Tryptose broth (commercial, dehydrated): 13.5 g
Yeast extract: 3 g
Distilled water: 1 litre

Dissolve ingredients in 1 litre water. Heat gently to dissolve. Dispense 5 ml portions into 16 x 150 mm test tubes. Autoclave 15 min at 121°. Final pH, 7.2 ± 0.2.

Tryptone Yeast Extract Agar

Tryptone: 10 g
Yeast extract: 1 g
Carbohydrate: 10 g (glucose and mannitol are the carbohydrates used for identification of *Staphylococcus aureus*)
Bromcresol purple: 0.04 g
Agar: 2 g
Distilled water: 1 litre

Dissolve agar with heat and gentle agitation. Adjust pH to 7.0 ± 0.2. Fill 16 x 125 mm tubes 2/3 full. Autoclave 20 min at 115°. Before use, steam medium 10-15 min. Solidify by placing tubes in ice water.

Tryptone (Tryptophane) Broth, 1%

Tryptone or trypticase: 10 g
Distilled water: 1 litre

Dissolve and dispense 5 ml portions into 16 x 125 or 16 x 150 mm test tubes. Autoclave 15 min at 121°. Final pH, 6.9 ± 0.2. For use with *V. parahaemolyticus*, add 30 g NaC1.

Tryptose Blood Agar Base

Tryptose: 10 g
Beef extract: 3 g
NaCl: 5 g
Agar: 15 g
Distilled water: 1 litre

Suspend ingredients in distilled water, mix thoroughly, and heat with occasional agitation. Boil about 1 min. Fill 16 x 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave 15 min at 121°. Before media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt.

Universal Preenrichment Broth

Tryptone: 5 g
Protease peptone: 5 g
KH_2PO_4: 15 g
Na_2HPO_4:7 g
NaCl: 5 g
Dextrose: 0.5 g
$MgSO_4$: 0.25 g
Ferric ammonium sulphate: 0.1 g
Sodium pyruvate: 0.2 g
Distilled water: 1 litre

Heat with gentle agitation to dissolve. Autoclave 15 min at 121°. Final pH, 6.3 ± 0.2.

Urea Broth

Urea: 20 g
Yeast extract: 0.1 g
K_2HPO_4 : 9.1 g
Na_2HPO_4: 9.5 g
Phenol red: 0.01 g
Distilled water: 1 litre

Dissolve ingredients in distilled water. Do not heat. Sterilize by filtration through 0.45 µm membrane. Aseptically dispense 1.5-3.0 ml portions to 13 x 100 mm sterile test tubes. Final pH, 6.8 ± 0.2.

Urea Broth (Rapid)

Urea: 20 g

Yeast extract: 0.1 g
KH_2PO_4: 0.091 g
Na_2HPO_4: 0.095 g
Phenol red: 0.01 g
Distilled water: 1 litre

Prepare as for urea broth, above.

Violet Red Bile Agar (VRBA)

Yeast extract: 3 g
Peptone or gelysate: 7 g
NaCl: 5 g
Bile salts or bile salts No. 3: 1.5 g
Lactose: 10 g
Neutral red: 30 mg
Crystal violet: 2 mg
Agar: 15 g
Distilled water: 1 litre

Suspend ingredients in distilled water and let stand for a few min. Mix thoroughly and adjust to pH 7.4 ± 0.2. Heat with agitation and boil for 2 min. Do not sterilize. Before use, cool to 45° and use as a plating medium. After solidification, add a cover layer above the agar of approximately 3.0 to 4.0 ml to prevent surface growth and spreading of colonies.

Xylose Lysine Desoxycholate (XLD) Agar

Yeast extract: 3 g
Ferric ammonium citrate: 0.8 g
L-lysine: 5 g
Sodium thiosulfate: 6.8 g
Xylose: 3.75 g
NaC1: 5 g
Lactose: 7.5 g
Agar: 15 g
Sucrose: 7.5 g
Phenol red : 0.08 g
Sodium desoxycholate: 2.5 g
Distilled water: 1 litre

Heat with agitation just until medium boils. Do not overheat. Pour into plates when medium has cooled to 50°. Let dry about 2 h with covers partially removed. Then close plates. Final pH, 7.4 ± 0.2. Do not store more than 1 day.

REAGENTS

The following reagents are used in the Microbiological Analyses Section of this Volume and are referenced in that Section. The listing is alphabetical.

Brilliant green solution

Brilliant green dye, sterile: 0.1 g
Distilled water, sterile: 100 ml

On day of use, add 20 ml I_2-KI solution and 10 ml brilliant green solution to 1 litre base. Re-suspend precipitate by gentle agitation and aseptically dispense 10 ml portions into 20 x 150 or 16 x 150 mm sterile test tubes. Do not heat medium after addition of I_2-KI and dye solutions.

Bromcresol Purple Dye Solution (0.2%)

Bromcresol purple dye: 0.2 g
Sterile distilled water: 100 ml

Butterfield's Phosphate-Buffered Dilution Water

KH_2PO_4: 34 g
Distilled water: 500 ml

Adjust pH to 7.2 with 1 N NaOH. Bring volume to 1 liter with distilled water. Sterilize 15 min at 121°. Store in refrigerator.

Dilution blanks

Take 1.25 ml of above stock solution and bring volume to 1 litre with distilled water. Dispense into bottles to 90 or 99 ± 1 ml. Sterilize 15 min at 121°.

Cellulase Solution

Dissolve 1 g cellulase in 99 ml sterile distilled water. Filter sterilize through a 0.45 µm filter. Cellulase solution may be stored at 2-5° for 2 weeks.

Chlorine Solution (200 ppm)

Commercial bleach (5.25% sodium hypochlorite): 8 ml
Distilled water containing 1 g sodium dodecyl sulphate: 992 ml

Dissolve 1 g sodium dodecyl sulfate in 992 ml distilled water. Add 8 ml commercial bleach and mix well. Make immediately before use.

Ethanol Solution (70%)

Ethanol (95%): 700 ml
Distilled water: add to final volume of 950 ml

Formalinized Physiological Saline Solution

Formaldehyde solution (36-38%): 6 ml
NaCl: 8.5 g
Distilled water: 1 litre

Dissolve 8.5 g NaCl in 1 liter distilled water. Autoclave 15 min at 121°. Cool to room temperature. Add 6 ml formaldehyde solution. Do not autoclave after addition of formaldehyde.

Hydrochloride Solution (1 N)

HCl (concentrated): 89 ml
Distilled water to make 1 litre

Kovac's Reagent

p-Dimethylaminobenzaldehyde: 5 g
Amyl alcohol (normal only): 75 ml
HCl (concentrated): 25 ml

Dissolve p-dimethylaminobenzaldehyde in normal amyl alcohol. Slowly add HC1. Store at 4°. To test for indole, add 0.2-0.3 ml reagent to 5 ml of 24 h bacteria culture in tryptone broth. Dark red colour in surface layer is positive test for indole. For enteropathogenic *E. coli*, also test at 72 h if negative at 24 h.

Lysostaphin Solution

Dissolve 2.5 mg of lysostaphin in 0.02M phosphate-saline buffer containing 1 % NaCl.

Methyl Red Indicator

Methyl red: 0.1 g
Ethanol (95%): 300 ml
Distilled water to make 500 ml

Nonfat dry milk

Nonfat dry milk: 100g
Distilled water: 1 litre

For *Salmonella*:

Suspend 100 g dehydrated nonfat dry milk in 1 liter distilled water. Swirl until dissolved. Autoclave 15 min at 121°.

Papain

Papain: 5 g
Distilled water: 1 litre

Add papain to sterile, distilled water and swirl to dissolve completely. Dispense 100 ml portion into bottles.

Physiological Saline Solution Sterile (0.85%)

NaCl: 8.5 g
Distilled water: 1 litre

Dissolve 8.5 g NaCl in water. Autoclave 15 min at 121°. Cool to room temperature.

Potassium Hydroxide Solution (40%)

KOH: 40 g

Distilled water to make 100 ml

Sodium Hydroxide Solution (1 N)

NaOH: 40 g
Distilled water to make 1 litre

Voges-Proskauer (VP) Test Reagents

Solution 1

alpha-Naphthol: 5 g
Alcohol (absolute): 100 ml

Solution 2

Potassium hydroxide: 40 g
Distilled water to make 100 ml

Voges-Proskauer (VP) test. At room temperature, transfer 1 ml of 48 h culture to test tube and add 0.6 ml solution 1 and 0.2 ml solution 2. Shake after adding each solution. To intensify and speed reaction, add a few creatine crystals to mixture. Read results 4 h after adding reagents. Development of eosin pink colour is a positive.

BUFFER SOLUTIONS

Buffer Test Solutions

Buffer TS (pH 2)

Combine 11.90 ml of 0.2 M hydrochloric acid and 88.10 ml of 0.2 M potassium chloride, and dilute to 200 ml with water.

Buffer TS (pH 5)

Add 51.5 ml of 0.2 M disodium hydrogen phosphate to 48.5 ml of 0.1 M citric acid.

Buffer TS (pH 5.45)

Dissolve 1.8360 g of citric acid and 3.198 g of disodium hydrogen phosphate in carbon dioxide-free water to make 200 ml.

Buffer TS (pH 6.5)

Combine 50 ml of 0.2 M potassium dihydrogen phosphate and 15.2 ml of 0.2 M sodium hydroxide, and dilute to 200 ml with water.

Buffer acetate TS (pH 5.0)

Add 4.6 g of anhydrous sodium acetate to 11.6 ml of 2 M acetic acid and dilute to 200 ml with water. Adjust the pH to 5.0 ± 0.1 with glacial acetic acid or 10% sodium hydroxide solution.

Barbital buffer solution (pH 7.6)

Dissolve 4.3 g of barbital sodium in 200 ml of water, adjust the pH to 7.6 with dilute hydrochloric acid, and filter.

Citric acid buffer solution

Dissolve 21 g of citric acid in water to make 1,000 ml (Solution A). Dissolve 28.4 g of disodium hydrogen phosphate in water to make 1,000 ml (Solution B). Combine 11 volumes of Solution A and 389 volumes of Solution B.

Formic acid buffer solution (pH 2.5)

Add 18 ml of water to 0.8 ml of formic acid, adjust the pH to 2.5 with strong ammonia TS, and dilute to 200 ml with water.

Phosphate buffer solution (pH 7.0)

Combine 50 ml of 0.2 M potassium dihydrogen phosphate and 29.54 ml of 0.2 M sodium hydroxide, and dilute to 200 ml with water.

Phosphate buffer solution (pH 7.3-7.4) (0.02M)

Stock solution 1:

Sodium phosphate dibasic anhydrous: 28.4 g
NaCl: 85 g
Distilled water: 1 litre

Stock solution 2:

Sodium phosphate monobasic monohydrate: 27.6 g
NaCl: 85 g
Distilled water: 1 litre

Make 1:10 dilutions of each stock solution. For example:

Stock solution 1	50 ml	Stock solution 2	10 ml
Distilled water	450 ml	Distilled water	90 ml
Approximate pH	8.2	Approximate pH	5.6

Using a pH meter, titer diluted solution1 to pH 7.3-7.4 by adding about 65 ml of solution 2. Use the resulting 0.02 M phosphate saline buffer solution in the lysostaphin susceptibility test on *S. aureus*.

Note: *Do not titer 0.2 M phosphate buffer to pH 7.3-7.4 and then dilute to 0.02 M strength. This results in a drop in pH of approximately 0.25. Addition of 0.85% salt after pH adjustment also results in a drop of approximately 0.2.*

Phosphate buffer solution (pH 7.5)

Dissolve 53.7 g of disodium hydrogen phosphate in water to make 1,000 ml (Solution A). Dissolve 20.4 g of potassium dihydrogen phosphate in water to make 1,000 ml (Solution B). Combine 21 volumes of Solution A and 4 volumes of Solution B, and adjust the pH to 7.5 with either Solution A or Solution B.

Standard Buffer Solutions

Reagent Solutions

Previously dry the crystalline reagents (except for boric acid), at 110° to 120°, and use water that has been previously boiled and cooled to prepare the solutions. Store the prepared reagent solutions in chemically resistant glass or polyethylene bottles, and use within 3 months. Discard if moulding is evident.

- *Boric acid/potassium chloride, 0.2 M*

  Dissolve 12.366 g of boric acid (H_3BO_3) and 14.911 g of potassium chloride (KC1) in water to make 1,000 ml.

- *Hydrochloric acid, 0.2 M*

  Dilute 19 ml of hydrochloric acid with water to make 1,000 ml and standardize the solution as follows: dissolve about 0.3 g, accurately weighed, of primary standard anhydrous sodium carbonate (Na_2CO_3), previously dried at about 270° for 1 h in 100 ml of water. Titrate with the hydrochloric acid using 2 drops of methyl red TS. When

the solution becomes faintly pink, boil to expel carbon dioxide, cool, and continue the titration until the faint pink colour is no longer affected by continued boiling. Each 10.60 mg of Na_2CO_3 is equivalent to 1 ml of 0.2 M hydrochloric acid.

- *Potassium chloride, 0.2 M*

  Dissolve 14.911 g of potassium chloride (KC1) in water to make 1,000 ml.

- *Potassium hydrogen phthalate, 0.2 M*

  Dissolve 40.844 g of potassium hydrogen phthalate [$KHC_6H_4(COO)_2$] in water to make 1,000 ml.

- *Potassium dihydrogen phosphate, 0.2 M*

  Dissolve 27.218 g of potassium dihydrogen phosphate (KH_2PO_4) in water to make 1,000 ml.

- *Sodium hydroxide, 0.2 M*

  Dissolve about 9 g of sodium hydroxide (NaOH) in about 950 ml of water, and add a freshly prepared saturated solution of barium hydroxide until no more precipitate forms. Shake the mixture thoroughly, and allow it to stand overnight in a stoppered bottle. Decant or filter the solution, and standardize the clear liquid as follows: Dissolve about 1 g, accurately weighed, of primary standard potassium hydrogen phthalate [$KHC_6H_4(COO)_2$], previously dried at 105° for 3 h in 75 ml of carbon dioxide-free water, and titrate with the sodium hydroxide solution to a permanent pink colour using 2 drops of phenolphthalein TS, as indicator. Each 40.84 mg of $KHC_6H_4(COO)_2$ is equivalent to 1 ml of 0.2 M sodium hydroxide.

Composition of Standard Buffer Solutions

To prepare a standard buffer solution having a pH within the range 1.2 to 10.0, combine the appropriate solutions, prepared above, as shown in the following table, and dilute with water to make 200 ml. The standard pH values given in this table are considered to be reproducible to within ± 0.02 of the pH unit specified at 25°.

