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HANDOUT FOR:

Physicochemical Analysis of Fruits and Vegetables

Intended for 2nd year Master's students, specializing in "Plant Production"

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Chapter I: Composition of fruits and vegetables

I.1. Generalities

Vegetables and fruits are a set of foods characterized nutritionally by high water content, a low fat and protein content and a wealth of phyto-microconstituents.

The words “fruits” and “vegetables” have different meanings depending on the context. Botanically, vegetables are plants parts entering the diet (leaf, root, tuber, bulb, seed, flower, stem). The fruits are the mature ovary of a flowering plant, typically containing seeds.

But on the culinary and gastronomic level, an essential source of distinction, the traditional meaning of these two words is often more precise «Vegetables are the products consumed in salty dishes, while fruits are the sweet products rather consumed in desserts; however with the evolution of tastes, some fruits are used as vegetables in sweet-salty dishes...».

I.2. Classification

Vegetables are classified according to their plant structure into:

- leafy vegetables: salads, spinach, sorrel, chard, parsley, celery, lamb's lettuce, purple cabbage, green cabbage, white cabbage, kale;
- flowering vegetables and bud vegetables: cauliflower, brussels sprouts, asparagus;
- bulbous vegetables: garlic, onion, shallots, leeks;
- root vegetables: carrots, turnips, radishes, celeriac, parsnips, beets, cassava, yams;
- tuber vegetables: potatoes, Jerusalem artichokes;
- vegetables without chlorophyll: mushrooms;
- fruit vegetables: pumpkin, zucchini, tomatoes, eggplants, peppers, cucumbers, banana, plantain, etc.
- legumes: green beans, peas, fresh beans;
- grain vegetables: sweet corn.

Fruits can be classified into:

- acidic fruits: citrus and berries;
- sweet fruit: apple, pears...
- starchy fruits that must be cooked to make them digestible: chestnut...
- dried fruits that have been dehydrated: apricots, prunes, figs...

- oleaginous fruits: nuts, hazelnuts, almonds, etc.



Figure 1. Examples of vegetables



Figure 2. Examples of fruits

I.3. Chemical Composition

It is recommended to consume 5 servings of fruits and vegetables per day, which corresponds to 5 portions of 80 to 100 g of fruits and vegetables.

I.3.1. Water

This food group has high water content, ranging between 75% and 95% of the total weight. Consequently, they make a significant contribution to the hydration intake.

Table 1. Water content in fruits and vegetables

Fruit	Water content (%)	Vegetable	Water content (%)
Apple	84	Asparagus	93
Avocado	76	Beans (green)	89
Banana (green)	76	Broccoli	90
Blue berry	83	Brussels sprouts	85
Cantaloupe	93	Cabbage	92
Cherry	80	Carrot	88
Citrus	89	Cauliflower	92
Grape	82	Lettuce	95
Grape fruit	89	Mushroom	91
Honeydew melon	93	Onion (dry)	88
Kiwifruit	82	Pepper (sweet)	92
Mango	82	Potato	78
Orange	86	Pumpkin	91
Peach	89	Spinach	93
Pear	83	Squash (summer)	94
Plum	87	Squash (winter)	85
Watermelon	93	Tomato (firm ripe)	94

I.3.2. Macronutrients

In terms of macronutrients, vegetables and fruits are characterized by low protein and fat content, with a carbohydrate content ranging from 5% to 20%. Dried fruits have the highest carbohydrate content (30% to 75%).

Table 2. Macronutrient composition of fruits or vegetables (per 100 g).

Vegetable	kcal	Carbohydrates (g)	Lipids (g)	Proteins (g)	Water
Root vegetables (Carrots)	31	6,6	Traces	0,8	89,2
Bulbous vegetables (Leeks)	23	3,7	Traces	1,6	90,5
Stem vegetables (Asparagus)	23	3,3	Traces	2,2	92
Leafy vegetables (Lettuce)	12	1,3	Traces	1,2	94,5
Flower vegetables (Artichokes)	18	1,2	Traces	2,9	85,3
Fruit vegetables (Tomato)	19	3,5	Traces	0,8	93,8
Banana	89	21	Traces	1,1	74
Grape	69	16,1	Traces	0,6	79,3
Fruit	kcal	Carbohydrates (g)	Lipids (g)	Proteins (g)	Water
Dried fruits	239	56,8	1,2	3,2	25
Starchy fruits	174	36,3	2,7	3	51,7
Oleaginous fruits					
-Nuts	674	10,5	63,8	14,5	3,3
-Olive	293	4	30	2	52
-Avocado	139	0,8	14,2	1,8	76,4

I.3.2.1. Carbohydrates

The levels are 5% for leafy and flowering vegetables, 5 to 10% for root vegetables, 10 to 15% for leguminous vegetables and 15-20% for tuberous vegetables.

Fruits are close to 5% for red fruits (currants, raspberries, strawberries, blackcurrants, blueberries), watermelon and melon; 5 to 10% for citrus fruits (oranges, grapefruit, lemons); 10% for stone fruits (cherries, apricots, peaches, plums) and kiwis; 12% for fresh pineapple or pome fruit (apples, pears), 15% for bananas and grapes, 18% for fresh figs.

With the exception of bananas, which are rich in starch, fruit carbohydrates are mostly simple carbohydrates; monosaccharides and disaccharides «sweeteners», including sucrose, free fructose and glucose (Table 3). The carbohydrate content and nature, on which the sweet taste of a fruit depends, changes with the maturity of the fruit (during ripening, the banana becomes richer in sucrose at the expense of starch).

Some fruits such as plums, prunes and cherry, contain sorbitol, which gives them special properties, including laxative effects. The presence of fructose justifies the moderation of fruits in case of metabolic syndrome patients, since this carbohydrate is more lipogenetic and therefore hypertriglyceridemic.

Table 3. Key sugars and sweeteners and their sweetening power

Natural sugar or derivatives		Food additives: Sweeteners		
Name	Sweetening power	Name	European number (EN)	Sweetening power
Sucrose	1	Sorbitol	E420	0.5
Glucose	0.7	Mannitol	E 421	0.5
Fructose	1,3	Isomalt	E 953	0.5
Galactose	0.3	Maltitol	E 955	0.9
Lactose	0.25	Lactitol	E 966	0.3
Maltose	0.6	Xylitol	E 967	1
Inverted sugar	1.15	Acesulfame K.	E 950	150 to 200
Honey	1.2 to 1.35	Aspartame	E 951	160 to 220
Isoglucose	0.9 to 1.2	Cyclamates	E 952	30
		Saccharin	E 954	300 to 500
		Thaumatococin	E 957	2000 to 3000
		Neohesperidin	E 959	1500 to 1.800

The presence of fructose and certain fibers gives fruits a low **glycemic index** (hyperglycemic power of a given food compared to that of a reference carbohydrate «glucose solution or white bread»). In addition, fruits and especially vegetables have a particularly low **caloric density** (number of calories per volume or weight of food) due to their richness in water, fiber and low energy content.

1.3.2.1.1. Starch

Starch is a polysaccharide of general chemical formula $(C_6H_{10}O_5)_n$ composed of a mixture of two homopolymers, amylose and amylopectin; themselves formed of D-glucose units. The bonds α -(1→4) and α -(1→6) of the D-glucose monomers, are respectively at the origin of the linear forms and ramifications in the structure of the molecule.

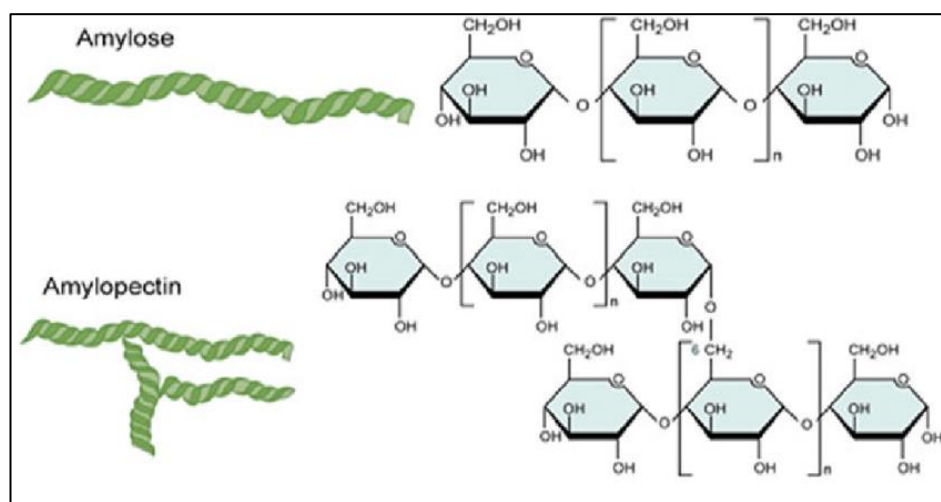


Figure 3. Structure of amylose and amylopectin

Starchy fruits (chestnut) and unripe banana are rich in starch. The potato is a vegetable in place apart from its higher starch content (19%), which often makes it classified as starchy food, a vague term corresponding to a heterogeneous and poorly identified set.

1.3.2.2. Vegetable dietary fiber

Fruits and vegetables are also characterized by their richness in dietary fiber, since the latter are components of the membranes of plant cells. The overall fiber content of plants (1-7%) varies with their age, origin and degree of maturation.

Fruits and vegetables provide less irritating fibers than cereals, as they have the advantage of facilitating intestinal transit, have a satiating effect, limit the peak of postprandial insulinemia by slowing the absorption of carbohydrates, without hindering the absorption of micronutrients and, for pectin, to reduce the level of LDL (*low density lipoprotein*)-cholesterol.

There are four main constituents: cellulose, hemicellulose, pectin and lignin

I.3.2.2.1. Cellulose

Cellulose is a highly hydrophilic polysaccharide with the formula $(C_6H_{10}O_5)_n$, consisting of a linear chain of several hundred to thousands of D-glucose units bound to $\beta(1\rightarrow4)$. It is abundant in green vegetables and fruit skin, and is partially degraded by intestinal flora (15%).

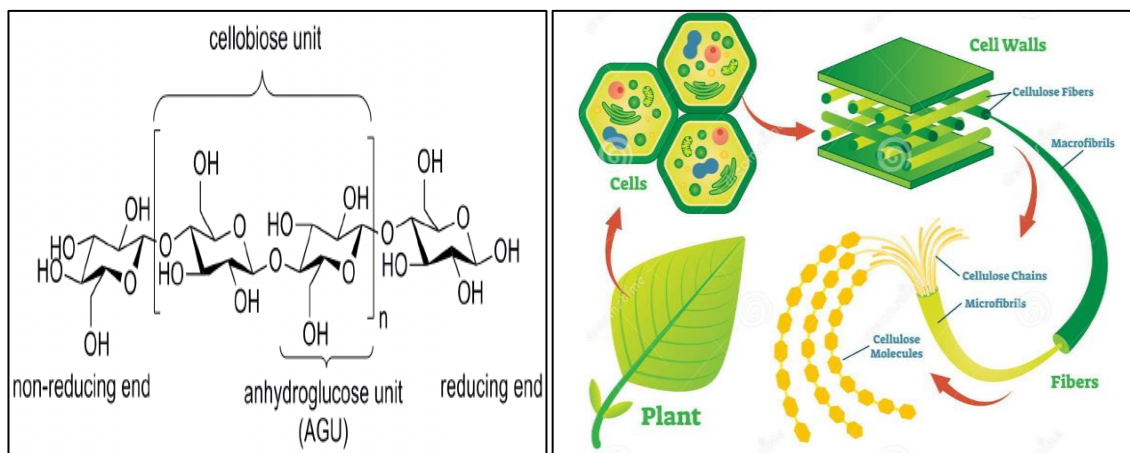


Figure 4. Structure of A) cellulose chain and B) cellulose fibers

I.3.2.2.2. Hemicelluloses A and B

Hemicellulosis is a non-starchy polysaccharide connected with different types of sugars «glucose, xylose, mannose, galactose, rhamnose or arabinose», but also their acidified forms «glucuronic acid and galacturonic acid». Various kinds of hemicelluloses are known like xylans, glucuronoxylans, arabinoxylans, glucomannans and xyloglucans. Hemicellulose accounts for about 1/3 of the fiber in fruits and vegetables, and is found mostly in young fruits and vegetables.

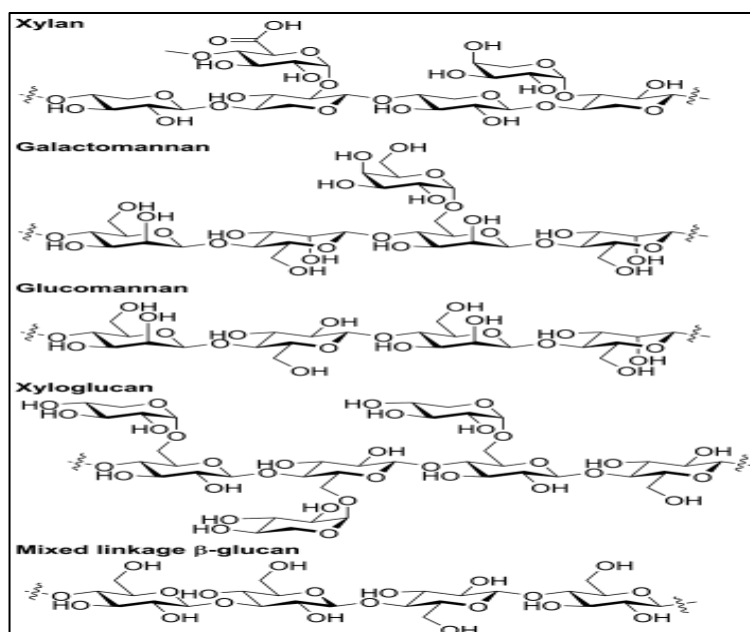


Figure 5. Some examples of hemicellulose

I.3.2.2.3. Lignin

This generic name includes a collection of highly heterogeneous polymers from the cross-linking of precursor lignols; all derived from phenylpropane (Figure 6). Its main functions are to impart rigidity and mechanical resistance to cell walls, as well as to provide water impermeability and resistance to decomposition.

Lignin is mainly located in the hard part of plants, the envelope of the grape grain, the fibrous part of asparagus, the grains of kiwi or strawberry. It is not metabolized in addition to being irritating to the intestine.

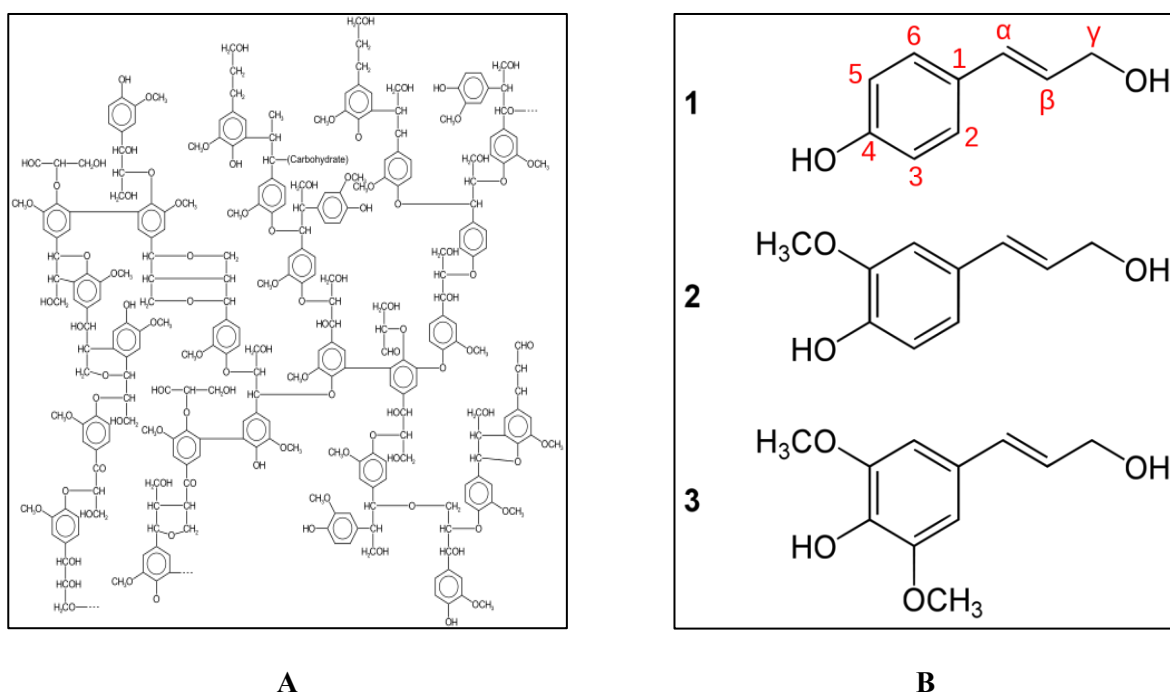


Figure 6. A) Possible structure of lignin, B) Structure of three monolignols "1: paracoumaryl alcohol; 2: coniferyl alcohol; 3: sinapyl alcohol"

I.3.2.2.4. Pectins

They are polysaccharides characterized by a skeleton of α -D-galacturonic acid and small amounts of more or less branched α -L-rhamnose. The generally accepted model describes pectins as a sequence of two major structures: a homogalacturonic main chain (or "smooth zone", called HG) and a rhamnogalacturonic chain (or "hairy zone", called RG).

These polymers are mainly present in berries (blueberries, currants, etc.) and pome fruits (apples, pears, quinces); giving them particular viscosity properties very useful for jams

production. By absorbing large amounts of water, they turn into a viscous gel that spreads into a thin layer on the intestinal mucosa and slows down the absorption of certain nutrients.

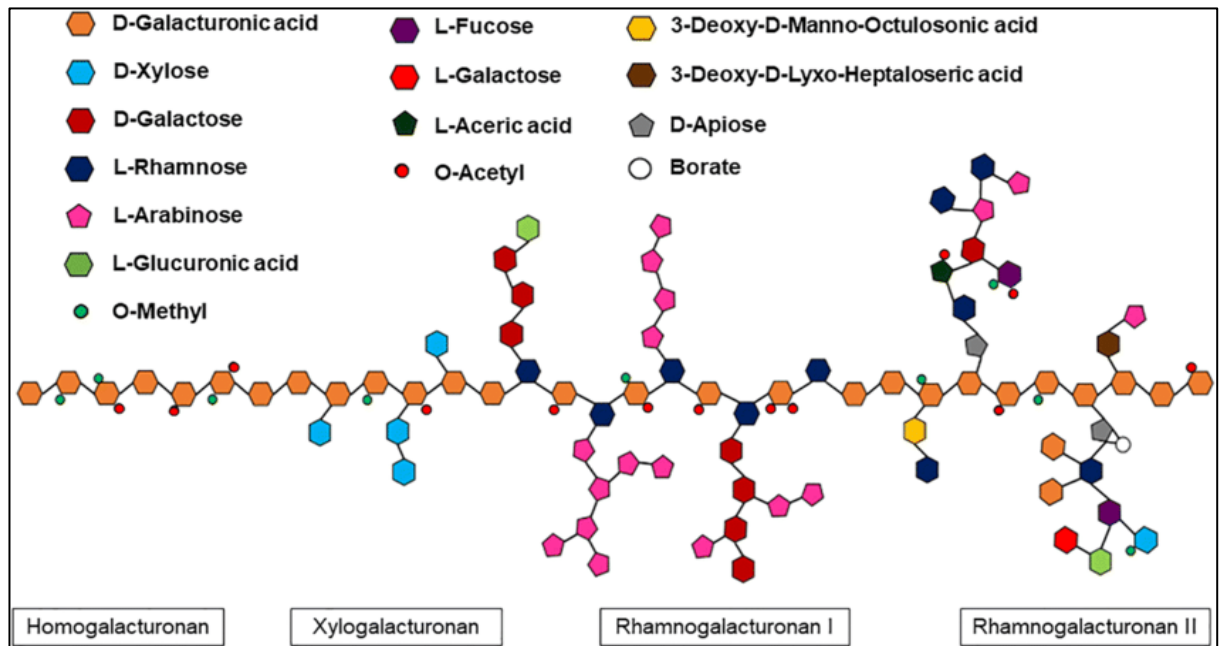


Figure 7. Structure of pectin

I.3.2.2.5. Prebiotic fibers

- Inulin: Salsify, artichokes, asparagus, Jerusalem artichokes are very rich in inulin, a fiber with characteristic prebiotic properties.
- Fructo-oligosaccharides or FOS are present in chicory root, onion, leek, salsify or artichoke. FOS stimulate the growth of bifidobacteria.

