1 Analytical Considerations

The analysis of natural flavors and colorants involves three different types of determinations: (1) chemical analysis of constituents, (2) analysis of residues, and (3) microbiological analysis.

Chemical Analysis

The most important determinations are the contents of the active components. Some information on the determinations is included, where in addition to conventional analysis, instrumental analysis may also be needed. For many of the components, estimations based on ultraviolet (UV) or visible spectrometric methods are appropriate. Furthermore, some volatile components can be determined by gas chromatography (GC) and nonvolatile components by high-performance liquid chromatography (HPLC). An advanced method is GC-MS where GC is combined with a mass spectrometer (MS) to identify compounds separated by GC.

In the case of many substances containing volatile oils, such as spices, the moisture content cannot be determined by loss of weight. For example, the American Spice Trades Association, Inc. (ASTA) describes the toluene distillation method, where the volatile oil content can be determined by distillation using a Clevenger trap.

Official Methods of Analysis of AOAC International is a veritable bible as far as analysis of plant products is concerned. The US Food and Drug Administration (FDA) and the European Union (EU) provide the Code of Federal Regulations (CFR) and the European Food Safety Authority (EFSA), respectively, where regulatory, specification-based, and analytical matters are described. Similarly, in the case of flavoring materials, the International Organization of the Flavor Industry (IOFI) provides some information. Codex Alimentarius also specifies and gives instructions for analysis. Good descriptions of a wide range of flavors, colorants, and test methods are given in the *Food Chemicals Codex* (*FCC*) (2008–2009).

Residue Analysis

In general, the residues that are unwelcome but likely to be present in natural flavors and colorants are: (1) solvents (in the case of extracts), (2) aflatoxins, (3) pesticides, and (4) heavy metals.

The **residual solvent** is limited according to food laws (see Chapter 8 on Solvent Extraction). This residue is determined by taking 50 g of the extract and collecting the residual solvent in 1 mL of toluene using water distillation under specified conditions.

Natural Food Flavors and Colorants, Second Edition. Mathew Attokaran.

The solvent present is then determined by GC. This is a method based on a paper by Todd (1960), on work carried out over half a century ago. Many attempts have been made to standardize an improved method but without success. Details of the determination are given in the *FCC*.

Aflatoxins are produced by the fungus *Aspergillus flavus* (from which the name is derived) and a few members of *Aspergillus* and *Penicillium* species. EU limits are 5 ppb for B1 and 10 ppb for total aflatoxin content. The FDA limit is 20 ppb for total aflatoxins. Methods are available from the AOAC and ASTA (for spices only).

The EU has included limits on ochratoxin contamination. The recommended limit is 30 ppb. The AOAC has provided methods of analysis. Aflatoxins are determined using HPLC with a fluorescence detector.

For the analysis of **pesticide residues**, detailed methods are given in the *Pesticide Analytical Manual* published by the FDA. The AOAC is also a good reference source. The residues are divided into organochlorine, organophosphorus, and pyrethroids, and can be determined using GC. For organochlorine compounds and pyrethroids, an electron capture detector (ECD) is required, while for organophosphorus compounds, a flame photometric detector (FPD) or nitrogen–phosphorus detector (NPD) is used.

Heavy metal residues, which are considered harmful and that are frequently found, include mercury, cadmium, arsenic, copper, lead, and zinc. Methods of testing are given by the AOAC. Atomic absorption spectrometry (AAS) is used for these determinations.

Artificial colors became the focus of attention when there was an attempt to adulterate red chili with Sudan dyes. However, this is not generally a problem with flavors and colorants, and its significance is gradually disappearing. For capsicum and turmeric, restrictions were introduced by the EU for the following dyes: butter yellow, fast garnet GBC, methyl yellow, metanil yellow, orange II, para red, *p*-nitro-analine, rhodamine, Sudan black B, Sudan orange, Sudan red B, Sudan red I to IV, and toluidine red. Bixin was also introduced more as a measure of preventing cross contamination.