Hydrochloric Acid Buffer		**Acid Phthalate Buffer**		**Neutralized Phthalate Buffer**		**Phosphate Buffer**		**Alkaline Borate Buffer**	
To 50.0 ml of 0.2 M KCl add the specified ml of 0.2 M HC1		To 50.0 ml of 0.2 M KHC_6H_4-$(COO)_2$ add the specified ml of 0.2 M HC1		To 50.0 ml of 0.2 M KHC_6H_4 - $(COO)_2$ add the specified ml of 0.2 M NaOH		To 50.0 ml of 0.2 M KH_2PO_4 add the specified ml of 0.2 M NaOH		To 50.0 ml of 0.2 M H_3BO_3KCl add the specified ml of 0.2 M NaOH	
pH	**ml**	**pH**	**ml**	**pH**	**ml**	**pH**	**ml**	**pH**	**ml**
1.2	85.0	2.2	49.5	4.2	3.0	5.8	3.6	8.0	3.9
1.3	67.2	2.4	42.2	4.4	6.6	6.0	5.6	8.2	6.0
1.4	53.2	2.6	35.4	4.6	11.1	6.2	8.1	8.4	8.6
1.5	41.4	2.8	28.9	4.8	16.5	6.4	11.6	8.6	11.8
1.6	32.4	3.0	22.3	5.0	22.6	6.6	16.4	8.8	15.8
1.7	26.0	3.2	15.7	5.2	28.8	6.8	22.4	9.0	20.8
1.8	20.4	3.4	10.4	5.4	34.1	7.0	29.1	9.2	26.4
1.9	16.2	3.6	6.3	5.6	38.8	7.2	34.7	9.4	32.1
2.0	13.0	3.8	2.9	5.8	42.3	7.4	39.1	9.6	36.9
2.1	10.2	4.0	0.1			7.6	42.4	9.8	40.6
2.2	7.8					7.8	44.5	10.0	43.7
						8.0	46.1		

STANDARD SOLUTIONS

Ammonium Standard Solution

Dissolve 296.0 mg of ammonium chloride, NH_4C1, in sufficient water to make 100 ml. Transfer 10.0 ml of this solution into a 1,000-ml volumetric flask, dilute to volume with water. Each ml of this solution contains 0.01 mg of NH^+_4.

Barium Standard Solution

Dissolve 177.9 mg of barium chloride, $BaC1_2 \cdot 2H_20$, in water in a 1,000-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 0.1 mg of Ba.

Barium Chloride Standard Solution

Dissolve 4.3 g of barium chloride in sufficient water to make 1,000 ml. Perform gravimetric analysis on the solution, and calculate the quantity of sodium sulfate (Na_2SO_4) corresponding to 1 ml of the solution. Each ml of this solution corresponds to about 2.5 mg of Na_2SO_4.

Chromium Standard Solution

To 0.934 g of potassium chromate, add 1 drop of 10% sodium hydroxide solution and water to 1,000 ml. To a 1.0 ml portion of the solution, add 1 drop of 10% sodium hydroxide solution and water to 1,000 ml. Each ml of this solution contains 0.25 μg of Cr.

Condensed Formaldehyde Standard Solution

Dilute 8.1 g of formalin (containing 37% of HCHO) with water to 1,000 ml. To a 10.0 ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 0.03 mg of HCHO. Prepare freshly before use.

Dithizone Standard Solution

Dissolve 10 mg of dithizone in 1,000 ml of chloroform. Store in a stoppered bottle lead free and in a cold place.

Formaldehyde Standard Solution

Dilute 2.7 g of formalin (containing 37% of HCHO) with water to 1,000 ml. To a 10 ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 0.01 mg of HCHO. Prepare the solution fresh.

Iron Standard Solution

Dissolve 8.63 g of ferric ammonium sulfate in 20 ml of dilute nitric acid, and add water to 1,000 ml. To 10 ml of the solution add 20 ml of dilute nitric acid and water to 1,000 ml. Each ml of this solution contains 0.01 mg of Fe. Store in a dark bottle.

Lead Standard Solution

Dissolve 159.8 mg of lead nitrate in 10 ml of dilute nitric acid, and add water to 1,000 ml. Prepare and store this solution in lead-free glassware. Dilute 10 ml of the solution with water to 100 ml. Each ml of this solution contains 0.01 mg of Pb. Prepare the solution fresh.

Lead Standard Solution for Dithizone test

To 10 ml of lead standard solution, add 1% nitric acid to 100 ml. Each ml of this solution contains 1 µg of Pb. Prepare the solution fresh.

Magnesium Standard Solution

Dissolve 50.0 mg magnesium metal, Mg, in 1 ml of hydrochloric acid in a 1,000-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 0.05 mg Mg.

Mercury Standard Solution

Dissolve 0.135 g of mercuric chloride in 10 ml of dilute nitric acid and sufficient water to make 1,000 ml. Dilute 10 ml of the solution with 10 ml of dilute nitric acid and water to make 1,000 ml. Dilute the second solution in same manner. Each ml of this final solution contains 0.1 µg of Hg in 1 ml. Prepare the solution fresh.

Methanol Standard Solution

To 5 ml of 0.1% methanol, add 2,5 ml of ethyl alcohol not containing methanol, and add water to 50 ml. Each ml of this solution contains 0.1 mg of CH_3OH.

Nitrate Standard Solution

Dissolve 1.63 g of potassium nitrate in water to make 1,000 ml. To a 10 ml portion of the solution, add water to 100 ml. Each ml of this solution contains 0.1 mg of NO_3.

Phosphate Standard Solution

Dissolve 143.3 mg of monobasic potassium phosphate, KH_2PO_4, in water in a 100 ml volumetric flask, dilute to volume with water, and mix. Transfer 10.0 ml of this solution into a 1,000-ml volumetric flask, dilute to volume with water, and mix. Each ml of this solution contains 10 µg phosphate.

Potassium Phosphate, Monobasic, Standard Solution

Dissolve 4.394 g of potassium phosphate monobasic in sufficient water to make 1,000 ml. Each ml of this solution contains 1 mg of phosphate.

Selenium Standard Solution

Add 10 ml of dilute sulfuric acid (1 in 2) to 1 g of selenium. Heat to dissolve, and evaporate to dryness on a water bath. Dissolve the residue in sufficient water to make 1,000 ml. To a 10

ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 0.01 mg of Se.

Thiamine Hydrochloride Standard Solution

Dissolve 0.1 g of vitamin B_1 hydrochloride reference standard previously dried at 105° for 2 h, in water to make 1,000 ml. To a 10 ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 1 μg of vitamin B_1 hydrochloride reference standard.

Zinc Standard Solution

Dissolve 4.4 g of zinc sulfate in water to make 1,000 ml. To a 10 ml portion of the solution, add water to 1,000 ml. Each ml of this solution contains 0.01 mg of Zn.

TEST SOLUTIONS

For the preparation of Test Solutions (TS), analytical grade reagents are to be used.

Certain of the following Test Solutions are intended for use as acid-base indicators in volumetric analyses. Such solutions should be adjusted so that when 0.15 ml of indicator solution is added to 25 ml of carbon dioxide-free water, 0.25 ml of 0.02 N acid or alkali, respectively, will produce the characteristic colour change.

The notation "PbT" indicates a lead-free solution.

In general, the directive to use a freshly prepared solution indicates that the solution is of limited stability and must be prepared on the day of use.

Acetic Acid TS

A solution containing approximately 30% w/v of CH_3COOH in water (approximately 5N).

Acetic Acid TS, Dilute

A solution containing approximately 6% w/v of CH_3COOH (approximately N).

Acetic Acid TS, Strong

See acetic acid TS.

Acetic Anhydride/Benzol TS

To 10 ml of acetic anhydride add sufficient benzol to make 100 ml.

Acetic Anhydride/Pyridine TS

To 25 g of acetic anhydride add sufficient dehydrated pyridine to make 100 ml. Prepare freshly before use.

Acetic Periodic Acid TS

Dissolve 5.4 g of periodic acid in 100 ml of distilled water and then add 1900 ml of glacial acetic acid and mix thoroughly. Store the solution in a dark glass-stoppered bottle or store in the dark in a clear glass-stoppered bottle.

Alcoholic Potassium Hydroxide TS

See potassium hydroxide TS, ethanolic.

Alizarin Yellow GG TS

Dissolve 0.1 g of alizarin yellow GG in 100 ml ethanol. Filter if necessary.

Alizarin Yellow GG/Thymolphthalein TS

Prepare by mixing 10 ml of alizarin yellow GG TS with 20 ml of thymolphthalein TS.

Alkaline Cupric Tartrate TS

(Fehling's TS). See cupric tartrate TS, alkaline.

Alkaline Mercuric-Potassium Iodide TS

(Nessler's TS). See mercuric-potassium iodide TS, alkaline.

Alkaline Tartrate Solution TS

See tartrate solution TS, alkaline.

1-Amino-2-Naphthol-4-Sulfonic Acid TS

Dissolve 0.2 g of 1-amino-2-naphthol-4-sulfonic acid in 195 ml of sodium bisulfite solution (3 in 20) and 5 ml of anhydrous sodium sulfite solution (1 in 5), and filter if necessary. Stopper tightly, and store in a dark, cold place. Use within 10 days of preparation.

Ammonia TS

A solution containing between 9.5% and 10.5% of NH_3 (approximately 6 N). Prepare by diluting 400 ml of ammonium hydroxide (28%) with sufficient water to make 1,000 ml.

Ammonia TS, Dilute

See ammonia TS.

Ammonia TS, Strong

A solution containing approximately 25% w/v of NH_3 in water (approximately 15 N).

Ammonia TS, Dilute (PbT)

Dilute ammonia TS, which complies with the following test: to 20 ml of ammonia TS add 1 ml of potassium cyanide TS (PbT), dilute to 50 ml with water and add 2 drops of sodium sulfide TS (PbT); no darkening should be produced.

Ammonia TS, Ethanolic

A 9 to 11% w/v solution of NH_3 in ethanol. A transparent, colourless liquid having a strong odour of ammonia. Specific gravity is about 0.80. Store in a rubber-stoppered container and in a cold place.

Ammonia/Ammonium Chloride Buffer TS

(Approx. pH 10). Dissolve 67.5 g of ammonium chloride (NH_4C1) in water, add 570 ml of ammonium hydroxide (28%) and dilute with water to 1,000 ml.

Ammoniacal Silver Nitrate TS

Add ammonia TS, dropwise, to a 1 in 20 solution of silver nitrate until the precipitate that first forms is almost, but not entirely, dissolved. Filter the solution, and store in a dark bottle.

(**Note:** *Ammoniacal silver nitrate TS forms explosive compounds on standing. Do not store this solution, but prepare a fresh quantity for each series of determination. Neutralize the excess reagent and rinse all glassware with hydrochloric acid immediately after completing a test.*)

Ammonium Acetate TS

A 10% w/v solution of ammonium acetate (CH_3COONH_4) in water.

Ammonium Acetate Citrate TS (PbT)

Dissolve 12.5 g of ammonium acetate (CH_3COONH_4) and 12.5 g of ammonium citrate [$C_3H_4OH(COOH)(COONH_4)_2$] in water, add strong ammonia TS until the solution is alkaline to thymol blue paper and add water to 100 ml. Purify with a 0.002% w/v solution of dithizone in chloroform, and finally shake the solution with chloroform to remove excess of dithizone.

Ammonium Carbonate TS

Dissolve 20 g of ammonium carbonate and 20 ml of ammonia TS in sufficient water to make 100 ml.

Ammonium Chloride TS

10.5% w/v of ammonium chloride in water (approximately 2 N).

Ammonium Chloride/Ammonium Hydroxide TS

Mix equal volumes of water and strong ammonia TS, and saturate with ammonium chloride.

Ammonium Citrate TS (PbT)

Dissolve 40 g of citric acid in 90 ml of water. Add 2 or 3 drops of phenol red TS, then cautiously add strong ammonia TS until the solution acquires a reddish colour. Remove any lead that may be present by extracting the solution with 20-ml portions of dithizone extraction TS until the dithizone solution retains its orange-green colour.

Ammonium Molybdate TS

Dissolve 6.5 g of finely powdered molybdic acid (85%) in a mixture of 14 ml of water and 14.5 ml of strong ammonia TS. Cool the solution, and add it slowly, with stirring, to a well-

cooled mixture of 32 ml of nitric acid and 40 ml of water. Allow to stand for 48 h, and filter through glass wool. This solution deteriorates upon standing and is unsuitable for use if, upon the addition of 2 ml of sodium phosphate TS to 5 ml of the solution, an abundant yellow precipitate does not form at once or after slight warming. Store it in the dark. If a precipitate forms during storage use only the clear, supernatant solution.

Ammonium Molybdate/Sulfuric Acid TS

Dissolve 18.8 g of ammonium molybdate in 300 ml of water, and add 150 ml of sulfuric acid and sufficient water to make 500 ml.

Ammonium Oxalate TS

A 3.0% w/v solution of ammonium oxalate [$(COONH_4)_2$] in water (approximately 0.5 N).

Ammonium Sulfanilate TS

To 2.5 g of sulfanilic acid add 15 ml of water and 3 ml of ammonia TS and mix. If necessary, add with stirring, more ammonia TS, until the acid dissolves. Adjust the pH of the solution to about 4.5 with dilute hydrochloric acid TS, using bromocresol green TS as an outside indicator, and dilute to 25 ml.

Ammonium Sulfide TS

Saturate ammonia TS with hydrogen sulfide (H_2S), and add two-thirds of its volume of ammonia TS. Residue on ignition: not more than 0.05%. The solution is not rendered turbid either by magnesium sulfate TS or by calcium chloride TS (carbonate). This solution is unsuitable for use if an abundant precipitate of sulfur is present. Store it in a small, well-filled, dark amber-coloured bottle, in a cold, dark place.

Ammonium Thiocyanate TS

A 7.6% w/v solution of ammonium thiocyanate (NH_4SCN) in water (approximately N).

Ammonium Thiocyanate/Cobalt Nitrate TS

Dissolve 17.4 g of ammonium thiocyanate and 2.8 g of cobalt nitrate in sufficient water to make 100 ml.

Amylase TS

To 0.2 g of amylase (crystal), add 100 ml of water, shake well and filter. Prepare freshly before use.

Anthrone TS

Dissolve about 0.1 g of anthrone in 100 g of sulfuric acid. Prepare freshly before use.

Antimony TS, Standard

Dissolve 2.742 g of antimony potassium tartrate in water, and dilute to 100 ml; dilute 5 ml of this solution to 500 ml with water. Each ml of the solution contains 0.001 mg of Sb.

Antimony Trichloride TS

Wash the surface of antimony trichloride with anhydrous chloroform until the washings become transparent. Add anhydrous chloroform to antimony trichloride to make a saturated solution. Store in a tight container shaded from light and in a cold place. Prepare freshly before use.

Arsenic TS, Dilute

Mix 1 ml of strong arsenic TS with sufficient water to produce 100 ml. The dilute solution of arsenic must be freshly prepared. 1 ml contains 0.01 mg of arsenic.

Arsenic TS, Strong

Dissolve 0.132 g of arsenic trioxide in 50 ml of hydrochloric acid (a 25% w/v solution of HC1 in water) and add sufficient water to 100 ml.

