Sections in This Document

1. Purpose

This chapter provides preliminary training to new analysts in sample preparation, analytical techniques, instrumentation, labeling, and other requirements for food standards, food additives, and color additives. The exercises in this chapter should be used to supplement in-house training.

2. Scope

This document applies to trainees and other analysts who analyze FDA regulated products for food standards, additives, and colors.

3. Responsibility

A. It is the responsibility of laboratory management and quality management staff to ensure that all analysts are properly trained and have the necessary knowledge and skills to analyze FDA-regulated

products to meet regulatory requirements of the agency, and that all training is documented.

B. This chapter is divided into three sections: Food Standards, Food Additives, and Color Additives. For each assigned training exercise, the trainee is required to read the background information, applicable methodology, instrument/equipment SOPs or work instructions, and relevant Safety Data Sheets before starting any sample analysis.

   The trainee will complete a worksheet for each assigned exercise.

C. The trainer will address any questions the trainee has prior to starting an exercise. Once the exercise is completed, the trainer will review the analysis, worksheet, and exercise questions with the trainee.

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4. Procedure

4.1. Preliminary documents for review

The trainee should review and become familiar with the following documents:

A. Definition of Terms and Explanatory Notes, Official Methods of Analysis of AOAC (current edition), Gaithersburg MD.

B. General Provisions and Requirements Applying to Specifications, Tests, and Assays of the Food Chemicals Codex (starting with General Specifications and Statements), Food Chemicals Codex, (current edition) United States Pharmacopeia, Rockville, MD.

C. ORA Laboratory Manual:

1. MAN-000037 Volume II: Methods, Method Verification and Validation (ORA-LAB.5.4.5).
2. MAN-000038 Volume II: Estimation of Measurement Uncertainty (ORA-LAB.5.4.6).
3. MAN-000043 Volume II: Ensuring the Quality of Test Results (ORA-LAB.5.9).
4. MAN-000048 Volume III: Basic Statistics and Data Presentation (III-04).

D. Local SOPs/Work Instructions:

1. Laboratory Safety Procedures.
2. Chemical Management and Hygiene.
3. Hazardous Waste.

4. Physical Reference Standards and Reference Materials.

E. Labeling:

1. Food, Drug, and Cosmetic Act, Section 201(k), (l), and (m). Washington DC: Library of Congress.
2. Code of Federal Regulations, Title 21, Pt. 101 – Food Labeling. Washington DC: Office of the Federal Register National Archives and Records Administration.

4.2. Food Standards

Planned work in the Food Standards Program area is infrequent. Therefore, the laboratory has the option of not completing this training section. However, it is recommended that trainee reviews this information to gain a basic understanding of food standards and related regulations.

A. Background

1. "Food Standards" is FDA's common designation for Standards of Identity, Standards of Quality, and Standards of Fill of Container enacted under Section 401 of the FD&C Act. Section 401 requires regulations be promulgated "fixing and establishing for any food, under its common or usual name so far as practicable, a reasonable definition and standard of identity, a reasonable standard of quality or reasonable standards of fill of container". The purpose of food standards is to maintain the integrity of food products, so consumers get what they reasonably expect.
2. Food standards were published and incorporated into law to prevent or mitigate the harm from economically motived adulteration. They are formulated in precise and definite terms, so they are defensible in court when challenged.
3. Economically motivated adulteration is a type of food fraud that has existed since man first started trading food products. It comprises a wide array of dishonest acts that are designed to deceive consumers about the content or true value of a food product.
    a. Early forms of economic adulteration included diluting alcohol or milk with water, increasing the density of flour by adding chalk powder, and substituting or mixing expensive imported spices with less expensive spices.
    b. An entirely new level of economic motivated adulteration came into existence with the Industrial Revolution. Less expensive synthetic ingredients replaced natural ingredients

in processed foods. Manufacturers used deceptive packaging practices and containers to hide the foods' quality or quantity.

c. Economically motived adulteration increased as foods became more complex. It became easier for unscrupulous manufacturers to add, substitute, or omit ingredients.

d. The impact of economically motivated adulteration affects more than just the economy. Depending on what was added, substituted, or removed, it can lead to health issues, and in some cases, death. Economically motivated adulteration continues to be a growing concern of the food industry, consumer advocacy groups and regulatory authorities.

4. Standards of Identity legally define what a food is. They establish the common or usual name and define the nature and essential characteristics of a food and the ingredients that must be used or may be used. Ingredients not recognized in the standard are not permitted.

   a. The standards of identity may specify minimum levels of valuable constituents and maximum levels for fillers. For example, oleomargarine must not have less than 80 percent fat as determined by the method specified in the Standard.

   b. They may designate a method of production or formulation if it is relevant to the identity of the finished food.

   c. They also describe the labeling requirements.

   d. Foods that have a legal standard of identity are considered "standardized" foods.

5. Not all foods have a Standard of Identity. For example, the Act does not require a standard of identity be established for fresh and dried fruits and vegetables.

   a. These foods are referred to as "non-standardized foods".

   b. 21 CFR Part 102 lists some non-standardized foods that have no Standard of Identify per se but are subject to specific regulatory requirements.

   c. Non-standardized foods without a specific naming requirement must be labeled using the common or usual name of the food.

6. Quality Standards establish minimum specifications for characteristics such as freedom from defects, tenderness, and

color along with objective methods for measuring these characteristics.

a. These standards are crucial for foods packaged in opaque containers where such characteristics would not be readily apparent to the consumer. For example, green beans that are highly fibrous do not have good eating quality.

b. The quality standard for canned green beans sets a limit for fiber and prescribes the method to be followed to determine the fiber content.

c. Section 403(h)(1) of the Act requires any food with an established quality standard whose quality falls below that of the standard to bear the statement that it falls below the quality standard on the label in a prescribed size and type or it is considered misbranded.

7. Fill of Container standards define how much product must be in the container. This standard is particularly important for foods packed in liquid and sealed in opaque containers.

a. This standard has been promulgated only for certain fruits, vegetables, fish, shellfish, and nuts that are canned, packed in glass, or packed in semi-rigid containers.

b. Fill standards vary widely, from those without methods (e.g. canned peaches) to those where both method and apparatus are specified in detail (e.g. press weight for tuna).

c. Some standards (e.g. canned applesauce) prescribe "sampling and acceptance" procedures, which state the number of units to be examined and the acceptable number of defective units permitted for a specified size lot. A lot is considered to fall below the standard of fill when the number of defective units exceeds the acceptable number.

d. Section 403(h)(2) of the Act requires any food with a prescribed fill of container standard whose contents fall below that standard to bear a statement that it falls below the fill of container standard on the label in a prescribed size and type or it is considered misbranded.

B. Questions

1. What consumer protection was presumed in the promulgation of food standards?

2. Differentiate between misbranding and adulteration.

3. What are the requirements for the fill of a container?
4. When is the packing liquid included in net weight calculations?
5. Define edible portion.
6. Can the net contents be satisfactory and fill of container fail?
7. What, if any, is the relationship between the vignette and the contents?
8. What is the maximum amount (in %) of peanuts allowed in mixed nuts?

4.2.1. Food Standards Exercises

This section is intended to acquaint the trainee with food standards, the law authorizing such standards, their basis, the procedures, techniques used in determining compliance with the standards, and the significance of the results. The trainee should carefully read sections 401, 403(g) and (h), and 701(e)(1) of the Act and review 21 CFR Parts 130-169 to become familiar with foods that have been standardized.

The analyst should be aware of the law in examining any product for standards.

NOTE: AOAC methods referenced in the exercises are the updated version of the methods listed in the CFR.

NOTE: Training samples may be used for more than one training exercise.

4.2.1.1. Cheese

A. The trainer will provide one or more types of cheese. Perform the following tests in duplicate.
   1. Determine moisture using AOAC Method 926.08 “Loss on Drying (Moisture) in Cheese Method I”.
   2. Determine fat content using – AOAC Method 933.05 “Fat in Cheese IDF-ISO-AOAC Method, Codex-Adopted –AOAC Method”.
   3. Compare results with established standards.

B. References
   1. Code of Federal Regulations. Title 21, Pt. 133-Cheeses, and Related Cheese Products. Washington DC: Office of the Federal Register National Archives and Records Administration.
   2. Official Methods of Analysis of AOAC International, AOAC International, (current ed.) Gaithersburg MD.

3. General Provisions and Requirements Applying to Specifications, Tests, and Assays of the Food Chemicals Codex – *Analytical Sample, Water and Loss on Drying, and Weighing Practices, Foods Chemical Codex (FCC)*, (current ed.). Rockville MD

C. Questions

1. Why is speed essential in sample preparation?
2. Differentiate between a cheese spread and cheese food.
3. List two kinds of cheese in which sorbic acid is permitted and include the conditions under which it might be used. List two kinds of cheese for which sorbic acid is not permitted.
4. What is a processed cheese?
5. In the AOAC procedure, is the moisture content directly determined?
6. In the cheese fat extraction, what other substances may be found?
7. Why is petroleum ether used for the extraction?

4.2.1.2. Jams and Jellies

Jams and jellies are highly susceptible to adulteration for economic gain. An economic cheat involves the substitution of a cheap, easily obtainable ingredient, such as water, for a more expensive one, such as fruit. Tests have been devised to check certain indices or ratios of indices related to product composition to determine their compliance with standards.

A. The trainer will provide one or more types of jams or jellies. Make the following determinations:

1. Net contents.
2. Soluble solids by refractometry – AOAC Method 932.12 “Solids (Soluble) in Fruits and Fruit Products: Refractometer Method”.
3. Compare results with established standards.

B. References

1. *Code of Federal Regulations*. Title 21, Pt. 150-Fruit Butters, Jellies, Preserves, and Related Products. Washington DC: Office of the Federal Register National Archives and Records Administration.
2. Official Methods of Analysis of AOAC International, AOAC International, (current ed.) Gaithersburg MD.

C. Questions

1. What is jam?
2. What is an imitation jelly?
3. Define “degrees Brix”.

4.2.1.3. Egg Noodles

Egg noodles are also susceptible to adulteration for economic gain.

A. The trainer will provide one or more types of noodles. Make the following determinations:

1. Prepare a well-mixed composite using AOAC Method 926.06 “Macaroni Products Preparation of Samples”.
2. Determine total solids and moisture in duplicate using AOAC Method 926.07 “Solids (Total) and Loss on Drying (Moisture) in Macaroni Products”.
3. Compare the results to the standards in 21 CFR 139.150 Noodle products.

B. References

1. Code of Federal Regulations. Title 21, Pt. 139 - Macaroni and Noodle Products. Washington DC: Office of Federal Register National Archives and Records Administration.
2. Official Methods of Analysis of AOAC International, AOAC International, (current ed.) Gaithersburg MD

C. Questions

1. Does the label have an ingredient statement? Why?
2. Do all noodle products contain eggs?
3. Compare the two drying methods. Which one is to be used to determine compliance with the standard for total solids?
4. What is the difference between macaroni and noodles?

4.3. Food Additives

A. Background

1. Section 201(s) of the FD&C Act defines a food additive as “any substance the intended use of which results or may reasonably be expected to result, directly or indirectly , in its becoming a component or otherwise affecting the characteristic of any food

(including any substance intended for use in producing, manufacturing, packing, processing, preparing, treating, packaging, transporting or holding food; and including any source of radiation intended for any such use), if such substance is not generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use..."

2. Food additives are used to modify chemical, biological, sensory, or physical characteristics of food and drink. They are added as preservatives, antioxidants, acid regulators, thickeners, stabilizers, emulsifiers, anti-caking agents, flavors, and flavor enhancers.
3. Food additives have been used for centuries to enhance the flavor of food and drink and preserve the quality of food. To prevent spoilage, salt was added to meat. Sugar, vinegar, and alcohol were used to preserve fruits and vegetables.
4. With the Industrial Revolution, food production moved from the farm to food factories as the population shifted from rural areas to the cities. New chemical preservatives such as borax and boric acid, sulfites, salicylic acid, benzoic acid, and formaldehyde were liberally added to food to increase food production, prevent spoilage, and increase profits.
5. As the food processing industry grew, new artificial thickeners, emulsifiers, and flavors were synthesized and used. The safety of these new additives was unknown, and regulations did not exist to prevent manufacturers from adding whatever they liked to their products and using deceptive labeling to sell them.
6. Department of Agriculture Chief Chemist, Dr. Harvey Wiley began researching the safety of chemical preservatives and published his assessment in a report to Congress in 1889. He acknowledged that it was probably not wise to absolutely prohibit the use of preservatives in foods, but it was "imperative" that any food containing a preservative should clearly state so on the label.
7. In 1902, Congress appropriated Dr. Wiley $5,000 to study the effects of chemical preservatives on health. Dr. Wiley established the "hygienic table trails" and assembled a squad of young men volunteers who would consume steadily increasing amounts of various preservatives carefully tracking the effects they had on

their bodies. This group of young men became known as the "Poison Squad". Of the substances studied, only benzoate of soda and sulfites were sanctioned for continued use in food. The use of sulfite was limited, and the use of benzoate had to be indicated on the label.

8. The first Pure Food and Drug Act was passed by Congress in 1906, prohibiting interstate commerce of adulterated food, drink, and drugs. This ACT advanced food safety but also had several shortcomings. Provisions were left out which would have established similar standards for foods as those established for drugs. The law did not include a scientific definition of harmfulness by which to judge substances used in foods as either safe or "poisonous or deleterious", and it only applied to a product once it was marketed.
9. Over the next several decades, USDA parameters were established for additives through court hearings and regulations. In 1938, the Pure Food and Drug Act was replaced by The Federal Food, Drug, and Cosmetic Act. Section 402(a)(1) considers a food that contains a poisonous or deleterious substance that was not added, not to be adulterated if the quantity of the substance is not injurious to health.
10. Under 402(a)(2)(C)(i), a food is deemed adulterated if it contains an unsafe food additive whose use is avoidable unless its use conforms to the terms of an exemption or regulation prescribing the conditions under which the additive can be safely used.
11. Section 406 allowed FDA to set tolerances for poisonous or deleterious substances required to be used during production that cannot be avoided. Any quantity used that exceeds the established limit will cause the food to be adulterated.
12. The processed food industry saw a dramatic growth surge after World War II. More processed foods became available in grocery stores and these foods used new chemical ingredients and packaging. By 1947, over 500 different chemical entities had been proposed for use in foods.
13. The Food Additives Amendment was passed in 1958 in response to concerns about the safety of additives used in food. It became Section 409 of the Act. This amendment and associated regulation established the requirement for a premarket review and approval by FDA, for any substance added to food unless the substance is

Generally Recognized as Safe (GRAS) or was sanctioned or approved by FDA or USDA prior to 1958.

14. A substance can be classified as GRAS if qualified experts have a reasonable certainty, it is not harmful under the conditions of its intended use based on appropriate scientific analyses, or its common use in food has demonstrated with reasonable certainty the substance is safe based on a substantial history of consumption by a significant number of people.
15. By definition, GRAS substances are not food additives and do not require premarket approval by FDA. This means that the determination of an ingredient as GRAS can be made by qualified experts outside of FDA. With the implementation of the GRAS Notification Program in 1997, a company can independently conclude an ingredient is GRAS, and they can voluntarily inform FDA of their conclusion.
16. The company is still required to ensure the ingredient meets the safety requirements and required regulations under the law. It is important to note whether a substance is deemed to be GRAS or approved as a food additive, safety determination is always limited to its intended conditions of use and not to the substance itself.
17. Incorporated within Section 409 is what is known as the “Delaney Clause”, which restricts FDA from approving food additives that have been found to cause cancer when ingested by man or animal or if it is found after appropriate tests to evaluate the safety of the additive, to induce cancer in people or animals
18. A stipulation to this clause allows for use of a carcinogen as a feed ingredient for animals raised for food production provided the additive will not adversely affect the animals under conditions of use and no residue of the additive can be found by methods prescribed or approved by regulation, in any edible portion of the animal after slaughter.
19. Additional amendments were made to Section 409 under the Food and Drug Administration Modernization Act (FDAMA) in 1977 when subsection (h) was added. This subsection established the requirement for premarket notification and approval of food contact substances. Subsection (k), food additives intended for use in animal food was added in 2018.

20. Any substance added to a food for a specific purpose in that food is referred to as a direct food additive and must be declared as an ingredient on the label.
21. Substances added to a food during processing are referred to as a secondary direct food additive. They have no technical effect in the finished food and do not have to be declared as an ingredient.
22. Indirect food additives are substances that come into contact with the food but are not to become a component of or have a technical effect in or on the food. Packaging, coatings, and adhesives are examples of indirect food additives. Indirect food additives are also not required to be listed on the label.
23. Both prior-sanctioned and GRAS substances are required to be listed as an ingredient on the product label.

B. References

1. *Code of Federal Regulations*, Title 21, Washington DC: Office of Federal Register National Archives and Records Administration
    a. Part 170 – Food Additives.
    b. Part 172- Food Additives Permitted for Direct Addition to Food for Human Consumption.
    c. Part 173 – Secondary Direct Food Additives Permitted in Food for Human Consumption.
    d. Part 174 – Indirect Food Additives: General.
    e. Part 175 – Indirect Food Additives: Adhesives and Components of Coatings.
    f. Part 176 – Indirect Food Additives: Paper and Paperboard Component.
    g. Part 177 – Indirect Food Additives: Polymers.
    h. Part 178 – Indirect Food Additives: Adjuvants, Production Aids, and Sanitizers.
    i. Part 180 – Food Additives Permitted in Food or in Contact with Food on an Interim Basis Pending Additional Study.
    j. Part 181 – Prior-Sanctioned Food Ingredients.
    k. Part 182 – Substances Generally Recognized as Safe.
    l. Part 184 – Direct Food Substances Affirmed as Generally Recognized as Safe.

m. Part 185 - Indirect Food Substances Affirmed as Generally Recognized as Safe.

n. Part 189 – Substances Prohibited from Use in Human Food.

o. Part 101 – Food Labeling.

2. US Food & Drug Administration, Compliance Program 7309.006 – Domestic and Import Food Additives and Color Additives (current edition).
3. US Food & Drug Administration, Center for Food Safety and Applied Nutrition, Food Additive Status List.
4. US Food & Drug Administration, Center for Food Safety and Applied Nutrition, Substances Added to Food (formerly EAFUS).
5. US Food & Drug Administration, Center for Food Safety and Applied Nutrition, GRAS Notices.
6. US Food & Drug Administration, Center for Food Safety and Applied Nutrition, Food Ingredient & Packaging Inventories.
7. US Food & Drug Administration, Center for Food Safety and Applied Nutrition, A Food Labeling Guide, Guide for Industry, January 2013.

C. Questions

1. What are the three conditions that are to be met for a preservative to be used according to good manufacturing practices?
2. List 10 standardized foods for which the addition of chemical preservatives has been permitted by regulation.
3. Are all substances added to food required to be declared on the product label? How should they be declared?
4. What is the term “Do.” an abbreviation for?

4.3.1. Food Additive Exercises

The exercises in this section will introduce the trainee to food additive analysis and associated regulations. The exercises do not need to be performed in order and can be abbreviated or omitted if the laboratory does not perform the analysis.

4.3.1.1. Water Phase Salt and Sodium Nitrite in Smoked Fish

This exercise has been developed to introduce the principles of analysis for common chemical preservatives such as sodium chloride and sodium nitrite, and related regulations.

A. The trainer will provide a sample of smoked fish. Determine water phase salt and nitrites as described.

1. Sample Preparation

   Prepare each subsample separately for analysis according to AOAC 937.07(e), except take the loin muscle as defined in 21 CFR 161.190(a)(3) for the sample portion. i.e. “the longitudinal quarter of the great lateral muscle (loin muscle) freed from skin, scales, visible blood clots, bones, gills and viscera and from the nonstriated part of such muscle." Grind the loin muscle according to AOAC 937.07(a), paragraph 2.