The initial limit for these artificial dyes was 10 ppb, which means the analysis requires the use of LC-MS-MS, in which a liquid chromatograph (LC) is connected with two mass spectrometers to quantify low levels of the target compounds. Now the limit may be increased to 500 ppb, which can be determined by HPLC. Readers will do well to check whether the increase in the limit is effected.

There is a general feeling that adulteration at these levels is not an advantage that can be readily exploited. Furthermore, contamination can result from many other means. For example, pesticide manufacturers use colors such as rhodamine for color coding their products to assist farmers in their identification, lubricants for machines used in farming operations and grinding are sometimes color coded, and farmers use dyes to write details such as weight, date, and lot number on bags of products.

Instrumental Chemical Analysis

Today chemical analysis has progressed from the initial days of volumetric and gravimetric determinations to chemical instrumentation. Instrumental analysis is more precise, reliable, and easier to carry out for both chemical analysis of constituents and, particularly, residue analysis. Initially colorimetric methods became the most dominant tools.

By taking advantage of the Beer–Lambert law of absorption, and comparing with a standard solution of known strength, the concentration of a chemical constituent can be determined at the absorption maxima in the visible region. Subsequently spectrophotometry, wherein either UV or visible spectra can be used, has become the major instrumental method for determinations.

In recent years, chromatography has become the most powerful tool for the determination of chemical compounds in plant products or extracts.

Gas Chromatography (GC)

For volatile constituents, gas–liquid chromatography, or more often simply called gas chromatography, has become very valuable for separating and analyzing chemical constituents that can be transformed into a volatile gaseous phase through controlled heating. The specific compound is then separated from other constituents while passing through a column. The mobile phase, which carries the volatile component through the column, is a carrier gas, usually an inert gas such as nitrogen or helium. The column is tubing made from glass, a polymer, or metal, which is coated with a stationary phase. The stationary phase is a microscopic layer of a suitable liquid or polymer on a nonreactive solid support. The volatile analyte of interest is then detected, using various types of detectors, depending on the class of compounds of interest.

Thermal Conductivity Detector (TCD) This is based on the change in thermal conductivity to the reference flow of a carrier gas as a result of the volatile compound. When the volatile compound emerges from the column, the thermal conductivity in the chamber, where one of the arms of a Wheatstone bridge is positioned, is reduced. This results in a detectable signal due to an upset in the electrical balance of the Wheatstone bridge.

Flame Ionization Detector (FID) The principle of an FID is based on the detection of ions formed during the combustion of organic compounds in a hydrogen flame. The ions generated are proportional to the concentration of organic compounds in the gas stream. Hydrocarbons generally have molar response factors corresponding to the carbon atoms in their molecules, whereas oxygenated and other heteroatoms tend to have a lower response factor. FID cannot detect inorganic molecules. Because it oxidizes organic molecules, it is not useful in preparatory work.

Electron Capture Detector (ECD) This is a device for detecting electron-absorbing components of high electronegativity, for example, halogenated compounds. It has a β-particle (electron) emitter, in conjugation with a make-up gas such as nitrogen flowing through the detection chamber. A typical electron emitter consists of a metal foil holding 10 μCi (370 MBq) of the radionuclide ^{63}Ni. The electrons from the electron emitter collide with the make-up gas molecules and move towards the positively charged anode, resulting in the production of a current. As the volatile compound is carried into the detector, electron-absorbing molecules of the volatile compound under analysis capture electrons, resulting in a proportionate reduction in the current.

These detectors are very sensitive to halogenated compounds such as chlorinated pesticides.

Nitrogen–Phosphorus Detector (NPD) In this type of detector, thermal energy ionizes a volatile compound. Nitrogen and phosphorus can be selectively detected with a high degree of sensitivity, and therefore an NPD is useful for analyzing phosphorus-containing pesticides.