Arsenous Acid TS

Dissolve 1 g of arsenous acid in 30 ml of sodium hydroxide solution (1 in 40), and heat. Cool, and slowly add sufficient glacial acetic acid to 100 ml.

Barium Chloride TS

A 12% w/v solution of barium chloride ($BaC1_2 \cdot 2H_2O$) in water (approximately N).

Barium Diphenylamine Sulfonate TS

A 0.3% w/v solution of p-diphenylamine sulfonic acid barium salt in water.

Benedict's Qualitative Reagent

See cupric citrate TS, alkaline.

Benzidine TS

Dissolve 50 mg of benzidine in 10 ml of glacial acetic acid, dilute to 100 ml with water and mix. (**Caution:** *benzidine is toxic*.)

Bertrand's TS, A

Dissolve 40 g of fine cupric sulfate in sufficient water to make 1,000 ml. Fill a glass-stoppered container almost to the top, and store.

Bertrand's TS, B

Dissolve 200 g of potassium sodium tartrate and 150 g of sodium hydroxide in sufficient water to make 1,000 ml. Store in a rubber-stoppered container.

Bertrand's TS, C

Dissolve 50 g of ferric sulfate (shall not reduce potassium permanganate solution) in sufficient water. Add 200 ml of sulfuric acid, and add sufficient water to make 1,000 ml.

Bertrand's TS, D

Dissolve 5 g of potassium permanganate in sufficient water to make 1,000 ml.

(**Standardization:** *Dissolve 0.25 g of ammonium oxalate in 100 ml of water, and add 2 ml of sulfuric acid. Heat this solution to the temperature of 60° to 70°, and titrate with Bertrand's TS, D. If the volume of Bertrand's TS, D consumed is designated as a ml, each 1 ml of Bertrand's TS, D is equivalent to (0.2238/a)g of Cu*).

2,2'-Bipyridine TS

Dissolve 0.100 g of 2,2'-bipyridine in 50 ml of purified absolute ethanol TS.

Bismuth Nitrate TS (I)

Reflux 5 g of bismuth nitrate ($Bi(NO_3)_3 \cdot 5H_2O$), in 7.5 ml of nitric acid and 10 ml of water until dissolved, cool, filter and dilute the solution to 250 ml.

Bismuth Nitrate TS (II)

Dissolve 5 g of bismuth nitrate ($Bi(NO_3)_3 \cdot 5H_2O$) in 25 ml of water and 25 ml of glacial acetic acid and dilute to 250 ml.

Borax Buffer (PbT)

Dissolve 3.0 g of borax in 90 ml of water, and extract with successive portions, each of 5 ml of 1 volume of diphenylthiocarbazone solution PbT and 4 volumes of chloroform, with vigorous shaking, until the extract is blue or purple in colour; continue the extraction with successive portions, each of 10 ml of chloroform, until the extract is colourless; reject the extracts, and dilute the solution to 100 ml with water.

Boric Acid TS

Dissolve 5 g boric acid in 500 ml distilled water in a 1,000-ml measuring flask. Add 25 ml alcoholic indicator solution (67 mg methyl red and 33 mg bromocresol green in 100 ml 96% ethanol) and 200 ml ethanol. Make up to volume with distilled water. The boric acid indicator solution is red. 5 ml must turn green with not more than 3 drops of 0.01 N NaOH.

Bromide/Bromate TS

(About 0.1 N bromine) (7.991 g Br per litre). Dissolve 3 g of potassium bromate ($KBrO_3$) and 15 g of potassium bromide (KBr) in sufficient water to make 1,000 ml and standardize the solution as follows: transfer about 25 ml of the solution, accurately measured, into a 500-ml iodine flask and dilute with 120 ml of water. Add 5 ml of hydrochloric acid, stopper the flask and shake it gently. Then add 5 ml of potassium iodide TS, re-stopper, shake the mixture, allow it to stand for 5 min and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS near the end of the titration. Calculate the normality. Store this solution in a dark amber coloured, glass-stoppered bottle.

Bromine TS

(Bromine water). A saturated solution of bromine, prepared by agitating 2 to 3 ml of bromine (Br_2) with 100 ml of cold water in a glass-stoppered bottled, the stopper of which should be lubricated with petrolatum. Store it in a cold place, protected from light.

Bromine/Acetic Acid TS

Dissolve 5 ml of bromine in 145 ml of potassium acetate in acetic acid TS. Prepare this solution fresh daily.

Bromine/Bromide TS

Add 1 ml of bromine to 300 ml of glacial acetic acid saturated with dry potassium bromide (5 g). 15 ml of this solution require about 50 ml of 0.05 N sodium thiosulfate. This solution is stored in a dark bottle and kept in the dark. It is standardized at least once a day during use.

Bromine/Glacial Acetic Acid TS

Dissolve about 1.5 g of bromine in sufficient glacial acetic acid to make about 100 ml. Each 1 ml of this solution is equivalent to about 2 ml of 0.1 N sodium thiosulfate.

Bromine/Hydrochloric Acid TS

Mix 1 ml of bromine/potassium bromide TS with 100 ml of hydrochloric acid, arsenic-free.

Bromine/Potassium Bromide TS

Dissolve 30 g of bromine and 30 g of potassium bromide in sufficient water to make 100 ml.

Bromocresol Blue TS

Use bromocresol green TS.

Bromocresol Green TS

Dissolve 0.05 g of bromocresol green in 100 ml of ethanol, and filter if necessary. For pH determinations, dissolve 0.05 g in 1.4 ml of 0,05 N sodium hydroxide, and dilute with carbon dioxide-free water to 100 ml.

Bromocresol Green/Methyl Red TS

Mix equal volumes of bromocresol green TS and methyl red TS.

Bromocresol Purple TS

Dissolve 0.25 g of bromocresol purple in 20 ml of 0.05 N sodium hydroxide, and dilute with water to 250 ml.

Bromophenol Blue TS

Dissolve 0.1 g of bromophenol blue in 100 ml of dilute ethanol (1 in 2), and filter if necessary. For pH determinations, dissolve 0.1 g in 3.0 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

Bromophenol Blue TS

(For citric acid). Mix bromophenol blue TS with equal volume of ethanol, adjust pH to 7.0 by adding 0.01 N sodium hydroxide solution.

Bromophenol Blue/Sodium Hydroxide TS

Dissolve 0.1 g of bromophenol blue in 3 ml of 0.05 N sodium hydroxide by mixing well, and add sufficient water to 25 ml.

Bromothymol Blue TS

Dissolve 0.1 g of bromothymol blue in 100 ml of dilute ethanol (1 in 2), and filter if necessary. For pH determinations, dissolve 0.1 g in 3.2 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

Buffer TS

See Buffer Test Solutions.

Calcium Chloride TS

A 7.5% w/v solution of calcium chloride ($CaC1_2 \cdot 2H_2O$) in water (approximately N).

Calcium Hydroxide TS

A solution containing approximately 0.14 g of $Ca(OH)_2$ in each 100 ml. To prepare, add 3 g of calcium hydroxide [$Ca(OH)_2$] to 1,000 ml of water, and agitate the mixture vigorously and repeatedly during 1 h. Allow the excess calcium hydroxide to settle, and decant or draw off the clear supernatant liquid.

Carr-Price TS

Weigh an unopened (100 g) bottle of antimony trichloride. Open the bottle and empty the contents into a wide-mouthed, glass-stoppered amber bottle containing approximately 100 ml

of chloroform. By difference, obtain the weight of antimony trichloride and then add sufficient chloroform to supply 100 ml for each 25 g. Dissolve by warming or shaking for several hours and filter through sodium sulfate into a clean, dry, amber bottle with ground glass stopper. This solution may be stored at room temperature but should be kept in the dark when not in use. The reagent is apparently stable for long periods of time, but it is convenient to make up sufficient amounts to last for one month. Rinse all glassware coming in contact with this reagent with chloroform, a mixture of ethanol and ether or dilute or concentrated hydrochloric acid before washing, since the antimony oxychloride which forms is insoluble in water.

Ceric Ammonium Nitrate TS

Dissolve 6.25 g of ceric ammonium nitrate [$(NH_4)_2Ce(NO_3)_6$] in 100 ml of 0.25 N nitric acid. Prepare the solution fresh every third day.

Chloral Hydrate TS

Dissolve 50 g of chloral hydrate in a mixture of 15 ml of water and 10 ml of glycerol.

Chlorine TS

(Chlorine water). A saturated solution of chlorine in water. Place the solution in a small, completely filled, light-resistant container. Chlorine TS, even when kept from light and air, is apt to deteriorate. Store it in a cold, dark place. For full strength, prepare this solution fresh.

Chromate TS, Standard

Dissolve 0.0566 g potassium dichromate ($K_2Cr_2O_7$) in 1,000 ml of water. Each ml contains 0.02 mg of Cr.

Chromic Acid TS

See Chromium trioxide TS.

Chromium Trioxide TS

A 3% w/v solution of chromium trioxide in water.

Chromotropic Acid TS

Dissolve 2.0 g of chromotropic acid (4,5-dihydroxy-2,7-naphthalene-disulfonic acid, disodium salt) in 40 ml of water in a 1-litre volumetric flask. Dilute to volume with 15 M sulfuric acid.

Citric Acid Buffer Solution

See standard buffer solutions.

Cobaltous Chloride TS

Dissolve 2 g of cobaltous chloride ($CoCl_2 \cdot 6H_2O$) in 1 ml of hydrochloric acid and sufficient water to make 100 ml.

Cobaltous Chloride TSC

Dissolve about 65 g cobaltous chloride ($CoCl_2 \cdot 6H_2O$) in enough of a mixture of 25 ml of hydrochloric acid and 975 ml of water to make 1,000 ml. Place exactly 5 ml of this solution in a 250-ml iodine flask, add 5 ml of hydrogen peroxide TS and 15 ml of 20% sodium hydroxide solution. Boil for 10 min, cool, and add 2 g of potassium iodide and 20 ml of 25% sulfuric acid. When the precipitate has dissolved, titrate the liberated iodine with 0.1 N sodium thiosulfate, using starch TS as indicator. Each ml of 0.1 N sodium thiosulfate is equivalent to 23.8 mg of $CoCl_2 \cdot 6H_2O$. Adjust the final volume of the solution by adding enough of the hydrochloric acid and water mixture so that each ml contain 59.5 mg of $CoCl_2 \cdot 6H_2O$.

Cobalt-Uranyl Acetate TS

Dissolve, with warming, 40 g of uranyl acetate [$UO_2(C_2H_3O_2)_2 \cdot 2H_2O$] in a mixture of 30 g glacial acetic acid and sufficient water to make 500 ml. Similarly, prepare a solution containing 200 g of cobaltous acetate [$Co(C_2H_3O_2)_2 \cdot 4H_2O$] in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 ml. Mix the two solutions while still warm, and cool to 20°. Maintain the temperature at 20° for about 2 h to separate the excess salts from solution, and then filter through a dry filter.

Congo Red TS

Dissolve 0.10 g of congo red (sodium diphenyl-diazo-bis-alpha-naphthylaminesulfonate) ($C_{32}H_{22}N_6O_6S_2Na_2$) in 20 ml of 90% ethanol and add sufficient water to make 100 ml.

Copper Sulfate Solution TS

Dissolve 34.639 g of $CuSO_4 \cdot 5H_2O$ in water, dilute to 500 ml, and filter through glass wool or paper. Determine the Cu content of the solution (preferably by electrolysis), and adjust the content to 440.9 mg Cu/25 ml.

Cresol Red TS

Triturate 0.10 g of cresol red in a mortar with 26.2 ml of 0.01 N sodium hydroxide until solution is complete, then dilute the solution with water to 250 ml.

Cresol Red/Thymol Blue TS

Add 15 ml of thymol blue TS to 5 ml of cresol red TS, and mix.

Crystal Violet TS

A 1% solution of methyl violet (methyl-rosaniline chloride; crystal violet) in glacial acetic acid.

Cupric Acetate TS, Strong

Dissolve 13.3 g of cupric acetate in 5 ml of acetic acid and 195 ml of water.

Cupric Citrate TS, Alkaline

(Benedict's qualitative reagent). With the aid of heat, dissolve 173 g of sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) and 117 g of sodium carbonate ($Na_2CO_3 \cdot H_2O$) in about 700 ml of water, and filter through paper, if necessary. In a separate container dissolve 17.3 g of cupric sulfate ($CuSO_4 \cdot 5H_2O$) in about 100 ml of water, and slowly add this solution, with constant stirring, to the first solution. Cool the mixture, dilute to 100 ml, and mix.

Cupric Nitrate TS

A 2.4% w/v solution of cupric nitrate [$Cu(NO_3)_2 \cdot 3H_2O$] in water.

Cupric Sulfate TS

A 12.5% w/v solution of cupric sulfate ($CuSO_4 \cdot 5H_2O$) in water.

Cupric Sulfate TSC

Dissolve about 65 g of cupric sulfate ($CuSO_4 \cdot 5H_2O$) in enough of a mixture of 25 ml of hydrochloric acid and 975 ml of water to make 1,000 ml. Pipet 10.0 ml of this solution in a 250-ml iodine flask, add 40 ml of water, 4 ml of acetic acid, and 3 g of potassium iodide. Titrate the liberated iodine with 0.1 N sodium thiosulfate, using starch TS as indicator. Each ml of 0.1 N sodium thiosulfate is equivalent to 24.97 mg of $CuSO_4 \cdot 5H_2O$. Adjust the final volume of the solution by adding enough of the mixture of hydrochloric acid and water to make each ml contain 62.4 mg of $CuSO_4 \cdot 5H_2O$.

Cupric Sulfate/Ammonia TS

Dissolve 0.4 g of cupric sulfate in 50 ml mixture of ammonia TS and solution of citric acid (1 in 5) in the ratio of 2:3.

Cupric Tartrate TS, Alkaline

(Fehling's TS). [The Copper Solution (A)]. Dissolve 34.66 g of carefully selected, small crystals of cupric sulfate ($CuSO_4 \cdot 5H_2O$) showing no trace of efflorescence or of adhering moisture, in sufficient water to make 500 ml. Store this solution in a small, tight container. [The Alkaline Tartrate Solution (B)]. Dissolve 173 g of crystallized potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) and 50 g of sodium hydroxide (NaOH) in sufficient water to make 500 ml. Store this solution in a small, alkali-resistant container. For use, mix exactly equal volumes of Solutions A and B at the time required.

Cyanogen Bromide TS

Dissolve 5 g of cyanogen bromide in water to make 50 ml. (**Caution:** *Prepare this solution under a hood, as cyanogen bromide volatilizes at room temperature and the vapour is highly irritating and poisonous*).

Denigès' Reagent

See mercuric sulfate TS.

4,4'-Diaminodiphenylamine TS

Triturate 4,4'-diaminodiphenylamine sulfate with a small amount of ethanol, and add ethanol again. Transfer this solution to a flask connected to a reflux condenser, heat on a water bath, and prepare a saturated solution.