Table 4. Classes of dietary fibers

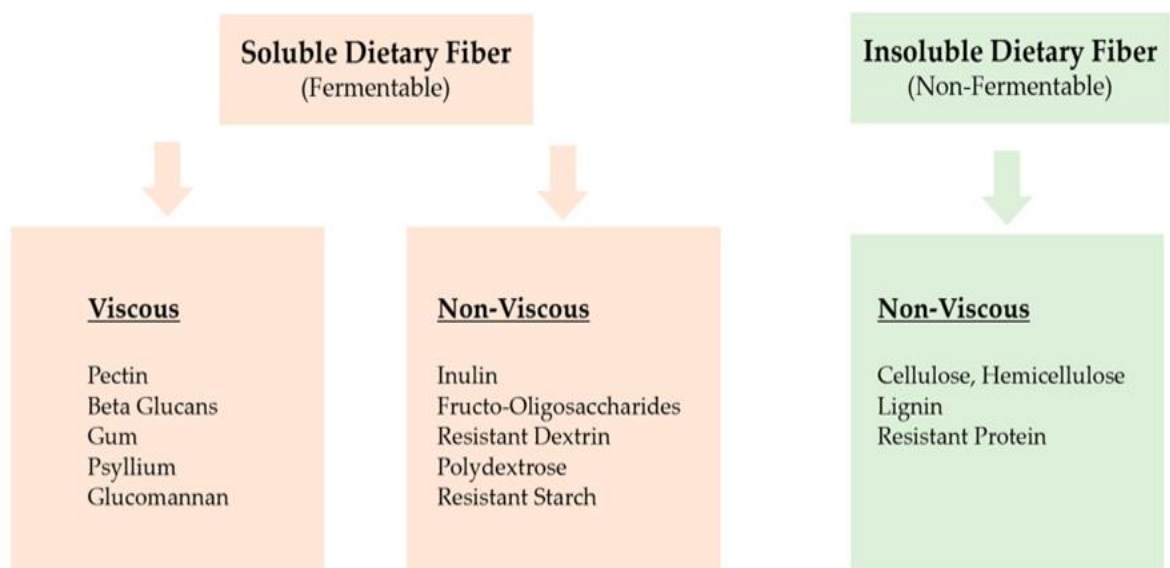


Table 5. Fiber content of certain fruits and vegetables per 100 g

Food	Fiber Content (g)
Prune	15 to 20
Almond, dried fig, dried apricot	10 to 15
Artichoke, salsify, Brussels cabbage, pea, quince, walnut, date, peanut, ripe raspberry, currant, raisin, dried banana, chestnut	5 to 10
Other vegetables (medium), olive, avocado, fig, kiwi	2.5 to 5
Fruits (medium), zucchini, lettuce, cucumber, turnip, onion, radish, pepper, pumpkin, tomato	1 to 2.5

I.3.2.2. Proteins

Their protein content is low (except dried fruits, oilseeds and starchy seeds): 1 to 2% variable depending on the supply of nitrogen fertilizer. The protein content of oleaginous fruits far exceeds that of fresh fruits: 8 to 11%. Peanuts contain 25 g of protein per 100g.

The proteins in fruits and vegetables are of good biological value and can, because of their good lysine content particularly, supplement partially the cereal-based diet. In total, the proteins in these foods make up 5-10% of the total protein intake.

I.3.2.3. Lipids

With the exception of oleaginous fruits with 50-65% lipid content (> 50% mono-unsaturated FA), fruits and vegetables are very limited sources of lipids, however, they are not free with about 1-2% lipid content in most vegetables. Polyunsaturated fatty acids (linoleic acid and α -linolenic acid) are very widely represented in leafy vegetables such as lamb's lettuce, spinach, and purple. Their **bioavailability** is low but they are able to enrich the flesh of animals that consume alpha-linolenic acid (snails, poultry).

I.3.3. Micronutrients

I.3.3.1. Mineral salts and trace elements

Fruits and vegetables have in common their richness in mineral salts and trace elements, with a content variation according to the product, the culture, the soils, the fertilizers used and the degree of maturity.

The main mineral salts are:

- potassium: 100 to 1500 mg/100 g (artichoke, chard, mushroom, grape, cherry, apricot, banana, dried fruits and oilseeds). These significant amounts of potassium coupled to the high potassium/sodium ratio, are important for the regulation of blood pressure;
- sodium (low): for vegetables, this content is 100 mg/100 g for celery and spinach, 50 mg/100 g for carrot and artichoke and less than 10 mg/100 g for endive, onion, leek, tomato, asparagus and green bean. It is even lower for fruits: apart from melon which contains approximately 20 mg/100 g, all other fruits contain less than 5 mg/100 g.
- magnesium: 20-200 mg/100 g (oilseeds fruits, bananas, kiwi);
- calcium: fruits and vegetables are thus the second source of calcium «20 to 40% of needs» after dairy products, with 10 to 50 mg in fruits (50 mg/ 100 g for rhubarb, prune and raspberry; 10 mg/ 100 g for peach, plum and tomato) and 50 to 100 mg in vegetables (cabbage), even 200 mg/ 100 g of watercress, dandelion or spinach. Calcium assimilation is reduced by the presence of oxalic and phytic acids, especially in sorrel, rhubarb, spinach and chard, but it is facilitated by the presence of vitamins C from fruits and vegetables.

The trace elements are mainly represented by iron (spinach, cabbage, lettuce, mushroom, artichoke, radish tops, dried fruits), copper, manganese, sulfur (responsible for the odor and least digestibility of onions, garlic, leek, turnip, radishes, cabbages except broccoli), iodine, cobalt, zinc and selenium; they are more concentrated in vegetables than fruits.

I.3.3.2. Vitamins

The most important nutritional feature of fruits and vegetables is their vitamin intake:

- Vitamin C is widely present in all fruits and vegetables, it is mainly present in citrus fruits, colored fruits, cabbage family and leafy vegetables. Berries, citrus fruits, exotic fruits, pepper, cauliflower, broccoli and Brussels cabbage are the first sources of vitamin C (blackcurrant: 200 mg/100 g), then guava and papaya (100-200 mg/100 g), pepper (130-150 mg/100 g), kiwi and lemon (70-90 mg/100 g).
- They are also major sources of folate (folic acid or vitamin B9), especially green leafy vegetables and cabbage (spinach).
- These are secondary sources of other B vitamins group, vitamins E (20% to 30% of requirements) and vitamin K.

I.3.4.1.2. Carotenoids

These tetraterpenes are fat-soluble plant pigments responsible for the red, orange and yellow colors of fruits and vegetables. Fruits and vegetables have a high content of carotenoids that belong to the carotene and xanthophyll families:

Carotenes:

- α - and β -carotene "Provitamin A": carrots, sweet potato, pumpkin, apricot, melon and exotic fruits
- lycopene: tomatoes

Xanthophylls:

- lutein and zeaxanthin, (spinach, cabbage, salad, mango, papaya, plum and orange, etc.).

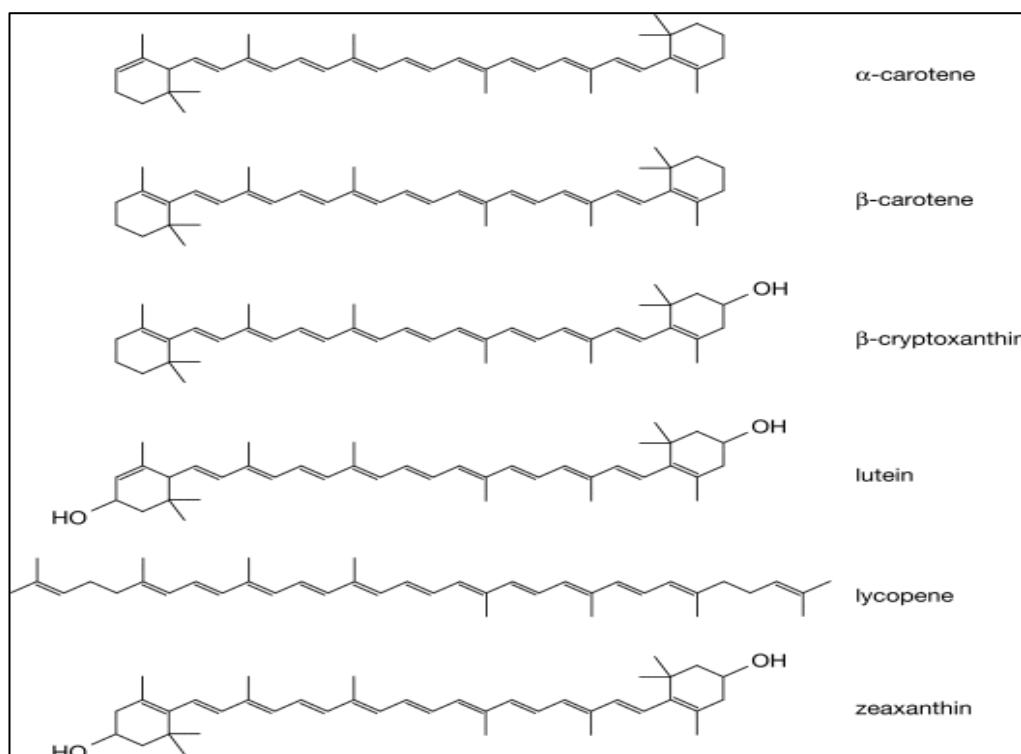


Figure 10. Structure of the main carotenoids.

I.3.4.1.3. Anthocyanins

These vacuolar water-soluble pigments take the form of glycosides responsible for the red, blue and purple colors of many fruits and vegetables. When the sugar fraction is removed by hydrolysis, the remaining aglycone is called anthocyanidin. They are found in root vegetables and fruit berries and grapes: blueberry, blackberry, cherry, black grape, blood orange, eggplant, vitelot potato, plum, blueberry, purple...

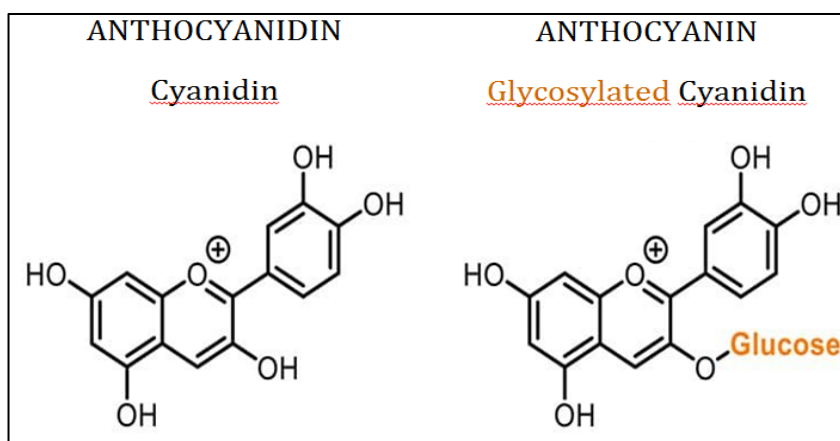


Figure 11. Structure of anthocyanin and anthocyanidin.

I.3.4.1.4. Betalains

Betalains are natural water-soluble pigments having a color shade varying from a dark yellow to an intense purple, present in a limited number of plant families, belonging to the order of caryophyllales such as red beetroot and prickly pear. These compounds have betalamic acid as a basic structural unit, which forms the class of:

- yellow-orange betaxanthins by condensation with different amino acids or free amino groups (indicaxanthin);
- red-violet betacyanins after conjugation with indoline derivatives (betanin).

The color and stability of betalains depends on the pH.

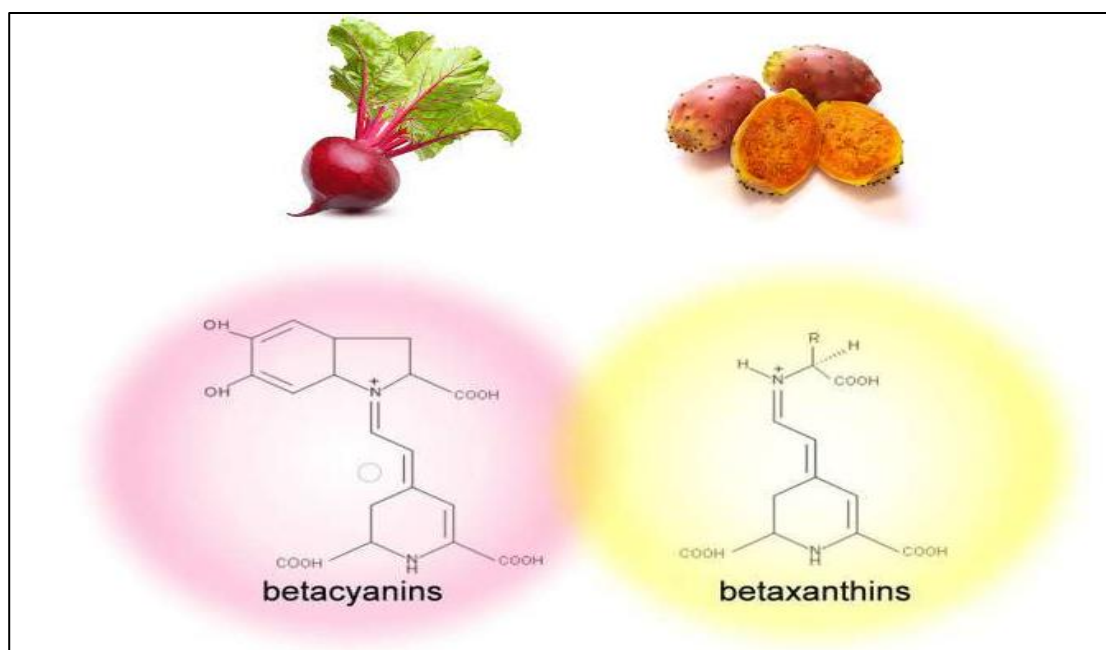


Figure 12. Chemical structure of betacyanins and betaxanthins

I.3.4.2. Antioxidants

Antioxidants are able to counter the accumulation of free radicals at the cellular level damaging the DNA and membranes, which promotes the processes of carcinogenesis and atherogenesis:

I.3.4.2.1. Polyphenols

They constitute one of the fundamental classes of plants secondary metabolites with 8000 different polyphenols listed today, already grouped into four main classes: phenolic acids, flavonoids, tannins and lignins. Their basic structure corresponds to a benzene nucleus substituted by one or more hydroxyl functions of various natures.

Flavonoids are the largest group in the polyphenol family. They are present in the family of cabbages, green vegetables and red fruits (spinach, lettuce, leek, parsley, blueberries, blackcurrant, raspberries, red grape, cherry but also apple and onion).

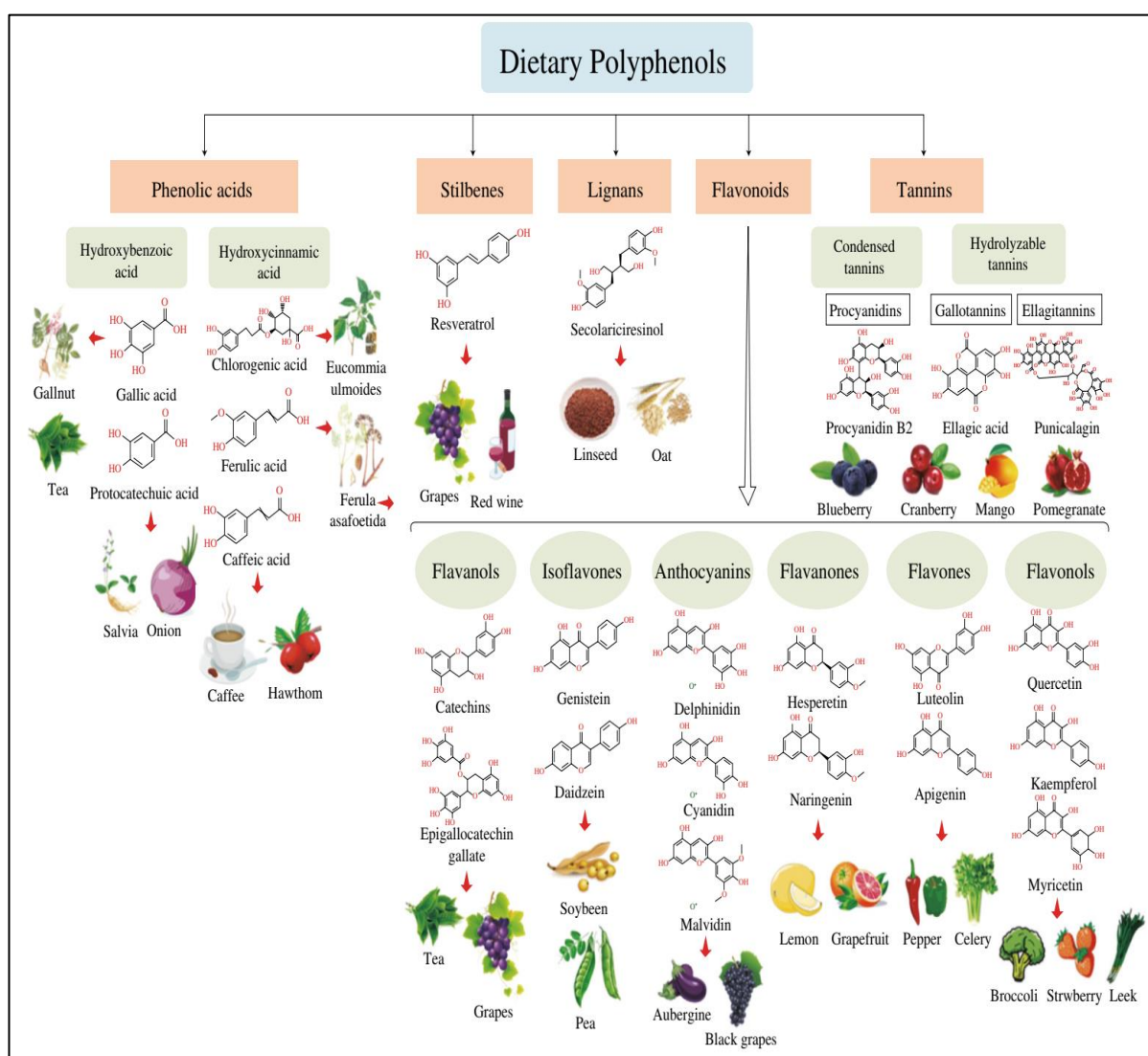


Figure 13. Polyphenols classes

I.3.4.2.2. Selenium

It is provided in part by dried fruits and oilseeds, while most vegetables and fruits contain less than 0.01 $\mu\text{g/g}$ (carrots: 0.022 $\mu\text{g}/100\text{ g}$, apples: 0.004 $\mu\text{g}/100\text{ g}$). Selenium activates glutathione peroxidase, which has a major antioxidant effect and prevents cell membrane alteration; this effect is potentiated in the presence of vitamin E. Other studies show that high intakes of selenium and vitamin E protect against cancer.

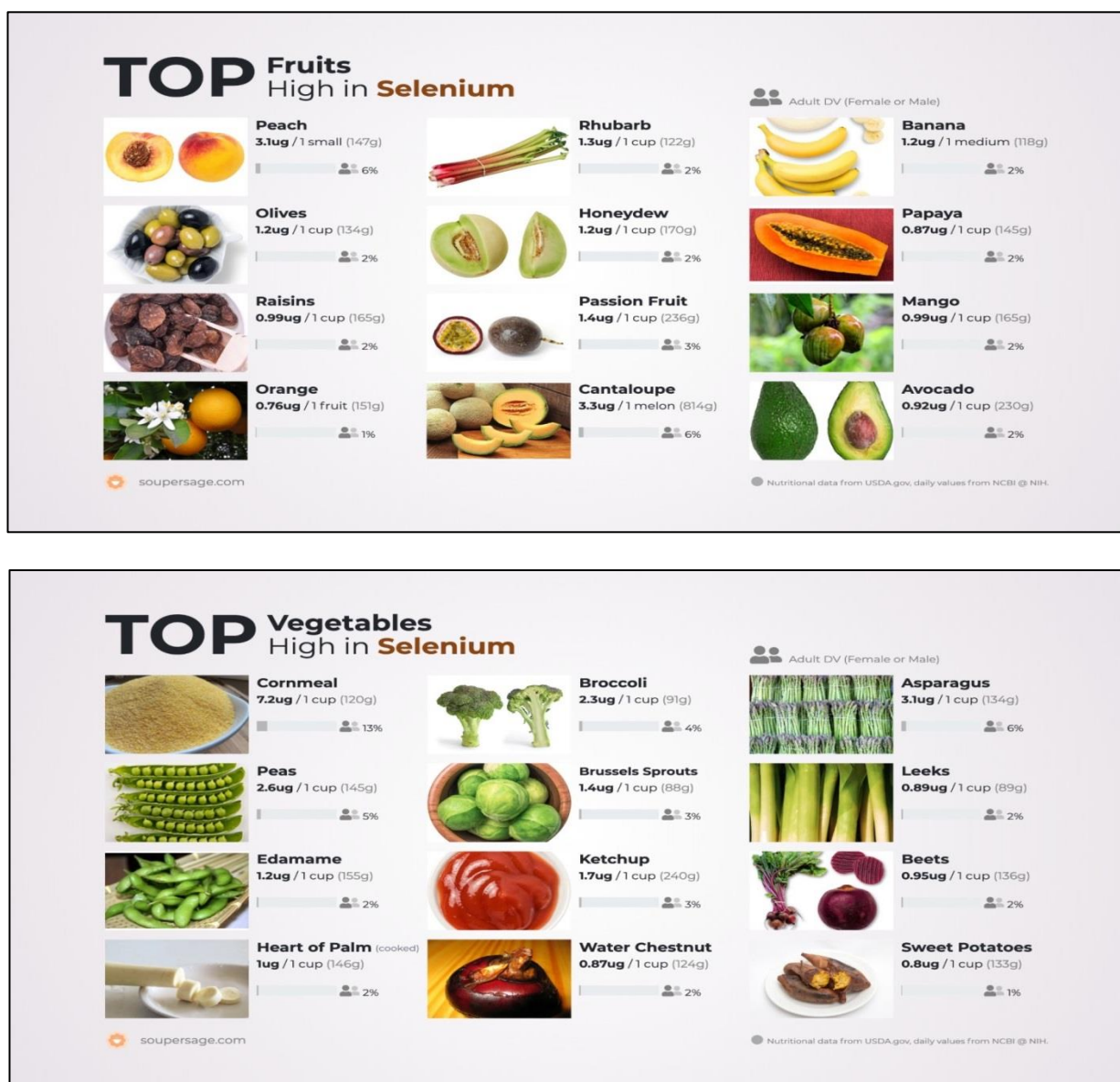


Figure 14. Fruits and vegetables rich in selenium.