A concentration of hydrogen gas below the minimum required for ignition is employed. A rubidium or cesium bead ignites the hydrogen and forms a cold plasma. When excited by an alkali metal, ejection of electrons results, which are detected as a current flow between an anode and a cathode in the chamber. A nitrogen or phosphorus volatile leaving the chromatography column causes an increase in current, which can be detected.

Flame Photometric Detector (FPD) Phosphorus-containing pesticides can also be determined using FPD. This allows sensitive and selective measurement of volatile sulfur and excited hydrogen phosphorus oxide species in a reducing flame. A photomultiplier tube measures the chemiluminescent emissions from these species. By using an appropriate filter, the FPD can determine either sulfur (394 nm) or phosphorus (526 nm).

High-Performance Liquid Chromatography (HPLC) Originally known as high-pressure liquid chromatography, HPLC is a technique to detect, quantify, and even identify nonvolatile components. Here the sample is dissolved in a suitable solvent and passed through a column packed with a stationary adsorbent material. Unlike in conventional column chromatography, where passage of the dissolved material through the adsorbent material occurs through the use of gravity, HPLC relies on pumps to pass a pressurized liquid solvent containing the sample mixture through long, thin columns filled with adsorbents. Each component of the sample moves at a different speed due to differences in the intensities of adsorption on the stationary phase. This results in separation of the components as they emerge from the column.

The active component of the stationary phase in the column is usually a granular material such as silica or a polymer, of 2–50 μm in size. The separated molecules leaving the column are detected by a suitable detector.

Typically, the columns were long and thin, of 4.5 mm diameter and 250 mm length. However, more recently, columns of 2.5 mm diameter and 50 mm length have been used. To increase the efficiency, sub-2 μm diameter particles, compared with the conventional 5 μm, are being used as adsorbents. Since very high pressures are used to pass the solution through the column, this technique is often referred to as ultra high-pressure liquid chromatography (UHPLC).

UV or UV/Visual Detector UV detectors, which are frequently used, use a deuterium discharge lamp with the wavelength ranging between 190 and 380 nm. When longer

wavelengths are required, an additional tungsten lamp (range 390–700 nm) is used. Combination detectors are currently available (photodiode array).

Almost all the chemical constituents that are analyzed may have absorptions in both ranges. It should be noted that not all components have similar spectra. The concentration may not be proportional to the peak size, as compounds with greater molar extinction coefficients can produce bigger peaks, even if present at a low dose.

Refractive Index Detector This can be considered as a universal detector for HPLC. The principle involved is the change in refractive index of the effluent when the compound under investigation passes the detector along with solvent. Naturally it is advantageous to have a large difference between the refractive index of the compounds and the mobile phase solvent.

Fluorescence Detector This is the most sensitive of all HPLC detectors. About 15% of all compounds fluoresce. Conjugated π-electrons in aromatic compounds produce the highest fluorescence activity. Fluorescence sensitivity is usually 10–1000 times greater than for UV detectors, even for strong UV-absorbing compounds. Moreover, fluorescence detectors are selective and specific. When compounds have specific functional groups that are excited by shorter wavelengths but emit at higher wavelengths, they are credited with having fluorescence. Aflatoxins, which can be excited in this manner and produce fluorescence emissions, are detected using fluorescence detectors as they are required to be measured at the parts per billion level.

Mass Spectrometer (MS) This produces an ion signal as a function of the mass-to-charge ratio. In order to do this, mass spectrometry works by ionizing chemical compounds to generate charged molecular fragments. The spectra are useful for determining the elemental or isotopic character of the sample and for elucidating the chemical structure of the molecule.

The ionization is achieved by bombarding a solid, liquid, or gaseous sample with electrons. The ions that emerge are separated according to their mass-to-charge ratio. The ions are detected by an electron multiplier. The atoms or molecules in the sample are then identified by correlating known masses with the identified masses or through a character fragmentation pattern.