Di-ß-Naphthylthiocarbazone/Chloroform TS

Add 0.1 g of di-ß-naphthylthiocarbazone to 100 ml of carbon tetra-chloride. Dilute this solution 1:40 with chloroform.

2,6-Dichlorophenol-Indophenol TS

Warm 0.1 g of 2,6-dichlorophenol-indophenol sodium ($C_{12}H_6C1_2NNaO_2$) with 100 ml of water and filter. The solution must be used within 3 days of preparation.

2,7-Dihydroxynaphthalene TS

Dissolve 0.1 g of 2,7-dihydroxynaphthalene in 1,000 ml of sulfuric acid and allow the solution to stand until the initial yellow colour disappears. If the solution is very dark, discard it and prepare a new solution from a different supply of sulfuric acid. This solution is stable for approximately one month if stored in a dark bottle.

p-Dimethylaminobenzaldehyde TS

Dissolve 0.125 g of p-dimethylaminobenzaldehyde in a cooled mixture of 65 ml of sulfuric acid and 35 ml of water, and add 0.05 ml of ferric chloride TS. Use within 7 days after preparation.

Dimethylglyoxime TS

A 1% w/v solution of dimethylglyoxime in ethanol.

2,4-Dinitrophenylhydrazine TS

Dissolve 0.2 g of 2,4-dinitrophenylhydrazine in 100 ml of 85% sulfuric acid. Filter through a glass-filter (G3) if necessary. Store in a light-shaded bottle and in a dark, cold place. Use within 2 weeks of preparation.

Diphenylamine TS

A 1% w/v solution of diphenylamine [$(C_6H_5)_2NH$] in sulfuric acid. The solution should be colourless.

Diphenylcarbazide TS

Dissolve 0.125 g of diphenylcarbazide [$(C_6H_5{\cdot}NH{\cdot}NH)_2CO$] in a mixture of 25 ml acetone and 25 ml water. To be prepared immediately before use.

Diphenylcarbazone TS

An approximately 1% w/v solution of diphenylcarbazone ($C_{13}H_{12}N_4O$) in ethanol. Store this solution in a brown bottle.

Diphenylthiocarbazone Solution (PbT)

Extract 15 ml of a 0.1% w/v solution of diphenylthiocarbazone ($C_6H_4{\cdot}N{:}N{\cdot}CS{\cdot}NH\text{-}NH{\cdot}C_6H_5$) in chloroform, with 2 successive portions, each of 50 ml of water containing 5 ml of dilute ammonia TS; acidify the combined extracts with dilute hydrochloric acid PbT, and extract with 100 ml of chloroform; wash the extract with 2 successive portions, each of 10 ml of water, and filter through a dry filter. Determine the approximate strength of this solution by the method for determination of zinc (see titanium dioxide monograph), using 5 ml of standard zinc sulfate TS diluted to 25 ml with water in place of the 25 ml of acid solution used in the determination; dilute with chloroform so that 3 ml is approximately equivalent to each ml of standard zinc sulfate TS. This solution must be freshly prepared.

α,α-Dipyridyl TS

A 0.2% w/v solution of alpha,alpha-dipyridyl ($C_{10}H_8N_2$) in absolute ethanol.

Dithizone TS

Dissolve 25.6 mg of dithizone in 100 ml of ethanol.

Dithizone TS, Extraction

Dissolve 30 mg of dithizone in 1,000 ml of chloroform, and add 5 ml of ethanol. Store the solution in a refrigerator. Before use shake a suitable volume of the dithizone extraction solution with about half its volume of 1% nitric acid, discarding the nitric acid. Do not use more than 1 month old.

Dithizone TS, Standard

Dissolve 10 mg of dithizone in 1,000 ml of chloroform. Keep the solution in a glass-stoppered, lead-free bottle, suitably wrapped to protect it from light, and store in a refrigerator.

Dragendorff TS

Solution 1: Weigh 0.85 g of basic bismuth nitrate, and dissolve in 10 ml of acetic acid and 40 ml of water.

Solution 2: Weigh 8 g of potassium iodide, and dissolve in 20 ml of water. Mix 5 ml of Solution 1, 5 ml of Solution 2, 20 ml of acetic acid, and 100 ml of water before use.

Eosin Y TS

(Adsorption indicator) A 0.5% solution of eosin Y in water.

Eriochrome Black TS

Dissolve 0.2 g of eriochrome black T and 2 g of hydroxylamine hydrochloride ($NH_2OH \cdot HC1$) in sufficient methanol to make 50 ml, and filter. Store the solution in a light-resistant container and use within 2 weeks.

Ethanol, Aldehyde-free

To 1,000 ml of ethanol, add 5 ml of sulfuric acid and 20 ml of water, and distil. Add 10 g of silver nitrate and 1 g of potassium hydroxide to a 1,000 ml portion of this distillate, boil for 3 h by connecting a reflux condenser, and recover the ethanol by distillation.

Ethanol TS, Purified Absolute

Add about 0.1% potassium permanganate and 0.1% potassium hydroxide to absolute ethanol and distil in an all-glass apparatus.

Ethanol TS, 72%

Mix 360 ml of purified absolute ethanol TS with 150 ml of water.

Ethanolic Potassium Hydroxide TS

See potassium hydroxide TS, ethanolic.

p-Ethoxychrysoidin TS

Dissolve 50 mg of p-ethoxychrysoidin monohydrochloride in a mixture of 25 ml of water and 25 ml of ethanol, add 3 drops of hydrochloric acid, stir vigorously, and filter if necessary to obtain a clear solution.

Fehling's TS

See cupric tartrate TS, alkaline.

Ferric Ammonium Sulfate TS

An 8% w/v solution of ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$] in water.

Ferric Ammonium Sulfate/Hydrochloric Acid TS

A 0.1% w/v solution of ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$] in hydrochloric acid.

Ferric Chloride TS

A 9% w/v solution of ferric chloride ($FeCl_3 \cdot 6H_2O$) in water (approximately N).

Ferric Chloride TSC

Dissolve about 55 g of ferric chloride ($FeCl_3 \cdot 6H_2O$) in sufficient of a mixture of 25 ml of hydrochloric acid and 975 ml of water to make 1,000 ml. Pipet 10 ml of this solution in a 250-ml iodine flask, add 15 ml of water and 3 g of potassium iodide, and allow the mixture to stand for 15 min. Dilute with 100 ml of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS. Each ml of 0.1 N sodium thiosulfate is equivalent to 27.03 mg of $FeCl_3 \cdot 6H_2O$. Adjust the final volume of the solution by the addition of enough of the mixture of hydrochloric acid and water, so that each ml contains 45.0 mg of $FeCl_3 \cdot 6H_2O$.

Ferric Chloride TS, Ethanolic

A 0.2% w/v solution of ferric chloride ($FeCl_3 \cdot 6H_2O$) in absolute ethanol. Prepare this solution fresh.

Ferric Chloride TS, Dilute

To 2 ml of ferric chloride TS, add sufficient water to make 100 ml. Prepare freshly before use.

Ferric Chloride/Hydrochloric Acid TS

Dissolve 5 g of ferric chloride ($FeCl_3 \cdot 6H_2O$) in 5 ml of hydrochloric acid and sufficient water to make 100 ml.

Ferric Sulfate TS

Add 500 ml of water to 50 g of ferric sulfate, and mix thoroughly. To this mixture, add 200 ml of sulfuric acid, dissolve by shaking well, and add sufficient water to make 1,000 ml.

Ferric Sulfate TS, Acid

Add 7.5 ml of sulfuric acid to 100 ml of water, and dissolve 80 g of ferrous sulfate in the mixture with the aid of heat. Mix 7.5 ml of nitric acid and 20 ml of water, warm, and add to this the ferrous sulfate solution. Concentrate the mixture until, with the sudden emission of a red coloured vapour, the black colour of the liquid changes to red. Test for the absence of ferrous iron, and, if necessary, add a few drops of nitric acid and boil again. When the solution is cold, add sufficient water to make 110 ml.

Ferrous Sulfate TS

Dissolve 8 g of clear crystals of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in about 100 ml of recently boiled and thoroughly cooled water. Prepare this solution fresh.

Ferrous Sulfate TS, Acid

Dissolve 7 g of ferrous sulfate crystals in 90 ml of recently boiled and thoroughly cooled water, and add sufficient sulfuric acid to make 100 ml. Standardize frequently with 0.1 N potassium permanganate (approximately 0.25 N).

Fluorescein TS

A 0.1% w/v solution of sodium fluorescein in 50% ethanol.

Folin-Ciocalteu TS

Into a 150-ml flask introduce 10 g of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), 2.5 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), 70 ml of water, 5 ml of phosphoric acid, and 10 ml of hydrochloric acid. Reflux the mixture gently for about 10 h, and add 15 g of lithium sulfate ($Li_2SO_4 \cdot H_2O$), 50 ml of water, and few drops of bromine. Boil the mixture, without the condenser, for 15 min or until the excess bromine is expelled. Cool, dilute with water to 100 ml, and filter. The filtrate has no greenish tint. Before use, dilute 1 part of filtrate with 1 part of water.

Formaldehyde TS

A solution containing approximately 37.0% w/v of HCHO. It may contain methanol to prevent polymerization.

Formalin/Sulfuric Acid TS

Mix 0.2 ml of formaldehyde TS with 10 ml of sulfuric acid. Prepare freshly before use.

Fuchsin/Sulfurous Acid TS

Dissolve 0.5 g of basic fuchsin in 300 ml of hot water, and cool. Add a solution of 5 g anhydrous sodium sulfite dissolved in 50 ml of water while stirring, and add 5 ml of hydrochloric acid with shaking. Dilute with water to 500 ml, and allow to stand for 5 h. Store in a light-shaded bottle, and in a cold place.

Hydriodic Acid TS

Distil hydriodic acid over red phosphorus, passing carbon dioxide through the apparatus during the distillation. The constant-boiling mixture distilling over a 126°-127°, which is colourless or nearly colourless, is used. Place the acid in a small, brown, glass-stoppered bottle previously swept out with carbon dioxide, seal with paraffin, and store in a cool, dark place.

Hydrochloric Acid TS, Brominated

Mix 1 ml of solution of bromine with 100 ml of hydrochloric acid.

Hydrochloric Acid TS, Dilute

A solution containing 10% w/v of HC1. Prepare by diluting 266 ml of hydrochloric acid (36%) with sufficient water to make 1,000 ml.

Hydrochloric Acid, Dilute (PbT)

A solution containing approximately 10% w/v of HCl; it complies with the following test: make 10 ml alkaline with ammonia dilute, PbT add 1 ml of potassium cyanide solution PbT, dilute to 50 ml with water, and add 2 drops of a 10% sodium sulfide solution in water. No darkening is produced.

Hydrochloric Acid TS, Stannated

Mix 1 ml of a solution of stannous chloride TS with 100 ml of hydrochloric acid (a 25% w/v solution of HC1 in water).

Hydrogen Peroxide TS

A solution containing between 2.5 and 3.5 g of H_2O_2 in each 100 ml. It may contain suitable preservatives, totalling not more than 0.05%.

Hydrogen Sulfide TS

A saturated solution of hydrogen sulfide made by passing H_2S into cold water. Store it in a small, dark, amber-coloured bottle, filled nearly to the top. It is unsuitable unless it possesses a strong odour of H_2S, and unless it produces at once a copious precipitate of sulfur when added to an equal volume of ferric chloride TS. Store in a cold dark place.

Hydroxylamine Hydrochloride TS

Dissolve 20 g of hydroxylamine hydrochloride ($HONH_2 \cdot HCl$) in sufficient water to make approximately 65 ml. Transfer to a separatory funnel, add a few drops of thymol blue pH indicator, then add strong ammonia TS until the solution assumes a yellow colour. Add 10 ml of a 4% solution of sodium diethyldithiocarbamate, mix well, and allow to stand for 5 min. Extract this solution with successive 10 to 15-ml portions of chloroform until a 5-ml portion of the chloroform extract does not assume a yellow colour when shaken with a dilute cupric sulfate solution. Add diluted hydrochloric acid PbT until the solution is pink, and then dilute with sufficient water to make 100 ml.

8-Hydroxyquinoline TS

A 5% w/v solution of 8-hydroxyquinoline (oxine) in ethanol.

Indigo Carmine TS

Dissolve a quantity of indigo carmine (sodium indigotindisulfonate) equivalent to 0.18 g of $C_{16}H_8O_8N_2S_2Na_2$, in sufficient water to make 100 ml. This solution should be used within 60 days of preparation.

Iodine TS

Dissolve 14 g of iodine in a solution of 36 g of potassium iodide in 100 ml of water, add 3 drops of hydrochloric acid, and dilute with water to 1,000 ml.

Iron Indicator TS

Place 62.5 g of ferric ammonium sulfate in a one-litre bottle, dissolve in 500 ml of water, add 450 ml of concentrated nitric acid, and mix.

Iron TS, Standard

Dissolve 0.70 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 50 ml of water and add 20 ml of dilute H_2SO_4(1:15). Dilute to 1,000 ml with water and mix thoroughly. Dilute 10 ml of this solution to 100 ml with water. Each ml contains 0.01 mg of Fe.

Lead TS, Standard

Dissolve 0.1598 g of lead nitrate [$Pb(NO_3)_2$] in water to which has been added 1 ml of nitric acid and dilute to 1,000 ml. Then dilute 10 ml of this solution to 100 ml. Each ml contains 0.01 mg of lead. This solution must be freshly prepared.

Lead Acetate TS

Dissolve 9.5 g of clear, transparent crystals of lead acetate [$Pb(COOCH_3)_2 \cdot 3H_2O$] in sufficient recently boiled water to make 100 ml. Store in a well-stoppered bottle.

Lead Acetate TS, Basic

Mix 10 parts of finely pulverized lead oxide (PbO) with 30 parts of lead acetate [$Pb(COOCH_3)_2 \cdot 3H_2O$] and 5 parts of water and heat gently in a closed vessel shaking repeatedly, until the mixture is white. Add 95 parts of water, heat for 1 h, shaking repeatedly, allow to cool and filter. Add water if necessary to obtain a solution of specific gravity 1.225-1.230.

Lead Subacetate TS

Triturate 14 g of lead monoxide (PbO) to a smooth paste with 10 ml of water, and transfer the mixture to a bottle, using an additional 10 ml of water for rinsing. Dissolve 22 g of lead acetate [$Pb(COOCH_3)_2 \cdot 3H_20$] in 70 ml of water, and add the solution to the lead oxide mixture. Shake it vigorously for 5 min, then set it aside, shaking it frequently over 7 days. Finally filter, and add enough recently boiled water through the filter to make 100 ml.

Lead Subacetate TS, Dilute

Dilute 3.25 ml of lead subacetate TS with sufficient water, recently boiled and cooled, to make 100 ml. Store in a small, well-filled, tight container.

Litmus TS

Boil 10 g of litmus of reagent purity with 40 ml of ethanol (90%) for 1 h and pour away the clear liquid, repeat this operation twice with 30 ml of ethanol (90%). Digest the washed litmus with 100 ml of boiling water for 1 h, cool and filter.