2. Water Phase Salt

   a. Analyze each of the prepared subs by AOAC 952.08A for moisture content (total solids) and AOAC 937.09 for salt content. (See 21 CFR 172.177.)

   b. Calculate salt content in the water phase of the loin muscle according to the formula:

      % Salt in water phase of loin muscle = [% Salt / (%Salt + % moisture)] x 100

3. Sodium Nitrite

   Analyze each of the prepared subs for nitrite using AOAC 973.31. Report nitrite as ppm sodium nitrite in loin muscle.

B. References

1. *Code of Federal Regulations*. Title 21, Pt. 110-Current Good Manufacturing Practice in Manufacturing, Packing, or Holding Human Food, and Sections 172.170 - Sodium Nitrate and 172.177-Sodium Nitrite Used in Processing Smoked Chub. Washington DC: Office of Federal Register National Archives and Records Administration
2. Official Methods of Analysis of AOAC International, AOAC International, (current ed.). Gaithersburg MD
3. Fish and Fishery Product Hazards and Controls Guidance (current ed.), US Food and Drug Administration.

4. FDA CPGM 7303.842 Seafood Processor Inspection Program-Domestic and Foreign Facilities (current ed.)
5. FDA CPGM 7303.844 Import Seafood Products (current ed.)
6. Appendix I: Apparatus for Tests and Assays – Volumetric Apparatus, Food Chemicals Codex (current edition). United States Pharmacopeia, Rockville, MD.
7. <1857> Ultraviolet-Visible Spectroscopy – Theory and Practice, USP-NF General Chapters, General Information (current edition).

C. Questions

1. Which fish or fish products may legally contain nitrates or nitrites? List the maximum legal level for each product and describe how the retail container must be labeled.
2. Where is "water phase salt" defined? How does water phase salt vary with moisture content? Write the equation used for calculating the salt concentration in the water phase.
3. Why is water added to the portion taken for analysis by AOAC 952.08A?
4. How would one show that all the volatile components had been evaporated from the tissue?
5. Show the reactions involved in the titrimetric analysis for total chloride.
6. Why is the HNO3 solution boiled before the indicator is added?
7. AOAC 937.09B(b) states “With 10 g sample each ml 0.1 N AgNO3 = 0.058% NaCl.” How is this factor obtained?
8. What is the structure of the colored product formed during the nitrite analysis? Show the reactions involved.
9. How has the order of addition of reagents been shown to affect this type of analysis?
10. What is the correlation between pH and color stability?

4.3.1.2. Nitrate and Nitrite in Bottled Water

A. Background

1. Nitrogen is an essential nutrient for all life and exits in several forms in nature. Bacteria in the soil convert nitrogen into ammonia and nitrate which are assimilated by plants. Nitrogenous

compounds not taken up by plants or converted to nitrous oxide by bacteria is converted to nitrate. Nitrate is readily soluble in water, passing through the soil into groundwater.

2. Man acquires nitrates and nitrites from consuming vegetables, processed meat, seafood, and water. When nitrate and nitrite are consumed, a little over half of the nitrate is absorbed and rapidly excreted as urea and ammonia in the urine. The remaining nitrate is converted by bacteria and stomach acid into nitrite and nitric oxide. Consuming high levels of these compounds has been shown to cause methemoglobinemia.
3. Methemoglobin is formed when nitrite oxidizes ferrous iron in hemoglobin to the ferric form. Methemoglobin is unable to bind and transport oxygen through the body causing weakness, cyanosis, coma, and in some cases death. In young children and infants, Methemoglobinemia (referred to as “blue baby syndrome”) can be caused by drinking water that is high in nitrates.
4. FDA is responsible for regulating the safety of bottled drinking water. 21CFR 165.110 defines bottled water as “water that is intended for human consumption and that is sealed in bottles or other containers with no added ingredients except that it may optionally contain safe and suitable antimicrobial agents”.
5. Flavored and nutrient-added water beverages are also required to meet bottled water regulations if it is declared as “water” on the label.
6. Artificially carbonated waters like soda water (club soda), tonic water, and seltzer water are not regulated as bottled water.
7. The Safe Drinking Water Act was passed in 1974 establishing the EPA drinking water standards. FDA regulations for bottled water are based on the EPA tap water standards.

B. The trainer will provide a sample of bottled water. Determine the amount of nitrate and nitrite in the water as described:

1. Prepare a composite as outlined in CPGM 7309.006 Part IV, Methodology, Food Additives.
2. Determine the amount of nitrate and nitrite using EPA method 300.1 - “The Determination of Inorganic Anions in Water by Ion Chromatography”.
3. Compare the results with 21 CFR165.110(b)(4)(iii)(A).

C. References

1. *Code of Federal Regulations*, Title 21, Part 165.110 - Bottled Water. Washington DC: Office of Federal Register National Archives and Records Administration
2. US Food & Drug Administration, Compliance Program 7309.006 – Domestic and Import Food Additives and Color Additives (current edition).
3. Method 300.1, Environmental Monitoring Systems Laboratory Office of Research and Development. US Environmental Protection Agency (current revision), Cincinnati, Ohio.
4. <1065> Ion Chromatography, USP-NF General Chapters, General Information (current edition).

D. Questions

1. What is the stability and storage conditions for the stock standards? Working standards?
2. What factors can affect trace analysis by IC?
3. How does diluting the eluate affect the analysis?
4. What does the analyst need to be aware of when using a pretreatment cartridge to eliminate certain types of matrix interferences?

4.3.1.3. Sulfites

A. Background

1. Sulfiting agents have been used as a food additive for centuries and are considered GRAS by FDA based on its extensive history of use.
2. Sulfites are added to many different types of foods for several technical purposes.
   a. Sulfur dioxide, sodium and potassium bisulfites, and metabisulfites are used in foods as antioxidants and bleaching agents.
   b. Sulfites are commonly used as preservatives in dried fruits, as a bleaching agent in the processing of maraschino cherries, on shrimp and lobster to prevent melanosis (“black spot”), to retain the color of cooked octopus, and prevent conch meat from darkening.

3. Sulfites were used for years on fresh fruit and vegetables to preserve the color and crispness but their use on fresh produce, except for potatoes, was prohibited in 1986, based on an increase of reported allergic-type reactions in individuals who are sensitive to sulfites.
4. To further protect these individuals, the labeling regulation requiring the presence of sulfites to be declared on any food containing at least 10 ppm sulfites was enacted in 1987.
5. This labeling requirement applies to foods containing added sulfites, not to foods with naturally occurring sulfur-containing compounds such as vegetables in the Brassica and Allium families.

B. The trainer will provide one or more samples of dried fruit preserved with sulfites. Determine the amount of sulfite, calculated as sulfur dioxide present in the food using both the AOAC and CAM methods.

1. Prepare a composite as outlined in CPGM 7309.006 Part IV, Methodology, Food Additives.
2. Determine the amount of sulfite using AOAC 990.28 “Sulfites in Foods Optimized Monier- Williams Method”. Report both the titrimetric and gravimetric results.
3. Determine the amount of sulfite using CAM Method C-004 “Determination of Sulfites in Food using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)” (Current version).
4. Compare your results to the regulations.

C. References

1. *Code of Federal Regulations*. Title 21, Part. 130.9 - Sulfites in Standardized Foods; Part 182, Subpart D – Chemical Preservatives. Washington DC: Office of Federal Register National Archives and Records Administration.
2. Official Methods of Analysis of AOAC International, AOAC International, (current ed.). Gaithersburg MD.
3. US Food & Drug Administration, Center for Food Safety and Applied Nutrition FDA Foods Program Compendium of Analytical Laboratory Methods: Chemical Analytical Manual (CAM).

4. US Food & Drug Administration, Compliance Program 7309.006 – Domestic and Import Food Additives and Color Additives (current edition).
5. <1738> Applications of Mass Spectrometry, USP-NF General Chapters, General Information (current edition).
6. Appendix 1: Apparatus for Tests and Assays – Volumetric Apparatus, Foods Chemical Codex (FCC), (current ed.). Rockville MD

D. Questions

1) Monier-Williams

1. Why doesn't the HCl distill into the sulfur dioxide absorber? What is an azeotrope?
2. What reaction occurs in the distilling flask? In the sulfur dioxide absorber?
3. What is the purpose of the gravimetric determination?
4. What purpose does the pyrogallol/KOH gas wash serve?

2) LC/MS/MS Sulfite Method

1. What is the purpose of the addition of 0.2% formaldehyde for sample extraction?
2. What volume of extracting solvent is used for the first extraction for the three different solid procedures (basic protocol, low moisture solids, and high moisture solids)
3. Why is the sample run through a C18 solid phase extraction (SPE) clean-up?
4. The method uses a HILIC column for addition separation of matrix constituents prior to MS/MS detection. What is a HILIC column?
5. Which type of calibration curve is used?
6. What are the monitored transitions?
7. What is the calculation to convert the concentration in vial from the calibration curve to concentration in sample (ppm)?
8. Can sulfites be declared using advisory statement such as “may contain sulfites”?

4.3.1.4. Sweeteners

A. Background

1. Artificial sweeteners have been used by the food industry since the 1800's. Saccharin was discovered in 1879 and by the early 1900s, was widely used in processed foods to replace sugar. By the 1970s, concerns were growing about saccharin's safety. Based on laboratory test results, FDA revoked its GRAS classification and imposed restrictions for the use of saccharin in foods. In 1977, Canada banned saccharin based on the Canadian Health Protection Branch Study. In the US, the Saccharin Study and Labeling Act of 1977 was passed. This ACT established criteria for the scientific study into the safety of saccharin and its impurities and required any food containing saccharin to carry a warning statement on its label. The requirement for warning statement was repealed in 2001.
2. Dulcin was discovered in 1883, but never gained the popularity saccharin had. Dulcin was removed from the market in 1954 due to studies linking it with cancer. Cyclamate was discovered in 1937 and by the 1960s, had become the sweetener of choice since it had less of a bitter aftertaste then saccharin. Cyclamate was banned for use in 1970 by FDA due to studies linking the sweetener to bladder cancer.
3. The development and use of sweeteners has increased dramatically since the 1970s. FDA approved six new synthetic sweeteners as food additives., Some plant- and fruit-based sweeteners, sugar alcohols, and sugars that are metabolized differently than sucrose have been classified as GRAS.

B. The trainer will provide at least one sample containing sweeteners. Identify any sweeteners present in the sample.

1. Prepare a composite as outlined in CPGM 7309.006 Part IV, Methodology, Food Additives.
2. Analyze the sample using the method designated by the trainer. If a validated method is not available, use AOAC Method 969.27, "Non-Nutritive Sweeteners in Nonalcoholic Beverages, Qualitative Thin-Layer Chromatographic Method".
3. Compare your results to the regulations.

C. References

1. *Code of Federal Regulations*. Title 21, Sections 172.800 - Acesulfame potassium; 172.803 - Advantame; 172.804 -

Aspartame; 172.829 - Neotame; 172.831 - Sucralose; 180.37 - Saccharin, Ammonium Saccharin, Calcium Saccharin, and Sodium Saccharin; 189.135 - Cyclamate and its Derivatives; 189.145 - Dulcin; 172.395 - Xylitol; 180.25 - Mannitol; 184.1835 Sorbitol. Washington DC: Office of Federal Register National Archives and Records Administration.

2. US Food & Drug Administration, Center for Food Safety and Applied Nutrition, GRAS Notices.
3. Official Methods of Analysis of AOAC International, AOAC International, (current ed.). Gaithersburg MD.
4. US Food & Drug Administration, Compliance Program 7309.006 – Domestic and Import Food Additives and Color Additives (current edition).
5. *Physical Tests and Determinations – Thin-Layer Chromatography, Food Chemicals Codex Appendix II (current edition).*

D. Questions

1. You have been given a dried fruit to analyze for cyclamates using AOAC method 969.27. Is this method suitable for the analysis? What should you do?
2. In terms of the TLC extraction procedure, what is the purpose of adding 5 ml of 50% NaOH solution?
3. Why do foods that contain sugar alcohols require a warning on the label? What is the warning?

4.3.1.5. Benzoates

A. Background

1. Sodium benzoate was the first preservative approved by FDA. Sodium benzoate is the salt of benzoic acid and unlike benzoic acid, is soluble in water. It is widely used in acidic foods to inhibit the growth of bacteria, yeast, and mold. It is also used as a flavoring agent and adjuvant. When used under acidic conditions, sodium benzoate is converted to benzoic acid. There is concern that benzoate can be transformed by decarboxylation into benzene when used in combination with vitamin C, so some manufacturers have replaced sodium benzoate with a different preservative.
2. This ingredient is used in food at levels not to exceed good manufacturing practice. Current usage results in a maximum level of 0.1 percent in food.

B. The trainer will provide at least one beverage sample. Determine if the sample contains benzoate. If present, determine the amount of benzoate the product contains.

1. Prepare a composite as outlined in CPGM 7309.006 Part IV, Methodology, Food Additives.
2. Analyze the sample using the method designated by the trainer. If a validated method is not available, use AOAC Method 979.08, “Benzoate, Caffeine, and Saccharin in Carbonated Beverages”
3. Compare your results to the regulations.

C. References

1. *Code of Federal Regulations*. Title 21, Sections 582.3733 - Sodium Benzoate. Washington DC: Office of Federal Register National Archives and Records Administration.
2. Official Methods of Analysis of AOAC International, AOAC International, (current ed.). Gaithersburg MD.
3. US Food & Drug Administration, Compliance Program 7309.006 – Domestic and Import Food Additives and Color Additives (current edition).
4. <621> Chromatography, USP-NF General Chapters, General Tests and Assays, (current edition).

D. Questions

1. The lab has a large supply of Silica Gel G plates that are used in several different analyses in the lab, including colors. Can these TLC plates be used for this analysis?
2. Is the pH of the mobile phase used in AOAC 979.08 critical? What affect can pH have on retention times?
3. What formula should be used to calculate % benzoate in the sample?
4. What is the salt of benzoic acid? What pH does sodium benzoate optimally function? Which is more soluble, sodium benzoate or benzoic acid?
5. In terms of HPLC, what may occur if there is a change in the composition of the mobile phase?
6. In terms of HPLC how critical is the detection wavelength?

4.4. Color Additives

A. Background

1. Section 201(t) of the FD&C Act defines a color additive as “a dye, pigment, or other substance made by a process of synthesis or similar artifice, or extracted, isolated, or otherwise derived, with or without intermediate, or final change of identity, from a vegetable, animal, mineral or other source, when added or applied to a food, drug or cosmetic, or the human body or any part thereof, is capable (alone or through reaction with other substance) of imparting color thereto”. The term “color” includes black, white, and shades of gray.
2. Colors are used to enhance a person’s appearance, offset color loss due to light, air, temperature extremes, moisture, and storage conditions, correct natural variations in color, decorate baked goods, and in certain products such as candy, can be associated with flavors. Unscrupulous companies will sometimes use color additives to deceive consumers by concealing damaged or inferior foods.
3. The earliest color additives were natural pigments from plants and minerals. This changed in 1885 when the first synthetic organic dye called Mauve was accidently discovered by William Henry Perkins while trying to synthesize quinine from aniline isolated from coal tar. This led to the discovery of other synthetic organic dyes produced from the by-product of coal processing. These colors became known as “coal-tar dyes”. By the 1900’s, most food, drugs, and cosmetics were artificially colored. Reported injuries and deaths associated with their use resulted in congress appropriating funds to examine these dyes. Subsequent testing found many of them contained dangerous and poisonous materials such as lead, arsenic, and mercury.
4. The Federal Food and Drugs Act of 1906 also known as the Pure Food and Drug Act was the start of federal color regulation. The 1906 Act prohibited the use of poisonous or deleterious colors in confectionary products and the staining or coloring of food to conceal inferior or damaged foods.
5. In 1907, USDA (FDA did not exist at this time) published the first list of synthetic organic dyes considered to be safe for use in foods. Out of the 80 dyes tested, seven were found to be safe and placed on this list. These were the first “approved” colors.
6. Over time, it was recognized the Pure Food and Drug Act of 1906 did not go far enough to protect the public from misbranded,

adulterated, and dangerous products. Marketed cosmetics contained hazardous ingredients such as arsenic, radium, mercury, and thallium.

7. In 1933, users of “Lash Lure” a new permanent mascara containing p-phenylenediamine, an oxidizing coloring agent, were injured and, in some cases, blinded. This incident has been linked to the passing of the first cosmetic regulations in the Food, Drug and Cosmetic Act of 1938. The 1938 Act increased government oversight of colors. “Harmless” coal-tar colors, other than those used in hair dye, were required to be listed in the recently established Code of Federal Regulations. Voluntary certification of listed color additive batches became mandatory and certification fees were established. Misbranding and adulteration provisions for colors were added.
8. In the same year, FDA developed labeling and record keeping provisions, established procedures for requesting color certification and adding new colors to the permitted list, and diluents that could be added to colors were identified.
9. A new nomenclature was formulated for certified coal-tar dyes consisting of a prefix (FD&C - Food, Drug and Cosmetic, D&C – Drug and Cosmetic, or Ext. D&C – External Drug and Cosmetic) followed by the specific color, a number, and the term “Lake” if applicable. A lake is an insoluble pigment formed by chemically reacting a straight dye with precipitants and substrata. Color additive lakes are provisionally listed under 21 CFR 81.1 and 21 CFR part 82.
10. Color regulations were further enhanced with the passing of the Color Amendments of 1960. The term “color additive” was defined. Factors to determine the safety of the color based on its use were established and specific conditions for their safe use had to be included in the regulations. The “Delany Clause” which prohibits the listing of a color additive that has been shown to be a carcinogen was added. The purity of the dye and an acceptable level of impurities had to be determined through scientific analysis.
11. A process for premarket approval of a new color additive or amendment for an existing color was created. Around 200 listed color additives, except those used to color hair, were moved to a provisional list until they were shown to be safe and permanently listed or their use was terminated. About half of these colors were approved and listed. Coal-tar colors used to color hair are still excluded from the regulations.

12. The passing of the Nutrition Labeling and Education Act (NLEA) in 1990 required all certified color additives to be declared on the product label with a few exceptions. CFR 101.22(k)(3) states color additives added to butter, cheese, and ice cream do not have to be declared as an ingredient unless it is required by regulation in parts 73 or 74 of the CFR.
13. Approved (listed) color additives are divided into two categories; those that are subject to batch certification and those that are exempt from batch certification. Color additives subject to batch certification are synthetic organic dyes, pigments, or lakes that are now derived from petroleum products instead of by-products of coal manufacturing. They are still referred to as “coal-tar colors”. They are classified into five main groups based on their chemical structure: azo, triphenylmethane, xanthene, indigoid, and quinoline.
14. Only a few synthetic organic dyes are permanently listed for use in foods; FD&C Yellow No. 5, FD&C Yellow No. 6, FD&C Blue No. 1, FD&C Blue No. 2, FD&C Red No. 3, FD&C Red No. 40, and FD&C Green No. 3. Except for FD&C Red No. 3, their aluminum lakes are permitted to be used in foods. Citrus Red No. 2 and Orange B are also listed for use in foods, but their use is restricted to specific foods.
15. Additional synthetic organic dyes are permanently listed for use in drugs and cosmetics. Lakes prepared from D&C colors are also permitted. Except for Ext. D&C Yellow No 7, lakes prepared from Ext. D&C colors are not permitted. Any use restrictions for D&C and Ext. D&C colors are specified in the CFR.
16. Color additives that are exempt from batch certification are obtained from natural sources such as plants and minerals. Cochineal extract (Carmine) is the only approved color derived from an insect. Certification exempt color additives must be approved by FDA before they can be used. They are required to comply with the identity, purity, and use restrictions listed in the CFR.
17. CFR 101.22(k)(1) requires certified color additives to be declared by their listed name in the CFR. For example, “FD&C Yellow No. 5” or “FD&C Blue No. 1 Lake” or abbreviated to “Yellow 5” or “Blue 1 Lake”.
18. CFR 101.22(k)(2) requires color additives not subject to certification to be declared by the common or usual name listed in the CFR. These colors may also be declared as “Artificial Color”. “Artificial Color Added”, “Color Added”, or “Colored with _____”, or “_____

color" with the blanks filled in with the name of the color additive. CFR 73.100(d)(2) requires food products that contain cochineal extract or carmine, including butter, cheese, and ice cream, declare its presence as an ingredient using its common or usual name; "Cochineal Extract" or "Carmine". Labeling of color additives not subject to certification can be challenging. For example, the color additive Turmeric cannot be declared as Curcumin which is a yellow pigment found in Turmeric. Also, only the carotenoids listed in part 73 of the CFR are approved color additives and must be declared by the listed name in the CFR. Vegetable Juice is a permitted color additive while Black Carrot juice is not.