I.3.4.3. Organic acids

Small amounts of various organic acids of the Krebs cycle or intermediates of the shikimic acid pathway (citric, malic, maleic acid, etc.) and various others not associated with specific metabolic cycles (oxalic and tartaric acids) are produced and accumulate in the vacuoles

of fruits and vegetables (0.5 to 1.5%) and give them a high acidity (pH = 3 to 4), all the better masked as the taste is sweeter (ripening process).

The acid content ranges from very low levels in some vegetables, such as sweet corn, to high levels in some fruits such as lemon, blackcurrant, grape and Logan blackberry. Among vegetables, spinach and rhubarb have an unusually high level of acidity due to the high content of oxalic acid.

Table 6. Major organic acids in selected fresh fruits and vegetables (mg/100g fresh weight).

Fruits and vegetables	Malic	Citric	Others
Grape	1095	61	Tartaric 1061
Lemon	228	5149	
Melon	14	1005	
Orange	131	2049	
Peach	2183	673	
Broccoli	120	210	
Cabbage, white	100	140	
Carrot	240	90	Oxalic 0–60
Cauliflower	190	10	
Green beans	112	34	Oxalic 20–45
Green pepper	190	77	
Lettuce	575	118	
Rhubarb	910	137	Oxalic 230–500
Tomato	81	1064	

1.3.4.3.1. Aliphatic acids

The most widespread and abundant acids in the tissues of fruit and vegetables are citric acid and malic acid, each of which may constitute more than 2% of the fresh weight:

- Citric acid is the main acid of citrus fruits (lemons > 3% citric acid), black and red currant, raspberries, Logan blackberries, strawberries, cranberries, pineapples, pomegranates and pears. In tomato, along with potato, many leafy vegetables and beetroot, citric acid is the main acid.
- Malic acid predominates in apples, most drupe fruits (plums, cherries, apricots, etc.) and cucurbit fruits, banana and rhubarb. Malic acid predominates in lettuce, cauliflower, carrot, artichoke, cucurbit, okra, onion, celery, parsnip, turnip, green pepper and green beans.
- The grapes are characterized by the presence of tartaric acid.
- The sour taste of spinach is due to uric acid, while oxalic acid acidifies sorrel and chard.

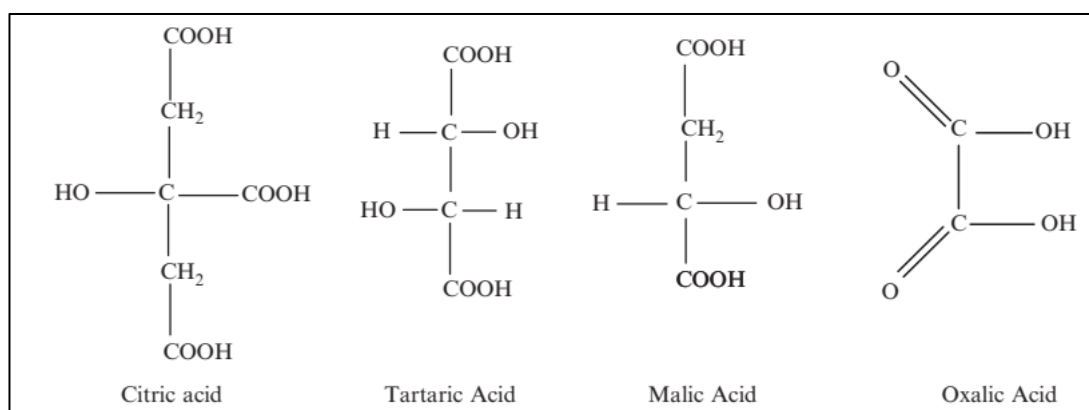


Figure 15. Chemical structures of common aliphatic organic acids in fruits and vegetables

Other aliphatic acids are: succinic, glycolic, gluoxylic, oxalo-acetic, fumaric, α -cetoglutaric, pyruvic, aconitic, lacto-isocitric, malonic, quinic and shikimic. In addition, various uronic, glutamic and short-chain free fatty acids are often present in small amounts.

1.3.4.3.2. Aromatic acids

Some phenolic acids contribute to the color and taste of the products. Hydroxycinnamic acids and their derivatives «p-coumaric, ferulic, caffeic and sinapic acids» are widespread in fruits and vegetables. These hydroxycinnamic acids are rarely free but are principally present as esters with quinic acids in the example of chlorogenic acid, ester of caffeic acid and quinic acid, which is mainly found in apples and pears, peach, potato and sweet potato.

Table 7. Chlorogenic acid content in common fruits

Fruit	Chlorogenic acid (mg/100 g fresh wt.)
Apples	
Empire	2.56
McIntosh	10.40
Golden Delicious	7.71
Granny Smith	3.2
Pear	
d'Anjou, Bosc	3–7
Red raspberry	1.5
Strawberry	2.9

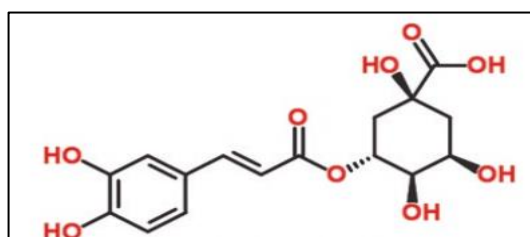
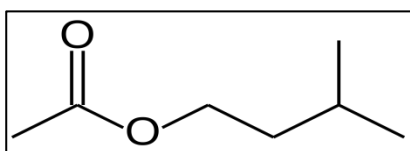


Figure 16. Chlorogenic Acid

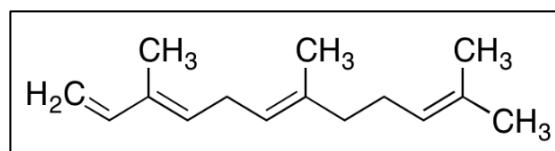
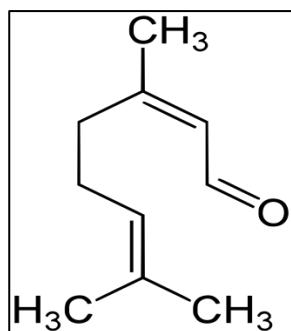
I.3.4.4. Aromas

The characteristic aroma of several ripe fruits is due to the evolution of many types of volatile components, including monoterpenes, esters, organic acids, aldehydes, ketones, alkanes, alcohols, lactones and sulfur compounds. Some of these ingredients specifically provide the characteristic aroma of fruits and are called character impact compounds:

- banana flavor is mainly from isoamyl acetate (amyl esters);
- Alpha farnesene is a major sesquiterpene (C₁₅) component developed by apples.
- Lime flavor is mainly due to limonene monoterpene, whereas citral is found in lemon;
- The main aromatic component of Bartlett pears has been identified as ethyl trans-2-cis-4-decadienate;
- Lactones characterize peaches.



Isoamyl acetate

 α - farnesene

Citral

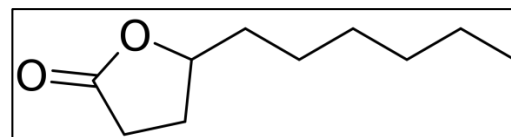
 γ -decalactone

Figure 17. Structures of aromas characteristic of certain fruits.

Citrus fruits are particularly rich in monoterpenes and derivatives:

- Monoterpenes such as limonene, myrcene, pinene, etc., are the main volatile components.
- Monoterpene derivatives such as geranium, neral (aldehydes), geraniol, linalool, terpineol (alcohols), geranyl acetate, neryl acetate (esters) etc. are also present.

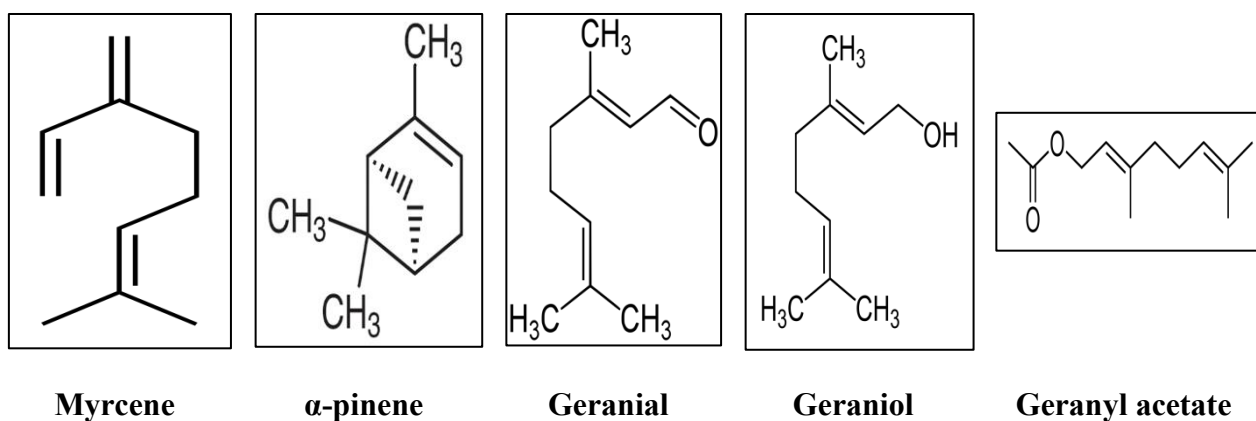


Figure 18. Structure of volatile aromatic compounds of citrus fruits

In melons the volatile components include esters, aldehydes, alcohols, terpenes and lactones:

- Hexyl acetate, isoamyl acetate and octyl acetate are the main aliphatic esters;
- Benzyl acetate, phenylpropyl acetate and phenylethyl acetate are also observed.

Depending on the variety, mangoes are characterized by high levels of:

- limonene, other monoterpenes, sesquiterpenes and ethyl esters of even fatty acids,
- or C6 aldehydes and alcohols (hexanal, hexanol).

Vegetables, with a few exceptions, tend to be bland. As for vegetables, the specific aroma is due to the amount of volatile compounds they contain (very small <100 $\mu\text{g/g}$ fresh weight), their diversity and interaction. The characteristic flavors and aromas are a combination of various compounds, mainly short-chain aldehydes, ketones and organic acids. Terpenes, phenolic compounds, glucosinolates and cysteine sulfoxides can also be found:

Plants of the genus *Allium* (onions, garlic, leek, chives and shallots) are characterized by powerful and penetrating aromas. These plants do not have the characteristic strong aroma unless the tissue is damaged and the enzymes are decompartmentalized so that the aroma precursors can be converted into odorous volatiles:

- In the case of onions, the precursor S-(1-propenyl)-l-cysteine sulfoxide hydrolyses by allinase to release the unstable sulfenic acid which undergoes further rearrangements to give the lachrymator, propanethial oxide «thiopropional-S-oxide», associated with the overall aroma of fresh onions.
- Garlic flavor (*Allium sativum* L.) is given by diallyl thiosulfinate (allicin).

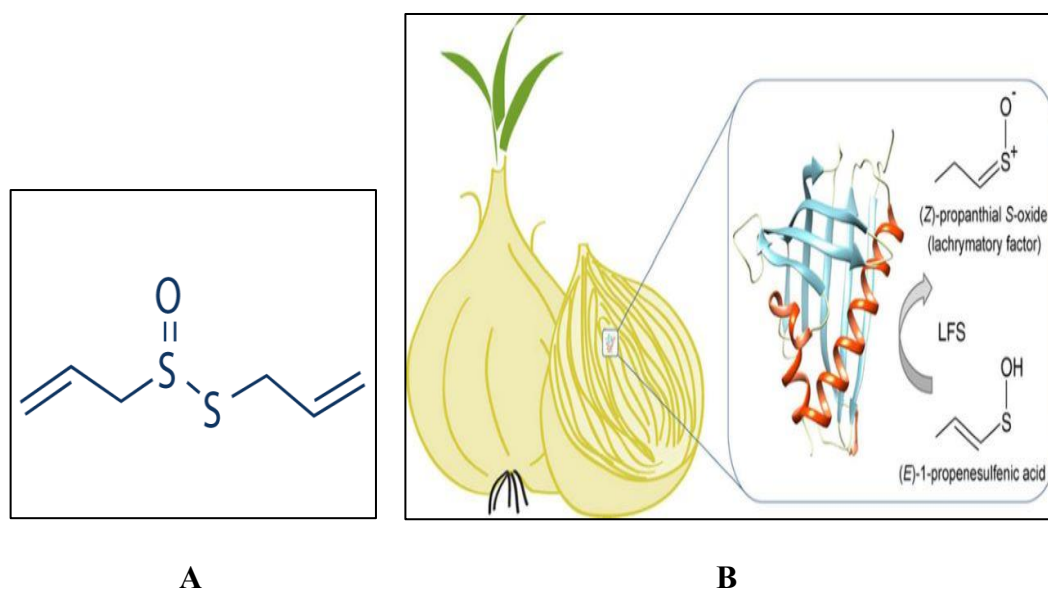


Figure 19. Structure of A) Allicin et de B) Propanethial oxide

The pungent aromas of the cruciferous family (cabbage, Brussels cabbage, turnip, brown mustard, watercress, radishes and horseradish) are formed by enzymatic processes in disturbed tissues and by cooking:

- Allyl isothiocyanate is the main source of pungency and aroma of horseradish and black mustard;
- Mild stinging of radishes is caused by 4-methylthio-3-t-butenylisothiocyanate;
- 2-phenylethyl isothiocyanate also provides a tingling sensation that influences the flavors of cress salads;
- Although they are generally not markedly pungent, fresh cabbage and Brussels sprouts contain both allyl isothiocyanate and allyl nitrile.
- 2-phenylethyl isothiocyanate also provides a tingling sensation that influences the flavors of cress salads ;

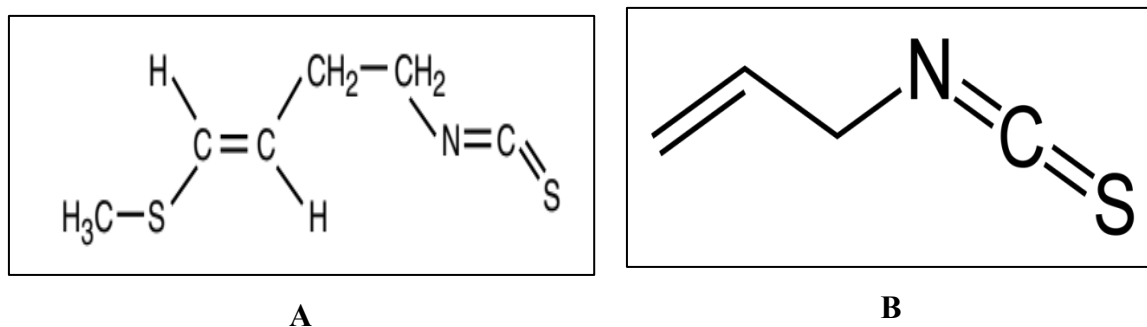


Figure 20. A) 4-methylthio-3-t-butenylisothiocyanate B) allyl isothiocyanate

It has been reported that the following elements play an important role in the aromatization of tomatoes: hexanal, trans-2-hexenal, cis-3-hexenal, cis-3-hexenol, trans-2-trans-4-decadienal, 2-isobutylthiazole, 6-methyl-5-hepten-one, 1-pentene-3-one and β -ionone.

Methoxy alkyl pyrazines are frequently responsible for the green earth aromas of many fresh vegetables:

- 2-methoxy-3-isobutylpyrazine has a strong pepper flavor detectable at a threshold of 0.002 ppb. This compound is responsible for much of the aroma of raw potatoes, green peas and pea pods.
- 2-methoxy-3-sec-butylpyrazine contributes to the aroma of raw red beet roots.

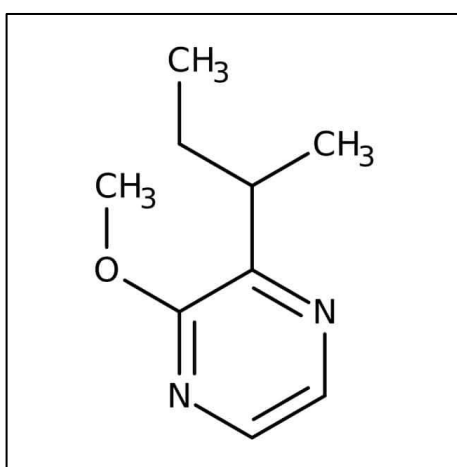


Figure 21. Structure of 2-methoxy-3-sec-butylpyrazine

Chapter II: Water and water activity a_w

II.1. Water

Water «H₂O» represents two hydrogen atoms covalently bound to an oxygen atom. Water is an odorless, tasteless and transparent liquid at room temperature. It is the most abundant molecule -but in extremely variable content- in food.

II.1.1 Abnormal physical properties

By comparing the properties of water with those of molecules of similar molecular weight and atomic composition, it is possible to determine that water behaves in an unusual way. These unique properties result from the structure of the water molecule and its dielectric qualities that allow it to form hydrogen bonds:

- Water has unusually high melting and boiling point temperatures;
- It has unusually high values for surface energy, permittivity, thermal capacity and phase transition heat (fusion, vaporization and sublimation)
- Its density is somewhat lower than expected: contrary to the normal case, the density of liquid water at 0°C is higher than that of ice at 0°C, and remains higher in the temperature range 0°C-100°C, with a maximum reached at 3.984°C. Accordingly, the ice floats on the water. If ice had a higher density than liquid water, the ice of the Arctic and Antarctic oceans would have sunk and frozen the oceans, making the planet uninhabitable for life.
- present the unusual property of expansion during solidification, and yet, despite these unusual properties, have a completely normal viscosity;
- The thermal conductivity of water is high compared to most other liquids,
- The thermal conductivity of the ice is greater than would be expected for a non-metallic solid; that of the ice at 0°C is approximately quadruple that of the liquid water at the same temperature, indicating that ice will drive thermal energy at a much higher rate than immobilized water (e.g., tissue);
- Since the thermal capacity of water is about twice that of ice, the thermal diffusivity of water and ice differ by about a factor 9: ice, in a given thermal environment, undergoes a temperature change at a speed 9 times that of liquid water.

N.B

Thermal conductivity characterizes the ability to diffuse heat in media without macroscopic displacement of matter.

Thermal capacity of a body measures the heat that must be transferred to increase its temperature by one kelvin.

Thermal diffusivity is the rate at which a material will undergo a temperature change.

Table 8. Physical properties of water

Property	Value
Molecular weight	18.0153
Melting point (at 101.3 kPa)	0.00°C
Boiling point (at 101.3 kPa)	100.00°C
Critical temperature	373.99°C
Critical pressure	22.064 Mpa
Triple point temperature	0.01°C
Triple point pressure	611.73 Pa
ΔH_{vap} at 100°C	40.647 kJ/mol
ΔH_{sub} at 0°C	50.91 kJ/mol
ΔH_{fus} at 0°C	6.002 kJ/mol

Table 9. Comparison of physical properties of water and ice

Other Temperature-Dependent Properties	Temperature (°C)			
	Ice		Water	
	-20	0	0	+20
Density (g/cm ³)	0.9193	0.9168	0.99984	0.99821
Vapor pressure (kPa)	0.103	0.6113	0.6113	2.3388
Heat capacity (J/g/K)	1.9544	2.1009	4.2176	4.1818
Thermal conductivity (W/m/K)	2.433	2.240	0.561	0.5984
Thermal diffusivity (m ² /s)	11.8×10^{-7}	11.7×10^{-7}	1.3×10^{-7}	1.4×10^{-7}
Compressibility (Pa ⁻¹)		2	4.9	
Permittivity	98	90	87.9	80.2

II.1.2. Structure

Many of the abnormal properties of water can be attributed to its simple but unique structure. The water molecule has a tetrahedral structure where the atoms are arranged at an angle of 104.5° and the distance between the hydrogen and oxygen nuclei is 0.96 °A. However, in the liquid and icy states, this angle is greater than 104.5°, probably due to the condensed water-water interaction.

A water molecule does not have a perfectly spherical shape and the molecular model indicates a diameter of about 3.12 °A. However, the average Van der Waals diameter of the

water (if one considers as a spherical quadruple where the oxygen nucleus forms the center) is considered to be approximately $2.8 \text{ \AA}$.

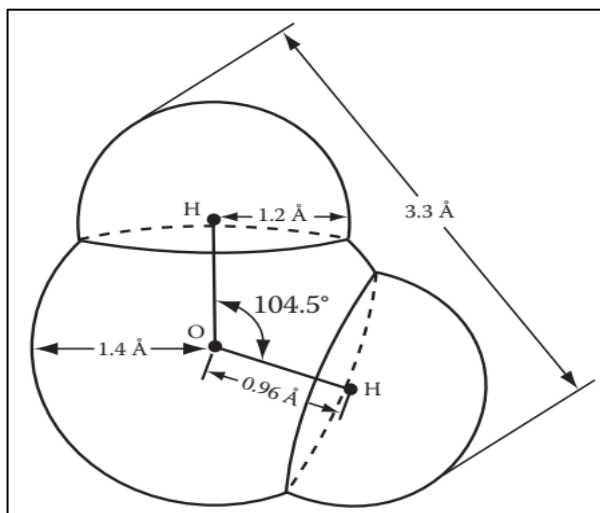


Figure 22. Schematic model of a single water molecule.

II.1.2.1. Chemical characteristics

II.1.2.1.1. Electronegativity and dipolar moment

Water is an oxygen dihydride where highly electronegative oxygen atom attracts and dislocates the electrons of the O – H bonds more towards it, and therefore the hydrogen atoms (+0.36) acquire a partial positive charge and the oxygen atom assumes a partial negative charge (-0.72).