Manganese Sulfate TS

Dissolve 90 g of manganese sulfate in 200 ml of water, 175 ml of phosphoric acid and 350 ml of diluted sulfuric acid (1 in 2). Add sufficient water to 1,000 ml.

Magnesia Mixture TS

Dissolve 5.5 g of magnesium chloride ($MgCl_2 \cdot 6H_2O$) and 7 g of ammonium chloride (NH_4Cl) in 65 ml of water, add 35 ml of ammonia TS, set the mixture aside for a few days in a well-stoppered bottle, and then filter. If the solution is not perfectly clear, filter again before use.

Magnesium Sulfate TS

Dissolve 12 g of crystals of magnesium sulfate ($MgSO_4 \cdot 7H_2O$), selected for freedom from efflorescence, in water to make 100 ml.

Malachite Green TS

A 1% w/v solution of malachite green oxalate in glacial acetic acid.

Mayer's TS

See mercuric-potassium iodide TS.

Mercuric Acetate TS

A 6% w/v solution of mercuric acetate [$Hg(COOCH_3)_2$] in glacial acetic acid. Store in a tight container protected from direct sunlight.

Mercuric Chloride TS

A 6.5% w/v solution of mercuric chloride ($HgCl_2$) in water (approximately 0.5 N).

Mercuric-Potassium Iodide TS

(Mayer's TS). Dissolve 1.358 g of mercuric chloride ($HgCl_2$) in 60 ml of water. Dissolve 5 g of potassium iodide (KI) in 10 ml of water. Mix the two solutions, and add water to make 100 ml.

Mercuric-Potassium Iodide TS, Alkaline

(Nessler's TS). Dissolve 10 g of potassium iodide (KI) in 10 ml of water, and add slowly with stirring, a saturated solution of mercuric chloride until a slight red precipitate remains undissolved. To this mixture add an ice-cold solution of 30 g of potassium hydroxide (KOH) in 60 ml of water, then add 1 ml more of the saturated solution of mercuric chloride. Dilute with water to 200 ml. Allow the precipitate to settle, and draw off the clear liquid. A 2-ml portion of this reagent, when added to 100 ml of a 1 in 300,000 solution of ammonium chloride in ammonia-free water, produces at once a yellowish brown colour.

Mercuric Nitrate TS

Dissolve 40 g of yellow mercuric oxide (HgO) in a mixture of 32 ml of nitric acid and 15 ml of water. Store in a light-shaded, glass-stoppered bottle (approximately 4 N).

Mercuric Sulfate TS

(Denigès' TS). Mix 5 g of yellow mercuric oxide (HgO) with 40 ml of water, and while stirring slowly add 20 ml of sulfuric acid, then add another 40 ml of water, and stir until completely dissolved (approximately 0.5 N).

Mercurous Nitrate TS

Dissolve 200 g of mercury in nitric acid and add sufficient water to produce 1,000 ml. Mercurous nitrate TS should be kept in a bottle containing a little metallic mercury.

p-Methylaminophenol Sulfate TS

Dissolve 2 g of p-methylaminophenol sulfate $[(HO{\cdot}C_6H_4{\cdot}NHCH_3)_2{\cdot}H_2SO_4]$ in 100 ml of water. To 10 ml of this solution add 90 ml of water and 20 g of sodium bisulfite. Confirm the suitability of this solution by the following test: add 1 ml of the solution to each of four tubes containing 25 ml of 0.5 N sulfuric acid and 1 ml of ammonium molybdate TS. Add 5 µg of phosphate (PO_4) to one tube, 10 µg to a second, and 20 µg to a third, using 0.5, 1.0, and 2.0 ml, respectively, of Phosphate Standard Solution, and allow to stand for 2 h. The solutions in the three tubes should show readily perceptible differences in blue colour corresponding to the relative amounts of phosphate added, and the one to which 5 µg of phosphate was added should be perceptibly bluer than the blank.

Methylene Blue TS

Dissolve 0.125 g of methylene blue in 100 ml of ethanol, and dilute with ethanol to 250 ml.

Methylene Blue TS, Diluted

To 1 ml of methylene blue TS, add sufficient water to make 100 ml.

Methyl Orange TS

Dissolve 0.1 g of methyl orange in 100 ml of water and filter if necessary.

Methyl Orange/Xylencyanol FF TS

Dissolve 1 g of methyl orange and 1.4 g of xylencyanol FF in 500 ml of 50% v/v ethanol.

Methyl Red TS

Dissolve 0.1 g of methyl red in 100 ml of ethanol, and filter if necessary. For pH determinations, dissolve 0.1 g in 7.4 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

Methyl Red/Methylene Blue TS

Add 10 ml of methyl red TS to 10 ml of methylene blue TS, and mix.

Methylrosaniline Chloride TS

See crystal violet TS.

Methyl Violet TS

See crystal violet TS.

Millon's TS

To 2 ml of mercury in an Erlenmeyer flask add 20 ml of nitric acid. Shake the flask under a hood to break up the mercury into small globules. After about 10 min add 35 ml of water and, if a precipitate or crystals appear, add sufficient dilute nitric acid (1 in 5, prepared from nitric acid free from the oxides which have been removed by blowing air through it until it is colourless) to dissolve the separated solid. Add sodium hydroxide solution (1 in 10), dropwise, with thorough mixing, until the curdy precipitate that forms after the addition of each drop no longer redissolves but is dispersed to form a suspension. Add 5 ml more of the dilute nitric acid, and mix well. Prepare this solution fresh.

Murexide Indicator Preparation

Add 0.4 g of murexide to 40 g of powdered potassium sulfate, and grind in a glass mortar to a homogeneous mixture. (Tablets containing 0.4 mg of murexide admixed with potassium sulfate or potassium chloride are available commercially.)

Naphthalenediol TS

Dissolve 0.1 g of 2,7-dihydroxynaphthalene in 1,000 ml of sulfuric acid and allow the solution to stand in the dark until the yellow colour has disappeared (at least 18 h).

1-Naphthol TS

Dissolve 1 g of 1-napthol in 25 ml of methanol. Prepare this solution freshly.

Naphthol Green TS

A 0.05% w/v solution of naphthol green in water.

alpha-Naphtholbenzein TS

A 1% w/v solution of alpha-naphtholbenzein in benzol.

Nessler's TS

See mercuric-potassium iodide TS, alkaline.

Neutral Red TS

A 0.1% w/v solution of neutral red in 50% ethanol.

Ninhydrin TS

A 0.2% w/v solution of ninhydrin (triketohydrindene hydrate, $C_9H_4O_3 \cdot H_2O$) in water. Prepare this solution fresh.

Nitric Acid TS, Dilute

A solution containing about 10% w/v of HNO_3. Prepared by diluting 105 ml of nitric acid (70%) with water to make 1,000 ml.

Nitric Acid/Sulfuric Acid TS

Prepare about 1,000 ml of nitric acid (32-35% w/v of HNO_3) by diluting 420 ml of nitric acid (70%) with 580 ml of distilled water, and add 30 ml of sulfuric acid.

Nitrite Standard TS

Dissolve 1.5 g of sodium nitrite ($NaNO_2$) in 1,000 ml of carbon dioxide-and ammonia-free water. Each contains 1 mg of NO_2.

o-Nitrobenzaldehyde TS

Saturate a 2 N sodium hydroxide solution with o-nitrobenzaldehyde ($NO_2C_6H_4CHO$).

Orthophenanthroline TS

Dissolve 0.15 g of orthophenanthroline ($C_{12}H_8N_2 \cdot H_2O$) in 10 ml of a solution of ferrous sulfate, prepared by dissolving 1.48 g of clear crystals of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 100 ml water. The ferrous sulfate solution must be prepared immediately before dissolving the orthophenanthroline. Store the solution in well-closed containers.

Oxalic Acid TS

A 6.3% w/v solution of oxalic acid ($C_2H_2O_4 \cdot 2H_2O$) in water (approximately N).

Oxalic Acid/Sulfuric Acid TS

Add an equal volume of sulfuric acid to water, and cool. To a 500 ml portion of the solution, add 25 g of oxalic acid.

8-Oxyquinoline TS

Dissolve 2 g of 8-oxyquinoline in 6 ml of glacial acetic acid. Add sufficient water to 100 ml. Prepare freshly before use.

Phenol Red TS

(Phenolsulfonphthalein TS). Dissolve 0.1 g of phenolsulfonphthalein in 100 ml of ethanol, and filter if necessary. For pH determinations, dissolve 0.1 g in 5.7 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

Phenolphthalein TS

Dissolve 0.2 g of phenolphthalein ($C_{20}H_{14}O_4$) in 60 ml of 90% ethanol and add sufficient water to make 100 ml.

Phenolphthalein/Thymol Blue TS

Dissolve 2 g of phenolphthalein and 0.1 g of thymol blue in 100 ml of absolute ethanol, and filter if necessary. Prepare freshly before use.

Phenolsulfonphthalein TS

See phenol red TS.

Phenylhydrazine Hydrochloride/Sodium Acetate TS

Dissolve 0.5 g of phenylhydrazine hydrochloride in 10 ml of sodium acetate TS, and filter if necessary. Prepare freshly before use.

p-Phenylphenol TS

On the day of use, dissolve 0.75 g of p-phenylphenol in 50 ml of sodium hydroxide TS.

Phloroglucin/Hydrochloric Acid TS

Dissolve 0.1 g of phloroglucin in 1 ml of ethanol, add 9 ml of hydrochloric acid, and mix well. Store in a dark place.

Phosphomolybdic Acid TS

Dissolve 5 g of phosphomolybdic acid ($20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$) in water, filter and dilute to 100 ml with water.

Phosphotungstic Acid TS

A 1% w/v solution of phosphotungstic acid (approximately $24WO_3 \cdot 2H_3PO_4 \cdot 48H_2O$) in water.

Picric Acid TS

See trinitrophenol TS.

Platinic Chloride TS

A 13% w/v solution of platinic chloride in water (approximately 0.5 N).

Platinum/Cobalt TSC

Transfer 1.246 g of potassium chloroplatinate (K_2PtC1_6), and 1.00 g of crystallized cobaltous chloride, ($CoCl_2 \cdot 6H_2O$), into a 1,000-ml volumetric flask, dissolve in about 200 ml of water and 100 ml of hydrochloric acid, dilute to volume with water, and mix. (Use this solution only when specified in an individual monograph.)

Potassium Acetate TS

A 10% w/v solution of potassium acetate ($KCOOCH_3$) in water.

Potassium Acetate in Acetic Acid TS

Dissolve 10 g of potassium acetate in 100 ml of a solution consisting of 90 ml of glacial acetic acid and 10 ml of acetic anhydride.

Potassium Bichromate TS

See potassium dichromate TS.

Potassium Bromate/Potassium Bromide TS

Dissolve 1.4 g of potassium bromate and 8.1 g of potassium bromide in sufficient water to make 100 ml.

Potassium Chloride/Hydrochloric Acid TS

Dissolve 25 g of potassium chloride in 0.85 ml of hydrochloric acid and 75 ml of water.

Potassium Chromate TS

A 10% w/v solution of potassium chromate (K_2CrO_4) in water.

Potassium Cyanate TS

Dissolve 1 g of potassium cyanate in 9 ml of water. Prepare freshly before use.

Potassium Cyanide TS (PbT)

Dissolve 50 g of potassium cyanide in sufficient purified water to make 100 ml. Remove the lead by shaking with portions of the dithizone extraction TS. Part of the dithizone remains in the aqueous phase but can be removed, if desired, by washing with chloroform. The strong potassium cyanide solution is then diluted to a concentration of 10 g per 100 ml.

Potassium Dichromate TS

A 7.5% w/v solution of potassium dichromate ($K_2Cr_2O_7$) in water.

Potassium Ferricyanide TS

Dissolve 1 g of potassium ferricyanide [$K_3Fe(CN)_6$] in 10 ml of water. Prepare this solution fresh.

Potassium Ferrocyanide TS

Dissolve 1 g of potassium ferrocyanide [$K_4Fe(CN)_6 \cdot 3H_2O$] in 10 ml of water. Prepare this solution fresh.

Potassium Hydroxide TS

A 6.5% w/v solution of potassium hydroxide (KOH) in water (approximately N).

Potassium Hydroxide TS, Ethanolic

Place a few g (5 to 10) of potassium hydroxide in a 2-litre flask, add 1 to 1.5 L of 95% ethanol and boil on a water bath under reflux condenser from 30 to 60 min. Distil and collect the ethanol. Dissolve 40 g of potassium hydroxide, low in carbonate, in 1,000 ml of the distilled ethanol keeping the temperature below 15.5° while the alkali is being dissolved. This solution should remain clear.

Potassium Iodate TS

A 0.71% w/v solution of potassium iodate in water. Preserve in the dark.

Potassium Iodide TS

A 16.5% w/v solution of potassium iodide (KI) in water (approximately N). Store in a light-resistant container.

Potassium Permanganate TS

A 1.0% w/v solution of potassium permanganate ($KMnO_4$) in water.

Potassium Permanganate/Phosphoric Acid TS

To 75 ml of phosphoric acid, add sufficient water to 500 ml, and dissolve 15 g of potassium permanganate in the solution.

Potassium Pyroantimonate TS

To 2 g of potassium pyroantimonate, add 100 ml of water. Boil the solution for about 5 min, cool quickly, and add 10 ml of 15% potassium hydroxide solution. Allow to stand for one day, and filter.

Potassium Sodium Tartrate TS

A 14.1% w/v solution of potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) in water.

Potassium Sulfate TS

A 1% w/v solution of potassium sulfate (K_2SO_4) in water.

Pyridine/Acetic Anhydride TS

Mix 3 volumes of pyridine with 1 volume of acetic anhydride. Prepare freshly before use.

Pyridinium Chloride/Chloroform TS

Place 75 g of anhydrous pyridine (C_5H_5N) and approximately 400 ml of chloroform in a 2-litre graduated cylinder. Weigh the cylinder and cool it in an ice water bath. Bubble dry hydrogen chloride slowly through the solution. At intervals of several min interrupt the flow of gas, remove, dry and weigh the cylinder and its contents to determine the rate of flow of the gas. When approximately 35 g have been added, stop the flow of gas, warm the mixture to room temperature and expel the vapours with a stream of dry air. Add 100 ml of anhydrous pyridine and dilute to 1,000 ml with chloroform. When most of the reagent has been used, discard the last 100 ml.

Quimociac TS

Dissolve 70 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) in 150 ml of water (Solution A). Dissolve 60 g of citric acid in a mixture of 85 ml of nitric acid and 150 ml of water, and cool (Solution B). Gradually add Solution A to Solution B, with stirring, to produce Solution C. Dissolve 5.0 ml of synthetic quinoline in a mixture of 35 ml of nitric acid and 100 ml of water (Solution D). Gradually add Solution D to Solution C, mix well, and allow to stand overnight. Filter the mixture, add 280 ml of acetone to the filtrate, dilute to 1,000 ml with water, and mix. Store in a polyethylene bottle. Caution. This reagent contains acetone. Do not use near an open flame. Operations involving heating or boiling should be conducted in a well-ventilated hood.