Chromatography Combined with Mass Spectrometry

A development is combining GC or HPLC with MS, providing both mass resolving and mass determining capability. MS can not only detect, but also indicate the properties of the molecule, so much so it can even be useful in identification. Thus a complex mixture of volatile or nonvolatile compounds can be separated effectively by GC or HPLC, respectively, and the structure of individual components can be arrived at by comparing with the corresponding data for standard reference compounds. Thus in GC-MS, a stream of separated compounds is fed into the ion source, which is a metallic filament to which a voltage is applied. The filament emits electrons,

which ionize the compounds that are being analyzed. The ions formed are further fragmented, yielding expected patterns of intact ions and fragments, which are passed on to the MS analyzer, resulting in the identification of the compounds.

However, in LC-MS ions are generated either by the loss or by the gain of charge from a neutral species. Here the ionization is effected by electron spray ionization.

Both GC and LC can be connected to a system with two MS instruments working in tandem to form GC-MS-MS or LC-MS-MS. Here the compounds are ionized in the first MS. The resulting chosen ion can be further fragmented by the second MS, resulting in daughter ions, which can be measured to quantify the original compound, even when present at very low levels of parts per billion or even parts per trillion.

Atomic Absorption Spectroscopy (AAS)

This is very useful for determining the concentration of an element, as a spectroscopic analytical procedure using the absorption of element-specific optical radiation (light) by free atoms in the gaseous state is utilized.

Atomic absorption is so sensitive that it can measure concentrations with an accuracy of parts per billion. The technique makes use of the wavelengths of light specifically absorbed by the element in question. This corresponds to the energies needed to promote electrons from one energy level to a higher level.

AAS is particularly useful for determining the level of heavy metals in plant products or their extracts. Besides food, it is also used in clinical, pharmaceutical, and environmental analysis.

Microbiological Analysis

For steam-distilled essential oils and solvent-extracted flavors and colorants, microbial contamination is not a major issue due to the sterilizing effect of processing. However, for plant products and aqueous extracts, microbial contamination is important. In ordinary cases where hygiene is well maintained, an evaluation of total plate count (TPC) or yeast and mold (Y&M) will suffice. However, in badly contaminated cases, the following pathogenic bacteria need to be tested for: coliforms, especially *Escherichia coli*; *Salmonella*; *Staphylococcus aureus*; and *Bacillus cereus*.

Analytical Procedure

To check the microbiology of samples, often the TPC or the Y&M count will give a fair idea.

For TPC and Y&M, the sample is diluted from 10^{-1} to 10^{-6} fold with peptone water depending on the level of infection. With relatively infection-free products, such as oleoresins, essential oils, and freshly made hot extracts, a dilution of 10^{-1} to 10^{-2} may be ideal. For clean products, such as spices or well-dried products, a dilution of 10^{-3} to 10^{-4} may be needed. For highly infected materials, a dilution of 10^{-5} to 10^{-6} would be a requirement.

When using TPC, 1 mL of the diluted solution is poured into a sterile petri plate containing 15 mL of melted plate count agar. It is then incubated at 35 ± 1 °C for 48 hours, at the end of which the number of colonies is determined.

For Y&M, 1 mL of the diluted solution is poured into a petri plate containing 15 mL of melted potato-dextrose-agar (PDA). In order to restrict the bacterial activity, a few drops of chlorophenicol (or tartaric acid solution) are added. This is to reduce the pH from 7–7.5 (ideal for bacterial growth) to 2.5–3.5 (suitable for yeast and mold). It is then incubated at 25 ± 1 °C for 5 days to determine the number of colonies. If there are only a few colonies, the incubation can be continued for up to 7 days before the counting of colonies is carried out.

Counting of colonies is best done using an Automatic Colony Counter. From the number of colonies counted, the actual TPC or Y&M can be determined by multiplying the number with the reciprocal of the dilution factor.

For pathogens, each determination requires a specific method. These can be obtained from the FDA-BAM (*Bacterial Analytical Manual*) or from the AOAC.