19. Labeling of colors in cosmetics is subject to additional regulations that are not applicable to foods. CFR 701.3(f)(3) permits color additives to be grouped together on the label without respect to order of predominance. CFR 701.3(g) states color additives can be declared using the phrase "may contain" if the same declaration of ingredients is used for other products similar in composition and all products that share the common declaration of ingredients are sold under a common trade name or brand designation. As an alternative to listing color additives on each product sold in a cosmetic assortment, CFR 701.3(h) allows color additives to be declared in a single composite list provided it is not misleading to the consumer and the list pertains to all the products in the assortment.
20. Other color designations such as the common name of the color, Colour Index Number (C.I. number), European Number (E code), or International Numbering System (INS) are used in other countries and can also be declared in parentheses along with the US approved name in the ingredients. INS numbers generally correspond with the E code for the same compound. For example, the common name for FD&C Yellow 5 is Tartrazine, C.I. number is C.I. 19140, E code is E102, and the INS number is INS102. Improperly declared color additives imply the color used may not be from a certified batch.
21. The analysis of colors has both a subjective and objective component. Three common attributes used to visually identify a color are hue, lightness, and chroma. Hue is the attribute of a color that allows it to be discerned as red, yellow, green, etc. and is dependent on its dominant wavelength. Lightness is the quality that distinguishes a lighter color from a darker one. Chroma is the purity or intensity of a color. Because of these attributes, colors cannot be solely described in spectrophotometric terms.

22. Color molecules contain a visible chromophore that is used to objectively analyze colors. A chromophore is a group of one or more unsaturated bonds that absorb light in the ultraviolet and visible region. Those that absorb light in the visible region impart color to the molecule. In most cases, they are covalent unsaturated bonds such as C=C, C=O, C=NH, CH=N, N=N, and aromatic rings.
23. An auxochrome is a group such as -OH, -NH2, -SO3H, and -COOH which by itself does not act as a chromophore, but when attached to a chromophore increases the absorption intensity and shifts the absorption wavelength.
24. A shift to a longer wavelength is a bathochromic effect (red shift). A shift to a shorter wavelength is a hypsochromic effect (blue shift). An increase in absorption is a hyperchromic effect and a decrease in absorption is a hypochromic effect.

B. References:

1. *Code of Federal Regulations*, Title 21, Washington DC: Office of Federal Register National Archives and Records Administration.
   a. Part 70 – Color Additives
   b. Part 73- Listing of Color Additives Exempt from Certification
   c. Part 74 – Listing of Color Additives Subject to Certification
   d. Part 81- General Specifications and General Restrictions for Provisional Color Additives for Use in Foods, Drugs, and Cosmetics
   e. Part 82 – Listing of Certified Provisionally Listed Color and Specifications
   f. Part 101 – Food Labeling
   g. Part 701 – Cosmetic Labeling
2. US Food & Drug Administration, Compliance Program 7309.006 – Domestic and Import Food Additives and Color Additives (current edition).
3. US Food & Drug Administration, Center for Food Safety and Applied Nutrition, Summary of Color Additives for Use in the United States in Foods, Drugs, Cosmetics, and Medical Devices.

C. Questions:

1. What is a color Additive?

2. Define these terms: straight color, lake, diluent.
3. What are “certified” colors?
4. Under what conditions may color additives be used in the area of the eye, In injections, or in surgical sutures?
5. List the restrictions, if any, for the following colors:
    a. Citrus Red No. 2
    b. Orange B
    c. Titanium Dioxide
    d. FD&C Blue No. 1
    e. FD&C Red No. 3
    f. FD&C Red No. 40
    g. FD&C Yellow No. 5
6. Give one example of a triphenylmethane color.
7. List the Colour Index Number (C.I. Number) and common name for:
    a. FD&C Red No. 1
    b. FD&C Red No. 3
    c. D&C Orange No. 3
    d. FD&C Green No. 3
    e. D&C Yellow No. 7
    f. D&C Yellow No. 9
    g. FD&C Blue No. 2
    h. FD&C Violet No. 1
8. A label of a food product declares “…E123…” as an ingredient. Is this product permitted in the U.S.?
9. A label of a food product declares “…Sunset Yellow FCF, Erythrosine Lake, Allura Red AC”. Is this product correctly labeled? If not, why? If the importer relabels the product, would it be permitted in the U.S.?
10. In terms of solubility, state whether the following color additives are water soluble or oil soluble.
    a. “Natural” colors such as riboflavin or saffron

b. Tartrazine

c. Sudan 1

d. D&C Red 19 or D&C Red 28

4.4.1. Color Additives Exercises

The purpose of the exercises in this section is to acquaint the trainee to analytical methods, techniques, and technologies used by FDA to test for colors in foods and cosmetics and familiarize the trainee with color additive regulations.

These exercises do not need to be performed in order and can be abbreviated or omitted if the laboratory does not perform the analysis.

The trainee must read and have a theoretical knowledge about the methods listed below. Attachment A contains a list of suggested color training samples.

4.4.1.1. Quantitative Determination of FD&C Colors in Foods (Graichen Method)

A. Background

This method was developed by Charles Graichen and John Molitor for analyzing colors in foods. The method was published in the Journal of Association of Agricultural Chemists (JAOAC) which later became the Journal of the Association of Official Analytical Chemists in 1963. The method was revised by Charles Graichen in 1973 and can be found in Attachment C.

In general, an extraction flows as follows:

1. A food is finely ground with an aqueous solution and Celite (diatomaceous earth) and packed into a chromatographic column.
2. Chloroform is added to remove oils/fats and natural colors.
3. An anion exchange resin solution is used to extract the colors.
4. Weak basic solutions are used to extract the colors from the resin.
5. All eluates collected are scanned by ultraviolet/visible (UV-VIS) spectroscopy to obtain an initial indication of color.
6. Eluates requiring further analysis are neutralized for further cleanup and isolation by SPE column using AOAC method 988.13.
7. Colors are identified by ultraviolet/visible (UV-Vis) spectroscopy.

B. The trainer will provide two or more samples to analyze for colors.

1. Extract and identify colors present in the sample using the Graichen method.
2. Determine if the color(s) detected meets labeling and color regulations.

C. References

1. Graichen and Molitor (1963). Quantitative Determination of FD&C Colors in Foods, Journal of the Association of Agricultural Chemists, Vol. 46, No. 2, pages 1022 – 1029. Revised in 1973. Attachment B contains a method copy including revisions.
2. *Physical Tests and Determinations – Column Chromatography*, Food Chemicals Codex Appendix II (current edition).
3. <1857> Ultraviolet-Visible Spectroscopy – Theory and Practice, USP-NF General Chapters, General Information (current edition).

D. Questions

1. What is the difference between Procedure I, II, and III?
2. A sample is prepared as per the method following Procedure I. In which eluate would the following colors be seen:
   a. Amaranth
   b. Green 3
   c. Beta Carotene

4.4.1.2. FD&C Color Additives in Foods: Rapid Cleanup for Spectrophotometric and Thin-Layer Chromatographic Identification (AOAC Method 988.13)

A. Background

1. This method was developed by Mary Young and published in the AOAC in 1988. It is a quick screening method that uses a reverse phase C-18 cartridge and different concentrations of isopropyl alcohol (the method includes an elution scheme to use as a guide) to separate, isolate, and concentrate colors present in the sample. The colors are then identified by visible spectrometry and confirmed by thin layer chromatography.
2. This method requires little expenditure of solvents and is capable of separating colors that are exempt from certification (i.e. “natural” colors) from synthetic colors and permitted colors from non-

permitted colors. It can be applied to a variety of products except high fat products such as baked goods.

B. The trainer will provide two or more samples to analyze for colors.

1. Extract and identify the colors present in the sample using AOAC Method 988.13.
2. Determine if the color(s) detected meets labeling and color regulations

C. References

1. Official Methods of Analysis of AOAC International, AOAC International, (current ed.). Gaithersburg MD
2. Physical Tests and Determinations – Thin-Layer Chromatography, Food Chemicals Codex Appendix II (current edition).
3. <1857> Ultraviolet-Visible Spectroscopy – Theory and Practice, USP-NF General Chapters, General Information (current edition).

D. Questions

1. How does the C18 sep-pak isolate and concentrate colors?
2. Yellow No. 5 is not retained as well as the other colors on the sep-pak. How can you improve its retention?
3. What are chromophores and auxochromes? Identify the chromophores and auxochromes in two of the following colors
   a. Citrus Red 2
   b. Orange B
   c. Titanium Dioxide
   d. FD&C Blue No. 1
   e. FD&C Red No. 3
   f. FD&C Red No. 40
   g. FD&C Yellow No. 5
4. What is hypsochromic shift? Give the effect of hypochromic shift for two of the colors in the question above.

4.4.1.3. Color Additive Analysis in Foods and Cosmetics using UPLC with Extended Photo-Diode Array Detection (LIB 4643)

A. Background

1. This method developed by Susan Clark, et al. describes a simple and efficient extraction and clean-up process applicable to a wide variety of different food and cosmetic sample types.
2. Colors are extracted/purified using three types of solid-phase extraction columns (cation-exchange (CBX), silica gel (SiOH), and hydrophilic-lipophilic balanced (HLB)) and identified by UPLC – PDA (ultra-performance liquid chromatography with extended photo-diode array detector).
3. This method has high sensitivity with minimal solvent use. Instrument run time is approximately 17 minutes. It utilizes a large spectral library and is capable of identifying over 200 color additives.

B. The trainer will provide two or more samples to analyze for colors.
   1. Extract and identify the colors present in the sample using LIB 4643.
   2. Determine if the color(s) detected meets labeling and color regulations

C. References
   1. Laboratory Information Bulletins (2000 – present), ORA Office of Regulatory Science SharePoint site.
   2. <621> Chromatography, USP-NF General Chapters, General Tests and Assays, (current edition).

D. Questions
   1. How versatile is this method?
   2. Describe how to determine the appropriate method section to use for a sample.
   3. When is an HLB SPE column used?
   4. Is it critical to filter both mobile phases?

4.4.1.4. Determination of Colors in Cosmetics

A. Background
   1. Chapter 19 in the Newburgers Manual of Cosmetic Analysis is a collection of methods for isolating and identifying colors in a variety of cosmetics. The methods use TLC, solvent-solvent extraction, and column chromatography to isolate and cleanup the colors, which are then identified by UV/VIS spectroscopy.
   2. This manual was last published in 1977. Attachment D contains a copy of Chapter 19 from Newburgers Manual of Cosmetic Analysis.

NOTE: The TLC solvents used in method 19.1 “Lipstick - Tube (Thin Layer Chromatographic Method)” are also used to analyze other powdered and liquid makeup. For the determination of colors, methylene chloride is used to move waxes and oils to the top of the plate and isolate oil-soluble colors. Water-soluble colors are scraped from the plate, dissolved in a suitable solvent, and analyzed using AOAC Method 988.13.

B. The trainer will provide at least two different types of cosmetics to analyze for colors.
    1. Extract and identify the colors present in the sample using the methods in Newburgers Chapter 19.
    2. Determine if the color(s) detected meets labeling and color regulations

C. References
    1. (1977). Newburgers manual of cosmetic analysis (2nd ed., chap. 19). Arlington, VA: Association of Official Analytical Chemists.
    2. Physical Tests and Determinations – Column Chromatography, Food Chemicals Codex Appendix II (current edition).
    3. Physical Tests and Determinations – Thin-Layer Chromatography, Food Chemicals Codex Appendix II (current edition).
    4. <1857> Ultraviolet-Visible Spectroscopy – Theory and Practice, USP-NF General Chapters, General Information (current edition).

D. Questions
    1. How many synthetic colors are approved for use in cosmetics? Which of these colors are permitted in eye area cosmetics?
    2. What colors or pigments are approved for use in tattoos? What about henna?
    3. A lipstick sample was analyzed using Newburgers, 19.1. One bright pink band of color, exhibiting orange fluorescence, was detected. Using Table 12 in Newburgers (page 119), what color(s) was(were) detected? Are these possible color(s) permitted for use?

5. Document History

Revision #	Status* (D, I, R)	Date	Author Name and Title	Approving Official Name and Title
1.3	R	06/06/2008	LMEB	LMEB
1.4	R	02/02/2010	LMEB	LMEB
1.5	R	02/06/2012	LMEB	LMEB
1.6	R	02/14/2013	LMEB	LMEB
1.7	R	05/02/2014	LMEB	LMEB
02	R	11/12/2020	LMEB	LMEB
03	R	See QMiS	COL, FAD, and DEC Operation Program Managers	LMEB

* - D: Draft, I: Initial, R: Revision

6. Change History

Revision #	Change
1.3	Answer Key 10.2.3, 2., straight color definition changed and mixture definition added
1.4	10.1.2.1 C., 10.1.2.2 C., 10.1.2.3 C., 10.1.2.4 C., 10.1.2.5 C., 10.2.2.1 C., 10.2.2.2 C, 10.2.2.3 C., 10.2.2.4 C., 10.2.2.5 C. – References updated or deleted 10.2.2.2 B. – “sulfides” changed to “sulfites” 10.2.3 B – revised 10.2.3. D. – “Neuberger” changed to “Newberger” Footer – web link updated
1.5	10.2.2.2 B. – updated method
1.6	Header – Division of Field Science changed to Office of Regulatory Science
1.7	10.2.2.2 D. 1. & 2. – deleted U tube 10.3 Answer Key 10.2.2.2. 1. & 2. – deleted U tube

Revision #	Change
02	Reformatted entire document which led to change in the numbering and bullets Revised the exercises and questions for Food Additives Updated answers to colors questions Removed obsolete links
03	Entire document was extensively revised with input obtained from COL, FAD, and DEC Operation committee members to capture current processes and references and must be read in its entirety. Document has been transferred to new TEMPLATE-000054 for formatting.

7. Attachments

List of Attachments

Attachment A - Color Additive Training Samples

Foods

- Bakery items (cookie, cake, bread)
- Candy (hard, soft)
- Gelatin
- Cheese
- Chocolate (candy, cocoa, powdered drink mix)
- Dried fruits and vegetables
- Spices
- Mukhwas (mouth freshener/digestive aid consisting of various seeds like fennel, anise, coriander, etc.)
- Gum

Cosmetics

- Lipstick, lip gloss or lip tint
- Powdered makeup (eyeshadow, blush, powder)
- Soap (liquid, bar)
- Liquid eyeliner
- Mascara
- Temporary hair color sprays and colored dry shampoo
- Lotions
- Liquid cosmetics (cologne, aftershave)
- Temporary tattoos

Attachment B - Answer Key

4.2. Food Standards

1. *What consumer protection was presumed in the promulgation of food standards?*

   Consumers are protected from adulterated and misbranded foods by use of standards.

2. *Differentiate between misbranding and adulteration.*

   A food shall be deemed to be “adulterated” if:

   a. It bears or contains any poisonous or deleterious substance which may render it injurious to health.

   b. It bears or contains any added poisonous or added deleterious substance (other than a substance that is a pesticide chemical in or on a raw agricultural commodity; or processed food, a food additive, a color additive, or a new animal drug) that is unsafe within the meaning of section 406 of The Act.

   c. It consists in whole or in part of any filthy, putrid, or decomposed substance, or if it is otherwise unfit for food.

   d. It has been prepared, packed, or held under unsanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health.

   A food shall be deemed to be “misbranded” if:

   a. Its labeling is false or misleading in any particular, or in the case of a food to which section 411 of the Act applies, its advertising is false or misleading in a material respect or its labeling is in violation of section 411(b)(2).

   b. It is offered for sale under the name of another food.

   c. Its container is so made, formed, or filled as to be misleading.

   d. In the case of nuts with misleading vignettes, misrepresentation of product, and

   e. incorrect net weight, misbranding charges apply.

3. *What are the requirements for the fill of container?*

   a. Food containers must be so made, formed, or filled so they are not misleading (FD&C Act, Sec. 403(d)).

   b. Fill of container is the minimum quantity of the solid food in the container after processing: not less than 90 percent of the total capacity of the container.

4. *When is the packing liquid included in net weight calculations?*

The packing liquid is included in the net weight calculations when it is also used by the consumer.

5. *Define edible portion.*

   Edible portion is any portion of a food product that is fit to be eaten.

6. *Can the net contents be satisfactory and fill of container fail?*

   When the volume of product settles during shipment the correct net contents may no longer properly fill the container. When this is expected, the manufacturer may include a comment on the label to that effect.

7. *What, if any, is the relationship between the vignette and the contents?*

   If the label bears any pictorial representation (vignette) of the mixture of nuts, it must depict the relative proportions of the nut ingredients contained in the finished food.

8. *What is the maximum amount (in %) of peanuts allowed in mixed nuts?*

   In mixed nuts, each nut ingredient shall be present in a quantity not less than 2 percent and not more than 80 percent by weight of the finished food.

4.2.1.1 Cheese

1. *Why is speed essential in sample preparation?*

   Speed is essential in sample preparation to prevent moisture loss, which would affect the results in the fat calculations of cheeses.

2. *Differentiate between a cheese spread and cheese food.*

   A cheese food is the food prepared by comminuting and mixing, without the aid of heat, one or more optional ingredients into a homogeneous mass.

   A cheese spread is the food prepared by comminuting and mixing, with the aid of heat, one or more optional ingredients into a homogeneous mass.

3. *List two kinds of cheese in which sorbic acid is permitted and include the conditions under which it might be used. List two kinds of cheese for which sorbic acid is not permitted.*

   a. Sorbic Acid is permitted for use in cold pack cheeses in an amount not to exceed 0.3 percent by weight and in pasteurized process cheeses in an amount not to exceed 0.2 percent by weight.

   b. Sorbic Acid is not permitted for use in Nuworld and Roquefort cheeses.

4. *What is a processed cheese?*

A processed cheese is the food prepared by comminuting and mixing, with the aid of heat, one or more of the optional cheese ingredients, into a homogeneous mass that is spreadable.

5. *In the AOAC procedure, is the moisture content directly determined?*

   The AOAC procedure uses an indirect method and moisture loss is determined, not content.

6. *In the cheese fat extraction, what other substances may be found?*

   Other product ingredients, such as oil-soluble natural colors, may be extracted if present.

7. *Why is petroleum ether used for the extraction?*

   Petroleum ether is used because fats and oils are readily soluble in this solvent, and it is easily evaporated.

4.2.1.2 Jams and Jelly

1. *What is jam?*

   A viscous or semi solid food, from a mixture of one or more fruits, mixed with or without water, and concentrated with or without heat. The soluble solids content of the finished product should not be less than 65 per cent. The standards of identity for jams and jellies (21 CFR 150) require that these products be prepared by mixing not less than 45 parts by weight of certain specified fruits (or fruit juice in the case of jelly), and 47 parts by weight of other designated fruits, to each 55 parts by weight of sugar or other optional nutritive carbohydrate sweetening ingredient.

2. *What is an imitation jelly?*

   A food is an imitation if it is a substitute for, and resembles another food, but is nutritionally inferior to that food (21 CFR 101.3(e)). Nutritional inferiority includes any reduction in the content of an essential nutrient that is present in a measurable amount.

3. *Define "degrees Brix."*

   A unit of measure on the Brix scale for measuring the density or concentration of sugar (sucrose) in solution, determined as percent by weight. It is used to measure the density of the packing medium of fruits and vegetables, or strength of juices.

4.2.1.3 Egg Noodles

1. *Does the label have an ingredient statement? Why?*

   The label of an egg noodle must have an ingredient statement when optional ingredients such as onions, garlic salt, gum gluten, or glyceryl monostearate are added.