Quinaldine Red TS

A 0.1% w/v solution of quinaldine red in glacial acetic acid.

Salicylaldehyde TS

A 20% v/v solution of salicylaldehyde in ethanol.

Schiff's TS

Aqueous solution of 0.125 g of crystalline rosaline chlorohydrate in 1,000 ml and discolourized with sulfurous acid.

Schiff's TS, Modified

Dissolve 0.2 g of rosaniline hydrochloride ($C_{20}H_{20}ClN_3$) in 120 ml of hot water. Cool, add 2 g of sodium bisulfite ($NaHSO_3$) followed by 2 ml of hydrochloric acid, and dilute to 200 ml with water. Store in a brown bottle at 15° or lower.

Silicotungstic Acid TS

Dissolve 10 g of silicotungstic acid ($SiO_2 \cdot 12WO_3 \cdot 26H_2O$) in water and neutralize with 10% sodium hydroxide solution to a methyl red endpoint. Dilute to approximately 100 ml.

Silver Ammonionitrate TS

See Silver ammonium nitrate TS.

Silver Ammonium Nitrate TS

Dissolve 1 g of silver nitrate in 20 ml of water. Add ammonia TS, dropwise, with constant stirring, until the precipitate is almost but not entirely dissolved. Filter, and store in a tight, light-resistant container.

Silver Nitrate TS

A 4.2% w/v solution of silver nitrate ($AgNO_3$) in water (approximately 0.25 N).

Silver Nitrate TS, Acid

Dissolve 15 g of silver nitrate in 50 ml of water, add 400 ml of ethanol and several drops of concentrated nitric acid. This solution is standardized against 0.05 N ammonium thiocyanate by the Volhard method. The solution is very stable.

Silver Nitrate Spray TS

Prepare the following two solutions:

(a) Dissolve 50 g of silver nitrate in 450 ml of distilled water. Store in an amber bottle.

(b) Add 120 ml of concentrated ammonium hydroxide to 330 ml of distilled water.

When required combine equal volumes of solutions (a) and (b) for use as spray reagent.

Sodium Acetate TS

A 13.6% w/v solution of sodium acetate in water (approximately N).

Sodium Azide TS

A 5% w/v solution of sodium azide in water.

Sodium Bisulfite TS

Prepare a solution of sodium bisulfite in water (approximately 0.5 N). Check the pH and if necessary, adjust to the range 3.0 to 4.5 with dilute sulfuric acid or sodium hydroxide.

Sodium Bitartrate TS

A 1% w/v solution of sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$) in water (approximately N). Prepare this solution fresh.

Sodium Borate TS

A 2% w/v solution of sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) in water.

Sodium Carbonate TS

A 10.6% w/v solution of anhydrous sodium carbonate (Na_2CO_3) in water.

Sodium Chloride TS

A 10% w/v solution of sodium chloride in water.

Sodium Cobaltinitrite TS

Dissolve 10 g of sodium cobaltinitrite [$Na_3Co(NO_2)_6$] in water to make 50 ml and filter if necessary.

Sodium Ethoxide TS

Dissolve 10 g of sodium in 120 ml of absolute ethanol, using the following method: remove surplus oil from the sodium metal with filter paper, weigh in benzol and again dry on a filter paper. Cut the weighed metal into small pieces about the size of a pea and carefully add one or two pieces at a time to a 500-ml conical flask which is fitted with a water-cooled reflux condenser and contains the 120 ml of ethanol.

Sodium Fluorescein TS

A 0.1% w/v solution of sodium fluorescein in 50% ethanol.

Sodium Fluoride TS

Dry about 0.5 g of sodium fluoride (NaF) at 200° for 4 h. Weigh accurately 0.222 g of the dried sodium fluoride, and dissolve it in sufficient water to make exactly 100 ml. Transfer 10.0 ml of this solution into a 1,000-ml volumetric flask, dilute to volume with water, and mix. Each ml of this final solution corresponds to 0.01 mg of fluorine (F).

Sodium Hydrogen Sulfite TS

A 33.3% w/v solution of sodium hydrogen sulfite in water. Prepare freshly before use.

Sodium Hydroxide TS

Dissolve 4.3 g of sodium hydroxide in water to make 100 ml (approximately N).

Sodium Hydroxide TS (5%), Methanolic

Dissolve 5 g of sodium hydroxide in 5 ml of water, then add sufficient methanol to make 100 ml. Use supernatant.

Sodium Indigotindisulfonate TS

See indigo carmine TS.

Sodium Nitrite TS

A 10% w/v solution of sodium nitrite in water. Prepare freshly before use.

Sodium Nitroferricyanide TS

A 5% w/v solution of sodium nitroferricyanide [$Na_2Fe(NO)(CN)_5 \cdot 2H_2O$] in water. Prepare this solution fresh.

Sodium Nitroprusside TS

See sodium nitroferricyanide TS.

Sodium Phosphate TS

See sodium phosphate TS, dibasic.

Sodium Phosphate TS, Dibasic

A 12% w/v solution of clear crystals of dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$) in water.

Sodium Phosphate TS, Monobasic

A 62.4% w/v solution of monobasic sodium phosphate ($NaH_2PO_4 \cdot 2H_2O$) in water (approximately 4 M).

Sodium Starch Glycolate TS (5%)

Moisten 5 g of sodium starch glycolate with a few drops of ethanol, add 100 ml of water and boil for 2-3 min, and cool.

Sodium Starch Glycolate TS (1%)

Dilute 10 ml of sodium starch glycolate TS (5%) to 50 ml with distilled water. Prepare freshly before use.

Sodium Sulfide TS

A 10% w/v solution of sodium sulfide ($Na_2S \cdot 9H_2O$) in water. Prepare this solution fresh.

Sodium Sulfide TS (PbT)

Dissolve 10 g of sodium sulfide (PbT) in sufficient water to make 100 ml and filter.

Sodium Thiosulfate TS

Use 0.1 N sodium thiosulfate.

Stannous Chloride TS

Dissolve 3.2 g of stannous chloride ($SnCl_2 \cdot 2H_2O$) in 40 ml of 0.3 N hydrochloric acid. Transfer the solution to a 100-ml volumetric flask and dilute to the mark with 0.3 N hydrochloric acid. Prepare fresh daily. The stannous chloride solution should be titrated with sulfuric periodic acid TS before use and adjusted so that 10.0 ml of the stannous chloride reagent will titrate 10.2 ml of the periodic acid reagent. For the titration, 5 ml of concentrated hydrochloric acid are added to 10 ml of stannous chloride plus 1 ml of starch indicator (a blue colour indicates the endpoint).

Starch TS

Triturate 1 g of arrowroot starch with 10 ml of cold water, and pour slowly, with constant stirring, into 200 ml of boiling water. Boil the mixture until a thin, translucent fluid is obtained. (Longer boiling than necessary renders the solution less sensitive.) Allow to settle, and use only the clear, supernatant liquid. Prepare this solution fresh.

Starch Iodide Paste TS

Heat 100 ml of water in a 250-ml beaker to boiling, add a solution of 0.75 g of potassium iodide (KI) in 5 ml of water, then add 2 g of zinc chloride ($ZnCl_2$) dissolved in 10 ml of water, and, while the solution is boiling, add with stirring a smooth suspension of 5 g of potato starch in 30 ml of cold water. Continue to boil for 2 min, then cool. Store in a well-closed container in a cool place. This mixture must show a definite blue streak when a glass rod dipped in a mixture of 1 ml of 0.1 M sodium nitrite, 500 ml of water, and 10 ml of hydrochloric acid, is streaked on a smear of the paste.

Starch Mucilage TS

See Starch TS.

Sulfanilic Acid TS

A 0.8% w/v solution of sulfanilic acid ($p\text{-}NH_2 \cdot C_6H_4SO_3H \cdot H_2O$) in acetic acid. Store in a tight container.

Sulfanilic Acid/α-Naphthylamine TS

Dissolve 0.5 g of sulfanilic acid in 150 ml of acetic acid. Dissolve 0.1 g of α-naphthylamine in 0.26 g of hydrochloric acid and 150 ml of acetic acid, and mix. When a pink colour is produced upon standing, add zinc dust to decolourize.

Sulfuric Acid TS

Add a quantity of sulfuric acid of known concentration to sufficient water to adjust the final concentration to between 94.5 and 95.5% of H_2SO_4.

Sulfuric Acid TS, Dilute

A solution containing 10% w/v of H_2SO_4. Prepare by cautiously adding 57 ml of sulfuric acid (95-98%) or sulfuric acid TS to about 100 ml of water, then cool to room temperature, and dilute with water to 1,000 ml (approximately 2 N).

Sulfuric Acid/Periodic Acid TS

Dissolve 3.42 g of periodic acid (H_5IO_6) in 100 ml of 0.25 M sulfuric acid. Transfer the solution to a 500-ml volumetric flask and dilute to the mark with 0.25 M sulfuric acid (approximately 0.03 M sulfuric acid/periodic acid).

Tannic Acid TS

Dissolve 1 g of tannic acid (tannin) in 1 ml of ethanol, and add water to make 10 ml. Prepare this solution fresh.

Tannic Acid/Glacial Acetic Acid TS

Dissolve 10 mg of tannic acid in 80 ml of glacial acetic acid while shaking, and add 32 ml of phosphoric acid. Prepare freshly before use.

Tartrate Solution TS, Alkaline

Dissolve 34.6 g of potassium sodium tartrate (Rochelle salt) and 10 g of sodium hydroxide in water, dilute to 100 ml, let stand two days, and filter through glass wool.

Thymol Blue TS

Dissolve 0.1 g of thymol blue in 100 ml of ethanol, and filter if necessary. For pH determinations, dissolve 0.1 g in 4.3 ml of 0.05 N sodium hydroxide, and dilute with carbon dioxide-free water to 200 ml.

Thiourea TS

A 10% w/v solution of thiourea in water.

Thymolphthalein TS

Dissolve 0.1 g of thymolphthalein in 100 ml of ethanol, and filter if necessary.

Tin (II) Sulfate TS

Add 10 g of tin (II) sulfate to 100 ml of 1% sulfuric acid. Agitate continuously for several hours decanting the solution from the insoluble fraction at frequent intervals.

Triketohydrindene Hydrate TS

See Ninhydrin TS.

Trinitrophenol TS

(Picric acid TS). Dissolve the equivalent of 1 g of anhydrous trinitrophenol in 100 ml of hot water. Cool the solution, and filter if necessary.

Uranyl Acetate TS

Dissolve 1 g of uranyl acetate in 20 ml of water by shaking well and filter.

Uranyl Zinc Acetate TS

Dissolve 10 g of uranyl acetate [$(CH_3COO)_2UO_2 \cdot 2H_2O$)] by heating with 50 ml of water and 5 ml of acetic acid (a solution containing approximately 30% w/v of CH_3COOH, in water, approximately 5 N). Dissolve 3 g of zinc acetate [$(CH_3COO)_2Zn$] in 30 ml of water and 3 ml of 30% w/v acetic acid (approximately 5 N). Mix the two solutions, allow to cool to room temperature, and remove by filtration any solid material which separates.

Vanadic Acid/Molybdic Acid TS

Dissolve 1.12 g of ammonium metavanadate in about 300 ml of warm water, add 250 ml of nitric acid. Combine the cooled solution with another solution of 27 g of ammonium molybdate in about 400 ml of warm water, then add sufficient amount of water to make 1,000 ml. Use after 3 to 4 days of preservation in a dark-coloured bottle.

Xylenol Orange TS

A 0.1% w/v of xylenol orange in ethanol.

Zinc Amalgam TS

Add about 10 g of granulated zinc to sufficient mercury, about 20 ml, to produce a liquid amalgam on cooling, and heat at 150°, with stirring, until the zinc is dissolved. Zinc amalgam may be used repeatedly until the content of zinc is reduced to 0.2% w/w, as determined by the following process. Fill a pycnometer with mercury at 25° ± 1°, and weigh. Repeat the operation, using the amalgam. Calculate the proportion of zinc from the formula:

Percentage w/w of zinc = (13.534 - A) / 0.000875

where A = (wt of amalgam - 13.534) / wt of mercury

Zinc Sulfate TS

A 10% w/w solution of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) in water.

Zinc Sulfate TS, Standard

Dissolve 0.440 g of zinc sulfate in sufficient water to produce 1,000 ml, and dilute 50 ml of the solution to 1,000 ml with water. Each ml of the solution contains 5 µg of zinc.

Zirconium/Alizarin TS

Dissolve 0.80 g of zirconium nitrate [$Zr(NO_3)_2 \cdot 5H_2O$] in water, add a few drops of 4 N nitric acid, and make up to 100 ml with water. Dissolve 0.10 g of alizarin sulfonate monohydrate in 20 ml of water, and make up to 100 ml with ethanol. Mix 1 ml of the first solution with 1 ml of the second solution and add 18 ml of water. This solution must be clear and the dilution should be freshly prepared.

Zwikker's TS

Mix 1 ml of pyridine with 4 ml of a 10% aqueous solution of copper sulfate and 5 ml of water.

VOLUMETRIC SOLUTIONS

Normal Solutions

A normal solution contains 1 g equivalent weight of the solute per litre of solution. The normalities of solutions used in volumetric determinations are designated as 1 N; 0.1 N; 0.05 N; etc.

Molar Solutions

A molar solution contains 1 g molecular weight of the solute per litre of solution. The molarities of such solutions are designated as 1 M; 0.1 M; 0.05 M; etc.

Preparation and Methods of Standardization

The details for the preparation and standardization of solutions used in several normalities are usually given only for those most frequently required. Solutions of other normalities are prepared and standardized in the same general manner as described. Solutions of lower normalities may be prepared accurately by making an exact dilution of a stronger solution, but solutions prepared in this way should be restandardized before use.

Dilute solutions that are not stable, such as 0.01 N potassium permanganate and sodium thiosulfate, are preferably prepared by diluting exactly the higher normality with thoroughly boiled and cooled water on the same day they are to be used.

All volumetric solutions should be prepared, standardized, and used at the standard temperature of 20°, if practicable. When a titration must be carried out at a markedly different temperature, the volumetric solution should be standardized at that same temperature, or a suitable temperature correction should be made. Since the strength of a standard solution may change upon standing, the normality or molarity factor should be redetermined frequently.

Although the directions provide only one method of standardization, other methods of equal or greater accuracy may be used. For substances available as certified primary standards, or of comparable quality, the final standard solution may be prepared by weighing accurately a suitable quantity of the substance and dissolving it to produce a specific volume solution of known concentration. Hydrochloric and sulfuric acids may be standardized against a certified primary standard.

In volumetric assays described, the number of mg of the test substance equivalent to 1 ml of the primary volumetric solution is given. In general, these equivalents may be derived by simple calculation.