Ready-made petri films are available, which saves the process of preparing the agar or PDA plates. Rapid petri films, as compared with ordinary films, are also available. These contain growth-promoting agents so that the time of incubation for both TPC and Y&M can be reduced to 24 hours. There are also rapid petri films available for specialized tests for pathogens. In these cases, the time of incubation can be reduced from 3–5 days to a mere 24 hours.

Automated testing devices, in particular to reduce the time taken for each test, are currently being developed. One such device is available to determine the degree of contamination in the case of a swab test. These hygiene meters depend on detecting adenosine triphosphate (ATP) molecules.

Important Agencies

The FCC has described a wide range of flavoring and coloring materials. The AOAC and ASTA (for spices only) have given analytical procedures for determining such components. Identification numbers of different natural flavorings and colorants have been given by the Flavor and Extract Manufacturers Association (FEMA) and the Chemical Abstracts Service (CAS). The EU allocates E-numbers to various items after all aspects that make them safe for use have been examined. To date, they have covered food colors and a few other items. Spices and their active components are yet to be given numbers. The FDA gives specifications and CFR numbers. FEMA, CAS, and CFR numbers, and E-numbers, whenever they are available, are given under each item.

The full names and addresses of each of these valuable agencies are given in the following:

American Spice Trade Association 1101 17th Street, NW Suite 700 Washington, DC 20036, USA Official Analytical Methods (for methods of analysis on spices)	**ASTA**

AOAC (Association of Official Agricultural Chemists) International 2275 Research Blvd, Ste 300 Rockville, MD 20850-3250, USA (for methods of analysis of plant products and impurities)	**AOAC**
Food Chemicals Codex Legal Department of United States Pharmacopeial Convention 12601 Twinbrook Parkway Rockville, MD 20852, USA (for specification and test methods)	**FCC**
European Union/European Food Safety Authority Via Carlo Magno 1A 43126 Parma, Italy (for food regulation, standards, and award of E-number)	**EFSA**
US Food and Drug Administration 10903 New Hampshire Avenue Silver Spring, MD 20993, USA (for regulatory matters and standards) Code of Federal Regulations (CFR)	**FDA**
Codex Alimentarius Secretariat Viale delle Terme di Caracalla 00153 Rome, Italy (for food safety, standards, and related matters)	**CODEX**
International Organization of the Flavor Industry Secretariat, 6 Avenue des Art 1210, Brussels, Belgium (consisting of the national associations of flavor manufacturers from several countries)	**IOFI**
Flavor and Extract Manufacturers Association 1101 17th Street NW, Suite 700 Washington, DC 20036, USA (generally recognized as safe [GRAS] list)	**FEMA**
Chemical Abstracts Service American Chemical Society 2540 Olentangy River Road Columbus, OH 43202, USA	**CAS**

FCC (FCC 6 2008–2009) is a body that provides descriptions, specifications, and testing methods for a wide range of food additives, including natural flavors and colorants. Today, the body has become an authority on food additives. It is operated by the US Pharmacopeial Convention (USP), and it is certain that the professionalism of the USP will also be extended to food chemicals.

The following are the abbreviations for **units of measurements** used:

%	percentage
°C	degree Celsius
µg	microgram (10^{-6} g)
µm	micrometer (10^{-6} m)
g	gram
kg	kilogram (1000 g)
km	kilometer (1000 m)
L	liter
m	meter
mg	milligram (10^{-3} g)
mL	milliliter (10^{-3} L)
mm	millimeter (10^{-3} m)
mt	metric tonne (1000 kg)
ng	nanogram (10^{-9} g)
nm	nanometer (10^{-9} m)
ppb	part per billion
ppm	part per million
v/w	volume/weight

References

FCC 6. 2008–2009. *Food Chemicals Codex*, 6th edn. Rockville, MD: United States Pharmacopeial Convention.

Todd, P.H. 1960. Estimation of residual solvents in spice oleoresin. *Food Technol.* **141**, 301–308.