2. *Do all noodle products contain eggs?*

   As defined in the standard, all noodle products should contain egg; in noodles, the total solids of egg or egg yolk are not less than 5.5 percent.

3. *Compare the two drying methods. Which must be used to determine compliance with the standard for total solids?*

   a. One method uses a vacuum oven with drying to a constant weight at temp 98-100 ºC (~ 5 hrs.) and the second uses an air oven at 130 ºC for 1 hour.

   b. CFR 21.139.150(a)4 specifies the vacuum oven method.

4. *What is the difference between macaroni and noodles?*

   The difference between macaroni and noodles is the egg content. In macaroni the total solids from egg white, frozen egg white, or dried egg white is not less than 0.5 and not more than 2.0 percent. In noodles, the total solids of egg or egg yolk are not less than 5.5 percent.

4.3 Food Additives

1. What are the three requirements that must be met for a preservative to be used according to good manufacturing practices? The three requirements for preservative use are the following: generally recognized as safe for such use (a food additive is covered by food additive regulations prescribing conditions of safe use); not used in such a way as to conceal damage or inferiority or to make the food appear better or of greater value than it is; and proper declaration on the food label in which used.
2. List 10 standardized foods for which the addition of chemical preservatives has been permitted by regulation. Milk and milk products, cereal, fish, macaroni, cheeses, canned vegetables, nuts, fruits bakery goods, and cacao.
3. Are all substances added to food required to be declared on the product label? Any substance directly added to food must be declared on the label. Substances added during processing that have no technical effect on the product and indirect food additives are not required to be declared. Substances added to foods should be declared as an ingredient by its common or usual name in descending order of predominance in the finished food. Ingredients cannot be listed “collectively” unless excepted from the requirement for using its specific name.
4. What is “Do.”? “Do.” Is the abbreviation for ditto.

4.3.1.1 Water Phase Salt and Sodium Nitrite in Smoked Fish

1. *Which fish or fish products may legally contain nitrates or nitrites? List the maximum legal level for each product and describe how the retail container must be labeled.*

The fish or fish products that may contain nitrates or nitrites are smoked cured sablefish, salmon, shad, and chubs at levels not to exceed (NTE) 200 ppm sodium nitrite and NTE 500 ppm sodium nitrate. In smoked tuna fish products, the level of sodium nitrite is NTE 10 ppm. The container label must bear the name and concentration of the additive.

2. *Where is "water phase salt" defined?*

   Water phase salt (WPS) is defined as the salt content of the water phase portion of the edible portion of the finished smoked product, as measured in the loin muscle (21 CFR 172.177). Generally, WPS should not be less than 3.5%. However, consult Compliance Programs 7303.844 and 7303.842 Domestic/Imported Fish and Fisheries Products for specific requirements, or the HACCP program (Fish and Fisheries Products Hazards & Controls Guide).

   How does water phase salt vary with moisture content?

   The water phase salt content is inversely proportional to the moisture content.

   Write the equation used for calculating the salt concentration in the water phase.

   % salt in water phase = [(% salt / (% salt + %water)] x 100.

3. *Why is water added to the portion taken for analysis by AOAC method 952.08A?*

   Water is added for the purpose of mixing and to help uniformly distribute the sample portion across the surface of the drying pan. A smooth layer of sample promotes even drying.

4. *How would one show that all the volatile components had been evaporated from the tissue?*

   If no significant change in weight is observed after an additional period of drying, then all volatiles have been evaporated.

5. *Show the reactions involved in the titrimetric analysis for total chloride.*

   The procedure uses a modification of the old Volhard Titration.

   For fish and fish products, the organic matter is oxidized by heating with HNO_3; the Cl^- is precipitated by the addition of Ag^+. This results in an excess of Ag^+.

   $Ag^+ + Cl^- \rightarrow AgCl$ ppt + excess Ag^+

   The excess Ag^+ is then titrated with SCN^- (thiocyanate).

   $Ag^+ + SCN^- \rightarrow AgSCN$ ppt

   One drop past the end point there is an excess of SCN^-. This excess SCN^- then reacts with the Fe^{+3} to produce a colored ferric thiocyanate complex:

   $Fe^{+3} + SCN^- \rightarrow FeSCN^{2+}$ (reddish-brown color)

So, the total amount of Ag^+ is known, and the excess Ag^+ after AgCl formation is known from the titration. Subtracting the moles of each yields the amount of Ag+ that reacts with the Cl^-, which in turn tells us the amount of Cl^-. The Fe^{+3} does not do anything until there is an excess of SCN^-; then it reacts and produces the colored $FeSCN^{2+}$ complex which is used for the end point. The reddish-brown $FeSCN^{2+}$ actually has a more complex formula. Bromide, iodide, and cyanide are interferences. Also, if one does not do the titration quickly, then there will be an interference from the AgCl (Ksp =1x10E-10) reacting with the SNC- since AgSCN has a Ksp = 1x10E-12). The analyst should get an RSD of < 5% and a relative error of < 2%.

6. *Why is the HNO_3 solution to be boiled before the indicator is added?*

   The HNO_3 solution is used to digest the protein and liberate the chloride in solution before the indicator is added.

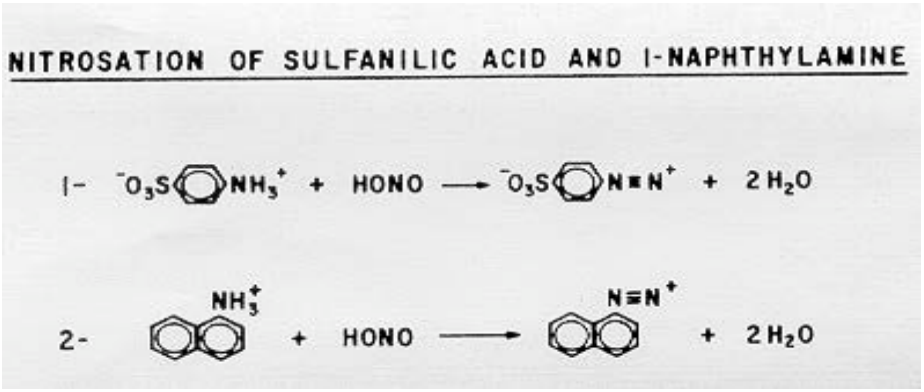
7. *AOAC method 937.09B(b) states "With 10g sample each mL 0.1N $AgNO_3$ = 0.058% NaCl." How is this factor obtained?*

   Assuming a 10.000 gram sample is used:

$$\frac{100\%\ X\ \frac{58.44\ mg\ NaCl}{meq}\ X\ 0.1\ N\ SCN^-}{10{,}000\ mg\ sample} = 0.058\%$$

8. *What is the structure of the colored product formed during the nitrite analysis? Show the reactions involved.*

   The nitrosation of sulfanilic acid form the diazonium salt of sulfanilic acid which then couples with 1-naphthylamine to form a pink azo compound.



9. *How has the order of addition of reagents been shown to affect this type of analysis?*

   Nitrite was added to sulfanilic acid, and the mixture was allowed to stand 20 minutes before 1-naphthylamine was added. The resulting amount of azo dye was

the same as that formed when nitrite was added to a mixture of sulfanilic acid and 1-naphthylamine.

10. *What is the correlation between pH and color stability?*

    Use of more acid and substitution of acids were investigated and the amount of dye compound formed was not affected.

4.3.1.2 Nitrate and Nitrite in Bottled Water

1. *What is the stability and storage conditions for the stock standards? Working standards?*

   The nitrate stock standard is stable for 6 months if stored at 4 °C. The nitrite stock standard is stable for 1 month if stored at 4 °C. Nitrite in water readily transforms into nitrate, so the working standards must be prepared fresh daily.

2. *What factors can affect trace analysis by IC?*

   Contamination control is important. The samples and solutions must be stored in a container made of polytetrafluoroethylene (PFTS), polypropylene (PP) or polystyrene. Glass has ion exchange properties and should be avoided. Deionized water used to prepare standards, eluents, and used to dilute samples must be the highest quality possible (< 0.1 µS·cm-1). Contamination can also come from the apparatus used to process the sample.

3. *How does diluting the eluate affect the analysis?*

   Diluting the eluate to separate co-eluting peaks results in a reduction in the overall response of the anion. Late eluting anions will be retained longer, lengthening the run time. Peaks will be shorter and broadened which will adversely affect the Method Detection Limit (MDL) of the anion. The QC must be repeated to show the dilution does not negatively impact the analysis.

4. *What does the analyst need to be aware of when using a pretreatment cartridge to eliminate certain types of matrix interference?*

   All method calibration standards must be treated in the same manner as the sample. Caution should also be exercised when using pretreatment cartridges because artifacts can be leached from the cartridge which can damage the guard and analytical columns causing a loss of column capacity resulting in shortened retention times and irreproducible results.

4.3.1.3 Sulfites

Monier-Williams Determination

1. *Why doesn't the HCl distill into the sulfur dioxide absorber? What is an azeotrope?*

   The risk of interfering substances reaching the hydrogen peroxide trap (sulfur dioxide absorber) by aerosolization, co-distillation, or steam distillation has been

reduced by condenser coolant temperatures, reflux rates, and nitrogen flow. The HCl is trapped in the reflux column.

An azeotrope is a mixture of liquids that has a constant boiling point and cannot be separated by distillation.

2. *What reaction occurs in the distilling flask? In the sulfur dioxide absorber?*

   An oxidation reaction: $SO_2 \rightarrow SO_3$.

3. *What is the purpose of the gravimetric determination?*

   The gravimetric determination serves as a confirmatory test specific for sulfites.

4. *What purpose does the pyrogallol/KOH gas wash serve?*

   The pyrogallol/KOH wash serves as an oxygen scrubbing solution.

LC-MS/MS Determination

1. *What is the purpose of the addition of 0.2% formaldehyde for sample extraction?*

   It converts free sulfite to a more stable adduct → hydroxymethylsulfonate (HMS).

2. *What volume of extracting solvent is used for the first extraction for the three different solid procedures (basic protocol, low moisture solids, and high moisture solids)?*

   Basic – 20 mL, low moisture – 30 mL, high moisture – 15 mL

3. *Why is the sample run through a C18 solid phase extraction (SPE) clean-up?*

   To remove all lipophilic matrix components

4. *The method uses a HILIC column for addition separation of matrix constituents prior to MS/MS detection. What is a HILIC column?*

   Hydrophilic Interaction Liquid Chromatography. Typically used when retention time in reverse phase chromatography is insufficient. Typically involves more polar or ionizable analytes.

5. *Which type of calibration curve is used?*

   Quadratic with $1/x^2$ weighting

6. What are the monitored transitions?
   a. HMS: 111-81 and 111-80
   b. Internal Std HMS (34S): 113-83 and 113-82

7. *What is the calculation to convert the concentration in vial from the calibration curve to concentration in sample (ppm)?*

$$ppm\ in\ sample = \left(\frac{x\ \mu g\ Na_2SO_3}{mL\ LC\ vial}\right) * \left(\frac{1\ mL\ vial}{v}\right) * \left(\frac{df}{m}\right) * \left(\frac{64\ gmol^{-1}\ SO_2}{126\ gmol^{-1} Na_2SO_3}\right)$$

Where x is the concentration (ug/mL) in vial from the calibration curve, df is the dilution factor for the sample, m is the mass (g) of sample analyzed, v is the volume (mL) of the extract added to the LC vial. The final term of the equation is used to convert from concentration Na_2SO_3 to SO_2. All values were reported as µg SO_2/g food sample.

8. *Can sulfites be declared using an advisory statement such as "may contain sulfites"?*

   No. Sulfiting agents is a food intolerance substance, not an allergen. The sulfiting agent used must be declared on the label as an ingredient.

4.3.1.4 Sweeteners

1. *You have been given a dried fruit to analyze for cyclamates using AOAC method 969.27. Is this method suitable for the analysis? What should you do?*

   No. A method extension or modification is needed prior to analyzing this sample.

2. *In terms of the TLC extraction procedure, what is the purpose of adding 5 ml of 50% NaOH solution?*

   Modification of the nature of the aqueous phase to basic changes the solubility of the 4 analytes. This will promote their extracting into the ethyl acetate organic phase.

3. *Why do foods that contain sugar alcohols require a warning statement on the label? What is the warning?*

   Sugar alcohols can product abdominal gas, bloating and diarrhea in some individuals.

   Labels must include the warning "excess consumption may have a laxative effect."

4.3.1.5 Benzoates

1. *The lab has a large supply of Silica Gel G plates that are used in different analyses in the lab, including colors. Can these TLC plates be used for this analysis?*

   No. Plates specified in LIB 3385 should be used.

2. *Is the pH of the mobile phase used in AOAC 979.08 critical? What affect can pH have on retention times?*

   Yes. Adjustments to pH can affect the retention time of certain analytes.

3. *What formula should be used to calculate % benzoate in the sample?*

% Benzoates = C^1 x (H/H^1) x (V^1/V) x 0.1

4. *What is the salt of benzoic acid? What pH does sodium benzoate optimally function? Which is more soluble, sodium benzoate or benzoic acid?*

   a. Sodium or potassium benzoate.

   b. pH 2.5 – 4.0

   c. Sodium benzoate is 180 times more soluble than benzoic acid.

5. *In terms of HPLC, what may occur if there is a change in the composition of the mobile phase?*

   The chromatographic elution pattern will change and possibly the peak shape; the resolution between peaks may not meet the performance criteria, components may elute in the void volume or greatly increase elution time resulting in poor chromatography and efficiency.

6. *In terms of HPLC how critical is the detection wavelength?*

   The wavelength at which the analyte of interest absorbs is critical information. When using HPLC with a UV/VIS fixed detector and attempting to chromatograph several analytes at once, a wavelength that is common for the group of analytes should be selected. However, modern instrumentation allows for different wavelengths to be simultaneously or sequentially monitored throughout the course of a chromatographic run.

4.4 Color Additives

1. *What is a color additive?*

   A color additive is defined as a dye, pigment, or other substance - whether synthetic or derived from a vegetable, animal, mineral, or other source - which is added to impart color to a food, drug, cosmetic, applied to the human body or medical device.

2. *Define these terms: straight color, lake, and diluent.*

   a. A *straight color* is a color additive that has not been mixed or chemically reacted with any other substance. Straight colors subject to certification are listed in 21 CFR Part 74. Straight colors exempt from certification are listed in 21 CFR Part 73. In addition, 21 CFR Section 81.10 and 81.30 identify straight colors whose listings or certain uses have been terminated or whose certificates have been cancelled.

   b. *Lakes* are pigments prepared by precipitating a soluble dye onto an insoluble reactive or adsorptive substratum or diluent. They are water-insoluble and more stable than straight dyes and are ideal for product in

which leaching of the color is undesirable (coated tablets and hard candies, for example).

c. A *diluent* is any component of a color additive mixture that is not of itself a color additive and has been added to facilitate the use of the mixture as a coloring agent. The term mixture means a color additive made by mixing two or more straight colors, or one or more straight colors and one or more diluents.

3. *What are "certified" colors?*

   Certified colors are listed color additives in Code of Federal Regulations (21 CFR Part 74) from certified batches analyzed by FDA's Color Certification Branch, CCB, to assure the listed specifications are met. These certifiable color additives are synthetic organic dyes, pigments, or lakes of a dye. In order to be used in foods, drugs, cosmetics, and medical devices manufactured or sold in the United States, the color additives must meet the following criteria: come from a certified batch, be allowed in the respective product, and the color additive name must be declared in the ingredients label.

4. *Under what conditions may color additives be used in the area of the eye, in injections, or in surgical sutures?*

   Color additives may be used in the area of eye, injections, or in surgical sutures only if the color additives are specifically listed for such use.

5. *List the restrictions, if any, for the following colors:*

   a. *Citrus Red No.2* - Permanently listed for use only in coloring of skins of mature oranges. Limit of 2 ppm calculated on basis of whole fruit weight.

   b. *Orange B* - Permanently listed for use in coloring surfaces and casings of frankfurters or sausages. Limit of 150 ppm by wt. of finished product.

   c. *Titanium dioxide* - Limit: of 1.0% by wt. of food.

   d. *FD&C Blue No. 1* - For food, drug, and cosmetic use, including drugs and cosmetics for eye area - GMP - 74.101, 74.1101, 74.2101, 82.101.

   e. *FD&C Red No. 3* - For food and ingested drugs - GMP - 74.303, 4.1303. Lake use terminated 2-1-90; All cosmetic uses terminated 2-1-90.

   f. *FD&C Red No. 40* - For food, drug, and cosmetic use, including drugs and cosmetics for eye area - GMP - 74.340, 74.1340, 74.2340.

   g. *FD&C Yellow No. 5* - For food, drug, and cosmetics, including drugs and cosmetics for eye area - GMP - 74.705, 74.1705, 74.2705, 82.705.

6. *Give one example of a triphenylmethane color.*

Brilliant Blue FCF (CI 42090, certifiable as FD&C Blue No.1) is an example of a triphenylmethane color. [Other triphenylmethane dyes, such as malachite green or gentian violet, are sometimes used as fungicides in the food industry.] Triphenylmethane colors consist of a common moiety of three phenyl groups attached to a central carbon (hence "methane"). Other functional groups are then attached to the phenyls, and these determine the different colors.

7. *List the Color Index Number (C.I. No.) and common name for FD&C Red No. I, FD&C Red No. 3, D&C Orange No. 3, FD&C Green No. 3. D&C Yellow No. 7, D&C Yellow No. 9, FD&C Blue No. 2, and FD&C Violet No. 1.*

Color	C.I. No.	Common
FD&C Red No.1	CI 16155	Ponceau 3R
FD&C Red No.3	CI 45430	Erythrosine
D&C Orange No.3	CI 16230	Orange G
FD&C Green No.3	CI	Fast Green
D&C Yellow No. 7	CI	Fluorescein
D&C Yellow No. 9	CI	Uranine K
FD&C Blue No.2	CI 73015	Indigotine
FD&C Violet No.1	CI 42640	Wool Violet 5BN

8. *A label of a food product declares "...E123..." as an ingredient. Is this product permitted in the U.S.?*

   E123 is the European code designation for Amaranth (a.k.a. CI 16185, former FD&C Red No.2) which is not permitted for use in the U.S. No E numbers are permitted in the US since we do not know that they meet the specifications listed in the CFR.

9. *A label of a food product declares "...Sunset Yellow FCF, Eythrosine Lake, Allura Red AC." Is this product correctly labeled? If not, why? If the importer re-labels the product, would it be permitted in the U.S.?*

   Artificial colors listed in 21 CFR part 74 must be declared on the labels by listing the color additive name (i.e. Red 40) among the list of ingredients (21 CFR 101.22(k)(1)). When colors are declared using their common names (i.e. Allura Red AC), it is assumed that the colors used were not from a certified lot and the product can be detained based on adulteration.

   Use of Erythrosine Lake is not permitted in the U.S. and the product would not be permitted to be sold even if relabeled.

10. *In terms of solubility, state whether the following color additives are water soluble or oil soluble:*

a. "*Natural" colors such as riboflavin or saffron* (water soluble)

b. *Tartrazine* (water soluble)

c. *Sudan 1* (oil soluble)

d. *D&C Red 19 or DC Red 28* (water soluble)

4.4.1.1 Quantitative Determination of FD&C Colors in Foods (Graichen Method)

1. *What is the difference between Procedure I, II, and III?*

   All use chloroform to remove FD&C Red 3, fats, and "natural" colors (i.e. carotenoids, chlorophyll, etc.). However,

   Procedure I uses 20% acetic acid as the aqueous phase and separately extracts azo colors from triphenylmethane colors.

   Procedure II uses pH 7.5 buffer as the aqueous phase and is required for the extraction of FD&C Blue 2.

   Procedure III uses 0.1 N Hydrochloric Acid and is required if the colors in the product were added as lakes.

2. *A sample is prepared as per the method following Procedure I. In which eluate would the following colors be seen:*

   a. *Amaranth* will elute in resin-in-hexane.

   b. *Green 3* will elute resin-in-butanol.

   c. *Beta Carotene* will elute in chloroform.