This asymmetric charge distribution with the angle of 104.5° gives a permanent dipolar character to the water molecule; the *dipolar moment*. The dipolar moment of water is about 1.85 Debye units (D) ($=6.2375 \times 10^{-30} \text{ cm}$) and it allows water molecules to engage in a hydrogen bond via dipole-dipole interactions.

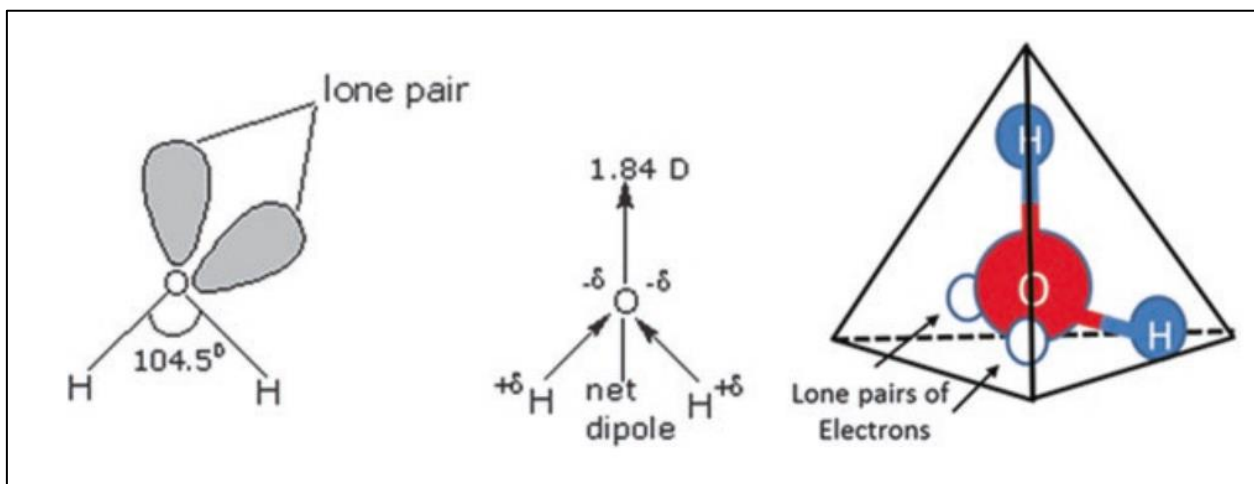


Figure 23. Tetrahedral arrangement of water molecule.

In addition to the pairs of electrons shared between oxygen and hydrogen that form simple bonds, H_2O has two pairs of isolated electrons (not shared with hydrogen). The shared pairs and the isolated pairs are arranged in the space around the central atom in a symmetrical or slightly deformed way; the *tetrahedral arrangement*. In addition, isolated electron pairs make H_2O molecules even more polar, but also angles smaller (104.5° for H_2O instead of 109.5° for methane).

Since a water molecule has two protons and two pairs of isolated electrons oriented along the axes of the tetrahedron, each water molecule can form four hydrogen bonds with four other water molecules, this allows water to form an extensive three-dimensional hydrogen bond network structure:

- Orbitals O – H act as donors of hydrogen bonds
- And the two-pair electron orbitals isolated from the oxygen atom act as acceptors of hydrogen bonds.

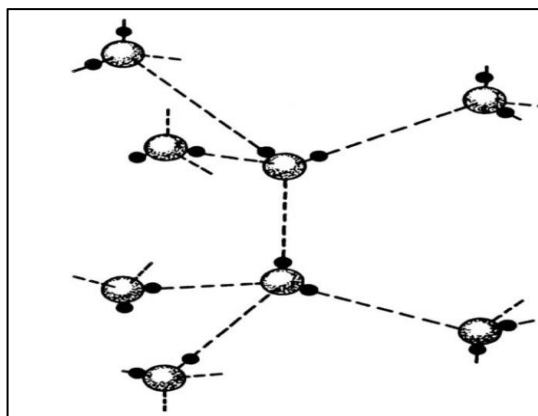


Figure 24. Hydrogen bonds of water molecules in the tetrahedral configuration.

This ability of water to engage in an extensive three-dimensional hydrogen bond provides a logical explanation for the anomaly of many of its properties. The large observed values of thermal capacity, melting point, boiling point, surface tension and phase transition enthalpies can be related to the additional energy required to break a large number of intermolecular hydrogen bonds.

The high permittivity (dielectric constant defined as the response of a given medium to an applied electric field) of water also seems influenced by the hydrogen bond. In addition to being a dipole, it also seems that hydrogen-bound molecular clusters give rise to multimolecular dipoles, effectively increasing permittivity.

II.1.3. Importance

The functional importance of water in food goes beyond its mere quantitative presence in its composition. Its interaction with lipids, carbohydrates and proteins in a food system profoundly changes their physical, chemical and microbiological properties, which has an impact on the sensory properties and acceptability of food by consumers:

- For food polymers, water serves as a structural component and plasticizer contributing to the attributes of protein, starch and dietary fiber. It is essential to the proper texture and appearance of fruits and vegetables, where water loss generally results in lower quality;
- Water also serves as a solvent or dispersal medium in a wide variety of foods. Its ability to dissolve a wide variety of materials makes it an almost universal solvent;
- Water is a major component even in manufactured food products (foam, emulsion and gel..) and its condition in these products strongly influences their texture, appearance and flavor.

On the other hand, water, being an essential requirement for the appearance and support of chemical reactions and microbial growth, is often responsible for the microbial, enzymatic and chemical deterioration of food. Food preservation techniques, such as freezing and dehydration, respectively involve the transformation of liquid water into ice or its elimination in the form of steam.

II.1.4. Classification of foods

Foods are classified into three groups according to their water content; high, medium and low humidity foods. Fruits and vegetables belong to the high humidity category, while the low humidity group includes dried fruits and dried vegetables.

II.2. Water Activity a_w

Water content alone is not a reliable indicator of food stability, as foods with the same water content differ in their perishability. The parameter reflecting both the quantity and *efficiency* of water is the water activity a_w .

Exp: peanut oil deteriorates if moisture content exceeds 0.6%, while potato starch is stable at 20% moisture.

The water activity (a_w) of a food, also known as relative vapor pressure (RVP), measures the availability of water for microbial, enzymatic or chemical activity that determines the shelf life of a food (food perishability). This is due, at least in part, to differences in association with

other food constituents; water closely associated with other food constituents is less available for microbial growth and chemical reactions causing decomposition.

Water activity is defined as the ratio of the water vapor pressure in a food to the saturated vapor pressure of pure water at the same temperature.

$$a_w = \frac{P}{P_o}$$

Where: a_w : water activity; P : partial pressure of water above the sample; P_o : pure water vapor pressure at the same temperature (specified).

The same ratio type also defines relative air humidity, RH (%):

$$RH = \frac{p'}{p_0} \times 100$$

p' : partial pressure of water vapor in the air.

If the food is in equilibrium with the air, then $p = p'$, it follows that the a_w of the food is equal to the relative humidity of the atmosphere (RH) in equilibrium with the food. Water activity is then expressed as the equilibrium relative humidity (ERH) as a percentage:

$$a_w = \frac{ERH}{100}$$

ERH: equilibrium relative humidity surrounding the product

II.2.1. a_w Prediction

The main mechanisms responsible for the depression of water vapor pressure in food are the solvent-solute interaction, the binding of water molecules to the polar sites of polymer constituents (exp: polysaccharides and proteins), water adsorption at the solid matrix surface and capillary forces.

The water contained in food exerts a vapor pressure; itself dependent on the amount of existing water, temperature and concentration of dissolved solutes (especially salts and sugars) in the water:

II.2.1.1. Water content

In foods with high moisture content, such as fruit juices, the depression can be entirely attributed to the water-solute interaction. If these foods are supposed to behave as ideal solutions, then their water vapor pressure is:

$$p = x_w p_0$$

x_w : is the water content (in molar fraction) of the food. It follows that the water activity of an ideal aqueous solution is equal to the molar concentration of water x_w . The water activity of foods with high water content ($a_w \geq 0.9$) can be calculated by this method.

As the water content is reduced, the binding of water by the solid matrix and capillary forces become increasingly important factors and overshadow the water-solute interaction. In addition, the ideal behavior of the solution is no longer applied due to the high concentration of the liquid phase. The relationship between water content and water activity, $a_w = f(X)$ becomes more complex.

II.2.1.2. a_w depends on temperature

For liquid phase and food with high humidity, a_w is very slightly, if not at all affected by temperature (p and p_0 affected identically, their ratio is not affected), the situation is different at lower levels of water content.

Temperature affects not only water molecules but also the solid matrix interacting with water. Therefore, temperature influences a_w at low humidity levels where adsorption and capillary effects are strong. The effects of temperature are unpredictable.

II.2.2. Water vapor sorption isotherms

The function representing the relationship between water content (g of water/g dry matter) and water activity at constant temperature is called water vapor sorption isotherm or moisture sorption isotherm of a food.

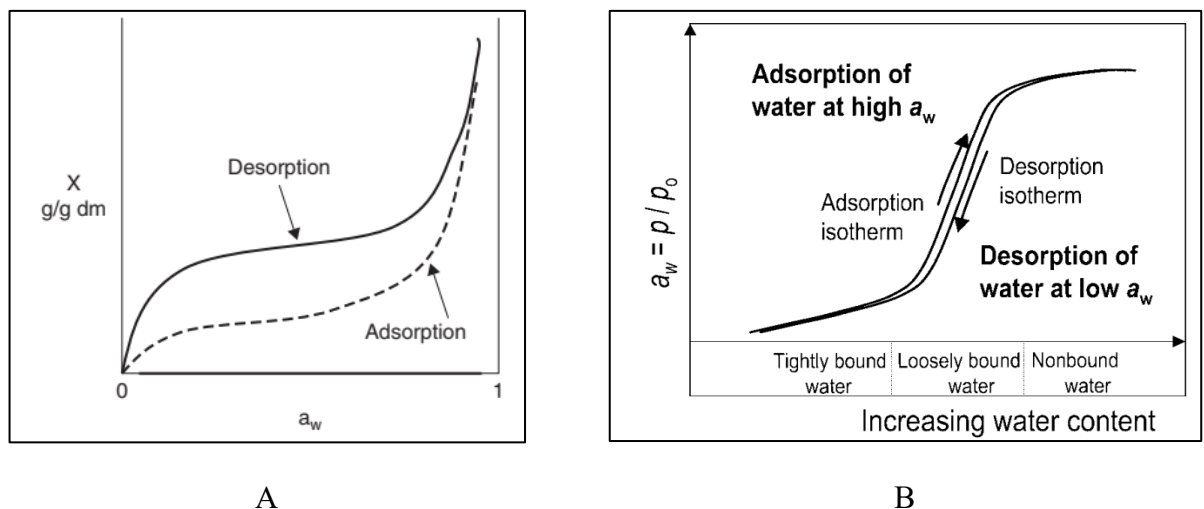


Figure 25. A) General form of sorption isotherm, B) Relationship between a_w and the form of water involved.

The general shape of an isotherm is sigmoid so that there are generally three zones depending on how the water binds to the product:

- zone 1: for low water activities ($a_w < 0.3$), it corresponds to the first layer of water (monolayer) that surrounds the dry food material. It corresponds to *strongly bound water*, also called *constitutive water*, which is intimately linked to biochemical components by hydrogen bonds and Van der Waals forces and can only be separated by very severe techniques. This water is not congealable and is practically not available as a solvent or reagent;
- zone 2: for medium water activities (a_w : 0.4 - 0.7), it corresponds to *loosely bound water*, adsorbed in poly-molecular layers covering the previous monolayer (multilayer). Although it is also available as both solvent and reagent, it is moderately reactive;
- zone 3: for important water activities ($a_w > 0.7$), it corresponds to *free water* or *liquid water* in the pores retained by capillary forces. This water is available as both solvent and reagent. Only in this form is water used by microorganisms and can allow enzymatic reactions.

II.2.2.1. Effect of a_w on food quality and stability

Bacterial growth does not occur at a_w levels below 0.9. With the exception of osmophilic species, the water activity limit for mold and yeast growth is between 0.8 and 0.9. Most enzymatic reactions require water activity levels of 0.85 or higher. The relationship between water activity and chemical reactions (Maillard browning, lipid oxidation) presents a more complex behavior with maxima and minima.

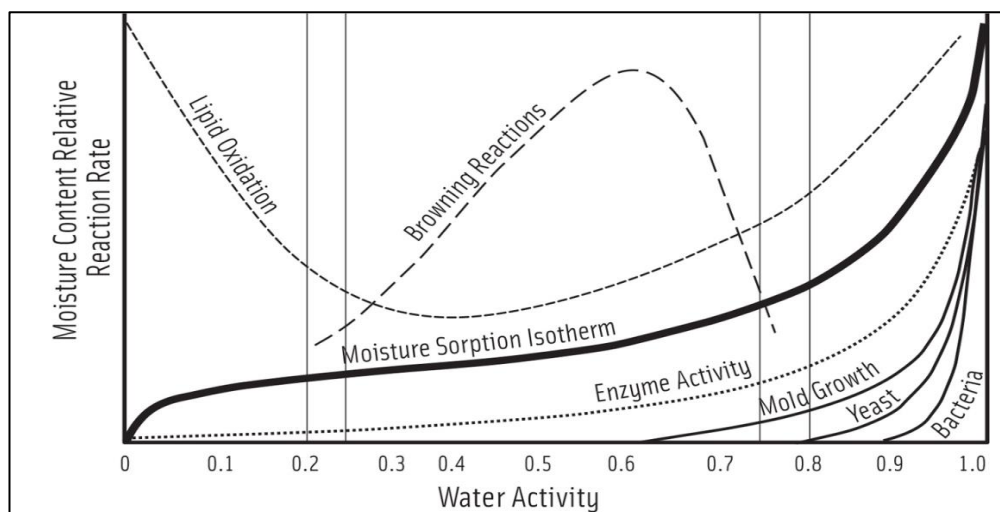


Figure 26. Effect of a_w on relative speed of deterioration mechanisms.

II.2.2.2. Determination of sorption isotherms

Sorption isotherms are determined experimentally, where samples are balanced at constant temperature with atmospheres at different known relative humidities. After balancing, the samples are analyzed for water content (moisture). Each ERH-moisture data pair gives a point on the isotherm. Basically, experimental methods are divided into two groups, namely static and dynamic procedures.

II.2.2.2.1. In static methods

Weighed samples are placed in jars, on aqueous solutions saturated with different salts (or sulphuric acid solutions (H_2SO_4) at different concentrations) and left to balance at constant temperature. At fixed temperature, the concentration of saturated solutions is constant as well as their water vapor pressure.

The sample is allowed to reach equilibrium (unchanged mass) for as long as necessary. This method is precise but slower: it can last from several days to several weeks.

Table 10. Saturated saline solutions commonly used in the determination of sorption isotherms

Salt	a_w at 25°C
Lithium chloride	0.11
Potassium acetate	0.22
Magnesium chloride	0.33
Potassium carbonate	0.44
Sodium chloride	0.75
Potassium sulfate	0.97

II.2.2.2.2. In dynamic methods

The sample is balanced with a gas flow whose relative humidity is continuously modified. Equilibrium is also achieved when the mass no longer varies, and the amount of moisture adsorbed or desorbed is determined by recording the change in the weight of the sample. The advantage of this method is the fast handling that lasts only a few hours.

II.2.3. Hysteresis

The two curves shown in Figure 26 indicate that one of them represents the achievement of equilibrium by the sample by losing moisture (desorption), while the other curve represents

the opposite path, that is, by gaining moisture (adsorption). This difference between the two curves reflects the phenomenon of non-reversibility often encountered and called *hysteresis*.

Sorption hysteresis is attributed to condensation of part of the water in the capillaries, in addition to changes in structure and porosity during drying (desorption) or humidification (adsorption).

Having two different values of water activity at the same moisture content according to the sorption path, casts doubt on the thermodynamic validity of the sorption equilibrium concept, and challenges attempts to develop mathematical models for the prediction of sorption isotherms. The best known models are:

II.2.3.1. The Brunauer-Emmett-Teller equation (BET)

$$\frac{X}{X_m} = \frac{C a_w}{(1 - a_w)[1 + a_w(C - 1)]}$$

X: water content (g water/g dry matter X)

X_m : the BET monolayer, interpreted as the value of X for saturation of a monomolecular layer of water on the adsorbent surface;

C: a constant, related to adsorption heat.

To find X_m and C from experimental sorption data, the BET equation is written as follows:

$$\frac{a_w}{(1 - a_w)X} = \Phi = \frac{1}{X_m C} + \frac{C - 1}{X_m C} a_w$$

If Φ is plotted as a function of a_w , a straight line is obtained. Thus X_m and C are calculated from the origin at y-axis and the slope.

The BET model was found to adjust sorption isotherms to water activity values of about 0.45.

II.2.3.2. The Guggenheim-Anderson-de Boer (GAB) model

This is another equation often used to predict sorption isotherms, with a wider field of application than that of the BET model:

$$\frac{X}{X_m} = \frac{CK a_w}{(1 - Ka_w)[1 - Ka_w + CKa_w]}$$

C and K: are constants, both related to temperature and adsorption heat.

II.3. Moisture and water activity analysis

III.3.1. Water content (moisture)

The moisture content of food can range from 0% for crystallized sugar or vegetable oil to more than 90% in raw watermelon or cucumbers.

The determination of water content of a food and, conversely, the dry matter or total solids (% solids = 100% - % moisture), is important not only to express the content of the other components on the basis of the wet weight (or dry), but also as an important factor in food stability and quality. Moisture dosing can be one of the most important analyses performed on a food product and yet one of the most difficult in terms of obtaining adequate precision and accuracy.

Beyond the importance of its total content, the determination of moisture in food depends on the state of water that may exist in food such as:

- Free water: this water retains its physical properties and thus acts as a dispersing agent for colloids and as a solvent for salts. It is readily evaporable and therefore available to act as a vector or chemical agent;
- Bound water «adsorbed»: this water is kept tightly or occluded in cell walls or protoplasm, as it is closely linked to proteins. It is retained by weak bonds, and generally requires heat treatment to be eliminated;
- Water of constitution «hydration»: this water is chemically linked, for example lactose monohydrate; also some salts such as $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$. It cannot be removed without causing damage (e.g., protein denaturation).

III.3.2. Analysis methods

The percentage of water measured depends largely on the method used for its analysis, which must always be indicated when reporting moisture content:

III.3.2.1. Sample Collection and handling

General sampling, handling, storage and sample preparation processes may be the greatest potential source of error in any analysis. Care should be taken to minimize accidental moisture loss or gain during these steps:

- Any exposure of a sample to the open air must be as short as possible;
- Any friction heating of a sample during grinding should be minimized;
- The headspace in the sample storage container should be minimal as moisture is lost from the sample to equilibrate the container environment with the sample;

- It is essential to control temperature fluctuations as moisture will migrate in a sample to the coldest part. To control this potential error, remove the entire sample from the container, mix again quickly, and then remove a test portion.

III.3.2.2. Analytical techniques

III.3.2.2.1. Oven drying methods

The principle of these "indirect" techniques is based on the evaporation of water from the sample and the calculation of moisture based on weight loss. The amount of moisture removed during oven drying is influenced by many variables, including drying time and temperature, the type of oven used (and the conditions inside it), and the type of sample.

The final choice of the appropriate method may depend on factors such as the need for speed, the subsequent use of the dried material or the availability of equipment.

III.3.2.2.1.1. General information

A. Removal of moisture

Any oven method is based on the fact that the boiling point of the water is 100 °C; however, this only takes into account pure water at sea level. In addition, open water is the easiest of the three forms of water to remove.

The presence of solutes raises the boiling point to >100°C (1 mole of a solute dissolved in 1L of water, increases the boiling point by 0.512 °C), and this rise continues throughout the drying process of the sample which becomes increasingly concentrated.

- In theory, the higher the drying temperature, the more complete the removal of moisture and the shorter the time required. The temperature of 250°C is required to obtain the true moisture content, eliminating all adsorbed water present;
- Practically, volatilization, deterioration and chemical reactions of constituents can occur at high temperatures, and it is prudent to use the minimum temperature that will allow the removal of moisture at an acceptable rhythm.

Additional steps prior to oven drying are recommended for certain sample types, and moisture content is calculated from moisture loss at the drying stages; preliminary and at oven:

- steam bath pre-drying for liquid products (juice..)
- air drying and grinding for field-dried grain since the surface and porosity of the sample influence the speed and efficiency of oven drying

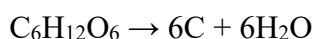
- inert material (e.g., sand) is added before drying foods that agglomerate together or to form a surface crust that prevents moisture from being removed: dehydrated fruits or sugar-rich foods

Generally, particle size, particle size distribution, sample size and surface area during drying influence the rate and efficiency of moisture removal.

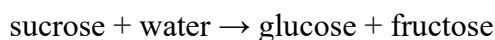
B. Decomposition of other food constituents

Decomposition occurs when time is too long or the temperature is too high. Thus, most analytical methods involve a compromise between time and a particular temperature at which limited decomposition could be a factor:

1. A major problem is that the physical process must separate all moisture without breaking down any of the constituents that could release water. For example, carbohydrates decompose at 100 °C and the moisture generated is not what we want to measure:



2. Other chemical reactions, such as the hydrolysis of sucrose, can lead to the use of moisture, which would reduce moisture for measurement:



3. The loss of volatile constituents, such as acetic, propionic and butyric acids; and alcohols, esters and aldehydes (aromatic compounds), is the source of a constant error.
4. While weight changes in these methods are believed to be due to moisture loss, weight gains can also occur due to the oxidation of unsaturated fatty acids and some other compounds.