0.1 N Ammonia, (3.505 g of NH_4OH per litre)

Add sufficient water to about 35 ml of ammonia TS to 1,000 ml. Standardize the solution with 0.1 N hydrochloric acid, using bromophenol blue TS as the indicator.

0.1 N Ammonium Thiocyanate, (7.612 g of NH_4SCN per litre)

Dissolve about 8 g of ammonium thiocyanate, NH_4SCN, in 1,000 ml of water, and standardize by titrating the solution against 0.1 N silver nitrate as follows: transfer about 30

ml of 0.1 N silver nitrate, accurately measured, into a glass-stoppered flask. Dilute with 50 ml of water, then add 2 ml ferric ammonium sulfate TS and 2 ml of nitric acid, and titrate with the ammonium thiocyanate solution to the first appearance of a red-brown colour. Calculate the normality, and, if desired, adjust the solution to exactly 0.1 N. If desired, 0.1 N ammonium thiocyanate may be replaced by 0.1 N potassium thiocyanate where the former is directed in various tests and assays.

0.01 M Barium Chloride

Dissolve 2.44 g of barium chloride in sufficient water, freshly boiled and cooled, to make 1,000 ml.

0.1 N Bromine, (7.990 g of Br per litre)

Dissolve 3 g of potassium bromate, $KBrO_3$, and 15 g of potassium bromide, KBr, in sufficient water to make 1,000 ml, and standardize the solution as follows: transfer about 25 ml of the solution, accurately measured, into a 500-ml iodine flask, and dilute with 120 ml of water. Add 5 ml of hydrochloric acid, stopper the flask, and shake it gently. Then add 5 ml of potassium iodide TS, re-stopper, shake the mixture, allow it to stand for 5 min, and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS near the end of the titration. Calculate the normality. Store this solution in dark, glass-stoppered bottles.

0.1 N Ceric Sulfate, (33.22 g of $Ce(SO_4)_2$ per litre)

Transfer 59 g of ceric ammonium nitrate, $Ce(NO_3)_4 \cdot 2NH_4NO \cdot 2H_2O$, to a beaker, add 31 ml of sulfuric acid, mix, and cautiously add water, in 20 ml portions, until solution is complete. Cover the beaker, let stand overnight, filter through a sintered-glass crucible of fine porosity, add water to make 1,000 ml, and mix.

Standardize the solution as follows: weigh accurately 200 mg of primary standard arsenic trioxide, As_2O_3, previously dried at 100° for 1 h, and transfer to a 500-ml Erlenmeyer flask. Wash down the inner walls of the flask with 25 ml of sodium hydroxide solution (2 in 5), swirl to dissolve the sample, and when solution is complete add 100 ml of water, and mix. Add 10 ml of dilute sulfuric acid (1 in 3) and 2 drops each of orthophenanthroline TS and a solution of osmium tetroxide in 0.1 N sulfuric acid (1 in 400), and slowly titrate with the ceric sulfate solution until the pink colour is changed to a very pale blue. Calculate the normality. Each 4.964 mg of As_2O_3 is equivalent to 1 ml of 0.1 N ceric sulfate.

0.01 N Ceric Sulfate, for Tocopherol Assay (3.322 g of $Ce(SO_4)_2$ per litre)

Dissolve 4.2 g of ceric sulfate, $Ce(SO_4)_2 \cdot 4H_2O$, or 5.5 g of the acid sulfate $Ce(HSO_4)_4$, in about 500 ml of water containing 28 ml of sulfuric acid, and dilute to 1,000 ml. Allow the solution to stand overnight, and filter.

Standardize this solution daily as follows: weigh accurately about 275 mg of hydroquinone, $C_6H_6O_2$, dissolve it in sufficient 0.5 N ethanolic sulfuric acid to make 500 ml, and mix. To 25 ml of this solution add 75 ml of 0.5 N sulfuric acid, 20 ml of water, and 2 drops of diphenylamine TS. Titrate with the ceric sulfate solution at a rate of about 25 drops per 10 sec until the red point is reached which persists for 10 sec. Perform a blank determination using 100 ml of 0.5 N ethanolic sulfuric acid, 20 ml of water, and 2 drops of diphenylamine TS, and make any necessary correction. Calculate the normality of the ceric sulfate solution by

the formula 0.05W/55.057V, in which W is the weight, in mg, of the hydroquinone sample taken, and V is the volume, in ml, of the ceric sulfate solution consumed in the titration.

0.05 M Disodium Ethylenediaminetetraacetate (EDTA), (16.811 g of $C_{10}H_{14}O_8N_2Na_2$ per litre)

Dissolve 18.7 g of disodium ethylenediaminetetraacetate in sufficient water, freshly boiled and cooled, to make 1,000 ml.

Standardize the solution as follows: weigh accurately about 0.2 g of chelometric standard calcium carbonate, $CaCO_3$, transfer to a 400-ml beaker, add 10 ml of water, and swirl to form a slurry. Cover the beaker with a watch glass, and introduce 2 ml of dilute hydrochloric acid TS from a pipet inserted between the lip of the beaker and the edge of the watch glass. Swirl the contents of the beaker to dissolve the calcium carbonate. Wash down the sides of the beaker, the outer surface of the pipet, and the watch glass, and dilute to about 100 ml with water. While stirring preferably with a magnetic stirrer, add about 30 ml of the disodium EDTA solution from a 50-ml buret, then add 15 ml of sodium hydroxide TS and 300 mg of hydroxynaphthol blue indicator, and continue the titration to a blue end-point. Calculate the molarity by the formula W/100.09V, in which W is the weight, in mg, of $CaCO_3$ in the sample of calcium carbonate taken, and V is the volume, in ml, of disodium EDTA solution consumed. Each 5.004 mg of $CaCO_3$ is equivalent to 1 ml 0.05 M disodium EDTA.

0.1 N Ferrous Ammonium Sulfate, (28.405 g of $FeSO_4(NH_4)_2SO_4$ per litre)

Dissolve 40 g of ferrous ammonium sulfate hexahydrate in a 100 ml portion of a mixture of 100 ml of sulfuric acid and 100 ml of water previously cooled, add water to 1,000 ml, and standardize as follows:

Titrate 25 ml of this solution with 0.1 N ceric sulfate, using 2 drops of orthophenanthroline TS as the indicator, until a red colour of the solution changes to pale blue. Calculate the normality from the volume of 0.1 N ceric sulfate consumed.

10 N Hydrochloric Acid

Prepare and standardize, as directed under 1 N hydrochloric acid, using 950 ml of hydrochloric acid.

6 N Hydrochloric Acid

Prepare and standardize, as directed under 1 N hydrochloric acid, using 570 ml of hydrochloric acid.

2 N Hydrochloric Acid

Prepare and standardize, as directed under 1 N hydrochloric acid, using 190 ml of hydrochloric acid.

1 N Hydrochloric Acid, (36.461 g of HC1 per litre)

Dilute 95 ml of hydrochloric acid with water to 1,000 ml. Standardize by one of the following methods:

Method I: Dissolve about 1.5 g sodium carbonate (standard reagent) previously dried at about 270° for 1 h and accurately weighed, in 100 ml of water, and titrate with hydrochloric acid, using 2 drops of bromophenol blue TS as the indicator. Near the endpoint, boil to expel carbon dioxide, cool and continue to titrate. Calculate the normality.

Method II: Add 130 ml of water and 5 drops of nitric acid to 20 ml of 1 N hydrochloric acid. While stirring constantly, add about 40 ml of silver nitrate solution (1 in 10) or even more if necessary, until the precipitation is completed. Boil the mixture gently for 5 min, allow to stand in the dark until the precipitate settles. Transfer the precipitate completely into a tared Gooch crucible, dry to constant weight at 110°, and wash with water, slightly acidified with nitric acid, until the washings give no reaction for silver. Dry to constant weight at about 110°. From the weight of silver chloride obtained, calculate the normality of hydrochloric acid.

0.5 N Hydrochloric Acid

Using 47.5 ml of hydrochloric acid, prepare and standardize, as directed under 1 N hydrochloric acid.

0.1 N Hydrochloric Acid

Prepare this solution by diluting 1 N hydrochloric acid with water to 10 volumes, or using 9.5 ml of hydrochloric acid, prepare as directed under 1 N hydrochloric acid. Standardize as directed under 1 N hydrochloric acid.

0.02 N Hydrochloric Acid

Dilute 0.1 N hydrochloric acid with water to 5 volumes, and standardize as directed under 1 N hydrochloric acid.

0.01 N Hydrochloric Acid

Dilute 0.1 N hydrochloric acid with water to 10 volumes, and standardize as directed under 1 N hydrochloric acid.

0.002 N Hydrochloric Acid

Dilute 0.1 N hydrochloric acid with water to 50 volumes.

0.001 N Hydrochloric Acid

Dilute 0.1 N hydrochloric acid with water to 100 volumes.

0.5 N Hydroxylamine Hydrochloride, (34.745 g of $NH_2OH \cdot HCl$ per litre)

Dissolve 35 g of hydroxylamine hydrochloride in 150 ml of water, and dilute to 1,000 ml with anhydrous methanol. To 500 ml of this solution add 15 ml of a 0.04% solution of bromophenol blue in ethanol, and titrate with 0.5 N triethanolamine until the solution appears greenish blue by transmitted light. Prepare this solution fresh before use.

0.1 N Iodine, (12.690 g of iodine per litre)

Dissolve 14 g of iodine in a solution of 36 g of potassium iodide dissolved in 100 ml of water. Add 3 drops of hydrochloric acid and water to 1,000 ml, and standardize as follows.

Weight accurately about 0.15 g of arsenic trioxide previously pulverized and dried to constant weight at 100°, and dissolve in 20 ml of 1 N sodium hydroxide by heating if necessary. Dilute with about 40 ml of water, add 2 drops of methyl orange TS, and add dilute hydrochloric acid until the yellow colour changes to pale pink. Add 2 g of sodium bicarbonate, dilute with 50 ml of water, and add 3 ml of starch TS. Titrate with 0.1 N iodine until a sustaining blue colour is produced. Store in a glass stoppered bottle and restandardize frequently. Calculate the normality. Each 4.946 mg of As_2O_3 is equivalent to 1 ml of 0.1 N iodine.

0.1 N Lithium Methoxide, (3.797 g of CH_3OLi per litre)

Dissolve 600 mg of freshly cut lithium metal in a mixture of 150 ml of absolute methanol and 850 ml of benzene. Filter the resulting solution if it is cloudy, and standardize it as follows: dissolve about 80 mg of benzoic acid, accurately weighed, in 35 ml of dimethylformamide, add 5 drops of thymol blue TS, and titrate with the lithium methoxide solution to a dark blue endpoint. (Caution. Protect the solution from absorption of carbon dioxide and moisture by covering the titration vessel with aluminium foil while dissolving the benzoic acid sample and during the titration.) Each ml of 0.1 N lithium methoxide is equivalent to 12.21 mg of benzoic acid.

0.1 N Magnesium Chloride, (4.761 g of $MgC1_2$ per litre)

Dissolve 10.5 g of magnesium chloride in freshly boiled and cooled water to make 1,000 ml.

0.1 M Mercuric Nitrate, (32.46 g of $Hg(NO_3)_2$ per litre)

Dissolve about 35 g of mercuric nitrate, $Hg(NO_3)_2 \cdot H_2O$, in a mixture of 5 ml of nitric acid and 500 ml of water, and dilute with water to 1,000 ml. Standardize the solution as follows: transfer an accurately measured volume of about 20 ml of the solution into an Erlenmeyer flask, and add 2 ml of ferric ammonium sulfate TS. Cool to below 20°, and titrate with 0.1 N ammonium thiocyanate to the first appearance of a permanent brownish colour. Calculate the molarity.

0.1 N Oxalic Acid, (4.502 g of $H_2C_2O_4$ per litre)

Dissolve 6.45 g of oxalic acid, $H_2C_2O_4 \cdot 2H_2O$, in sufficient water to make 1,000 ml. Standardize by titration against freshly standardized 0.1 N potassium permanganate as directed under Potassium Permanganate 0.1 N. Store this solution in glass-stoppered bottles, protected from light.

0.1 N Perchloric Acid, (10.046 g of $HClO_4$ per litre)

Transfer about 8.5 ml of 70% perchloric acid into a 1,000-ml flask, add 950 ml of glacial acetic acid, and shake well. Add 15 ml of acetic anhydride gradually by dividing into 1 ml portions, and then dilute with acetic acid to 1,000 ml. Allow the solution to stand overnight.

Standardize as follows: add 50 ml of glacial acetic acid to 0.4 g of potassium biphthalate, previously dried at 120° for 1 h and accurately weighed, and heat to dissolve on a water bath. Titrate with 0.1 N perchloric acid to the end point, at which the colour changes from violet to blue, using 1 ml of 0.05% acetic anhydride solution of crystal violet as the indicator, and calculate the normality by the following formula:

Normality factor = (Weight of potassium biphthalate (g) x 1,000 x 10) / (The number of ml of 0.1 N perchloric acid consumed x 204.22)

0.1 N Potassium Acid Phthalate, (20.42 of $KHC_6H_4(COO)_2$ per litre)

Dissolve 20.42 g of primary standard potassium biphthalate, $KHC_6H_4(COO)_2$, in glacial acetic acid in a 1,000-ml volumetric flask, warming on a steam bath if necessary to effect solution and protecting the solution from contamination by moisture. Cool to room temperature, dilute to volume with glacial acetic acid, and mix.

0.1 N Potassium Bromate, (2.784 g of $KBrO_3$ per litre)

Dissolve 2.8 g of potassium bromate in sufficient water to make 1,000 ml.

Standardize as follows: transfer 40 ml of the solution into a glass-stoppered flask, and add 3 g of potassium iodide and 3 ml of hydrochloric acid. Stopper tightly, and allow to stand for 5 min in the dark. Titrate the free iodine with 0.1 N sodium thiosulfate, using starch TS as the indicator. Perform a blank test in the same manner as the sample.

0.1 N Potassium Dichromate, (4.903 g of $K_2Cr_2O_7$ per litre)

Dissolve 4.904 g of potassium dichromate previously pulverized and dried to constant weight at 120°, in sufficient water to make 1,000 ml.

0.5 N Potassium Hydroxide, Ethanolic

Dissolve about 35 g of potassium hydroxide, KOH, in 20 ml of water, and sufficient aldehyde-free alcohol to make 1,000 ml. Allow the solution to stand in a tightly stoppered bottle for 24 h. Then quickly decant the clear supernatant liquid into a suitable, tight container, and standardize as follows: transfer quantitatively 25 ml of 0.5 N hydrochloric acid into a flask, dilute with 50 ml of water, add 2 drops of phenolphthalein TS, and titrate with the ethanolic potassium hydroxide solution until a permanent, pale pink colour is produced. Calculate the normality. Store this solution in a tightly stoppered bottle protected from light.

1 N Potassium Hydroxide, (56.109 g of KOH per litre)

Using about 70 g of potassium hydroxide, prepare and standardize as directed under 1 N sodium hydroxide. Each 204.2 mg of $KHC_6H_4(COO)_2$ is equivalent to 1 ml of 1 N potassium hydroxide.