4.4.1.2 FD&C Color Additives in Foods: Rapid Cleanup for Spectrophotometric and Thin-Layer Chromatographic Identification (AOAC Method 988.13)

1. *How does the C_{18} sep-pak isolate and concentrate colors?*

   The C_{18} sep-pak is a small reverse phase liquid chromatography column. Colors are adsorbed onto the C_{18} while more polar compounds (sugars, flavors, etc.) are not retained. The colors separate into bands based on their polarity.

2. *Yellow No. 5 is not retained as well as the other colors on the sep-pak. How can you improve its retention?*

   The C_{18} sep-pak is flushed with 5 ml of acetic acid to improve adsorption of the colors on the sep-pak. Yellow 5 is more polar and has a lower affinity for the sep-pak. Acidifying the sample solution will strengthen its adsorption on the sep-pak.

3. *What are chromophores and auxochromes? Identify the chromophores and auxochromes in two of the following colors: Citrus Red 2, Orange B, Titanium*

Dioxide, FD&C Blue No. 1, FD&C Red No. 3, FD&C Red No. 40, and FD&C Yellow No. 5.

Chromophores are groups of one or more unsaturated bonds that absorb light (in the ultraviolet or visible region) and thus produce colored substances. They are, in most cases, covalent unsaturated groups such as C=C, C=O, C=NH, CH=N, and N=N.

Examples are FD&C Yellow No. 5 (N=N) and FD&C Blue No. 1 (N=CH).

Auxochromes are functional groups that do not confer color to a substance themselves but increase the coloring power of a chromophore. They contain functional groups, such as –OH, $–NH_2$, $–SO_3$ H, –COOH, that don't exhibit absorption in visible wavelengths (however, auxochromes may absorb strongly in the far-ultraviolet region). When an auxochrome is combined with a chromophore in the same molecule, the chromophore absorption will typically shift to a longer wavelength (a bathochromic shift) and show an increase in intensity (a hyperchromic shift). Auxochromes provide an unshared pair of elections in conjunction to the chromophore.

Examples are FD&C Blue No. 1 ($–SO_3$) and Citrus Red 2 (–OH).

4. *What is hypsochromic shift? Give the effect of hypochromic shift for two of the colors in the question above.*

Hypsochromic shift is a change in wavelength maxima to a shorter wavelength, and thus change in color.

For FD&C Red No. 40 and Citrus Red 2, the addition of base produces a change in color from red to orange and a wavelength maximum shift of approximately 50 nanometers.

4.4.1.3 Color Additive Analysis in Foods and Cosmetics using UPLC with Extended Photo-Diode Array Detection (LIB 4643)

1. *How versatile is this method?*

LIB 4643 was designed to be a suite of techniques to analyze a broad spectrum of synthetic and natural colors in a vast variety of foods, cosmetics, and other marketed products. This method can analyze water and some oil-soluble colors. The extraction and cleanup is fast and simple, and the colors are identified by UPLC-PDA analysis. Over 150 different synthetic dyes and natural pigments have been characterized and stored in a spectral library on the instrument to assist in identifying undeclared colors.

2. *Describe how to determine the appropriate method section to use for a sample.*

Method selection is based on whether the sample is a liquid or solid, water or oil-based, or considered a “crossover” product. A method selection flowchart is included in the LIB.

3. *When is an HLB SPE column used?*

   The HLB column is a secondary column that can retain most synthetic dyes or natural pigments not bound by the CBX. If color is observed passing through the CBX SPE when the sample is loaded onto the column, the eluate can be collected for further analysis following the HLB column protocol.

4. *Is it critical to filter both mobile phases?*

   No, only mobile phase A must be filtered. Mobile phase B is a mix of high-grade organic solvents and does not require filtering.

4.4.1.4 Determination of Colors in Cosmetics

1. *How many synthetic colors are approved for use in cosmetics? Which of these colors are permitted in eye area cosmetics?*

   Presently there are 36 synthetic colors approved for use in cosmetics, of which only six are permitted in eye area cosmetics: FD&C Yellow No.5, FD&C Red No. 40, FD&C Blue No.1, D&C Green No.5, D&C Black No. 2, and D&C Black No. 3.

2. *What colors or pigments are approved for use in tattoos? What about henna?*

   Color additives are not approved for tattoos since tattoos require the color to be injected under the skin. No color additives are approved for direct injection.

   The color additive henna is not permitted to be applied to the skin and is only approved for coloring hair.

3. *A lipstick sample was analyzed using Newburgers, 19.1. One bright pink band of color, exhibiting orange fluorescence, was detected. Using Table 12 in Newburgers (page 119), what color(s) was(were) detected? Are these possible colors permitted for use?*

   A bright pink band of color exhibiting orange fluorescence under UV light can be due to either D&C Red No. 27, which is permitted for use in cosmetics, or it can be due to D&C Red No. 19, which is not permitted for use in the U.S.

Attachment C - Quantitative Determination of FD&C Colors (Graichen Method)

Quantitative Determination of FD&C Colors in Foods

CHARLES GRAICHEN ~~8-202-245-1143~~

Division of Colors and Cosmetics Technology, Food and Drug Administration, Washington, D.C. 20204

ABSTRACT

The bases of this updated procedure remain the published liquid anion exchange resin solution extraction of colors from a ground sample-celite-aqueous phase column, and subsequent separation of color mixtures by chromatography on cellulose columns. A Brinkman Polytron or Tekmar equipment gives more complete grinding of difficult to grind foods, and therefore a more complete extraction of colors. A more acid aqueous phase and a delayed extraction step have been added for foods colored with the lakes of colors. Shorter cellulose columns give quicker separations of colors. Different diameters are used according to the amount of color. Five eluants were selected to enable the separation of any combination of water soluble FD&C colors. Two previously used eluants are 5% alcohol-1% NaCl and 5% NaCl-1% ammonium hydroxide. A new eluant, 60% alcohol-1% NaCl is used to separate combinations of FD&C Reds No. 2, 4, and 40. A fourth eluant, 1% NaCl-0.1% ammonium hydroxide is a change from 1% ammonium hydroxide to lessen fading of FD&C Blue No. 2. The fifth eluant, 10% NaCl-0.1% ammonium hydroxide is used for a column separation of FD&C Green No. 3 and FD&C Blue No. 1. Many improvements in technique are included.

CORRECTION TO PROCEDURE

Delete the first paragraph under (a), page 5, and substitute:

(a) Spectra in resin solutions. Prepare the solutions in the three resin-butanol solvents by direct dilution as follows: Pipet 1 ml of color solution into a 200 ml flask. Add 40 ml of 1-butanol and mix. (Some colors will not completely dissolve). Add 150 ml of the resin-butanol solvent, and dilute to volume with butanol, and mix until solution is complete.

For spectra in resin-in-hexane, prepare solutions using a column to dissolve color in manner similar to use for extraction of color from food (See pages 9, 10 and 11). Mix 20 g celite with 11 ml of 20% acetic acid. Pack ca 3/4 of this mixture in chromatographic tube No. 1. In a beaker, mix 1 ml of the color solution with 15 g celite and 8 ml of 20% acetic acid. Transfer to tube and pack. Rinse out beaker with the remaining 1/4 of the first 20 g portion, transfer to column and pack. Elute color with resin-in-hexane. The elution of color is usually complete in less than 150 ml. Collect 150 ml and dilute to 200 with butanol. If elution is not complete at 150 ml collect larger volume and dilute 3 parts with 1 part butanol.

INTRODUCTION .

The procedure described in this paper is based on two publications (1)(2). Since then, some colors have been deleted from the permitted list, and some new colors have been added. Another change is that the aluminum or calcium lake of an FD&C color may now be certified for use in foods (3). These changes have created new separation and extraction problems which have made necessary this updating of the original procedures.

Principle

The food is finely ground and mixed with an appropriate aqueous phase and celite. This mixture is packed into a chromatographic column. The column is eluted with chloroform to remove fats and chloroform soluble natural colors. The FD&C colors are then eluted from the column with a solution of liquid Amberlite anion exchange resin. The colors are extracted from the resin solution into water. Mixtures of colors are separated by column chromatography on cellulose. The separated colors are identified and quantitatively determined from the visible range absorbance spectra.

METHOD

Apparatus

(a) Chromatographic tubes.--Figure 1. Four sizes are used. No. 1 is used for the resin extraction step. It is 52 mm id with 40 mm fritted disk, and is ca 200 mm long above disk. Nos. 2, 3, and 4 are used for separation of mixtures of colors. These three are all ca 23 inches

long above the fritted disks. No. 2 is 44 mm id with a 40 mm disk. No. 3 is 31 mm id with a 30 mm disk. No. 4 is 22 mm id with a 20 mm disk.

(b) Plunger.--Figure 2. Solid aluminum plunger weighing ca. 1,300 g. If this diameter is varied, the id of chromatographic tube No. 1 is also varied.

(c) Top disks.--Cut 50 mm disks from teflon coated fiber glass cloth or other suitable material. The disks are reused. Their use is solely to prevent mechanical disturbance of celite-sample column.

(d) Mortar and pestle.--Pyrex, 16 and 32 oz. capacities.

(e) Tissue blender.--Brinkman Polytron with PT 35 ST Generator or equivalent. Tekmar's Super Dispax system, model SD-45, seems to be comparable.

(f) Glass cylinder.--Cylinder is a container for the grinding and must fit the blender. One used with the PT 35 Generator is 48 mm id and about 210 mm in depth. Other sizes may be used; the size will regulate sample size and volume of aqueous phase.

(g) Spectrophotometer.--Recording, visible range, equipped with 1, 2.5, 5.0, and 10 cm cells.

Reagents

(a) Amberlite, LA-2.--A Rohm and Haas liquid anion exchange resin.

(b) Solka-Floc.--Grade BW-40, from Brown Co., Berlin, N.H.

(c) Whatman powdered cellulose.--Grade CF 11.

(d) Celite-545.

(e) Salt solutions.

(1) 25% NaCl.--(W/V)

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(2) 20% NaCl.--(W/V)

(3) 1%NaCl.--(W/V)

(4) 10% NaCl-0.1% ammonium hydroxide.--(W/V/V)

(5) 5% NaCl-1% ammonium hydroxide.--(W/V/V)

(6) 1% NaCl-1% ammonium hydroxide.--(W/V/V)

(7) 1% NaCl-0.1% ammonium hydroxide.--(W/V/V)

(f) Alcohol solutions

(1) 75% Alcohol.--(V/V)

(2) 60% Alcohol.--(V/V)

(3) 5% Alcohol.--(V/V)

(4) 60% Alcohol-1% NaCl.--(V/W/V)

(5) 5% Alcohol-1% NaCl.--(V/W/V)

(g) Acetic acid solutions.

(1) 20% Acetic acid.--(V/V)

(2) 1% Acetic acid.--(V/V)

(h) Dilute HCl solutions.

(1) 1N HCl.--(9 + 91)

(2) 0.1N HCl.--Dilute 1N HCl (1 + 9)

(i) 10% Ammonium hydroxide.--(V/V)

(j) 1N NaOH.--Dissolve 40 g NaOH in 1 L water.

(k) Buffer, pH 7.5.--McIlvain's. Mix 75 ml 0.1M citric acid solution with 925 ml 0.2M Na_2HPO_4 solution.

(l) Resin solutions.

(1) Resin-in-Hexane.--Mix 50 ml LA-2 resin and 950 ml hexane (95% practical grade). Shake the solution with 200 ml 20% acetic acid. Discard lower phase.

Page 5 -

(2) Resin-in-butanol.--Mix 50 ml LA-2 resin and 950 ml 1-butanol; shake the solution with 400 ml 20% acetic acid and 10 ml 20% NaCl solution. Discard lower phase

(3) Resin-in-butanol, pH 7.5.--Mix 50 ml LA-2 resin and 950 ml 1-butanol. Add 3.0 ml glacial acetic acid. Shake the solution with 400 ml pH 7.5 buffer. Discard lower phase. Shake with 200 ml buffer. Discard lower phase.

(4) Resin-in-butanol, 0.1N HCl.--Mix 50 ml LA-2 resin and 950 ml 1-butanol. Shake the solution with 400 ml water and 19 ml concd HCl. Discard lower phase.

Reference spectra

Prepare water solutions of colors such that 1 ml diluted to 200 ml will yield suitable spectra in 1 cm cells. Do not dry the color samples. Calculate concentration of solutions using certified strength as labeled.

(a) Spectra in resin solutions.--Pipet 1 ml of color solution into 200 ml flask. Add ca 40 ml 1-butanol and mix. Add 150 ml of the particular resin solution. Dilute to 200 ml with butanol. For a blank, dilute 150 ml of resin solution to 200 ml with butanol. Record spectrum from where absorption begins down to 370 nm.

Record the spectra of FD&C Blue No. 2 and FD&C Red No. 3 in resin-in-butanol, pH 7.5. Record the spectra of FD&C Blue No. 1, FD&C Green No. 3, and FD&C Violet No. 1 in the three resin-butanol solutions. Record the spectra of all other FD&C colors and of Orange B in all four resin solutions.

(b) Spectra in water or water and alcohol.--For colors other than FD&C Red No. 3, FD&C Violet No. 1, and Orange B, pipet 1 ml aliquots

into three 200 ml flasks, add a few mg of ammonium acetate, and dilute to volume with each of water, 5% alcohol-1% NaCl solution, and 60% alcohol-1% NaCl solution. Record these neutral, qualitative, and quantitative spectra from where absorption begins down to 350 nm. At the same time, make a second dilution of each color in each solvent but with no added ammonium acetate. From each, transfer 90 ml to each of two 100 ml glass stoppered graduated cylinders. To one, add 10 ml of 1N HCl, mix and draw the qualitative acidic spectrum. To the second, add 10 ml of 1N NaOH, mix and draw the qualitative basic spectrum.

Record the spectrum of FD&C Red No. 3 in only 0.5% ammonium hydroxide in water. This spectrum or the spectrum in resin-in-butanol, pH 7.5 is used for quantitative work.

Record the spectra of FD&C Violet No. 1 in only 60% alcohol, and record only the quantitative neutral and the qualitative acidic spectra. The basic spectrum fades to colorless very rapidly. By itself, the acidic spectrum of FD&C Violet No. 1 is essentially stable. There is some evidence that the presence of unidentified food components which carry through the separation procedures with the FD&C Violet No. 1, the color is not always stable in the 0.1N HCl conditions for the acidic spectrum.

Orange B saponifies to Orange K during extraction from resin solution. Therefore, the reference spectra by which Orange B is identified and determined are those of Orange K.

For each solvent, pipet 1 ml of Orange B solution into each of two 200 ml flasks. Add 1 ml of 0.1N NaOH, mix, and allow to stand at room temperature for 30 minutes. Add 1 ml of 0.1N HCl, mix, and continue

as above for the neutral and quantitative spectrum, and for the qualitative acidic and basic spectra. Calculate the A per mg per L on the basis of the starting Orange B. Record these spectra in 60% alcohol-1% NaCl, 5% alcohol-1% NaCl, and in water.

Preparation of Solka-Floc columns

The columns are prepared in advance, and are reused several times. The preparation of a 80 g column in a 44 mm id tube is described. It takes about 3 hours to prepare. The 40 g column in the 31 mm tube and the 20 g column in the 22 mm tube are the same length as the 80 g column and take the same time to prepare.

Close the end of tube with a short piece of rubber tubing and a screw clamp. Add ca 200 ml of water. Weigh 40 g Solka-Floc into each of two L beakers. Add ca 900 ml of water to each, and stir to make a thin slurry. If the slurry is too thick, the column will not pack evenly, and in subsequent use, the bands of color will not remain sufficiently straight. Add some of the slurry to the tube, open clamp, and as water drains, continue to add slurry until all is transferred. Towards the end of this addition, keep a few inches head of water-slurry above the settled bed, so that top surface of bed remains level. Add ca 300 ml water wash in three or four portions, keeping a few inches head,to settle most of the Solka-Floc. Weigh 4 g Whatman cellulose into 100 ml beaker, add ca 20 ml of water, stir briefly, and transfer to tube. The Whatman cellulose settles the remainder of the Solka-Floc and caps the top of column.

Add 75% alcohol from a pipet keeping the tip of the pipet moving around the inside of tube and controlling the flow so that a thin film,

rather than drops, runs down the inside of tube. (All additions of light over dense solvents are done in this manner. There will be virtually no mixing.) Add about 200 ml of the 75% alcohol. Let drain to about 1 inch above cap. Do not start this 75% alcohol wash unless the following water wash and salt solution wash can be completed before column flow is stopped.

Add ca 300 ml of water from a pipet about 1/2 inch above the liquid surface, keeping the pipet moving so that a flow pattern does not establish and dig into cap. (All additions of dense to light solvents are done in this manner.) Let drain to about 1 inch above cap. Add ca 800 ml of 25% NaCl solution. Let drain to about 1 inch above cap and close clamp.

After a column has been used, if it is clean, resalt. If there are food components or traces of colors on column, wash the column with 75% alcohol, water, and resalt. When build-up of cellulose that is added with each sample becomes excessive, discard column.

Choice of procedure

Procedure I, using 20% acetic acid aqueous phase, is the quickest and cleanest method of extracting the sulfonated azo colors, and has the additional advantage of separately extracting the triphenylmethane colors. Procedure II, using pH 7.5 buffer aqueous phase, is required for a quantitative extraction of FD&C Red No. 3 and FD&C Blue No. 2. Procedure III, using 0.1N HCl, is required to be used if the colors were added as lakes. In addition to the above criteria for selection of procedure, we have some examples where the nature of the food affected ease of grinding or efficiency of extraction of colors and thereby selection of procedure.

Hand grinding

The sample must be ground fine, and uniformly mixed into the celite if the colors are to be quantitatively extracted. Most are ground by hand with a mortar and pestle. If no more than 8 g of sample is to be ground with 15 g of celite, use the 16 oz mortar. If more than these quantities are taken, use the 32 oz mortar.

Weigh a sample which is estimated to contain 0.2 mg or more of the color present in least amount. (This is not always practical.) Weigh a 5 g or larger sample to $\pm$ 0.1 g to permit at least a 2% accuracy. Weigh a smaller size sample to $\pm$ 0.01 g. A gelatin dessert or beverage mix with coarse components (granulated sugar) may not be uniform. Weigh a large sample, dissolve, and take an aliquote. For up to 8 g of sample add 8 ml of the aqueous phase. If the food is one which will slowly dissolve, let stand for a while. Add 5 g of celite and grind until fine and uniformly mixed. Add 10 g of celite and grind until uniformly mixed. The texture should be such that the mixture will pack under pressure and crumble when disturbed as will a mixture of 8 ml of aqueous phase and 15 g of celite. Add a little more celite or aqueous phase if required for proper texture. If more than 8 g of sample is taken, increase the celite and aqueous phase proportionately. For foods which are predominately water such as apple sauce, beverages, estimate the water content, add 1/4 as much glacial acetic acid, and an appropriate amount of celite. Chewing gums are hand ground. Do not exceed a ratio of 2.5 g of gum to 15 g of celite, and take the time required to get the sample-aqueous phase and first 5 g of celite uniform before the next 10 g is added.

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Mechanical grinding

Use a mechanical grinder for difficult to grind foods such as maraschino cherries or colored pineapple. The following is written for the 48 mm cylinder and blender described in "Apparatus".

Tare the cylinder. Add 25 to 28 g sample and weigh (Difference = S). For each g, add 2 ml aqueous phase, and reweigh (Difference = A). Stand cylinder and contents in a water-ice bath and allow about 10 minutes to cool contents. Lower Generator into sample-aqueous phase mixture and run at low speed until chunks are reduced to small size. (About 2 minutes). Lower Generator to full depth and run at high speed for about 1/2 minute.

Meanwhile, tare the mortar. At end of grinding period, raise Generator, and before mixture settles, pour 12 g or larger suitable sample into mortar. Weigh mortar plus mixture (Difference = F).

Weight sample = (F)(S)/(A+S).

Add 15 g or more of celite and complete the grinding by hand.