Remark

Decomposition products CO, CO₂, CH₄ and H₂O can be released at all temperatures but at different rates.

C. Temperature control

There may be considerable temperature variability within a furnace, whose extent is to be determined before relying on the data collected during its use:

- The greatest temperature variation exists in a convection oven (a differential of 10°C is not unusual). Without a fan, the hot air circulates slowly and is even more obstructed by the trays placed in the oven. It also depends on the load placed in the oven and the room

temperature. This type of oven should not be used when precise and accurate measurements are required;

- Forced draft furnaces have the least internal temperature differential (not greater than 1 °C). The air is circulated by a fan that forces the movement of the air throughout the oven cavity; then it does not matter if the shelves are completely or only half filled with moisture bins.

D. Trays (capsules)

Pans used for moisture determination are of various shapes and may or may not have a lid:

- The AOAC moisture container measures approximately 5.5 cm in diameter with an insert cover;
- Other reusable pans have covers that slide on the outside edge, although they are costly in terms of appropriate cleaning to allow reuse.

Capsule covers are required to control sample loss by spattering:

- Slipping of metal covers to allow evaporation of moisture, also creates an area of product loss by spattering; this produces erroneous results and large standard deviations;
- Consider using fiberglass discs for covers; they prevent spattering while allowing the surface to breathe.



Figure 27. Fiberglass Trays and Filters

Handling and preparation of trays

The preparation and handling of the trays before use must be taken into account:

- Use only tongs to handle any tray (even fingerprints have weight);
- All pans must be oven treated to prepare them for use : the weight of these containers may vary and gives a balance error of 0.0001 g;
- Store dried wet pans in a functional dessicator;
- Fiberglass covers must be dried for 1 h before use

Control of surface crust formation (sand pan technique)

Some food materials tend to form a semi-permeable crust or agglomerate during drying, which will contribute to erratic and erroneous results. To control this problem, analysts use the sand pan technique for two reasons:

- prevent surface crust from forming
- disperse the sample so evaporation of moisture is less impeded.

Clean, dry sand, the amount of which depends on the size of the sample (20 to 30 g sand/3 g sample), and a short glass stirring rod are preweighed in a moisture container and then mixed with the sample (preweighed), before drying to a constant weight. Other heat-stable inert materials such as diatomaceous earth can be used, especially for sticky fruits.

However, the inert matrix used must not give erroneous results for the test due to decomposition or by trapping moisture lost by the sample.

E. Calculations

The moisture and total solids content of food can be calculated using the oven drying procedures as follows:

$$\begin{aligned} \text{\%Moisture (wt/wt)} &= \frac{\text{wt H}_2\text{O in sample}}{\text{wt of wet sample}} \times 100 & \text{Ou} & \begin{aligned} \text{\%Moisture (wt/wt)} \\ &= \frac{\text{wt of wet sample} - \text{wt of dry sample}}{\text{wt of wet sample}} \times 100 \end{aligned} \\ \text{\%Total solids (wt/wt)} &= \frac{\text{wt of dry sample}}{\text{wt of wet sample}} \times 100 \end{aligned}$$

II.3.2.2.1.2. Types of oven**A. Convection oven**

This type operating by electricity is built in double metal wall cabinet insulated by ceramic fibers. It is designed for the heating and drying process, providing precise temperature control and safety.



Figure 28. Convection oven

B. Forced draft oven

When using a forced-draft oven, it is a fan that sets the air in motion. In this case, the sample is rapidly weighed in a covered pre-dried container and placed in the oven for an arbitrarily chosen time if there is no standardized method. Drying times for this method are 0.75 to 24 hours, depending on the food sample and its pre-treatment; some liquid samples are first dried on a steam bath at 100°C to minimize spattering (AOAC method 990.19, 990.20).

An alternative to selecting a time period for drying is to weigh and reweigh the dried sample and pan until two successive weighings taken 30 min apart agree within a specified limit, for example, 0.1–0.2 mg for a 5-g sample. The user of this second method must be aware of sample transformation, such as browning which suggests moisture loss of the wrong form. Lipid oxidation and a resulting sample weight gain can occur at high temperatures in a forced draft oven. Samples high in carbohydrates should not be dried in a forced draft oven but rather in a vacuum oven at a temperature no higher than 70°C.

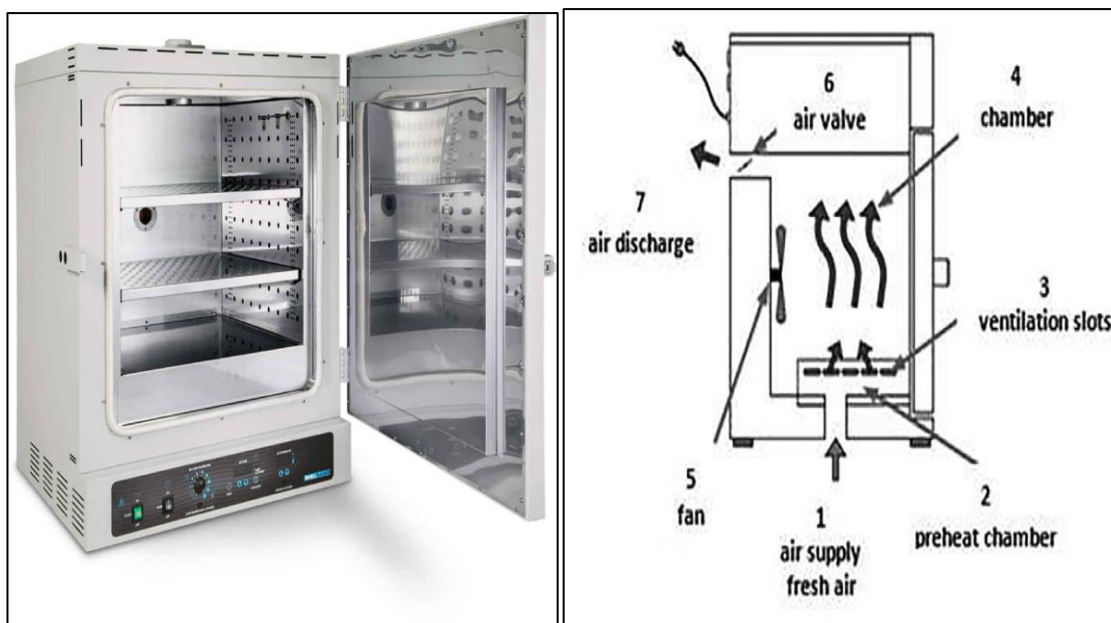


Figure 29. Forced draft oven.

C. Vacuum ovens

Vacuum ovens need a dry air purge in addition to temperature and vacuum controls to operate within method definition. Currently, an air trap that is filled with calcium sulfate containing an indicator to show moisture saturation is used. A rotameter with appropriate size is placed between the trap and the vacuum oven to measure air flow (100–120 ml/min) into the oven.

By drying under reduced pressure (25–100 mm Hg), one is able to obtain a more complete removal of water and volatiles without decomposition within a 3–6-h drying time. The drying time is a function of the total moisture present, nature of the food, surface area per unit weight of sample, whether sand is used as a dispersant and the relative concentration of sugars and other substances capable of retaining moisture or decomposing. The drying interval is determined experimentally to give reproducible results.

The following are important points in the use of a vacuum drying oven:

1. Temperature used depends on the product, such as 70°C for fruits
2. If the product to be assayed has a high concentration of volatiles, you should consider the use of a correction factor to compensate the loss.
3. Analysts should remember that in a vacuum, heat is not conducted well. Thus pans must be placed directly on the metal shelves to conduct heat ;
4. The pronounced cooling observed during drying (evaporation is an endothermic process) should not be compensated by increasing the temperature, otherwise the samples overheat during the last stages.

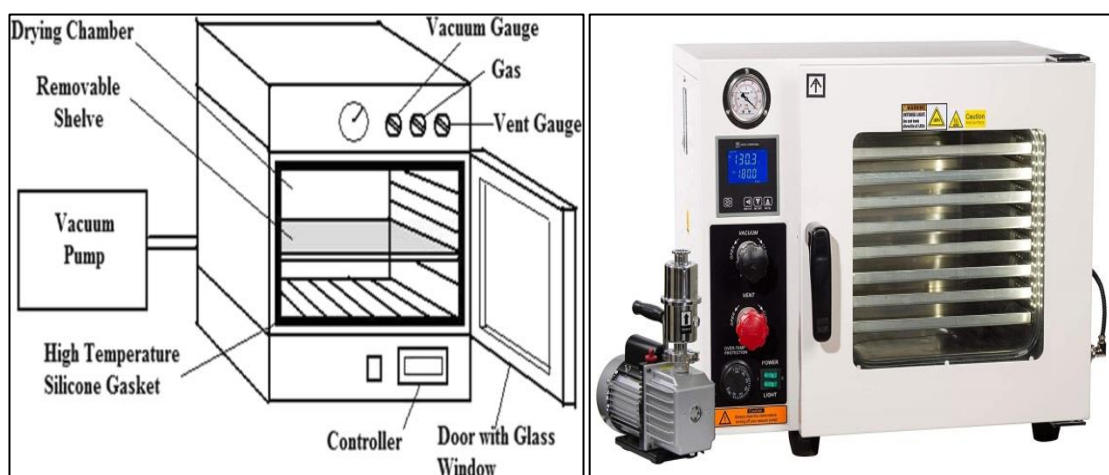


Figure 30. Vacuum oven.

Drying in a vacuum oven may be preferred for some samples like those high in fat, because the boiling point is lower under vacuum and the absence of air can minimize oxidative reactions.

D. Microwave Analyzer

These microwave moisture/solids analyzers have the distinct advantage of desired accuracy and speed of analysis, which far outweighs their limitation of testing only single samples. This drying mode allows adjusting the moisture content of food products during the

process before final packaging, thereby reducing production costs, meeting regulatory requirements and ensuring product consistency (coherency).

The general operating procedure is to adjust the microwave power according to the type of sample, then tare the internal balance with two sampling pads. Then, the sample is placed as quickly as possible between these two pads which are centered on the pedestal, and weighed against the tare weight. Time for the drying operation is set by the operator and “start” is activated.

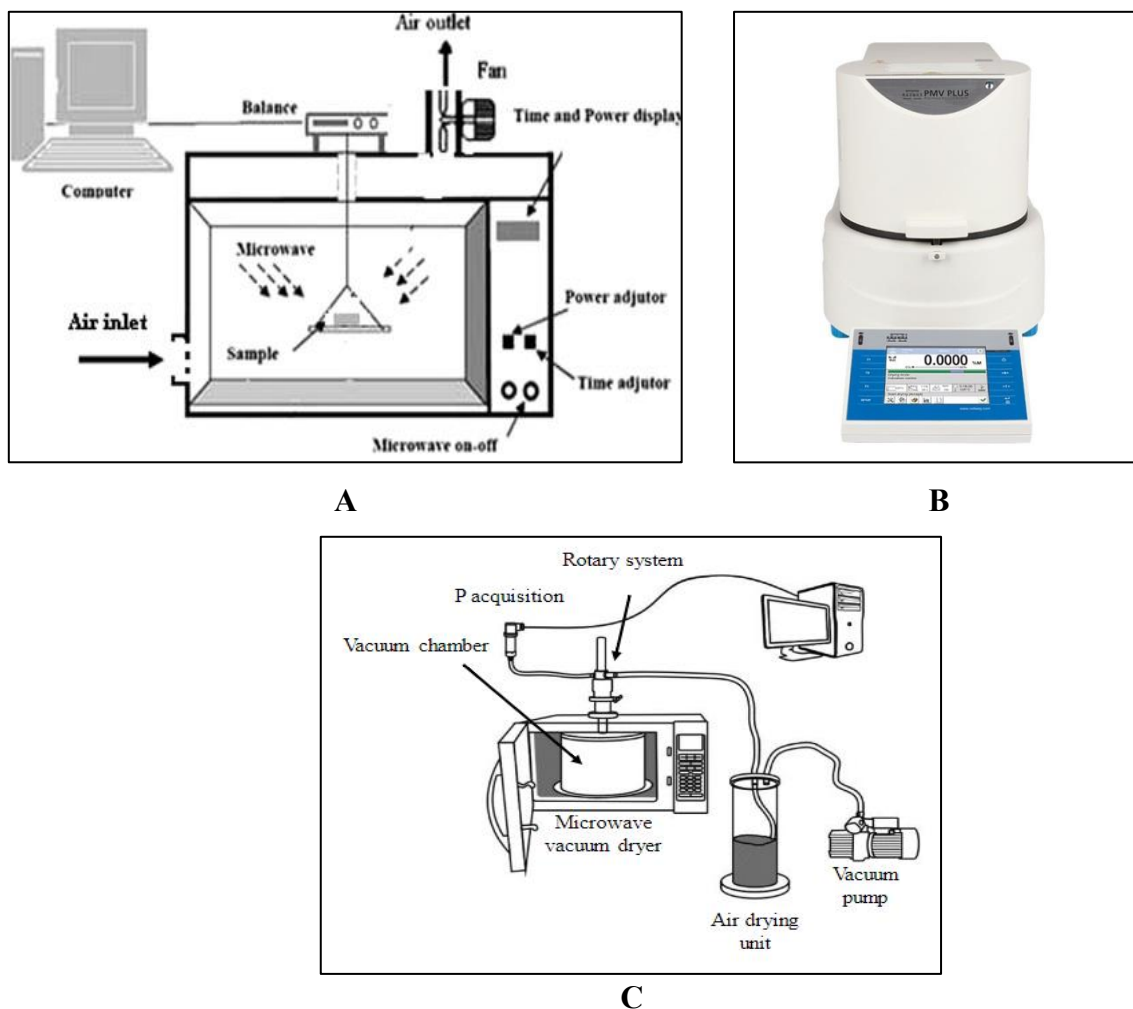


Figure 31. A and B) Microwave oven, C) Vacuum microwave oven

Newer models have a temperature control function, thus removing the need to guess appropriate (time, power) settings for specific applications. These new models also focus microwave energy directly on the sample. There are some considerations:

1. the sample must be of a uniform, appropriate size to provide for complete drying ;
2. the sample must be centrally located and evenly distributed, so some portions are not burned and other areas are under-processed ;

3. the amount of time used to place an appropriate sample weight between the pads must be minimized to prevent moisture loss or gain before weight determination ;
4. Sample pads also should be considered (fiberglass and quartz fiber pads), should not absorb microwave energy, as this can cause the sample to burn, nor should they fray easily, as this causes them to lose weight and can affect the analysis. In addition, they should absorb liquids well.

There are vacuum microwave ovens that can accommodate either a single sample in triplicate or three different samples at once and can yield in 10 minutes results similar to what would take 5 hours in a vacuum oven at 100°C.

E. Infrared drying

Unlike thermal conductivity and convection with conventional ovens, this technique (not currently approved by AOAC) involves heat penetration into the sample, which greatly reduces the required drying time to 10-25 min, using an infrared lamp that provides a temperature of 2000 to 2500 K (degrees Kelvin).

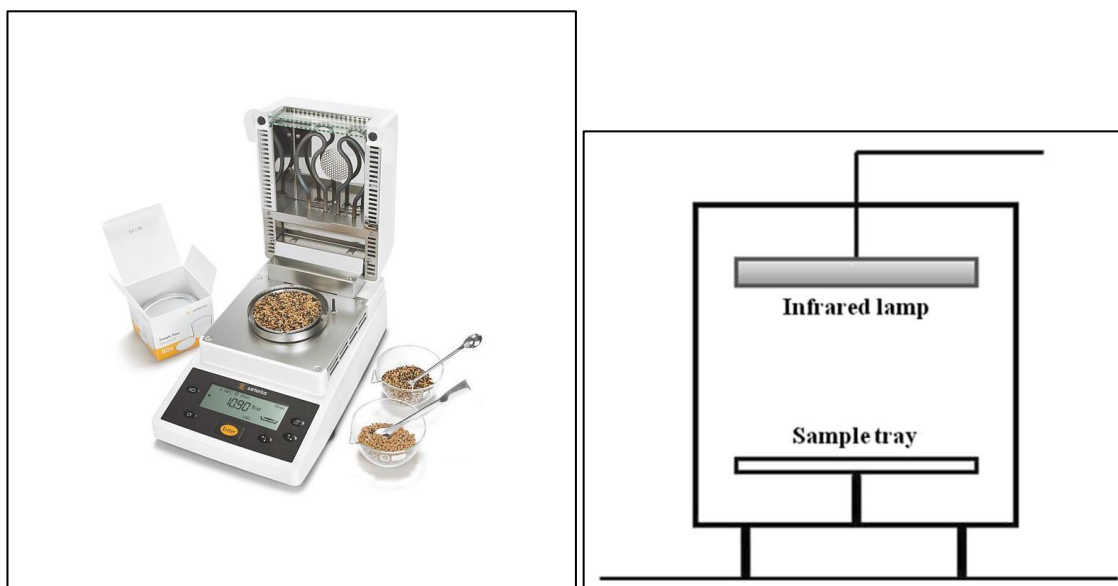


Figure 32. Schematic diagram of infrared drying.

F. Rapid moisture analyzer technology

This technology provides fast and accurate results comparable to reference methods in just a few minutes, which justifies its use in production and in the laboratory. These compact instruments detect moisture levels from 50 ppm to 100% using sample weights from 150 mg to 40 g.

Using a digital scale, the test sample is placed on an aluminum tray or filter paper and the heat control program (from 25 to 275°C) heats the sample to a constant temperature. As moisture

is removed, the instrument automatically weighs and calculates the percentage of moisture or solids.

Remark

In order to determine the optimal temperature and time or by using new equipment not described in the official methods, analysts must perform their own tests and establish the appropriate conditions based on the resulting drying curves.

II.3.2.2.2. Distillation methods

Distillation procedures involve separating moisture from solids and then quantifying it directly in volume. This direct measurement method is more suitable for foodstuff with relatively low moisture content: nuts, spices... (AOAC 986.21 for spices).

The water is co-distilled with a high boiling point, immiscible in water solvent; distilled water is condensed, collected and measured in a collecting vessel or trap. Since this is a direct measure of water rather than weight loss, distillation methods can:

- Provide better accuracy and precision than oven drying methods, especially for samples with low humidity. Although, the water volume reading in a receiving tube may be less accurate than using a weight measurement.
- Not suitable for the analysis of temperature-sensitive food, as samples are always exposed to high temperatures;
- Undesirable chemical reactions are not eliminated but can be minimized by using a lower boiling point solvent.
- In addition, distillation techniques are not easily amenable to routine testing of multiple samples.

Two distillation procedures are used today:

II.3.2.2.2.1. Direct distillation

With miscible solvents having a boiling point higher than water, the sample is heated in mineral oil. Other miscible liquids with a boiling point slightly higher than water can be used (for example, toluene, xylene and benzene).

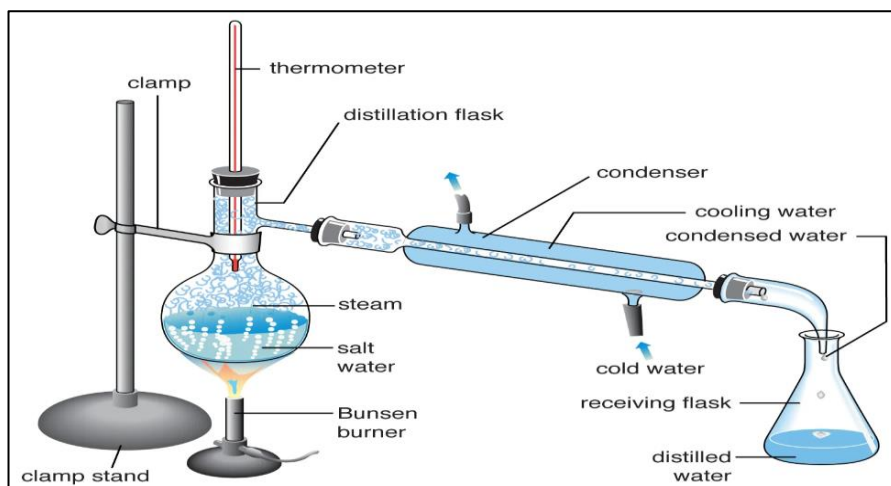


Figure 33. Direct distillation

II.3.2.2.2. Reflux distillation with an immiscible solvent

Reflux involves the return of condensate (condensed vapors) to the system from which it comes. It uses either a less dense solvent than water such as toluene, (boiling point 110.6°C) or xylene, (boiling range $137\text{-}140^{\circ}\text{C}$), or a solvent denser than water such as tetrachloroethylene (boiling point 121°C). The advantage of using this last solvent is that material to be dried floats; therefore it will not char or burn. In addition, there is no fire hazard with this solvent.

A Bidwell-Sterling moisture trap is commonly used with a solvent less dense than water; toluene (the most widely used method):

- When the toluene in the distillation just starts to boil, a hazy cloud (a vaporous emulsion of water in toluene) will be observed rising in the distillation flask.
- Condensation occurs when vapors rise to form water droplets on the condenser.

Three potential sources of error with distillation should be eliminated if observed:

1. Formation of emulsions that will not break: usually this can be controlled by allowing the apparatus to cool after distillation is completed and before reading the amount of moisture in the trap;
2. Clinging of water droplets to dirty apparatus: a burette brush, with the handle end flattened so it will pass down the condenser, is needed to dislodge moisture droplets;
3. Decomposition of the sample with production of water: This is principally due to carbohydrate decomposition to generate water, if this is a measurable problem, discontinue method use and find an alternative procedure.