0.5 N Potassium Hydroxide

Dilute 1 N potassium hydroxide with water freshly boiled and cooled to 5 volumes, or using about 35 g of potassium hydroxide, prepare as directed under 1 N potassium hydroxide.

0.1 N Potassium Hydroxide

Dilute 1 N potassium hydroxide with water, freshly boiled and cooled, to 10 volumes, or using about 7 g of potassium hydroxide, prepare as directed under 1 N potassium hydroxide. Standardize as directed under 1 N potassium hydroxide.

0.05 M Potassium Iodate, (10.70 g of KIO_3 per litre)

Dissolve 10.700 g of potassium iodate of primary standard quality, KIO_3, previously dried at 110° to constant weight, in sufficient water to make 1,000 ml.

0.1 N Potassium Permanganate, (3.161 g of $KMnO_4$ per litre)

Dissolve about 3.3 g of potassium permanganate in 1,000 ml of water, and boil for about 15 min. Allow to stand in a tightly closed flask for at least 2 days, and filter through a fine porosity sintered glass crucible. Store in a glass-stoppered, light-resistant bottle, and restandardize before use.

Standardize as follows: dissolve 0.2 g of sodium oxalate previously dried at 110° to constant weight and accurately weighed, in about 250 ml of water. Add 7 ml of sulfuric acid, heat to about 70° and titrate with 0.1 N potassium permanganate while hot. Each 6.700 mg of $Na_2C_2O_4$ is equivalent to 1 ml of 0.1 N potassium permanganate.

0.01 M Potassium Sulfate, (1.743 g of K_2SO_4 per litre)

Dissolve 1.743 g of potassium sulfate, previously dried at 110° for 4 h, in sufficient water, freshly boiled and cooled, to make 1,000 ml.

0.1 N Silver Nitrate, (16.99 g of $AgNO_3$ per litre)

Dissolve about 17.5 g of silver nitrate, $AgNO_3$, in 1,000 ml of water, and standardize the solution as follows: dilute about 40 ml, accurately measured, of the silver nitrate solution with about 100 ml of water, heat the solution, and add slowly, with continuous stirring, dilute hydrochloric acid TS until precipitation of the silver is complete. Boil the mixture cautiously for about 5 min, then allow it to stand in the dark until the precipitate has settled and the supernatant liquid has become clear. Transfer the precipitate completely to a tared filtering crucible, and wash it with small portions of water slightly acidified with nitric acid. Dry the precipitate at 110° to constant weight. Each 14.332 mg of silver chloride obtained is equivalent to 1 ml of 0.1 N silver nitrate. Protect the silver chloride from light as much as possible during the determination.

0.05 N Sodium Arsenite, (3.248 g of $NaAsO_2$ per litre)

Transfer 2.4725 g of arsenic trioxide, which has been pulverized and dried at 100° to constant weight, to a 1,000-ml volumetric flask, dissolve it in 20 ml of 1 N sodium hydroxide, and add 1 N sulfuric acid or 1 N hydrochloric acid until the solution is neutral or only slightly acid to litmus. Add 15 g of sodium bicarbonate, dilute to volume with water, and mix.

0.1 N Sodium Chloride, (5.844 g of NaCl per litre)

Dissolve 5.845 g of sodium chloride, previously dried at 110° for 2 h, in sufficient water to make 1,000 ml.

1 N Sodium Hydroxide, (39.997 g of NaOH per litre)

Dissolve 45 g of sodium hydroxide in about 950 ml of water, and add a saturated barium hydroxide solution, freshly prepared, until not further precipitate is formed. Shake the mixture thoroughly, and allow to stand overnight in a stoppered bottle. Decant the supernatant liquid or filter the solution, and standardize by one of the following methods. Store in a well-fitted, rubber-stoppered bottle, or in a bottle with a soda-lime tube, and restandardize frequently.

Method I: Dilute 25 ml of 1 N hydrochloric acid or 1 N sulfuric acid with 50 ml of water, freshly boiled and cooled, and titrate with 1 N sodium hydroxide, using 2 drops of phenolphthalein TS as the indicator.

Method II: Dissolve about 5 g of potassium biphthalate previously powdered, dried at 100° for 3 h and weighed accurately, in 75 ml of water, freshly boiled and cooled, and titrate with 1 N sodium hydroxide solution, using drops of phenolphthalein Ts as the indicator. Each 204.2 mg of potassium biphthalate is equivalent to 1 ml of 1 N sodium hydroxide.

0.5 N Sodium Hydroxide

Using about 22 g of sodium hydroxide, prepare, standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

0.2 N Sodium Hydroxide

Dilute 1 N sodium hydroxide with water, freshly boiled and cooled, to 5 volumes, or use about 9 g of sodium hydroxide and prepare as directed under 1 N sodium hydroxide. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

0.1 N Sodium Hydroxide

Dilute 1 N sodium hydroxide with water, freshly boiled and cooled, to 10 volumes, or use about 4.5 g of sodium hydroxide and prepare as directed under 1 N sodium hydroxide. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

0.05 N Sodium Hydroxide

Dilute 1 N sodium hydroxide with water, freshly boiled and cooled, to 20 volumes. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

0.02 N Sodium Hydroxide

Dilute 0.1 N sodium hydroxide with water, freshly boiled and cooled, to 5 volumes. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

0.01 N Sodium Hydroxide

Dilute 0.1 N sodium hydroxide with water, freshly boiled and cooled, to 10 volumes. Standardize and store, as directed under 1 N sodium hydroxide. Restandardize frequently.

0.1 N Sodium Methoxide, in Pyridine, (5.40 g of CH_3ONa per litre)

Weigh 14 g of freshly cut sodium metal, and cut into small cubes. Place about 0.5 ml of anhydrous methanol in a round-bottom 250-ml flask equipped with a ground-glass joint, add 1 cube of the sodium metal, and when the reaction subsides, add the remaining sodium metal to the flask. Connect a water-cooled condenser to the flask, and slowly add 100 ml of anhydrous methanol, in small portions, through the top of the condenser. Regulate the addition of the methanol so that the vapours are condensed and do not escape through the top of the condenser. After addition of the methanol is complete, connect a drying tube to the top of the condenser, and allow the solution to cool. Transfer 17.5 ml of this solution (approximately 6 N) into a 1,000-ml volumetric flask containing 70 ml of anhydrous methanol, and dilute to volume with freshly distilled pyridine. Store preferably in the reservoir of an automatic buret suitably protected from carbon dioxide and moisture. Standardize the solution as follows: weigh accurately about 400 mg of benzoic acid, transfer it into a 250 ml wide-mouth Erlenmeyer flask, and dissolve it in 50 ml of freshly distilled pyridine. Add a few drops of thymolphthalein TS, and titrate immediately with the sodium methoxide solution to a blue endpoint. During the titration, direct a gentle stream of nitrogen into the flask through a short piece of 6-mm glass tubing fastened near the tip of the buret. Perform a blank determination, correct for the volume of sodium methoxide solution consumed by the blank, and calculate the normality. Each 12.21 mg of benzoic acid is equivalent to 1 ml of 0.1 N sodium methoxide in pyridine.

0.1 M Sodium Nitrite, (7.900 g of $NaNO_2$ per litre)

Dissolve 7.5 g of sodium nitrite, $NaNO_2$, in sufficient water to make 1,000 ml, and standardize the solution as follows: Weigh accurately about 500 mg of U.S.P. Sulfanilamide Reference Standard, previously dried at 105° for 3 h, and transfer to a beaker or a casserole. Add 50 ml of water and 5 ml of hydrochloric acid, and stir well until dissolved. Cool to 15°, and add about 25 g of crushed ice, then titrate slowly with the sodium nitrite solution, stirring vigorously until a blue colour is produced immediately when a glass rod dipped in the titrated solution is streaked on a smear of starch iodide paste TS. When the titration is complete, the endpoint should be reproducible after the mixture has been standing for 1 min. Calculate the molarity. Each 17.22 mg of sulfanilamide is equivalent to 1 ml of 0.1 M sodium nitrite.

0.1 N Sodium Thiosulfate, (15.82 g of $Na_2S_2O_3$ per litre)

Dissolve about 26 g of sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$, and 200 mg of sodium carbonate, Na_2CO_3, in 1,000 ml of recently boiled and cooled water. Standardize the solution as follows: weigh accurately about 210 mg of primary standard potassium dichromate, previously pulverized and dried at 120° for 4 h, and dissolve in 100 ml of water in a 500 ml glass-stoppered flask. Swirl to dissolve the sample, remove the stopper and quickly add 3 g of potassium iodide, KI, and 5 ml of hydrochloric acid. Stopper the flask, swirl to mix, and let stand in the dark for 10 min. Rinse the stopper and inner walls of the flask with water, and titrate the liberated iodine with the sodium thiosulfate solution until the solution is only faint

yellow in colour. Add starch TS, and continue the titration to the discharge of the blue colour. Calculate the normality.

0.01 N Sodium Thiosulfate

Dilute 0.1 N sodium thiosulfate with water, freshly boiled and cooled, to 10 volumes. Standardize as directed under 0.1 N sodium thiosulfate before use.

4 N Sulfuric Acid

This solution contains 196.155 g of H_2SO_4 per 1,000 ml.

Using 120 ml of sulfuric acid, prepare and standardize, as directed under 1 N sulfuric acid.

1 N Sulfuric Acid, (49.039 g of H_2SO_4 per litre)

While stirring, slowly add 30 ml of sulfuric acid to about 1,000 ml of water, allow to cool to 20°, and standardize with sodium carbonate (standard reagent) as directed under 1 N hydrochloric acid. Each 52.99 mg of Na_2CO_3 is equivalent to 1 ml of 1 N sulfuric acid.

0.5 N Sulfuric Acid

Using 15 ml of sulfuric acid, prepare and standardize, as directed under 1 N sulfuric acid.

0.2 N Sulfuric Acid

Using 6 ml of sulfuric acid, prepare and standardize, as directed under 1 N sulfuric acid.

0.1 N Sulfuric Acid

Dilute 1 N sulfuric acid with water to 10 volumes, or using 3 ml of sulfuric acid, prepare as directed under 1 N sulfuric acid. Standardize as directed under 1 N sulfuric acid.

0.02 N Sulfuric Acid

Dilute 0.1 N sulfuric acid with water to 5 volumes, and standardize as directed under 1 N sulfuric acid.

0.01 N Sulfuric Acid

Dilute 0.1 N sulfuric acid with water to 10 volumes, and standardize as directed under 1 N sulfuric acid.

0.5 N Sulfuric Acid, Ethanolic

Add cautiously, with stirring, 13.9 ml of sulfuric acid to a sufficient quantity of absolute ethanol to make 1,000 ml. Alternatively, this solution may be prepared by diluting 100 ml of 5 N sulfuric acid with absolute ethanol to make 1,000 ml.

0.1 M Thorium Nitrate, (48.01 g of $Th(NO_3)_4$ per litre)

Weigh accurately 55.21 g of thorium nitrate $Th(NO_3)_4 \cdot 4H_2O$, dissolve it in water, dilute to 1,000 ml, and mix. Standardize the solution as follows: transfer 50 ml into a 500-ml volumetric flask, dilute to volume with water, and mix. Transfer 50 ml of the diluted solution into a 400-ml beaker, add 150 ml of water and 5 ml of hydrochloric acid, and heat to boiling. While stirring, add 25 ml of a saturated solution of oxalic acid, then digest the mixture for 1 h just below the boiling point and allow to stand overnight. Decant through Whatman No. 42, or equivalent, filter paper, and transfer the precipitate to the filter using about 100 ml of a wash solution consisting of 70 ml of the saturated oxalic acid solution, 430 ml of water, and 5 ml of hydrochloric acid. Transfer the precipitate and filter paper to a tared tall-form porcelain crucible, dry, char the paper, and ignite at 950° for 1.5 h or to constant weight. Cool in a desiccator, weigh, and calculate the molarity of the solution by the formula 200W/264.04, in which W is the weight, in g, of thorium oxide obtained.

0.1 N Titanous Chloride, (15.426 g of $TiC1_3$ per litre)

Mix 200 ml of 15% titanous chloride and 150 ml of hydrochloric acid, and dilute with freshly boiled and cooled water to 2,000 ml. Transfer into a light-shaded bottle equipped with a buret, replace the air in the bottle with hydrogen, and allow to stand for 2 days before use. Standardize as follows: place 3 g of ferrous ammonium sulfate in a wide-mouthed 500 ml flask, dissolve in 50 ml of freshly boiled and cooled water in an atmosphere of carbon dioxide and add 25 ml dilute sulfuric acid (27 in 100). Pass carbon dioxide through the solution, then quickly add 40 ml of 0.1 N potassium permanganate and add 0.1 N titanous chloride until the endpoint is nearly reached. Immediately add 5 g of ammonium thiocyanate, and titrate the solution with 0.1 N titanous chloride to the end point, when the colour of the solution disappears. Perform a blank test in the same manner as the sample.

Normality factor = Volume of 0,1 N potassium permanganate added (ml) / The number of ml of 0.1 N titanous chloride consumed

0.5 N Triethanolamine, (74 g of $N(CH_2CH_2OH)_3$ per litre)

Transfer 65 ml (74 g) of 98% triethanolamine into a 1,000 ml volumetric flask, dilute to volume with water, stopper the flask, and mix thoroughly.

0.01 M Zinc Acetate, (1.835 g of $Zn(CH_3COO)_2$ per litre)

Dissolve 2 g of zinc acetate in sufficient water to make 1,000 ml. Standardize as follows: to 25 ml of 0.01 M zinc acetate, add 2 ml of ammonia/ammonium chloride buffer solution and sufficient water to about 100 ml. Titrate the solution with 0.01 M disodium ethylenediaminetetraacetate, using 3 drops of eriochrome black TS as the indicator.

0.025 M Zinc Chloride, (3.407 g of $ZnC1_2$ per litre)

Place about 1.6 g of zinc in a beaker, add 30 ml of dilute hydrochloric acid, cover with a watch glass, and allow to stand. Dissolve by heating gently on a water bath after the initial rapid release of hydrogen gas. Wash the watch glass and the inside wall of the beaker with water, evaporate to almost dryness on a water bath, cool, and add water to 1,000 ml. Standardize as directed for 0.05 M zinc sulfate. Calculate the molarity.

0.05 M Zinc Sulfate, (8.072 g of $ZnSO_4$ per litre)

Dissolve about 15 g of zinc sulfate, $ZnSO_4 \cdot 7H_2O$ in sufficient water to make 1,000 ml, and standardize the solution as follows: dilute about 35 ml 0.05 M zinc sulfate accurately measured, with 75 ml of water, add 5 ml of ammonia/ammonium chloride buffer TS and 0.1 ml of eriochrome black TS, and titrate with 0.05 M disodium ethylenediaminetetraacetate until the solution is deep blue in colour. Calculate the molarity.