Procedure I

Grind sample with 20% acetic acid aqueous phase and celite. In a 400 ml beaker, mix 11 ml 20% acetic acid with 20 g celite. Transfer ca 3/4 of this mixture to a 52 mm tube, level the mixture, and pack by the weight of the plunger. Transfer the ground sample-celite-aqueous phase in amounts equivalent to not more than 15 g of celite, level each portion and pack each portion by the weight of plunger. Flush out mortar with the remaining 1/4 of celite-aqueous phase, add to column and pack. Lay a 50 mm disk on surface. Place a powder funnel in tube so that stem is centered over disk. Solutions may be poured in rapidly. The column will not be disturbed.

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Add 100 ml of chloroform. As last of chloroform enters column, add 50 ml hexane. As last of hexane enters column, add 300 ml of resin-in-hexane. As last of resin-in-hexane enters column, add as required up to 300 ml of resin-in-butanol.

Collect a 100 ml fraction of chloroform. If the color mixture has a red component, always shake some of this chloroform with water and sufficient ammonium hydroxide to make alkaline. The appearance of a red color indicates FD&C Red No. 3, in which case a new start using Procedure II is required for a quantitative determination of this color. However, Procedure I can be continued for the determination of other colors.

Collect a 25 ml hexane fraction. By changing receivers 25 ml in advance of the calculated resin-in-hexane solvent front, no color will be lost in the hexane fraction.

Collect resin-in-hexane fractions of 75 ml in 100 ml glass stoppered graduated cylinders. Dilute each to 100 ml with butanol and combine those with extracted color.

Collect resin-in-butanol fractions of 75 ml. Transfer the first to a 125 ml separator, and allow to stand for 10 to 15 minutes. Drain and discard the water layer. Transfer the butanol layer back to cylinder, measure volume, and add 1/3 as much butanol. Dilute the 75 ml fractions (which contain extracted color) to 100 ml with butanol, and combine.

If because of insufficient grinding or mixing, colored particles can be seen in the column, allow the column to stand for three hours or up to over night. Elute another column volume (75 ml or more) of resin-in-butanol. Additional color will usually be extracted. If the column

now looks clean, dilute the resin-in-butanol as above and combine. If the column still contains the colored particles and the amount of color is judged to be of a significant amount, make a new start, and make sure that grinding and mixing is more complete.

If only approximately 1/2 of the sulfonated azo colors extract in the resin-in-hexane, and perhaps 10% or 20% fails to extract with 3 or 4 fractions of resin-in-butanol, this indicates that lakes of the colors were used. Make a new start using Procedure III.

If in the use of Procedure I, a blue component is observed to fade, or if no blue or green color is found in a chocolate or caramel shade which is due to added colors, examine the food for FD&C Blue No. 2. Only when a large amount of FD&C Blue No. 2 is present will it be detected by the use of Procedure I. Procedure II is used for the determination of FD&C Blue No. 2 with the precautions that the spectrum of the resin-in-butanol, pH 7.5 extract of the colors is always drawn without delay, and is always used for the quantitative datum for FD&C Blue No. 2. The sulfonated azo colors will not interfere where FD&C Blue No. 2 has its absorption maximum. Combinations of FD&C Blue No. 2 with FD&C Violet No. 1, FD&C Blue No. 1 or FD&C Green No. 3 are not very likely to occur, but can be determined by the use of simultaneous equations.

Procedure II

Grind the sample with pH 7.5 buffer and celite. Mix 11 ml of buffer with 20 g of celite and pack the column as in Procedure I.

Add 125 ml of chloroform. When last of chloroform enters the column, add 300 ml or more of resin-in-butanol, pH 7.5. Collect a 100 ml fraction

of chloroform. Collect 75 ml fractions of resin-in-butanol, pH 7.5 extracts of the colors. Dilute each to 100 ml with butanol, and combine the colored fractions.

Procedure III

Grind the sample with 0.1N HCl and celite. Mix 11 ml of 0.1N HCl with 20 g celite and pack column as in Procedure I. Elute column with 125 ml of chloroform. When last of chloroform enters the column, follow with resin-in-butanol, 0.1N HCl. Collect a 100 ml chloroform fraction and 75 ml fractions of resin solution. When not much color is being eluted, let the column stand for 3 hours or up to over night. Elute another column volume (75 ml or more) of resin-in-butanol, 0.1N HCl.

If the color mixture has a red component, examine the chloroform extract for FD&C Red No.3. When lakes are used, a negative test for FD&C Red No. 3 in the earlier use of Procedure I will not prove the absence of this color. If present, recover the color from the chloroform by extractions with 1% NaCl-1% ammonium hydroxide. There will be problems with emulsions. Our experience includes one sample where this had to be followed by a water wash of the chloroform to recover all of the FD&C Red No. 3. Normally, the bulk of the FD&C Red No. 3 will be in the resin solution extract. Some may remain on the column although it will not be seen because of the acid conditions. From laboratory baked cookies, the recovery of FD&C Red No. 3 was 80 to 90%. The recovery will probably vary with the composition of the food.

Dilute each colored resin-in-butanol, 0.1N HCl extract to 100 ml with butanol and combine.

Spectrophotometric examination of resin solution extracts of color

Record the spectra in appropriate cell lengths against appropriate blanks. These spectra enable the analyst to estimate the composition of the color mixture and to make a better selection of column size in subsequent processing to separate colors from each other. If the extracts are clean, the analyst may return to these spectra for quantitative data. The resin-in-hexane extract of the sulfonated azo colors is often clean.

If the sample was completely ground and mixed into the celite, all of the sulfonated azo colors extract in the resin-in-hexane. If grinding and mixing was not complete, 1 or 2% may be found in the resin-in-butanol along with the triphenylmethane colors. It is seldom that any of the 3 butanol-resin solvents give clean extracts.

FD&C Red No. 3 does not yield a suitable spectrum in resin-in-butanol, 0.1N HCl. When used as a lake, it must be extracted by Procedure III, but then extracted into water and determined in alkaline solution.

Extraction of colors from resin-in-hexane yellow

Wash the solution with two 0.5 volumes of 1% NaCl solution. Extract the colors with three to five 0.1 volumes of 1% NaCl-1% ammonium hydroxide solution plus sufficient (about 0.1 volume) 10% ammonium hydroxide to make alkaline in the first. Wash each extract in series through 75 ml of ether. Combine the extracts in a 400 ml beaker. Add glacial acetic acid to neutral or weakly acid. Add ca 0.5 g Whatman cellulose and sufficient NaCl to make a 25% solution. Stir until the salt seems to be dissolved. Place mixture under vacuum in a vacuum desiccator (25 to 30 cm Hg pressure is sufficient) for 20 minutes to remove dissolved ether. Color mixture is now ready to be absorbed on a Solka-Floc column for separation and/or purification.

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Extraction of colors from resin-in-butanol

Wash the resin-in-butanol solution with two 0.5 volumes of 1% NaCl solution. Add an equal volume of hexane. Extract the colors with 0.1 volumes of 1% NaCl-1% ammonium hydroxide plus sufficient 10% ammonium hydroxide in the first (about 0.2 volume) to make alkaline. Wash each extract in series through 75 ml of ether. Combine in a 400 ml beaker. Add sufficient acetic acid to make neutral or weakly acid. This should be done without delay if the sample contains FD&C Violet No. 1. Add about 1 g of Whatman cellulose, sufficient NaCl to make a 25% solution and treat in vacuum for 20 minutes.

Extraction of colors from resin-in-butanol, pH 7.5

Add an equal volume of hexane. Extract the colors with 0.1 volumes of 1% NaCl-1% ammonium hydroxide. Wash each extract in series through 75 ml of ether.

(a) For color mixtures with FD&C Blue No. 2, neutralize immediately with acetic acid and combine in a 400 ml beaker. Add about 1 g of Whatman cellulose, sufficient NaCl to make a 25% solution, and treat in vacuum for 20 minutes.

(b) For color mixtures with FD&C Red No. 3, neutralize with acetic acid, then add 3 ml per 100 additional acetic acid. Extract the FD&C Red No. 3 from the water solution with two 75 ml portions of ether. Wash the ether layers in series with two 10 ml portions of 1% acetic acid. Add these washes to the water solution containing the remaining colors. Add 1 g Whatman cellulose, sufficient NaCl to make a 25% solution, and treat in vacuum for 20 minutes.

Combine the two ether layers. Extract the FD&C Red No. 3 from the ether with 10 ml portions of water plus sufficient 10% ammonium hydroxide to make alkaline in the first (25 to 30 ml) and 1 ml in the remaining. Combine the extracts in a 400 ml beaker. Treat in vacuum for 20 minutes. Add a few drops of 10% ammonium hydroxide, measure the volume, and draw the spectrum. There may be some background, but it is simple to correct for background since only FD&C Red No. 3 is present. If it is felt to be necessary to purify this color by chromatography on cellulose, see directions in section further on.

Extraction of colors from resin-in-butanol, 0.1N HCl

Proceed as under "Extraction of colors from resin-in-butanol." If FD&C Red No. 3 is present, add to this the extraction of acid solution with ether to isolate the FD&C Red No. 3. Recover the color from the chloroform, and combine with that from the resin solution.

Chromatographic separation of color mixtures

Select column size according to amount of color. If 0.5 to 1.0 mg of one or more colors are present in a mixture, use the 80 g column. Use the 40 g and 20 g columns for proportionally smaller quantities. With extreme range of composition, exceed these column loadings as needed, so that the color present in least amount can be isolated and determined. This may require variations in procedure. If necessary, a longer column can be prepared. A column of about 120 g can be prepared in the 44 mm id tube.

Open the outlet of column. Stir the color mixture to suspend the cellulose, and pour the mixture slowly down a stirring rod held with the

end just above the liquid. Rinse out the beaker with small portions of 25% NaCl solution and add to column. Float down a layer of 20% NaCl solution (about 50 ml for the 80 g column). Float down a layer of appropriate lighter developing solvent which will make the required separations.

Figures 3 to 6 show the elution patterns of certain colors with four eluants. Two are those used by Ms Sclar (1). The other two eluants are new. These chromatograms were obtained using 0.5 mg of color on a 30 g column in a 31 ml tube. The separation of a mixture will be better with the 80 g, 40 g, and 20 g columns than indicated in these chromatograms.

Perhaps 90% of the time, 5% alcohol-1% NaCl will be used. This solvent (figure 4) will elute if present, first FD&C Yellow No. 5, then FD&C Yellow No. 6. A change to 5% alcohol at this point will elute the red color or colors with less tailing.

When two or more reds are present, absorb the mixture on a second column and elute with 60% alcohol-1% NaCl (figure 3). FD&C Red No. 40 will elute first followed by FD&C Red No. 4. Change to 60% alcohol to elute FD&C Red No. 2 with less tailing.

Most but not all samples of FD&C Yellow No. 6 contain significant amounts of a higher sulfonated isomer. This isomer will usually be collected with the FD&C Yellow No. 5 if also present. Only occassionally will it be eluted ahead of the FD&C Yellow No. 5.

With columns of these lengths, FD&C Blue No. 1 or FD&C Green No. 3 will if present be about 85% separated ahead of FD&C Yellow No. 5 when 5% alcohol-1% NaCl is used. These two colors are better resolved from

FD&C Yellow No. 5 by 60% alcohol-1% NaCl. They and FD&C Violet No. 1 will elute about 85% separated ahead of FD&C Red No. 40. The separation from FD&C Yellow No. 5 will be complete.

FD&C Violet No. 1 will remain near the top of column when 5% alcohol-1% NaCl is used. Continue with this solvent until the reds have eluted. Change to 60% alcohol to elute the FD&C Violet No. 1.

The chromatograms with 5% NaCl-1% ammonium hydroxide (Figure 5) are included as this solvent might be needed to resolve certain combinations of color including Orange K.

Figure 6 shows the chromatograms of a few colors using 1% NaCl-0.1% ammonium hydroxide eluant. This change from the 1% ammonium hydroxide used by Ms. Sclar, was made to minimize fading of FD&C Blue No. 2. The four component color mixture of a chocolate shade, FD&C Yellow No. 5, FD&C Yellow No. 6, FD&C Red No. 2, and FD&C Blue No. 2 would first be chromatographed with this solution. The FD&C Blue No. 2 would elute separately after the red and yellow colors. This mixture is neutralized, absorbed on another column, etc.

FD&C Green No. 3 and FD&C Blue No. 1 is an unlikely combination, but they have been found together. When isolated from a food, the analyst has a dilute solution which is not convenient to spot on paper for separation by the systems mentioned by Ms. Sclar. It is more convenient to absorb the dilute color mixture on a column. Develop the chromatogram with 10% NaCl-0.1% ammonium hydroxide. The FD&C Green No. 3 is converted to the purple (basic) form which moves ahead of the FD&C Blue No. 1. The column loading should not exceed 0.5 mg of either of the colors for the 80 g column. Most of the FD&C Green No. 3 will elute free of any FD&C Blue No. 1. Some subsidiary colors in the FD&C Green No. 3 will elute

with the FD&C Blue No. 1, but if nearly equal amounts were present in the mixture, the spectra will be essentially those of FD&C Blue No. 1. After the main band of FD&C Blue No. 1 has eluted, there will still be bands of subsidiary colors from both left on the column. Since the separation is not complete, and since it has not been determined that the colors are stable for this period of time in the alkaline solution, draw the spectrum of the mixture before absorbing on column, and after identification of the two colors, solve for each by the use of simultaneous equations.

Purification and concentration of a color

Normally, the separation procedure also serves to purify the color. Occasionally, further purification is needed. The same procedure is used to concentrate a color solution from a few hundred ml to 20 or 30 ml.

Absorb the color on column from neutral 25% NaCl solution. Wash the color into the column with a layer of 20% NaCl solution. Float down a layer of dilute salt solution of such concentration that the color will move slowly. Depending on mobility of color, and length of column below the color, 100 to 200 ml of this solution is used for the 80 g column. Float down a layer of water to strip the color, In the case of FD&C Red No. 3, all solutions are alkaline with about 0.5% ammonium hydroxide.

Qualitative and quantitative spectra of separated color

Measure the volume. Divide the solution into three portions. To one, add a few crystals of ammonium acetate, and record the neutral quantitative spectrum. (If the analyst uses an earlier resin solution spectrum for quantitative purposes, record the neutral water or water and alcohol solution spectrum as a qualitative one.) Add 1N HCl to the second

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portion in ratio of 1 to 9, mix, and draw the acidic qualitative spectrum. Add 1N NaOH to the third portion in ratio of 1 to 9, mix, and draw the basic qualitative spectrum. Compare these spectra to those of the reference compounds. (As directed earlier where FD&C Red No. 3 is isolated by extraction, only the spectrum of this color in dilute ammonium hydroxide is obtained.)

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Calculations

For each separated color, calculate the amount as follows:

ppm = (A)(V)(F)/(S)(L)(W)

Where:

A = absorbance reading

V = volume of solution in ml

F = factor to be applied if color solution measured represents only a fraction of sample.

S = the absorption per mg per liter per cm cell length of the reference compound.

L = cell length in cm

W = weight of sample in g

REFERENCES

(1) Sclar, R.N. and Freeman, K.A., JAOAC, 38, 797 (1955)

(2) Graichen, C. and Molitor, J.C., JAOAC, 46, 1022 (1963)