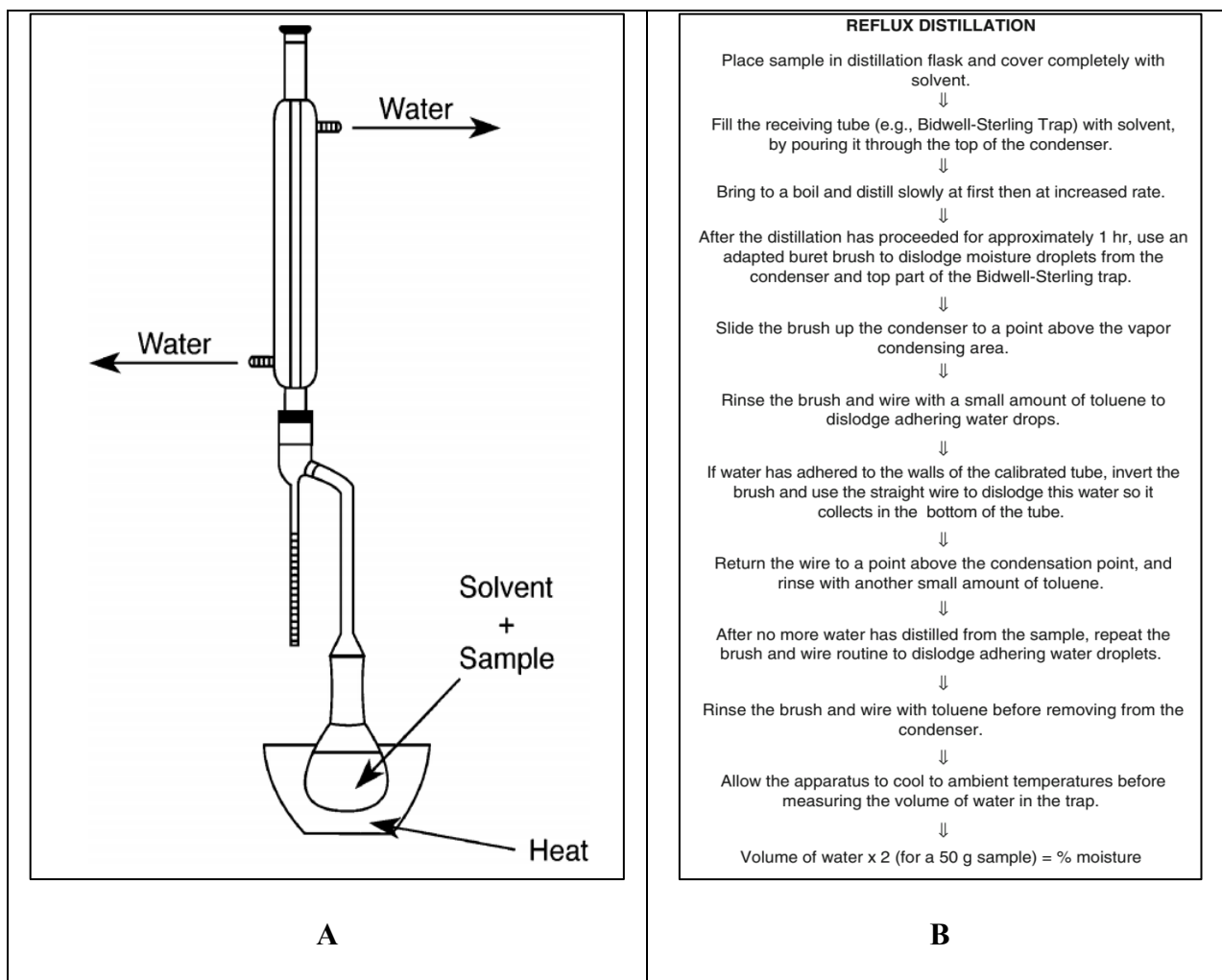
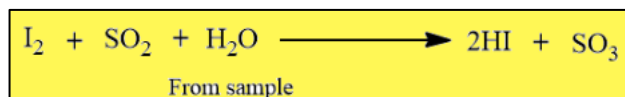


Figure 34. A) Reflux distillation apparatus with Bidwell-Sterling receiver, B) Reflux distillation procedures with toluene using a Bidwell – Sterling trap.

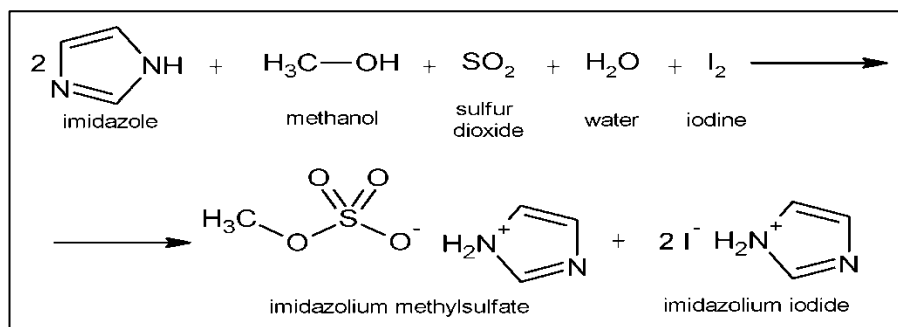
II.3.2.2.3. Chemical method: Karl Fischer titration

The Karl Fischer titration method is quite fast, accurate and does not use heat. It is suitable for low humidity foods that are sensitive to decomposition or volatilization under vacuum or high temperatures, such as dried fruits and vegetables (official method AOAC 967.19).

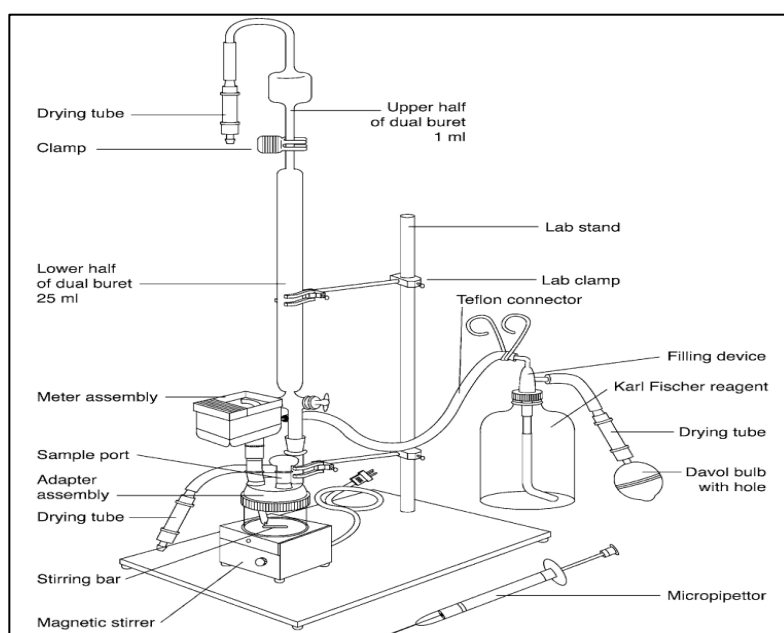
Its principle is based on the colorimetric detection of a stoichiometric reaction involving the reduction of iodine by sulfur dioxide in the presence of water:



This was modified to include methanol and pyridine in a four-component system to dissolve iodine and SO_2 :



These reactions show that for each mole of water, 1 mol of iodine, 1 mol of SO₂, 3 mol of pyridine, and 1 mol of methanol are used. For general work, a methanolic solution is used that contains these components in the ratio of 1 iodine:3 SO₂:10 pyridine, and at a concentration so that 3.5 mg of water = 1 ml of reagent.



A



B

Figure 35. A) Manual Karl Fischer titration unit, B) Automated Karl Fischer volumetric titration unit.

II.3.2.2.3.1. Volumetric titration procedure

It is appropriate for samples with moisture content greater than ~ 0.03 . Iodine and SO₂ are added to the sample in a closed chamber protected from atmospheric moisture, the excess I₂ that did not react with water can be visually determined by the dark red-brown color indicating the endpoint.

Karl Fischer reagent (KFR) is added directly as a titrant if the sample moisture is accessible. However, if moisture in a solid sample is inaccessible to the reagent, it is extracted with a suitable solvent (methanol) and the methanol extract is titrated with KFR.

Before determining the amount of water in a sample, a KFR water (moisture) equivalence (KFReq) must be determined. The KFReq value represents the equivalent amount of moisture that reacts with 1 ml of KFR.

Standardization must be checked before each use because the KFReq will change with time. Sodium tartrate dihydrate ($\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O}$), very stable and contains 15.66% water under all expected conditions, is the ideal primary standard for the determination of KFReq :

$$\begin{aligned} \text{KFReq (mg H}_2\text{O/ml)} \\ = \frac{36 \text{ g H}_2\text{O/mol Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O} \times S \times 1000}{230.08 \text{ g/mol} \times A} \end{aligned}$$

Where:

KFReq = Karl Fischer reagent moisture equivalence S = weight of sodium tartrate dihydrate (g)

A = ml of KFR required for titration of sodium tartrate dehydrate

Once the KFReq is known, the moisture content of the sample is determined as follows:

$$\% \text{H}_2\text{O} = \frac{\text{KFReq} \times K_s}{S} \times 100$$

Where:

KFReq = Karl Fischer reagent water (moisture) equivalence

K_s = ml of KFR used to titrate sample,

S = weight of sample (mg)

II.3.2.2.3.2. Coulometric titration

Sensitivity and accuracy are improved by the inclusion of a potentiometer (conductometry) to electronically determine the end point by applying a current and measuring the potential to detect excess iodine. It is ideal for products with very low humidity, from 0.03% to parts per million (ppm) levels. In this method, the amount of iodine needed to titrate moisture is determined by the current needed to generate iodine electrolytically ($2\text{I}^- \rightarrow \text{I}_2 + 2\text{e}^-$).

Automated units (used for 100 ppm of water at very high concentrations) use a pump for the mechanical addition of titrant and use the conductivity method for the determination of the endpoint.

II.3.2.2.3.3. Difficulties and sources of error

The main ones are as follows:

1. Incomplete moisture extraction: fineness of grind (i.e., particle size) is important ;
2. Atmospheric moisture: external air must not be allowed to infiltrate the reaction chamber.
3. Moisture adhering to walls of unit: All glassware and utensils must be carefully dried.
4. Interferences from certain food constituents: “oxidation of ascorbic acid to dehydroascorbic acid by KFR, release of water during the formation of acetals by reaction of carbonyl compounds with methanol, reaction of unsaturated fatty acids with iodine” overestimates moisture content.

II.3.2.2.4. Physical methods

Physical methods of moisture determination are based on the measurement of various physical properties of food samples, such as dielectric constant, conductivity, specific gravity or density, refractive index, freezing point or energy absorption in the medium or near infrared spectrum, followed by comparison with the corresponding properties of standards with known moisture content:

It is important to keep in mind that:

- Physical methods often provide rapid analysis of a large number of samples;
- But the relationship between the measured physical property and the moisture content of the specific food sample must be known, either by referring to the published tables of standard composition for that food, or by the construction of standard curves by the analyst.

II.3.2.2.4.1. Dielectric method

The dielectric method is based on the electrical properties of water. These instruments measure the change in capacity or resistance to an electrical current passing through a sample, and require calibration against samples of known moisture content. These techniques are very useful where continuous measurement is required, while they are limited to samples at less than 30-35% humidity. Note that the water dielectric constant is 80.37 to 20°C.

II.3.2.2.4.2. Hydrometry

Hydrometric methods are based on the relationship between specific gravity or density and moisture content. Hydrometry is best applied to the analysis of solutions consisting of a single solute in a water medium:

A. Hydrometer

It is a standard weight at the end of a spindle that moves a weight of liquid equal to its own weight (Archimedes principle). The weight per unit volume of a liquid is determined by measuring the volume moved by a standard weight object. Hydrometers, available in narrow and wide ranges of specific gravity, are calibrated to read specific gravity directly at 15.5 or 20°C. A hydrometer is not as accurate as a pycnometer, but its speed of analysis is a decisive factor.

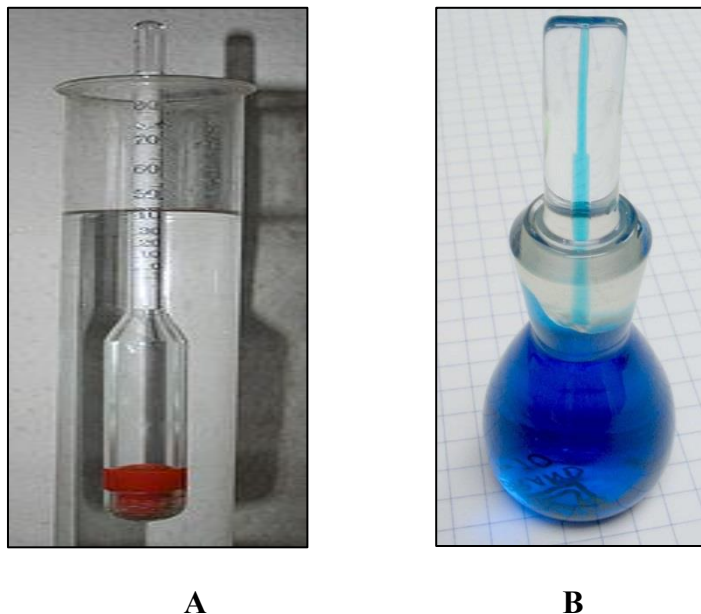


Figure 36. A) The hydrometer and B) the pycnometer.

The rudimentary but surprisingly accurate hydrometer is equipped with various modifications depending on the fluid to be measured. The Brix hydrometer is a type of sucrose meter (percentage of sucrose) used for sweet solutions such as fruit juices and syrups at 20 °C. The term Brix is interpreted as the percentage by weight of pure sucrose.

B. Pycnometer

The specific gravity measurement consists in comparing the weights of equal volumes of a liquid and water in standardized glassware, a pycnometer; this will give the density of the liquid in relation to the water.

Using a clean, dry pycnometer at 20°C, the analyst weighs it empty, fills it to the full point with distilled water at 20°C, inserts the thermometer to seal the fill opening, and puts on the cap for the overflow tube. The pycnometer is wiped dry in case of any spillage from filling and is reweighed. The density of the sample is calculated as follows:

$$\text{Density of sample} = \frac{\text{weight of sample filled pycnometer} - \text{weight of empty pycnometer}}{\text{weight of water filled pycnometer} - \text{weight of empty pycnometer}}$$

This method is used to determine solids in sugar syrups (method AOAC 932.14B).

Rq: the 20/20 mark after the specific gravity number indicates that the measuring temperature of both fluids was 20°C.

II.3.2.2.4.3. Refractometer

The refractive index method is based on how water in a sample affects the refraction of light. The refractive index (RI) of a liquid is a dimensionless constant that can be used to describe the nature of the food. If done correctly and no crystalline solid is evident, the refractometer procedure is fast and surprisingly accurate (soluble solids in fruits and fruit products AOAC 932.12; 976.20; 983.17).

The refractometers provide the refractive indices, but they are (mainly portable) equipped with scales calibrated -according to the product concerned- to read the percentage of solids, the percentage of sugars, etc., by exploiting the fact that the RI of a solution increases with concentration.

These refractometers are calibrated in °Brix (g sucrose/100 g sample) in the analysis of fruit products and tomato products, which is equivalent to the percentage of sucrose on a w/w basis. Tables are provided with the instruments to convert values and adjust temperature differences.

Since all chemical compounds have a refractive index, this measurement is used for the qualitative identification of an unknown compound by comparing its RI with the values in the literature. The RI varies with the concentration of the compound, the temperature and the wavelength of the light.



A



B

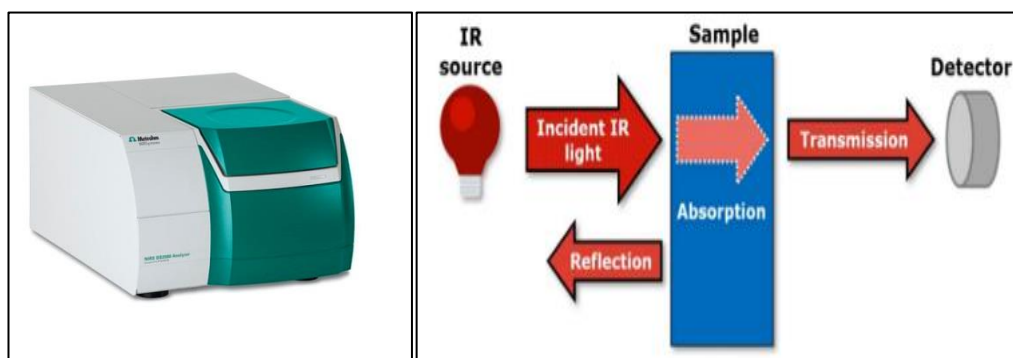
Figure 37. Abbe bench top refractometer and hand-held refractometer.

The RI of standard fluids is given under the writing n_D^{20} ; a value between 1.3000 and 1.7000; n is the symbol of RI; 20 refers to the temperature in °C; and D is the wavelength of the light beam, the line D of the sodium spectrum.

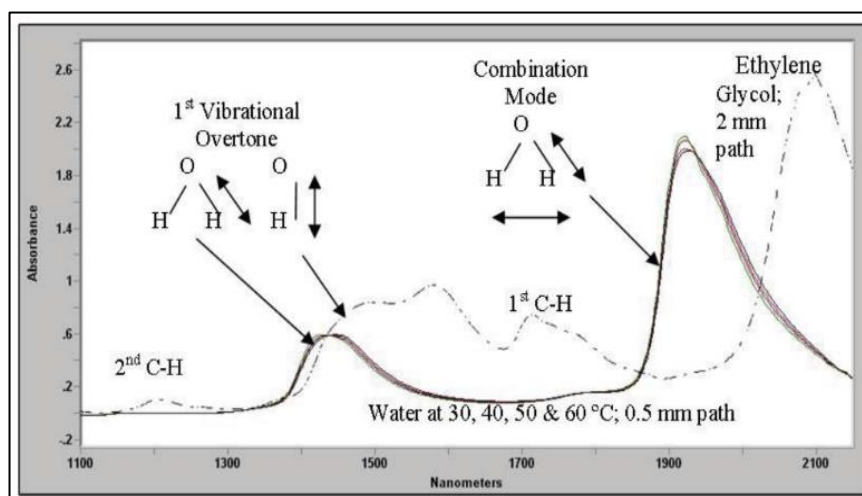
II.3.2.2.4.4. Infrared analysis

The principle is based on the measurement of absorption at wavelengths characteristic of water molecular vibration. Similar to UV-Vis spectroscopy, infrared spectroscopy consists of irradiating the sample with «near or medium» infrared radiation specific to the constituent to be dosed, and measures absorption by its molecules. For water, the near-infrared (NIR) bands (1400–1450; 1920–1950 nm) are characteristic of the stretching –OH of the water molecule and can be used to determine the moisture content of a food.

The concentration of this constituent is determined by measuring the energy reflected or transmitted by the sample, which is inversely proportional to the absorbed energy. The infrared spectrometers must be calibrated for each analyte that must be uniformly distributed throughout the sample. Near-infrared spectrophotometry is used for the moisture content of dried vegetables (official method AOAC 967.19);



A



B

Figure 38. Principle and apparatus for water dosage by infrared, B) the characteristic near infrared bands of the water molecule.

II.3.3. Measuring water activity a_w

There are different techniques to measure a_w :

- The commonly used approach is based on measuring the amount of moisture in the balanced headspace above a sample of the food product, which is directly correlated with the a_w of the sample. The sample is placed in a small closed chamber at constant temperature, and the ERH of the sample atmosphere after balancing is measured by a relative humidity sensor.
- A simple and precise variant of this approach is the cooled mirror technique in which the water vapor in the headspace condenses on the surface of a mirror that is cooled in a controlled manner. The dew point (dependent on water vapor) is determined by the temperature at which condensation takes place, which determines the relative humidity in the headspace.

Two other general approaches to measuring a_w are:

- using the freezing point depression and sample moisture content to calculate a_w ;
- equilibrating a sample in a chamber held at constant relative humidity (by means of a saturated salt solution) and then using the water content of the sample to calculate a_w .



Figure 39. Measurement device for w_a .

Chapter III: Macronutrients measurement

III.1. Organic acids

There are two interrelated concepts in food analysis that deal with acidity: pH and titratable acidity. Each of these quantities is analytically determined in separate ways and each has its own particular impact on food quality:

- Titratable acidity (total acidity): measures the total concentration of acid contained in a food by exhaustive titration of intrinsic acids with a standard base. Titratable acidity is a better predictor of acid's impact on flavor than pH;
- The need to quantify only the free H_3O^+ concentration leads to the second major concept of acidity, that of pH (active acidity). The ability of a microorganism to develop in a specific food is an example of dependence on hydronium ion concentration than on titratable acidity.

III.1.1. Units of concentration

The following table summarizes the terms used to express the concentration:

Table 11. Concentration Expressions Terms

<i>Unit</i>	<i>Symbol</i>	<i>Definition</i>	<i>Relationship</i>
Molarity	<i>M</i>	Number of moles of solute per liter of solution	$M = \frac{\text{moles}}{\text{liter}}$
Normality	<i>N</i>	Number of equivalents of solute per liter of solution	$N = \frac{\text{equivalents}}{\text{liter}}$
Percent by weight (parts per hundred)	wt%	Ratio of weight of solute to weight of solute plus weight of solvent $\times 100$	$\text{wt} = \frac{\text{wt solute} \times 100}{\text{total wt}}$
	wt/vol%	Ratio of weight of solute to total vol. $\times 100$	$\text{wt}\% = \frac{\text{wt solute} \times 100}{\text{total volume}}$
Percent by volume	vol%	Ratio of volume of solute to total volume	$\text{vol}\% = \frac{\text{vol solute} \times 100}{\text{total volume}}$
Parts per million	ppm	Ratio of solute (wt or vol) to total wt or vol. $\times 1,000,000$	$\begin{aligned} \text{ppm} &= \frac{\text{mg solute}}{\text{kg solution}} \\ &= \frac{\mu\text{g solute}}{\text{g solution}} \\ &= \frac{\text{mg solute}}{\text{liter solution}} \\ &= \frac{\mu\text{g solute}}{\text{ml solution}} \end{aligned}$
Parts per billion	ppb	Ratio of solute (wt or vol) to total wt or vol. $\times 1,000,000,000$	$\begin{aligned} \text{ppb} &= \frac{\mu\text{g solute}}{\text{liter solution}} \\ &= \frac{\mu\text{g solute}}{\text{kg}} \\ &= \frac{\text{ng solute}}{\text{ml}} \\ &= \frac{\text{ng solute}}{\text{g}} \end{aligned}$

III.1.1.1. Molarity (M)

Is the number of moles of the solute per liter of solution,

III.1.1.2. Normality (N)

Is the number of equivalents (Eq) of a solute per liter of solution:

- In acid and base solutions, the normality represents the concentration or moles of H^+ or OH^- per liter that will be exchanged in a neutralization reaction when taken to completion.
- For oxidation–reduction reagents, the normality represents the concentration or moles of electrons per liter to be exchanged when the reaction is taken to completion. The following are some examples of molarity vs. normality (equivalents):

Table 12. Examples of molarity versus normality (equivalents)

Reaction	Relation molarity /normality	
acid-base	$1\text{ M } H_2SO_4 = 2\text{ N } H_2SO_4$	2 equivalents of H^+ per mol of acid
	$NaOH\ 1\text{ M} = NaOH\ 1\text{ N}$	1 equivalent of OH^- per mol of base
	$1\text{ M } CH_3COOH = 1\text{ N}$ acetic acid	1 equivalent of H^+ per mol of acid
	$1\text{ M } H_2C_4H_4O_5 = 2\text{ N}$ malic acid	2 equivalents of H^+ per mol of acid
oxidation-reduction	$HSO_3^- + I_2 + H_2O = SO_4^{2-} + 2I^- + 3H^+$ $1\text{ M } I_2 = 2\text{ N}$ Iodine	2 equivalents of electrons gained per mol of I_2
	$1\text{ M } HSO_3^- = 2\text{ N}$ bisulfite	2 equivalents of electrons lost per mol of bisulfate

III.1.1.3. Equivalent weight

It can be defined as the molecular weight divided by the number of equivalents in the reactions. In working with normality and milliliters, the term milliequivalents (mEq) is usually preferred. Milliequivalent weight is the equivalent weight divided by 1000.

Table 13. Molecular and Equivalent Weights of Common Food Acids

<i>Acid</i>	<i>Chemical Formula</i>	<i>Molecular Weight</i>	<i>Equivalents per Mole</i>	<i>Equivalent Weight</i>
Citric (anhydrous)	$\text{H}_3\text{C}_6\text{H}_5\text{O}_7$	192.12	3	64.04
Citric (hydrous)	$\text{H}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$	210.14	3	70.05
Acetic	$\text{HC}_2\text{H}_3\text{O}_2$	60.06	1	60.05
Lactic	$\text{HC}_3\text{H}_5\text{O}_3$	90.08	1	90.08
Malic	$\text{H}_2\text{C}_4\text{H}_4\text{O}_5$	134.09	2	67.05
Oxalic	$\text{H}_2\text{C}_2\text{O}_4$	90.04	2	45.02
Tartaric	$\text{H}_2\text{C}_4\text{H}_4\text{O}_6$	150.09	2	75.05
Ascorbic	$\text{H}_2\text{C}_6\text{H}_6\text{O}_6$	176.12	2	88.06
Hydrochloric	HCl	36.47	1	36.47
Sulfuric	H_2SO_4	98.08	2	49.04
Phosphoric	H_3PO_4	98.00	3	32.67
Potassium acid phthalate	$\text{KHC}_8\text{H}_4\text{O}_4$	204.22	1	204.22

Exp : the molecular weight of H_2SO_4 is 98.08 g. Since there are 2 equivalents per mole of H_2SO_4 , the equivalent weight of H_2SO_4 is 49.04 g.

III.1.1.4. Percentage concentration

It is the mass amount of solute or analyte per 100 ml or 100 g of material. Percentage can be expressed for solutions or for solids (on a volume basis or mass basis).

III.1.1.5. Other expressions

When the percentage becomes a number less than 1%, **parts per million** (ppm), **parts per billion** (ppb) and even **parts per trillion** (ppt) usually are preferred (very diluted solutions).

III.1.2. pH

In contemporary food analysis, pH is usually determined instrumentally with a pH meter; however, chemical pH indicators also exist.

III.1.2.1. pH-meter

This potentiometer determines the concentration of hydrogen ions $[\text{H}^+]$ (or more precisely, their activity) by measuring the voltage that develops between the two electrodes of an electrolytic cell dipped in the solution to be tested.

Four main parts of the pH system are required:

- reference electrode, having a constant and known potential independently of the H^+ concentration;
- pH sensitive indicator electrode through an H^+ ion selective glass membrane;
- voltmeter or amplifier capable of measuring small voltage differences in a very high resistance circuit;
- the sample under analysis

H₃O⁺ ions in solution contribute a new potential across an ion-selective glass membrane built into the indicating electrode. This modifies the potential difference between the two electrodes in a way that is proportional to H₃O⁺ concentration. The new potential resulting from the combination of all individual potentials is called the **electrode potential** and is readily convertible into pH readings.

The Nernst equation links the response of the electrode to the activity:

$$E = E^0 - \frac{RT}{nF} \ln \frac{[C]^c [D]^d \dots}{[A]^a [B]^b \dots}$$

E° = standard electrode potential, or standard redox potential: is a characteristic constant of the reaction

R = universal gas constant : 8.314 J K⁻¹ mol⁻¹

T = absolute temperature (Kelvin)

n = the number of moles of electrons that appear in the electrode half-reaction

F = Faraday constant: 96 485 C (coulombs)

ln = natural logarithm: 2,303 x log₁₀

E = measured electrode potential,

By replacing constants with their numerical values and moving to decimal logarithms, we obtain for the temperature of 25°C:

$$E = E^0 - \frac{0.0592}{n} \log \frac{[C]^c [D]^d \dots}{[A]^a [B]^b \dots}$$

III.1.2.2. Activity vs. Concentration

In using pH electrodes, the concept of activity vs. concentration must be considered. Activity is a measure of expressed chemical reactivity, while concentration is a measure of all forms (free and bound) of ions in solution. Because of the interactions of ions between themselves and with the solvent, the effective concentration or activity is, in general, lower than the actual concentration, although activity and concentration tend to approach each other at infinite dilution.

Activity and concentration are related by the following equation:

$$A = \gamma C$$

where:

A = activity; γ = activity coefficient; C = concentration

The activity coefficient is a function of ionic strength ($I = 1/2 \sum C_i z_i^2$). Ionic strength is a function of the concentration of, and the charge on, all ions in solution. Activity issues can become significant for hydronium ions below pH 1 and for hydroxyl ions at pH 13 and above

III.1.2.3. Guidelines for Use of pH Meter

It is very important that the pH meter be operated and maintained properly. For maximum accuracy, the meter should be standardized using two buffers (two-point calibration) of pH values about 3 pH units apart, bracketing that of the anticipated sample pH.

The three standardization buffers used most widely in laboratories are a pH 4.0 buffer, a pH 7.0 buffer, and a pH 9.0 buffer (at 25 °C). The electrode should be rinsed thoroughly with distilled water and blot dry, before being immersed in the second buffer and perform a second normalization.

III.1.2.4. Determination procedure

The official method for determining the pH of fruits and vegetables (pH measurement in fruit and vegetables ISO 1842:1991) consists of the following steps

III.1.2.4.1. pH meter calibration

Calibrate the pH meter according to the manufacturer's instructions using at least two buffer solutions, while stirring with the magnetic stirrer. If the pH meter does not include a temperature correction system, the buffer solution temperature should be 20 ± 2 °C.

III.1.2.4.2. Preparation of samples

The pH is determined on a homogenized sample.

- Liquid products: Carefully mix the laboratory sample until homogeneous;
- Thick or semi-thick products: Mix the sample and grind if necessary with a blender or mortar. If the product obtained is still too thick, add an equal mass of distilled water and mix well.

III.1.2.4.3. Sampling

Use as a test sample a volume of the prepared sample sufficient for immersion of the electrodes, depending on the device used.

III.1.2.4.4. Determination

Insert the electrodes into the test sample and adjust the pH meter temperature correction system to the measuring temperature. When the constant value is reached, read the pH directly, at 0.05 pH unit at least. Perform two determinations on two separate test takes.

III.1.2.4.5. Expression of results

Take as result the arithmetic mean of the results of the two determinations.



Figure 40. Benchtop pH meter and portable pH meter

III.1.3. Titratable acidity

The titratable acidity measures the total acid concentration in a food. Food acids are usually organic acids, with citric, malic, lactic, tartaric, and acetic acids being the most common. However, inorganic acids such as phosphoric and carbonic (arising from carbon dioxide in solution) acids often play an important and even predominant role in food acidulation.

The organic acids present in foods influence:

- the flavor: tartness,
- the color: through their impact on anthocyanin and other pH-influenced pigments,
- the microbial stability: via inherent pH-sensitive characteristics of organisms,
- keeping quality: arising from varying chemical sensitivities of food components to pH.
- The titratable acidity of fruits is used, along with sugar content, as an indicator of maturity.

While organic acids may be naturally present in the food, they also may be formed through fermentation or they may be added as part of a specific food formulation.

III.1.3.1. Principe

Titrateable acidity is determined by neutralizing the acid present in a known quantity (weight or volume) of food sample using a standard base. The endpoint for titration “equivalence point” is achieved when the amount of base added is chemically equivalent to the amount of acid present in the sample:

$$(\text{ml of X})(N \text{ of X}) = (\text{ml of Y})(N \text{ of Y})$$

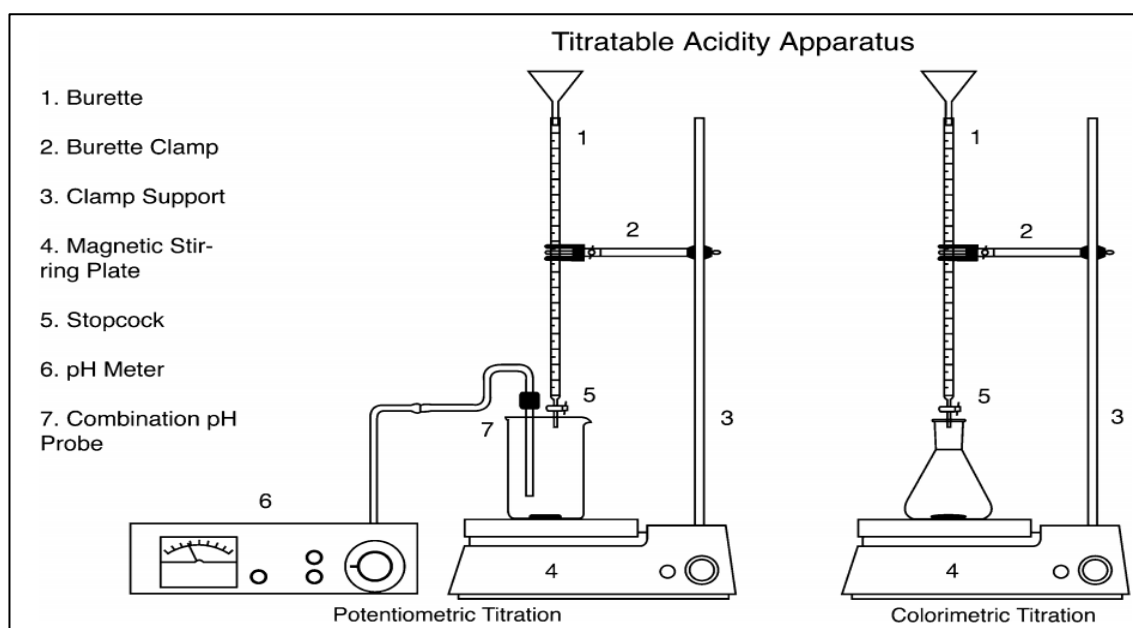


Figure 41. Two variants of titration acidity.

The endpoint for titration is usually either a target pH or the color change of a pH-sensitive dye, typically phenolphthalein. The volume of titrant used, along with the normality of the base and the volume (or weight) of sample, is used to calculate the titrateable acidity, expressed in terms of the predominant organic acid.

III.1.3.1.1. Potentiometric Titration (by pH-meter)

At the equivalence point in a titration, the total neutralization of the acid is achieved and the pH of the solution increases rapidly to finally approach the pH of the titrant. The exact equivalent point is the halfway mark on this slope of abrupt pH increase.

The advantage of this method is the identification of the precise equivalence point, in addition to being effective for highly colored samples. However, the need for careful pH

recording in relation to the titrant and the slow response with some electrodes make the potentiometric approach somewhat cumbersome.

III.1.3.1.2. Color titration (by indicators)

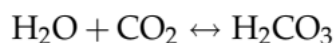
For routine work, an indicator solution is often used to approximate the equivalence point. This approach tends to slightly overshoot the equivalence point, which emphasizes that the resulting values are approximate and depend on the specific indicator.

Colored indicators are weak acids or bases, which can influence the titration by conferring their own acid/base character to the sample analyzed if they are added too generously. Typically, two to three drops of indicator are added to the solution to be titrated:

- Phenolphthalein is the most common indicator for food use. It changes from clear to red in the pH region from 8.0 to 9.6.
- Bromothymol blue is sometimes used as an alternative indicator for low acid solutions (plant extracts). It changes from yellow to blue in the pH range from 6.0 to 7.6.

N.B :

- Sodium hydroxide (NaOH) is the most commonly used base in titratable acidity determinations.
- CO₂ interferes with acidity by formation of carbonic acid in the presence of water:



CO₂ free water (by purging water with CO₂-free gas for 24 h or by boiling distilled water for 20 min and allowing it to cool before use), should be used to prepare the base or dilute the sample.

III.1.3.2. Approach

Determining titratable acidity on most samples is relatively routine, and various procedures share many common steps. An aliquot of sample (often 10 ml) is titrated with a standard alkali solution (often 0.1 N NaOH) to a phenolphthalein endpoint. Potentiometric endpoint determination is used when sample pigment makes use of a color indicator impractical:

- Erlenmeyer is preferred when indicators are used and the magnetic bar ensures homogenization of the reaction mixture. Titrant is dispensed at a slow rate until the endpoint is approached and then added dropwise until the endpoint does not fade after 5–10 s;

- The pH electrode usually demands that beakers be used instead of Erlenmeyer flasks when samples are analyzed potentiometrically. Mixing is almost always achieved through magnetic stirring. Otherwise, titration practices are identical to those described previously.

III.1.3.2.1. Calculation

Food acids are usually reported as percent of total sample weight. Thus, the equation for titratable acidity is as follows:

$$\% \text{ acid (wt/wt)} = \frac{N \times V \times \text{Eq wt}}{W \times 1000} \times 100$$

Where:

N = normality of titrant, usually NaOH (mEq/ml)

V = volume of titrant (ml)

Eq. wt. = equivalent weight of predominant acid (mg/mEq)

W = mass of sample (g)

1000 = factor relating mg to grams (mg/g) ($1/10 = 100/1000$)

For routine titration of fruit juices, milliliters can be substituted for sample weight in grams: depending on the soluble solids content of the juice, the resulting acid values will be high by 1–6%. However, this is common practice.

$$\% \text{ acid (wt/vol)} = \frac{N \times V_1 \times \text{Eq wt}}{V_2 \times 1000} \times 100 \quad \text{or} \quad \% \text{ acid (wt/vol)} = \frac{N \times V_1 \times \text{Eq wt}}{V_2 \times 10}$$

where:

N = normality of titrant, usually NaOH (mEq/ml)

V_1 = volume of titrant (ml)

Eq. wt. = Equivalent weight of predominant acid (mg/mEq)

V_2 = volume of sample (ml)

1000 = factor relating mg to grams (mg/g) ($1/10 = 100/1000$)

III.1.3.3. Volatile Acidity

In acetic acid fermentations, it is sometimes desirable to know how much acidity comes from the acetic acid and how much is contributed naturally by other acids in the product.

This can be achieved by first performing an initial titration to measure titratable acidity as an indicator of total acidity. The acetic acid is then boiled off, the solution is allowed to cool, and a second titration is performed to determine the fixed acidity. The difference between fixed and total acidity is the volatile acidity.

III.1.3.4. Other Methods

High-performance liquid chromatography (HPLC) and electrochemistry both allow identification of specific acids. HPLC uses refractive index, ultraviolet, or for some acids electrochemical detection. Ascorbic acid has a strong electrochemical signature and significant absorbance at 265 nm.

In ideal cases, the sensitivity and selectivity of electrochemical methods such as voltammetry and polarography are exceptional. However, interfering compounds often reduce the practicality of electrochemical approaches.

Unlike titration, chromatographic and electrochemical techniques do not differentiate between an acid and its conjugate base, existing inevitably side by side as part of the inherent food-buffer system. As a result, acids determined by instrumental methods may be 50% higher than values determined by titration. It follows that Brix/acid ratios can be based only on acid values determined by titration.

III.2. Lipids

An accurate and precise quantitative and qualitative analysis of lipids in foods is important for accurate nutritional labeling, determination of whether the food meets the standard of identity, and to ensure that the product meets manufacturing specifications. Inaccuracies in analysis may prove costly for manufacturers and could result in a product of undesirable quality and functionality.

III.2.1. General considerations

By definition, lipids are soluble in organic solvents and insoluble in water. Therefore, water insolubility is the essential analytical property used as the basis for the separation of lipids from proteins, water, and carbohydrates in foods.

The wide range of relative hydrophobicity of different lipids makes the selection of a single universal solvent impossible for lipid extraction of foods. Glycolipids are soluble in alcohols and have a low solubility in hexane. In contrast, triacylglycerols are soluble in hexane and petroleum ether, which are nonpolar solvents.

Some lipids in foods are components of complex lipoproteins and liposaccharides; therefore, successful extraction requires that bonds between lipids and proteins or carbohydrates be broken so that the lipids can be freed and solubilized in the extracting organic solvents.

This chapter will focus on some of the main methods commonly used; solvent extraction methods and instrumental methods:

III.2.2. Solvent extraction methods

III.2.2.1. Sample Preparation

The validity of the fat analysis of a food depends on proper sampling and preservation of the sample before the analysis. An ideal sample should be as close as possible in all of its intrinsic properties to the material from which it is taken. The sample preparation for lipid analysis depends on the type of food and the type and nature of lipids in the food.

To analyze the lipids in foods effectively, knowledge of the structure, the chemistry, and the occurrence of the principal lipid classes and their constituents is necessary. Therefore, there is no single standard method for the extraction of all kinds of lipids in different foods. For the best results, sample preparation should be carried out under an inert atmosphere of nitrogen at low temperature to minimize chemical reactions such as lipid oxidation.

The common preparatory steps in lipid analysis act to aid in extraction by removal of water, reduction of particle size, or separation of the lipid from bound proteins and/or carbohydrates:

Predrying Sample

The organic solvent cannot easily penetrate into moist tissues due to its hydrophobic nature or hygroscopic properties, which saturate it with water and render it ineffective for lipid extraction. Predrying makes the sample easier to grind for better extraction, breaks fat–water emulsions to make fats dissolve easily in the organic solvent, and helps to free fat from the tissues of foods.

However, drying at high temperatures is not desirable because it binds lipids to proteins and carbohydrates and prevents their extraction by organic solvents. Low-temperature vacuum oven drying or lyophilization provides better extraction.

Particle Size Reduction

The extraction efficiency of lipids from dried foods depends on particle size; therefore, adequate grinding is very important. The classical method of determining fat in oilseeds involves the extraction of the ground seeds with selected solvent after repeated grinding at low temperature to minimize lipid oxidation.

Extraction of fat from finished products can be a challenge, based on the ingredients (e.g., energy bars with nuts, caramel, protein, granola, soybean oil). Such products may best be ground after freezing with liquid nitrogen.

Exp : It can be difficult to extract lipids from whole soybeans because of the limited porosity of the soybean hull, whereas extraction is easily accomplished if the beans are broken mechanically by grinding.

For better extraction, the sample and solvent are mixed in a high-speed comminuting device such as a blender.

Acid Hydrolysis

A significant portion of the lipids in foods is bound to proteins and carbohydrates, and direct extraction with nonpolar solvents is inefficient. Such foods must be prepared for lipid extraction by acid hydrolysis which can break both covalently and ionically bound lipids into easily extractable lipid forms.

The sample can be predigested by refluxing for 1 h with 3 *N* hydrochloric acid. Ethanol and solid hexametaphosphate may be added to facilitate separation of lipids from other components.

Solvent Selection

Ideal solvents for fat extraction should:

- have a high solvent power for lipids and low or no solvent power for proteins, amino acids, and carbohydrates,
- evaporate readily and leave no residue, have a relatively low boiling point, and be nonflammable and nontoxic in both liquid and vapor states,
- penetrate sample particles readily, be in single component form to avoid fractionation, and be inexpensive and nonhygroscopic.