(3) CFR 21, Parts 8 and 9

Figure 1 - Chromatographic Tubes

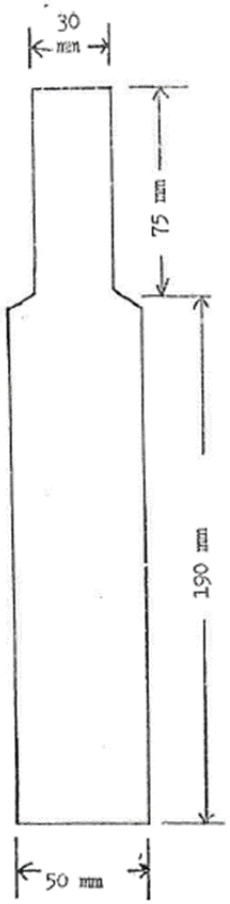


Figure 2 - Aluminum Plunger

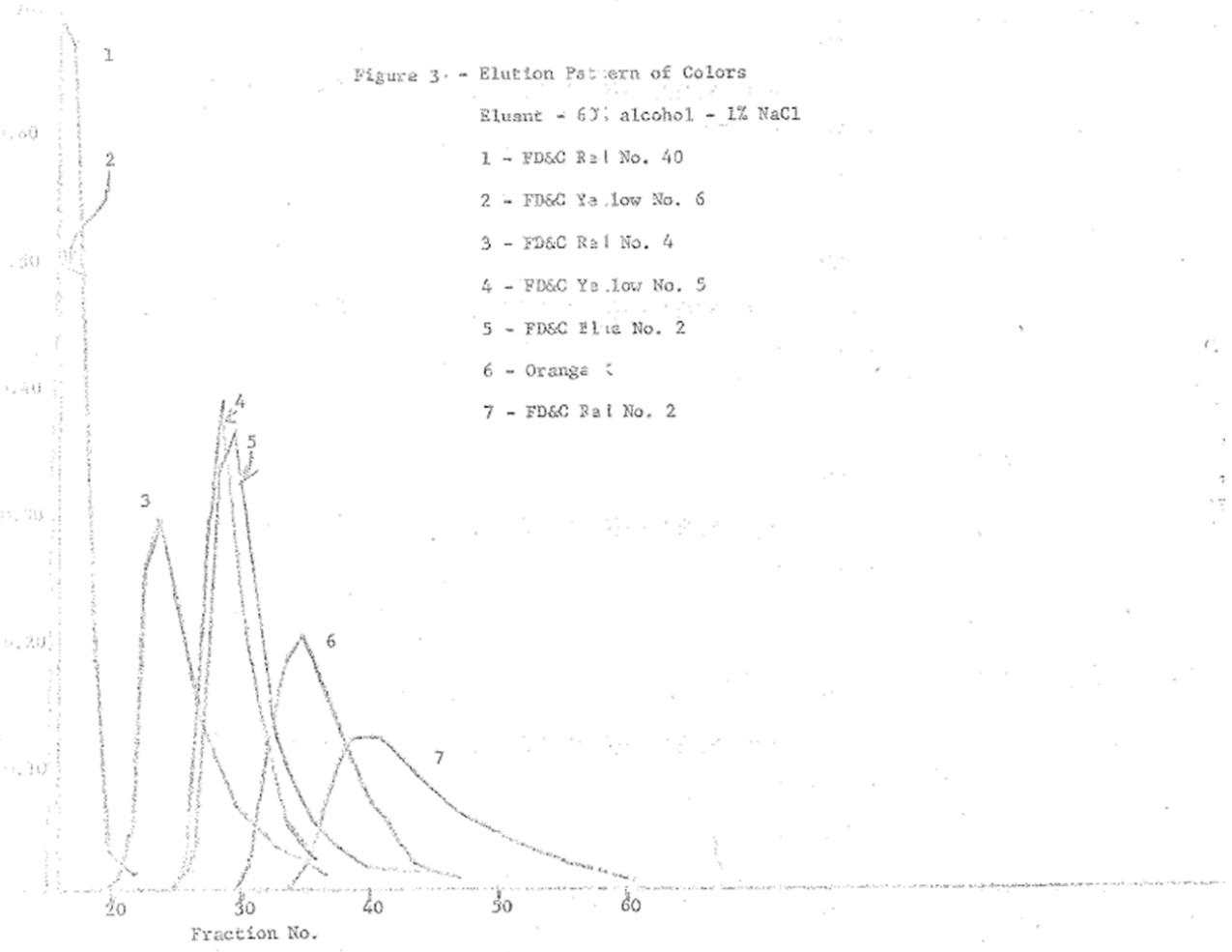


Figure 3. - Elution Pattern of Colors

Eluant - 6% alcohol - 1% NaCl

1 - FD&C Red No. 40

2 - FD&C Yellow No. 6

3 - FD&C Red No. 4

4 - FD&C Yellow No. 5

5 - FD&C Blue No. 2

6 - Orange I

7 - FD&C Red No. 2

Figure 4 - Elution Pattern of Colors

Eluant - 5% NaCl - 1% NH_4OH

1 - FD&C Red No. 40

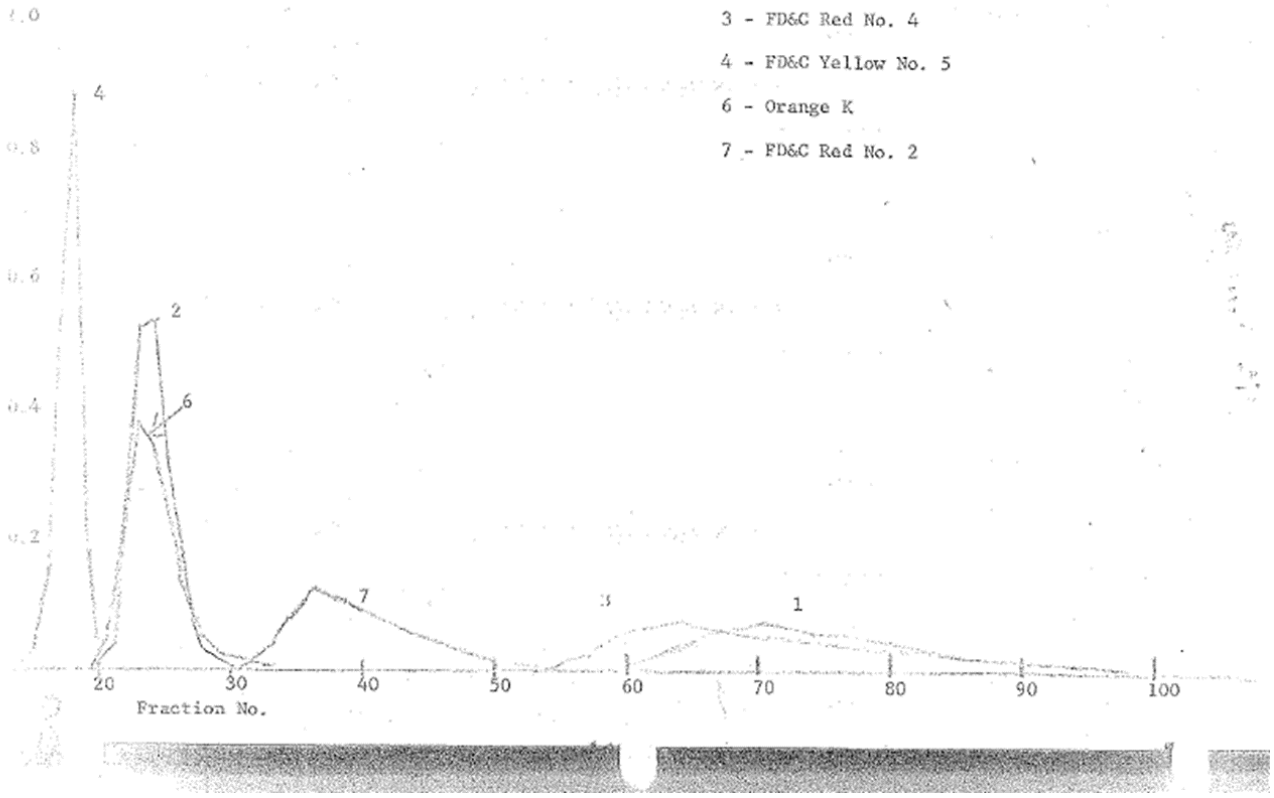
2 - FD&C Yellow No. 6

3 - FD&C Red No. 4

4 - FD&C Yellow No. 5

6 - Orange K

7 - FD&C Red No. 2



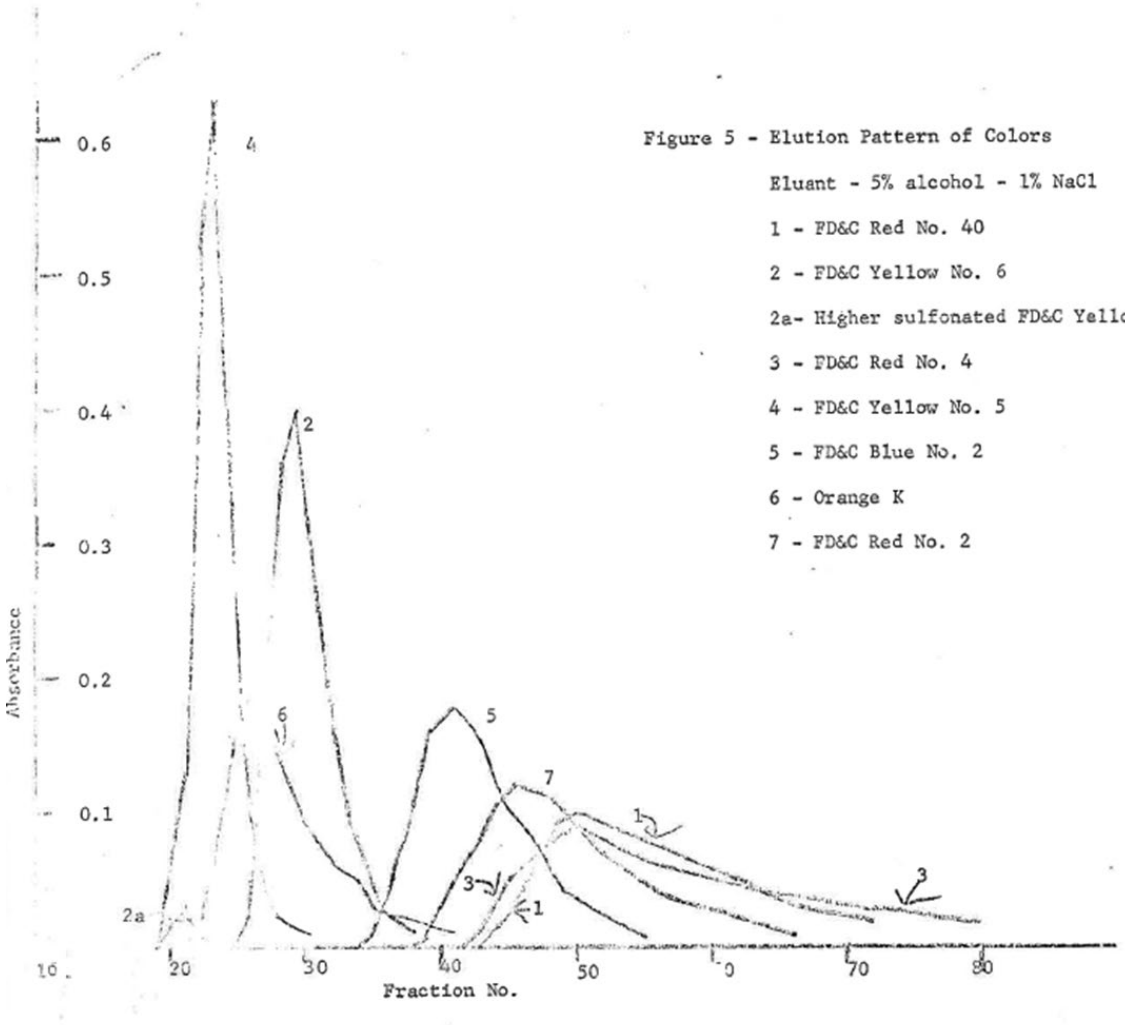


Figure 5 - Elution Pattern of Colors

Eluant - 5% alcohol - 1% NaCl

1 - FD&C Red No. 40

2 - FD&C Yellow No. 6

2a- Higher sulfonated FD&C Yellow No. 6

3 - FD&C Red No. 4

4 - FD&C Yellow No. 5

5 - FD&C Blue No. 2

6 - Orange K

7 - FD&C Red No. 2

Figure 6 - Elution Pattern of Colors

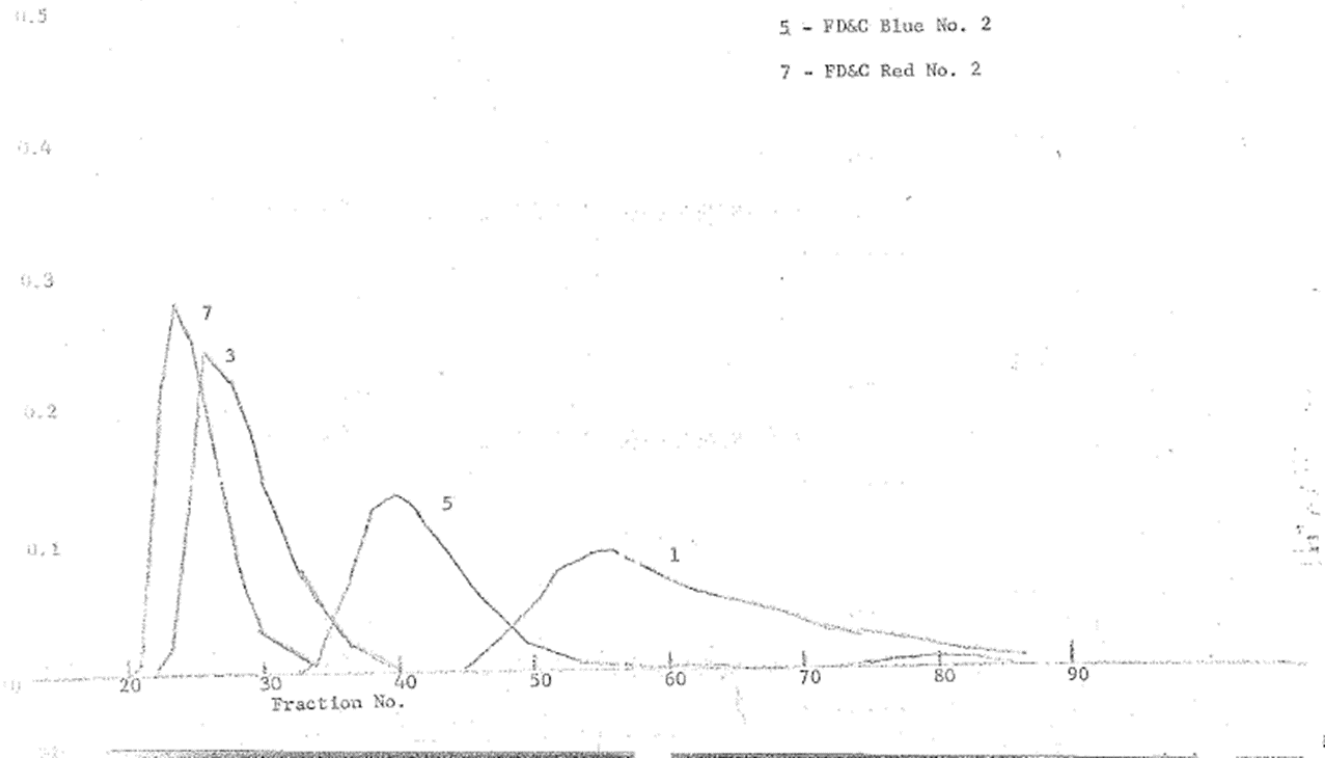
Eluant - 1% NaCl - 0.1% NH_4OH

1 - FD&C Red No. 40

3 - FD&C Red No. 4

5 - FD&C Blue No. 2

7 - FD&C Red No. 2



Attachment D - Newburger's Chapter 19

CHAPTER 19. Determination of Colors in Cosmetics

Contributed by
SANDRA J. BELL
Division of Color Technology, Food and Drug Administration

19.1 Lipstick—Tube (Thin Layer Chromatographic Method)

The Code of Federal Regulations (1) lists the color additives permitted for use in lipsticks. Since lipsticks usually contain a more complex mixture of colors than other cosmetics, the separation and quantitation of individual colors has been the subject of much developmental work. Extraction and column chromatographic methods (2–6) are either too time consuming or inadequate for analyzing the many combinations of colors which may be present in a lipstick. A faster thin layer chromatographic (TLC) method (7) separates and quantitates most of the colors present in commercial lipsticks. This TLC method was developed during the investigation of several lipsticks which presented either a comprehensive range of colors or particular problems of separation. Colors found in lipsticks in that study are listed in Table 11.

Using this method, most lipstick colors are separated on one plate with two consecutive developments using different solvent systems. Total development time is approximately 2½ hours. After development, individual bands of color are scraped from the plate, dissolved in suitable solvents, filtered, and determined spectrophotometrically. The presence of D&C Red No. 7 requires a modified procedure. A plate containing a buffered zone retards the development of D&C Red No. 7 while allowing other colors to pass through.

Table 11. Colors Found in Lipsticks in Silk's Study (2)

Color	Colour Index No.
D&C Red No. 7	15850
D&C Red No. 8	15585
D&C Red No. 9	15585
D&C Red No. 10	15630
D&C Red No. 12	15630
D&C Red No. 19	45170
D&C Red No. 21	45380
D&C Red No. 27	45410
D&C Red No. 35[a]	12120
D&C Red No. 36	12085
FD&C Red No. 3	45430
D&C Orange No. 4	15510
D&C Orange No. 5	45370
D&C Orange No. 17 RED 17	26100
FD&C Blue No. 1	42090

[a] Not permitted in the United States at present.

(a) Apparatus

(1) Spectrophotometer.—Visible range.

(2) TLC equipment.—20 × 20 cm plates coated with 250 mm Silica Gel G; developing tank lined with Fisher No. 77 paper, or equivalent.

(3) Ultraviolet light.—Long wave.

(b) Reagents

(1) Methylene chloride.

(2) Phosphate buffer.—0.05*M*, pH 8.0. Prepare a 0.1*M* K_2HPO_4 solution, and add 2–3 drops of toluene as a preservative. Prepare a 0.1*M* KH_2PO_4 solution, and add 2–3 drops of toluene. Mix 5.3 ml of K_2HPO_4 solution and 94.7 ml of KH_2PO_4 solution together and dilute the resulting solution to 200 ml with water.

(3) Solvent systems.—*(I)* Mix 20 ml of *n*-butanol, 4 ml of 95% ethanol, and 3 ml of ammonium hydroxide. *(II)* Mix 15 ml of ethyl acetate, 3 ml of methanol, and 3 ml of ammonium hydroxide (3+7). Prepare fresh daily.

(c) Application of Sample

Remove the shiny surface from the rounded end of a lipstick with tissue. Weigh the tube of lipstick (without cap). Warm a TLC plate in a 100°C oven for 5 minutes to soften the lipstick when it is applied. Apply 10–20 mg of lipstick directly to the plate with several light overlapping streaks ca 2 cm from the bottom of the plate. Reweigh the lipstick to determine the amount applied.

(d) Determination

(1) Oil-soluble colors.—Line the developing

Table 12. Appearance of Colors Under Normal and Fluorescent (Long Wave UV) Light

Color	Appearance Under Normal Light	Appearance Under Fluorescent Light
D&C Orange No. 4	orange	dark dull band
D&C Red No. 7	dark purplish red	dark dull band
D&C Red No. 8	orange	dark dull band
D&C Red No. 19	bright purplish pink	very bright orange-pink
D&C Red No. 27	bright purplish pink	slightly fluorescent orange-pink
Fluoresceins	orange-pink to yellow	bright yellow-green

tank with paper. Pour enough methylene chloride over the paper to reach a depth of 1 cm in the tank. Place the warm plate in the tank, and develop. Remove the plate, dry, and redevelop 2–4 times. Oils and waxes will move toward the top of the plate and will not interfere with subsequent separations. Methylene chloride separates unsulfonated oil-soluble colors in zones below the waxes and oils in the following descending order: D&C Red No. 36, D&C Orange No. 17, and the former D&C Red No. 35. Scrape the individual bands into a beaker, slurry with $CHCl_3$, and filter off the silica gel. Obtain spectrophotometric curves. Compare the curves with those of standards to obtain quantitative results.

(*2*) *Other colors.*—After removing the oil-soluble colors, use the same plate to develop the colors remaining at the baseline. Line the tank with paper. Pour ca 315 ml of solvent system II over the paper into the tank and equilibrate for 10 minutes. Place the plate in the tank to develop. If the bands are not well resolved, remove the plate, dry, and redevelop.

Tentatively identify the colors on the plates by relative position, color, and fluorescence. Solvent system II separates the remaining colors in the following descending order: D&C Red No. 19; D&C Red No. 8 and D&C Red No. 10 (these two do not separate from one another); D&C Orange No. 4; D&C Red No. 27; FD&C Red No. 3; D&C Red No. 21 (tetrabromofluorescein); tribromofluorescein; D&C Orange No. 5 (dibromofluorescein); monobromofluorescein; fluorescein. D&C Red No. 21 usually contains ca 10% tribromofluorescein, which is calculated separately. D&C Orange No. 5 typically contains ca 30% tribromofluorescein and ca 10% tetrabromofluorescein and may also contain fluorescein and monobrominated fluorescein in smaller amounts. The fluoresceins are calculated individually to obtain good quantitative results, since their wavelengths of maximum absorbance and absorptivities vary.

(*3*) *Visualization of colors.*—Examine the TLC plate under a long wave ultraviolet (UV) light. Table 12 lists the appearance of the various colors under normal and long wave UV light.

Scrape the bands from the plate, and dissolve D&C Red No. 8 and D&C Red No. 10 in alcohol. Dissolve D&C Red No. 19 in 30% acetic acid solution. Dissolve the halogenated fluorescein fractions, including D&C Red No. 27, in 10% ammonium hydroxide (1+9). If a significant amount of color remains at the baseline and does not appear to be D&C Red No. 7 (*see* (d)(*4*)), dissolve in alcohol. Obtain spectrophotometric curves for all solutions. Use the absorptivity values for standard solutions to calculate the amount of color present.

(*4*) *When D&C Red No. 7 interferes.*—D&C Red No. 7 may be present if a lipstick is dark red or purple. D&C Red No. 7 usually separates with solvent system II into 3–4 bands, with a dark red zone remaining at the baseline. If no fluoresceins are present, combine these bands and extract with 30% acetic acid. In the presence of fluoresceins, one or more of the D&C Red No. 7 bands may overlap them, making quantitation of either impossible. In this case, use the following modified method:

Spot a series of small overlapping drops of phosphate buffer solution across the TLC plate ca 3 cm above the lower edge. The band should be ca ¼" wide. Let it dry and apply the lipstick as in (c), just below the buffered band. Develop with methylene chloride as in (d)(*1*). After drying, develop to a height of 6 cm, using solvent system I in an unlined tank. Air dry or gently heat the plate. Repeat once or twice until a colorless zone ca ¼" wide develops above the band of D&C Red No. 7, which will remain close to the baseline, trapped by the buffer zone. Other colors will pass

through the buffer zone. Scrape the zone containing D&C Red No. 7 and extract with 30% acetic acid. Determine the amount of color present spectrophotometrically. Continue the development of colors remaining on the plate with solvent system II. Make certain that the solvent is above the scraped area. Determine the other colors as in (d)(*2*).

D&C Red No. 19 and D&C Red No. 27 will often contain a subsidiary color similar in appearance to, and directly below, the principal color. Combine the subsidiary color with the appropriate parent color for the determination. Traces of unsulfonated subsidiary colors may appear at the solvent front with the waxes and oils after development in methylene chloride. They are generally not included unless present in large amounts, which is unusual. FD&C Red No. 3 will overlap the principal subsidiary color of D&C Red No. 27.

19.2 Lipstick—Tube (Additional TLC Solvent Systems)

Besides the colors covered in **19.1**, lipsticks conceivably can contain many colors legally permitted in various parts of the world but prohibited in the United States. Perdih lists a number of solvent systems tested for the separation of over 150 colors of this type (8, 9). Other authors have developed TLC solvent systems with additional separating ability for various types of colors; in every case Silica Gel G is the adsorbent. The following principal solvent systems are listed under the types of colors they are used to separate.

(a) Oil-Soluble Colors

(*1*) Benzene-$CHCl_3$ (4+1) (10).
(*2*) Benzene-$CHCl_3$ (5+1) (11).
(*3*) $CHCl_3$-tetrahydrofuran (25+1).
(*4*) Petroleum ether-diethyl ether (25+1).
(*5*) Toluene-benzene-$CHCl_3$ (1+1+1) (10).

(b) Fluorescein-Type Colors

(*1*) Acetone-$CHCl_3$-butylamine-water (22+5+2+2).[1]
(*2*) Acetone - $CHCl_3$ - triethylamine - water (30+45+5+1) (12).
(*3*) Carbon tetrachloride-$CHCl_3$-acetic acid (5+3+1).
(*4*) Tetrahydrofuran - methanol - ammonium hydroxide-water (10+2+1+1).

[1] Bell, S. J., U.S. Food and Drug Administration, Washington, DC (1975).

(c) Sulfonated Colors

(*1*) Isoamyl alcohol - acetone - water - ammonium hydroxide (13+10+4+1) (13).
(*2*) Isoamyl alcohol - dioxane - water - ammonium hydroxide (10+10+4+1) (14).

(d) Basic Colors

Carbon tetrachloride - $CHCl_3$ - formic acid-acetic acid (60+40+5+5) and (30+70+5+5).

19.3 Lipstick—Other Forms (Gels, Paste, Roll-on, etc.)[2]

Most of these products on the market today contain a base soluble in methylene chloride. Add 2–3 ml of solvent to ca 15–30 mg of sample in a small beaker. Slurry well. Using a small pipet or capillary tube, apply the slurry to a TLC plate in a streak ca 3 cm from the bottom. Rinse the beaker with additional solvent and apply the washing to the plate. Repeat if necessary. Proceed as in 19.1(d).

19.4 Blushers—Powder and Stick; Make-up—Liquid and Cream

Slurry the product in methylene chloride, $CHCl_3$, or alcohol. Apply the slurry in a streak across the TLC plate. Develop the plate as in 19.1(d). Certifiable colors will ascend the plate with either methylene chloride or solvent system II, 19.1(b)(*3*)(*II*). Pigments will remain at the baseline.

19.5 Nail Lacquer[2]

Proceed as in 19.1 with the following modifications: It is not necessary to heat the TLC plate before sample application. Apply the nail lacquer to the plate in a thin line. Use the brush to smooth the surface of the line with short, overlapping strokes. Do not develop in methylene chloride or the line of sample will curl up and fall off the plate when it dries. Begin with development in solvent system II, 19.1(b)(*5*)(*II*), and proceed as in 19.1(d). The bands will not be as even as those for lipstick; subsequent developments in solvent system II are usually necessary. Although the bands are uneven, fewer colors are normally present in a nail lacquer than in a lipstick, and separations can still be achieved.

19.6 Eye Products—Shadow, Liner, Mascara, Eyebrow Pencil, etc.

The Regulations (1) require that all color additives in a cosmetic for use in the area of

[2] Bell, S. J., U.S. Food and Drug Administration, Washington, DC (1976).

the eye be specifically listed for that purpose. Presently only certain inorganic pigments are listed. This method does not determine these inorganic pigments. It is a screening method to determine the presence of non-permitted synthetic organic colors.

(a) Application of Sample

(1) Water-soluble products (eye shadow, mascara, etc.).—Slurry in a small amount of water. Apply to a Silica Gel G plate with a small pipet or capillary tube.

(2) Oil base products.—Slurry with a small amount of methylene chloride or $CHCl_3$. Apply to a Silica Gel G plate as in (a)(*1*).

(3) Gel-type products.—Some of these products contain a base soluble in alcohol. Slurry and apply.

(b) Determination

Develop all plates with methylene chloride, 19.1(b)(*1*). Prohibited unsulfonated organic colors will appear near the solvent front. Let the plate dry. Develop with solvent system II as in 19.1(d). Prohibited sulfonated and halogenated organic colors separate above the baseline. Permitted inorganics and pigments remain at the baseline.

19.7 Clear Liquid Products—Colognes, Aftershave, Hair Setting Solutions[2]

Some clear products contain only one color. The color can often be determined directly with no separation of the color from the product.

Pour the sample into a spectrophotometric cell of appropriate path length, depending upon color intensity. Obtain the spectrophotometric curve and compare it to those of standards of the same pH. Since these products usually contain >50% alcohol, standards should be prepared in alcohol solution. If mixtures are present, evaporate a weighed amount of sample to a small volume. Streak the entire sample across a TLC plate containing Silica Gel G. Develop the plate, using methylene chloride to separate any oil-soluble colors, and using solvent system II as in 19.1(d). Since any combination of FD&C, D&C, or External D&C colors may be present, an additional solvent system capable of separating many combinations of certified colors may be necessary, as in 19.2(c)(*1*).

19.8 Shampoo and Other Liquid Detergent Solutions (15)

This method is designed to separate small amounts of colors from anionic surface-active agents such as sodium lauryl sulfate and sodium dodecylbenzenesulfonate.

(a) Apparatus and Reagents

(1) DEAE-Sephadex A-25.

(2) Chromatographic tube.—With fritted glass disk in base, or equivalent.

(b) Preparation of Column

Prepare a loose slurry of DEAE-Sephadex A-25 in 30% alcohol. Pour the mixture into the chromatographic tube to fill ca 5/6 of the column length. Let the bottom drain until the packing settles about halfway down the column.

(c) Determination

Accurately weigh 3–5 g of shampoo into a beaker. Heat on a steam bath to evaporate the volatile material. Dissolve the residue in 20 ml of alcohol. Carefully transfer the sample solution to the top of the column, directing it down the side with a glass stirring rod. Wash the beaker with an additional small amount of alcohol and add it to the column again. When the sample solution is level with the column surface, gently add two 5 ml portions of HCl-30% alcohol (1+9) with the same technique, washing down the sides of the column in each case. When the solvent is again level with the column surface, carefully fill the column with the same solvent system, and let the column develop. The various colors will form bands; collect these separately. Obtain spectrophotometric curves for each band. If a mixture of colors is indicated from the visible curve, evaporate the solution to a small volume, spot it on a Silica Gel G TLC plate, and develop as in 19.7.

19.9 Deodorants[2]

(a) Creams and Lotions

Slurry ca 1 g of product with a solution of 20 ml of acetone and 4 ml of HCl. (Some products precipitate more readily with 20 ml of alcohol and 4 ml of HCl.) Filter off the precipitate. Obtain visible spectrophotometric curves of the filtrate. Most deodorants contain only one color. If a mixture is present, it can be separated either by TLC as in 19.2(c)(*1*) or by column chromatography as in 19.14.

(b) Stick Deodorants

Dissolve an accurately weighed sample in carbon tetrachloride by gently heating ca 5 minutes on a steam bath. Extract the water-soluble colors with water (upper layer). Obtain visible spectrophotometric curves for both the

aqueous and organic layers. Separate the mixtures as in 19.2(c)(*1*), 19.2(c)(*2*), or 19.14.

19.10 Bath Oils[2]

Add 1 part of water and 1 part of $CHCl_3$ to an accurately weighed sample of oil. Shake gently in a separatory funnel. Add ca 20 ml of saturated sodium chloride solution to break the emulsion that may form with some "foaming bath oils." Extract the water-soluble colors into the aqueous (upper) layer. Obtain visible spectrophotometric curves for both phases. Separate the mixtures as in 19.2(c)(*1*), 19.2(c)(*2*), or 19.14.

19.11 Hand Creams and Lotions[2]

Slurry 2 g of product with ca 20 ml of alcohol. (Some products precipitate more readily with 20 ml of alcohol and 4 ml of HCl.) Filter off the precipitate. Obtain visible spectrophotometric curves of the filtrate. Separate the mixtures as in 19.2(c)(*1*), 19.2(c)(*2*), or 19.14.

19.12 Detergent Bars and Soaps[2]

Colors in detergent bars are subject to color additive regulations. Detergent bars usually contain several detergents and a soap, and may contain one or more color additives. Ion exchange column chromatography, 19.8, is usually not directly applicable to the isolation of colors. The large relative amount of surfactants to color makes an increased sample size necessary, which results in a clogged column. Various approaches may be used, depending on the composition of the sample.

(*a*) *Direct Determination*

A few bars are strongly colored with a single water-soluble color. A direct determination may be possible. Dissolve a weighed portion in water and obtain the visible range absorption spectrum. Determine the pH of the solution. Compare the spectrum with the spectra of the reference compounds at the pH of the sample solution. If the sample solution is too cloudy or if a mixture of colors is present, dilute with water and proceed as in (c).

(*b*) *Dimethylformamide Isolation*

Some detergent bars will contain dimethylformamide (DMF)-soluble colors in a base partially insoluble in the DMF. The colors may be oil-soluble or may be certain water-soluble colors.

Weigh an appropriate-size sample of shavings from the bar, add some DMF, and heat the mixture on a steam bath for 15 minutes with frequent stirring. Let the mixture cool and filter to remove any precipitate. If the filtrate is clear, obtain the visible spectrum, and compare with spectra of likely oil-soluble or water-soluble colors in solutions of DMF. If the solution is cloudy, proceed as in (e). If a mixture of colors is present, separate by TLC, 19.2.

(*c*) *Immiscible Solvent Extraction*

Some bars will contain an oil-soluble color, a water-soluble color, and an insoluble pigment. Weigh an appropriate-size sample of shavings from the bar, add water, and heat to dissolve. If insoluble pigments are present, they will not dissolve. For each 9 volumes of mixture, add 1 volume of concentrated HCl. Extract with 2 or 3 portions of ether to remove the oil-soluble colors. Soap will also extract into the ether. If the sample contains insoluble pigments, they will generally form a layer at the interface between the ether and water layers. Filter the separated layers, as both will probably contain some of the insoluble pigments. Obtain spectra of the two solutions, and if each contains only one color, compare to spectra of likely colors in these solvents. Usually only one oil-soluble color and one water-soluble color will be present. If more than one of either is present, separate by TLC, 19.2.

This chapter is concerned only with synthetic organic colors. The analyst may wish to proceed as necessary to identify the insoluble pigment.

(*d*) *Precipitation of Surfactants and Soap*

Some bar bases will be relatively insoluble in an acid medium. Proceed as in (c), and if a precipitate forms on the addition of acid, filter the mixture to remove the precipitate.

(*e*) *Supplementary Extraction*

Cloudy fractions obtained by the above methods may be further separated as follows: Acidify the solution with enough HCl to give $\geq 5\%$ acid concentration. Extract with 2–3 portions of isoamyl alcohol. Wash the alcohol extract again with 5% HCl, and dilute with two parts of hexane. Extract the isoamyl alcohol-hexane layer with 2–3 small portions of water and then with 1% ammonium hydroxide. Sulfonated colors are extracted into the isoamyl alcohol (upper) layer from acid solution, and upon dilution with hexane, they are back-extracted into water. With some colors, ammonium hydroxide must be added to the aqueous layer for the extraction to be completed. Oil-soluble colors remain in the organic (upper) layer.

Further separate any fraction which remains

cloudy as in 19.8 or 19.13 (not necessary with most samples). If the mixture still does not separate, proceed as in 19.2(c)(2).

19.13 Ion Exchange Cleanup for Colors (16)

This method, designed for foods, can be used as a cleanup method for some cosmetics. The method also separates some colors from one another.

The sample is ground with Celite and dilute acetic acid, and the mixture is packed into a column. Oil-soluble colors are extracted with $CHCl_3$. Water-soluble colors are extracted with solutions of a liquid anion exchange resin in hexane, or a resin in butanol. The water-soluble colors are extracted from the resin solution with dilute NH_4OH.

(a) *Apparatus*

(1) Chromatographic tube. — Glass tube 290 × 52 mm id with 40 mm coarse fritted glass disk in base.

(2) *Plunger.*—Solid aluminum, designed to fit the column, weighing 1300 g to give reproducible packing.

(3) *Top disk.*—Glass fiber filter circle, 5 cm (No. 934H, H. Reeve Angel & Co., Inc., 9 Bridewell Pl, Clifton, NJ 07014, or equivalent).

(b) *Reagents*

(1) *Celite 545.*—Available from Johns-Manville Products Corp., Greenwood Plaza, Denver, CO 80217, or equivalent.

(2) *Ion exchange resin.*—Amberlite LA-2 (Rohm & Haas Co., Independence Mall West, Philadelphia, PA 19105), or equivalent.

(3) *Resin-in-hexane.*—Mix 50 ml of Amberlite LA-2 resin and 950 ml of *n*-hexane (practical). Add 200 ml of 20% acetic acid (v/v) and shake for ca 15 seconds. Allow to settle, and discard the lower phase.

(4) *Resin-in-butanol.*—Mix 50 ml of Amberlite LA-2 resin and 950 ml of *n*-butanol. Add 10 ml of 20% NaCl solution (w/v) and 400 ml of 20% acetic acid (v/v), and shake for ca 15 seconds. Allow to settle, and discard the lower phase.

(c) *Preparation of Column and Sample*

Thoroughly mix 20 g of Celite and 10 ml of 20% acetic acid (v/v). Add half of the resulting mixture to the chromatographic tube, (a)(1), and level the mixture. Pack by the weight of the plunger, (a)(2). Repeat with the remaining mixture. If the sample is in a solution, evaporate it to ca 20 ml and add enough acetic acid to make a 20% (v/v) solution. If the sample is not in solution form, add 1 ml of 20% acetic acid and 2 g of Celite 545 for each gram of sample. Grind the sample in a mortar and pestle to give a uniform mixture. Use a powder funnel to add a portion of the moist powder to the chromatographic tube. Level and pack the mixture with the plunger. Repeat with the remaining sample mixture. Place a 5 cm circular glass fiber filter on top of the packed column to prevent disturbances when eluting solutions are added. Pour 200 ml of $CHCl_3$ into a funnel placed to direct the flow to the center of the glass fiber filter. Fats, oils, and any oil-soluble colors will elute with $CHCl_3$. A larger volume of $CHCl_3$ may be necessary to completely elute oil-soluble materials. When the last of the $CHCl_3$ enters the column, add 50 ml of hexane. As the last of this enters the column, add 200 ml of resin-in-hexane. As the last of this enters the column, add sufficient resin-in-butanol to elute the remainder of the colors from the column. Collect the chloroform fraction, and change the receiver to collect the resin-in-hexane fraction 25 ml in advance of this front. Change the receiver to collect the resin-in-butanol fraction 25 ml in advance of this front.

If FD&C Red No. 3 is present, some will be in a colorless form and will elute in the $CHCl_3$ fraction. It may be detected by shaking some of the $CHCl_3$ solution with water plus an excess of base. Some of the FD&C Red No. 3 will be in all fractions, and some will remain on the column. The system is not suitable for this color.

Sulfonated azo colors will elute with the fraction containing the resin solution in hexane. Wash with two portions of sodium chloride solution (½ the volume of the resin-in-hexane solution) to remove some of the acid. Extract the colors with a ⅕ volume of 10% ammonium hydroxide containing 1% sodium chloride solution followed by ⅒ volumes of 1% ammonium hydroxide containing 1% sodium chloride solution. Wash the aqueous solution with 75 ml of ether to assure complete removal of the resin.

The somewhat amphoteric colors (FD&C Blue No. 1, FD&C Green No. 3, Wool Violet 4BN—formerly FD&C Violet No. 1) will not extract with resin-in-hexane, but will extract with resin-in-butanol. To recover the colors from resin-in-butanol, add an equal volume of hexane and proceed as directed for the recovery of the colors from the resin-in-hexane solution.

Obtain visible range spectra, and if only one color is present, compare the spectra to those of likely reference compounds. If more than

one color is present, proceed as in 19.2(c)(*1*), 19.2(c)(*2*), or 19.14.

19.14 Column Chromatographic Separation of Sulfonated Water-Soluble Colors (17)

Sclar first reported the use of cellulose columns and several salt solutions to resolve mixtures of the water-soluble FD&C colors (17). Graichen used the systems for part of a systematic procedure for the determination of colors in foods (16).

If any fluorescein colors are included, first remove them from the color mixture by extraction with ether from an acid solution. If any basic colors are included, remove them from the color mixture by extraction with ether from basic solution. The colors remaining in solution can probably be resolved by cellulose column chromatography.

A variety of column diameters can be used. They will require appropriate changes in the weight of the cellulose, the volumes of the solutions used, and the amount of the color mixtures absorbed on the column for separation. The specific systems suitable for particular mixtures of food colors may be useful in separating the permitted cosmetic colors. The analyst may have to devise his own solvent system. The specific procedures most used for food color combinations are summarized as follows:

(*a*) *Apparatus*

Glass columns with coarse fritted disks, and delivery tips closed with rubber tubing and pinch clamps. (*1*) 44 mm id and ca 58 cm length. (*2*) 55 mm id and ca 44 cm length.

(*b*) *Reagents*

(*1*) *Solka-Floc®.*—BW-40 (Brown Co., Berlin, NH 02570).

(*2*) *Whatman cellulose.*—Grade CF 11.

(*3*) *60% Alcohol.*—(v/v).

(*4*) *60% Alcohol (v/v)-1% NaCl (w/v).*—Mix equal volumes of the two solutions.

(*5*) *5% Alcohol.*—(v/v).

(*6*) *5% Alcohol (v/v)-1% NaCl (w/v).*—Mix equal volumes of the two solutions.

(*7*) *20% NaCl solution.*—(w/v).

(*8*) *25% NaCl solution.*—(w/v).

(*c*) *Preparation of Column*

Slurry 100 g of Solka-Floc in ca 1800 ml of water. Close the clamp and add ca 300 ml of water to the column. Add the slurry, and open the clamp. As the slurry drains, add more water until all is transferred. Add water and keep the column flowing until most of the cellulose has settled. Mix 4 g of Whatman cellulose in ca 20 ml of water and add the mixture to the tube. The coarser Whatman cellulose will form a cap on top of the level settled bed of Solka-Floc. A disturbance that does not dig through this cap will not materially affect the performance of the column.

Allow the water to drain to ca 2 cm above the cellulose cap, and carefully add 150 ml of alcohol from a pipet, moving the tip around the inside wall of the tube. If the addition is done carefully, the alcohol layer can be floated on top of the water layer with very little mixing. This technique is used in all additions of a lighter solvent over a denser one.

When the alcohol has drained to ca 2 cm above the cap, wash the column with ca 300 ml of water. Mixing must occur. Rotate the pipet around the inside wall of the tube, and the disturbance will not dig through the cap. Wash the column with ca 1000 ml of 25% NaCl solution.

Columns perform better if they are prepared in advance and allowed to set. They are used repeatedly. Do not allow the liquid to drain below the cap at any time, as the column will separate from the side of the tube. Discard any such column.

(*d*) *Absorption of Colors on Column*

The 100 g column will normally be suitable for 1.5 mg of each of the sulfonated azo colors and 0.5 mg of the sulfonated triphenylmethane colors.

Adjust the pH of the solution to neutral or weakly acid. If needed, heat on the steam bath to lower the alcohol concentration to below 10%. Add ca 0.5 g of Whatman cellulose/100 ml, and saturate the solution with salt. Open the clamp and transfer the sample mixture to the tube. Rinse the sample beaker into the tube with a little 25% NaCl solution. Float down a layer of 50 ml of 20% NaCl solution to wash the color into the column bed.

(*e*) *Separation No. 1*

(Use to resolve FD&C Yellow No. 5, FD&C Yellow No. 6, and one or more of the former FD&C Red No. 2, the former FD&C Red No. 4, and FD&C Red No. 40.)

Absorb the color mixture on a 100 g column in the 44 mm tube. Elute with 650 ml of 5% alcohol-1% NaCl solution, followed by 300 ml of 5% alcohol, then water. FD&C Yellow No. 5 and FD&C Yellow No. 6 will be eluted sepa-

rately. The three red colors, if present, will elute together. If the mixture includes FD&C Blue No. 1 or FD&C Green No. 3, a little of the blue or green color will elute ahead of the FD&C Yellow No. 5. Many color combinations can be resolved.

(f) Separation No. 2

(Use to resolve the former FD&C Red No. 2, the former FD&C Red No. 4, and FD&C Red No. 40.)

Absorb the color mixture on a 100 g column in a 55 mm tube. Elute with 650 ml of 60% alcohol-1% NaCl solution, followed by 300 ml of 60% alcohol, then water. The 3 colors are eluted separately. Many mixtures not resolved by Separation No. 1 will be resolved by Separation No. 2.

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