It is difficult to find an ideal fat solvent to meet all of these requirements. Ethyl ether and petroleum ether, pentane and hexane are the most commonly used solvents. A combination of

two or three solvents is frequently used. The solvents should be purified and peroxide free and the proper solvent-to-solute ratio must be used to obtain the best extraction of lipids from foods

III.2.2.2. Extraction methods

III.2.2.2.1. Continuous solvent extraction method: Goldfish method

Solvent from a boiling flask continuously flows over the sample held in a ceramic thimble. Fat content is measured by weight loss of the sample or by weight of the fat removed.

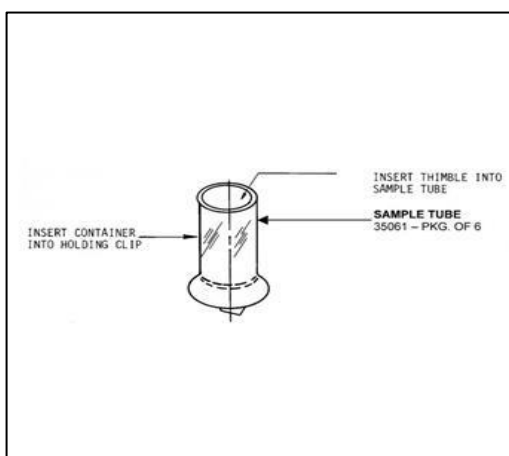
The continuous methods give faster and more efficient extraction than semicontinuous extraction methods. However, they may cause channeling (the solvent can preferentially take certain pathways through the sample) which results in incomplete extraction.



A



B



C



D

Figure 42. A) Goldfish fat extractor, B) glass holding tube; C) extraction beaker; D) extraction cartridge.

The Goldfish method is similar to the Soxhlet method, except that the extraction chamber is designed in a way that the solvent flows through the sample rather than accumulating around it. The procedure involves :

1. Weigh predried porous ceramic extraction thimble. Place vacuum oven dried sample in thimble and weigh again. Sample could instead be combined with sand in thimble and then dried;
2. Weigh predried extraction beaker;
3. Place ceramic extraction thimble into glass holding tube and then up into condenser of apparatus;
4. Place anhydrous ethyl ether (or petroleum ether) in extraction beaker and put beaker on heater of apparatus;
5. Extract for 4 h;
6. Lower the heater and let sample cool;
7. Remove the extraction beaker and let air dry overnight, then at 100°C for 30 min. Cool beaker in desiccator and weigh.

The percentage of fat content is calculated using the following equation (based on dry weight):

$$\text{Weight of fat in sample} = (\text{beaker} + \text{fat}) - \text{beaker}$$

$$\% \text{ Fat on dry weight basis} = \frac{\text{g of fat in sample}}{\text{g of dried sample}} \times 100$$

III.2.2.2.2. Semicontinuous solvent extraction method: Soxhlet method

It is the reference method used for determining fat content in dehydrated solid foods. The solvent builds up in the extraction chamber for 5–10 min and completely surrounds the sample and then siphons back to the boiling flask. Fat content is measured gravimetrically by weight loss of the sample or by weight of the fat removed. This method provides a soaking effect of the sample and does not cause channeling. However, this method requires more time than the continuous method.

Exp : AOAC Method 920.39C for Cereal Fat.

The procedure is carried out as follows:

1. Preparation of Sample : If the sample contains more than 10% H₂O, dry the sample to constant weight at 95–100°C under pressure $\leq$ 100 mm Hg for about 5 h;

2. Weigh, to the nearest mg, about 2 g of predried sample into a cellulosic predried extraction thimble, with porosity permitting a rapid flow of ethyl ether. Cover sample in thimble with glass wool;
3. Weigh predried boiling flask and put anhydrous ether (or petroleum ether) in boiling flask;
4. Assemble boiling flask, Soxhlet flask, and condenser;
5. Extract for about 4 h : the solvent vapors formed by heating the flask pass through the adduction tube, condense in the condenser and fall back into the extractor, thus macerating the solid in the solvent. The condensed solvent accumulates in the extractor and then returns to the flask after reaching the top of the siphon tube, along with the extracted substances. The solvent evaporates again, while the extracted substances remain in the flask (their boiling point must be significantly higher than that of the extracting solvent);
6. Dry boiling flask with extracted fat in an air oven at 100°C for 30 min (or by rotary evaporator), cool in desiccator, and weigh.

The percentage of fat content based on dry weight is calculated by:

$$\% \text{ Fat on dry weight basis} = \frac{\text{g of fat in sample}}{\text{g of dried sample}} \times 100$$

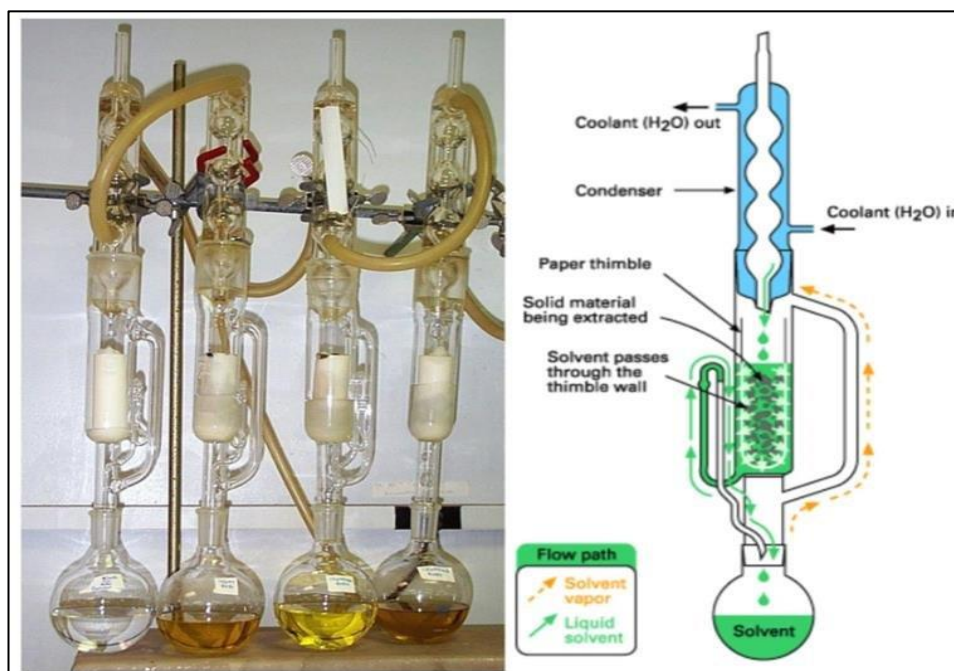


Figure 43. Semi-continuous Soxhlet extraction method.

III.2.2.2.3. Discontinuous solvent extraction methods: Chloroform–Methanol procedure

The various methanol–chloroform extraction procedures are rapid, well suited to low-fat samples, and can be used to generate lipid samples for subsequent fatty acid compositional analysis.

Both the Folch extraction and its modified method, known as the Bligh and Dyer extraction, use this solvent combination to recover lipids from food. In these methods:

- Food samples are mixed/homogenized in a chloroform–methanol solution, and the homogenized mixture is filtered into a collection tube;
- A 0.88% potassium chloride aqueous solution is added to the chloroform–methanol mixture containing the extracted fats. This causes the solution to break into two phases: the aqueous phase (top) and the chloroform phase containing the lipid (bottom);
- The phases are further separated in a separatory funnel or by centrifugation;
- After evaporation of the chloroform, the fat can be quantitated by weight

N.B : A cautionary note is that chloroform and methanol are highly toxic, so the extraction procedure must be done in well-ventilated areas.

Practically:

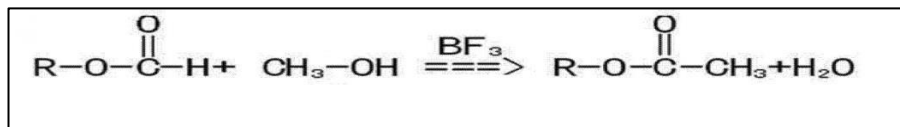
1. Weigh 1 g accurately and homogenize 1 min in 10 ml of methanol, then add 20 ml of chloroform and homogenize for 2 min;
2. Filter on a Buchner funnel and reextract cake and filter paper with 20 ml of chloroform plus 10 ml methanol;
3. Filter and combine two filtrates and measure volume. Calculate 25% of the total volume and add this amount of 0.88% KCl. Let separate in a separatory funnel;
4. Remove the lower lipid-containing layer and measure the volume. Calculate 25% of the volume and add this volume of water to wash the sample;
5. Remove water from the sample by adding sodium sulfate. Filter to separate sample from hydrated sodium sulfate. Use chloroform to wash the sodium sulfate on the filter paper ;
6. Place the chloroform–lipid solution in a weighed round bottom flask and remove chloroform from the lipid using a rotary evaporator with water temperature below 50°C (If the extract is cloudy, add 20 ml of acetone and 20 ml of chloroform and reevaporate.);
7. Weigh the flask to determine the % lipid in the sample.

III.2.2.2.4. Total Fat by GC for Nutrition Labeling (AOAC Method 996.06)

Triglyceride, triundecanoin (C11:0), is added as internal standard. Fat is extracted into ether, then methylated to *fatty acid methyl esters (FAMES)* using BF₃ (boron trifluoride) in methanol. FAMES are quantitatively measured by capillary gas chromatography (GC) against C11:0 internal standard. Total fat is calculated as sum of individual fatty acids expressed as triglyceride equivalents. Saturated and monounsaturated fats are calculated as sum of respective fatty acids. Monounsaturated fat includes only *cis* form.

The principle of the method is:

- With the exception of 100% lipid products, fat and fatty acids are extracted from food by hydrolytic methods (acidic, alkaline hydrolysis, or even a combination of both and the Soxhlet extraction method depending on the type of food);
- Pyrogalllic acid is added to minimize oxidative degradation of fatty acids during analysis;
- Before any samples are subjected to acid or alkaline hydrolysis, 1.0 ml of triundecanoin internal standard solution is added to the sample. In the case of a sample extracted by the Soxhlet method, or if the sample is 100% fat, 1.0 ml of the internal standard is added directly to the flask used as the sample is methylated;
- Fat is extracted into ether, then methylated to fatty acid methyl esters (FAMES) using BF₃ (boron trifluoride) in methanol.



- FAMES are quantitatively measured by capillary gas chromatography (GC) against C11:0 internal standard;
- Total fat is calculated as sum of individual fatty acids expressed as triglyceride equivalents.
- Saturated and monounsaturated fats (In the *cis* form only) are calculated as sum of respective fatty acids. Trans fat can be quantified utilizing this method in conjunction with identification criteria established by the American Association of Oil Chemists (AOCS Method Ce 1h-05).

Chromatographic Conditions

The following is a set of conditions that might be used, but conditions may be modified as needed to optimize separation:

Column	Any 0.25 mm ID × 100 m long, 0.20 µm film thickness 100% biscyanopropanol (nonbonded) capillary column such as SP-2560, HP-88, CP-Sil 88, etc.
Column temperature	170°C, hold 11.10 min; increase 7.9°C/min to 200°C, hold 1.27 min; increase 7.9°C/min to 210°C, hold 16 min
Injection port temperature	250°C
Detector temperature	300°C
Carrier gas (hydrogen) flow	1.2 ml/min (26 cm/s)
Split ratio	100:1
Hydrogen flow	30 ml/min
Air flow	300 ml/min
Makeup gas flow	30 ml/min

Calculations

Calculations can be made by either peak height or integrated area by entering data for sample weight (mg) and final volume (ml) in a current validated computer software.

$$\% \text{ Fatty acid on a lipid basis} = \text{mg/mg of fatty acid} \times 100$$

Or

$$\% \text{ Fatty acids on a sample basis} = \text{mg/mg of fatty acid} \times \% \text{ lipid}$$

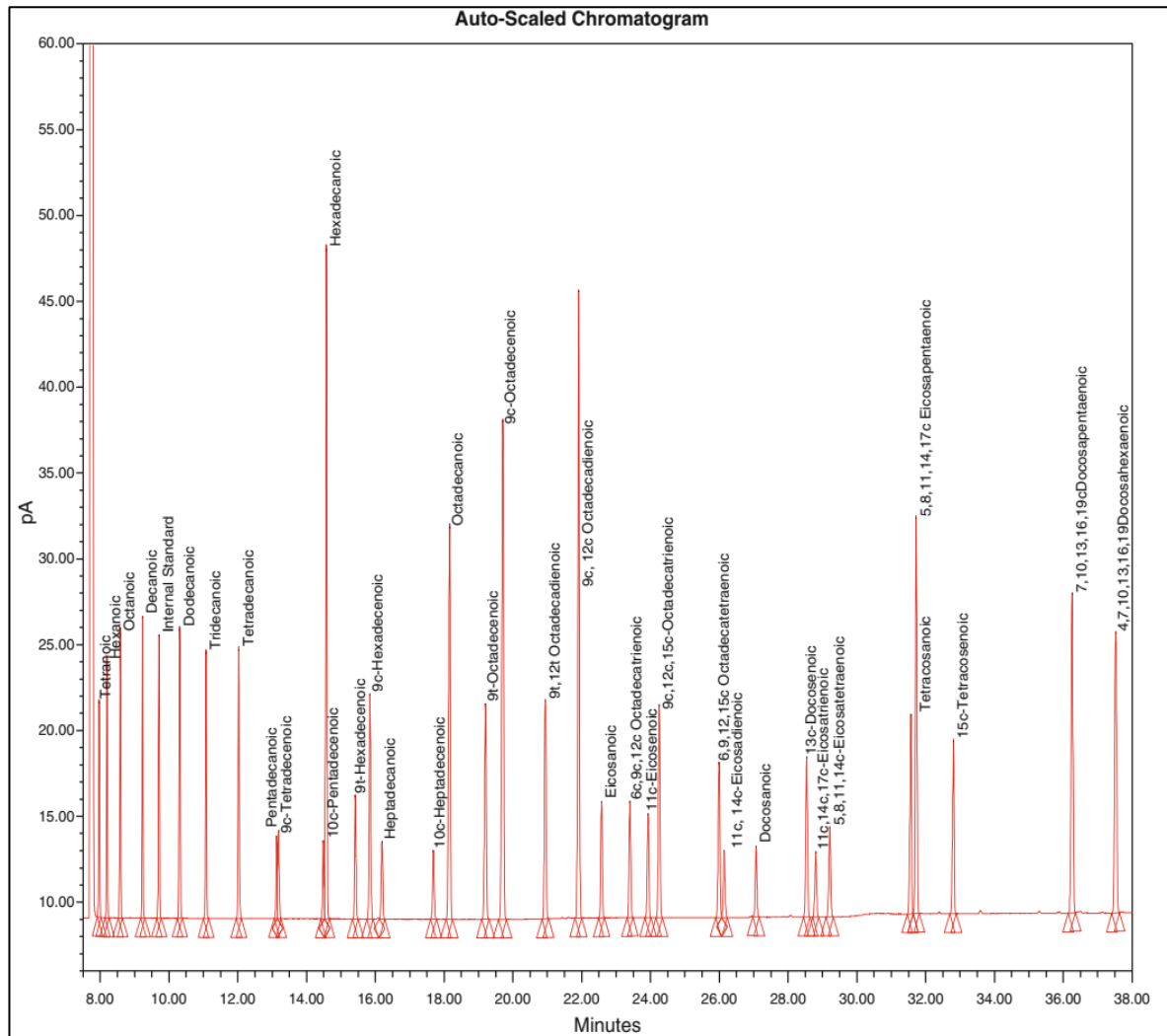


Figure 44. Example of chromatogram from gas chromatography analysis.

Alternative calculations can be made using peak areas:

- Calculate the response factor (RF) for each standard (by dividing the peak area by the internal standard area) and the standard factor (F) (by dividing the response factor by the internal standard concentration, mg/ml);
- Calculate the sample response factor (SRF) by dividing the sample fatty acid peak area by the internal standard peak area.

$$\% \text{ Fatty acid} = \frac{\text{SRF}}{F} \times \frac{\text{final sample volume (ml)}}{\text{sample weight (mg)}} \times 100$$

where:

SRF = sample response factor

F = standard factor

Table 14. Fatty acid conversion table

Fatty Acid ^a	Molecular Weight				Conversion Factors ^b		
	Acid	Methyl Ester	Triglyceride	1/3 Triglyceride	Triglyceride/Methyl Ester	Acid/Methyl Ester	Acid/Triglyceride
4:0 Butyric	88.11	102.14	302.38	100.79	0.9868	0.8627	0.8742
5:0 Valeric	102.40	116.43	345.25	115.08	0.9885	0.8795	0.8898
6:0 Caproic	116.16	130.19	386.53	128.84	0.9897	0.8923	0.9016
7:0 Heptanoic	130.19	144.22	428.62	142.87	0.9907	0.9027	0.9112
8:0 Caprylic	144.21	158.24	470.69	156.90	0.9915	0.9114	0.9192
10:0 Capric	172.27	186.30	554.85	184.95	0.9928	0.9247	0.9314
12:0 Lauric	200.35	214.38	639.02	213.01	0.9937	0.9346	0.9405
13:0 Tridecanoic	214.35	228.38	681.10	227.03	0.9941	0.9386	0.9441

Fatty Acid ^a	Molecular Weight				Conversion Factors ^b		
	Acid	Methyl Ester	Triglyceride	1/3 Triglyceride	Triglyceride/Methyl Ester	Acid/Methyl Ester	Acid/Triglyceride
14:0 Myristic	228.38	242.41	723.18	241.06	0.9945	0.9421	0.9474
14:1 Myristoleic	226.38	240.41	717.18	239.06	0.9944	0.9417	0.9469
15:0 Pentadecanoic	242.41	256.44	765.26	255.09	0.9948	0.9453	0.9503
15:1 Pentadecenoic	240.40	254.43	759.26	253.09	0.9947	0.9449	0.9499
16:0 Palmitic	256.43	270.46	807.34	269.11	0.9950	0.9481	0.9529
16:1 Palmitoleic	254.43	268.46	801.46	267.11	0.9950	0.9477	0.9525
17:0 Heptadecanoic	270.48	284.51	849.42	283.14	0.9953	0.9507	0.9552
17:1 Heptadecenoic	268.48	282.51	843.42	281.14	0.9952	0.9503	0.9549
18:0 Stearic	284.48	298.51	891.50	297.17	0.9955	0.9530	0.9573
18:1 Oleic	282.48	296.51	885.50	295.17	0.9955	0.9527	0.9570
18:2 Linoleic	280.48	294.51	879.50	293.17	0.9954	0.9524	0.9567
18:3 Gamma Linolenic	278.48	292.51	873.50	291.17	0.9954	0.9520	0.9564
18:3 Linolenic	278.48	292.51	873.50	291.17	0.9954	0.9520	0.9564
18:4 Octadecatetraenoic	276.48	290.51	867.50	289.17	0.9954	0.9517	0.9561
20:0 Arachidic	312.54	326.57	975.66	325.22	0.9959	0.9570	0.9610
20:1 Eicosenoic	310.54	324.57	969.66	323.22	0.9959	0.9568	0.9608
20:2 Eicosadienoic	308.53	322.56	963.66	321.22	0.9958	0.9565	0.9605
20:3 Eicosatrienoic	306.53	320.56	957.66	319.22	0.9958	0.9562	0.9603
20:4 Arachidonic	304.52	318.55	951.66	317.22	0.9958	0.9560	0.9600
20:5 Eicosapentaenoic	302.52	316.55	945.66	315.22	0.9958	0.9557	0.9598
22:0 Behenic	340.59	354.62	1,059.82	353.27	0.9962	0.9604	0.9641
22:1 Erucic	338.59	352.63	1,053.82	351.27	0.9962	0.9602	0.9639
22:5 Docosapentaenoic	330.50	344.53	1,029.55	343.18	0.9961	0.9593	0.9630
22:6 Docosahexaenoic	328.57	342.60	1,023.82	341.27	0.9961	0.9591	0.9628
24:0 Lignoceric	368.64	382.67	1,143.98	381.33	0.9965	0.9633	0.9667
24:1 Nervonic	366.63	380.66	1,137.98	379.33	0.9965	0.9632	0.9666

^a: Top number indicates carbon chain length and degree of unsaturation. Bottom number(s) indicate position of double bond(s) on the carbon chain.

^b: Conversion factors: To convert methyl ester to triglyceride: multiply by T/M ratio; To convert methyl ester to acid: multiply by A/M ratio; To convert triglyceride to acid: multiply by A/T ratio; To convert tridecanoic to methyl ester: multiply by 1.065 or divide by A/M ratio.

III.2.2.2.5. Instrumental methods

Instrumental methods offer numerous attractive features compared with the previously described extraction methods. In general, they are rapid, nondestructive, and require minimal sample preparation and chemical consumption. However, the equipment can be expensive and measurements often require the establishment of calibration curves specific to various compositions:

III.2.2.2.5.1. Infrared Method

The infrared (IR) method is based on absorption of IR energy by fat at a wavelength of 5.73 μm . The more the energy absorption at 5.73 μm , the higher is the fat content of the sample. Near-infrared (NIR) spectroscopy has been used to measure the fat content of cereals and oilseeds.

III.2.2.2.5.2. Specific Gravity (Foss-Let Method)

Fat content by the Foss-Let method is determined as a function of the specific gravity of a sample solvent extract (measurement of the variation in solvent density). A sample of known weight is extracted for 1.5–2 min in a vibration-reaction chamber with perchloroethylene. The extract is filtered, and using a In a magnetic floating cell controlled by a digital potentiometer, its specific gravity is determined. The reading can then be converted to oil or fat percentage using a conversion chart.

III.3. Proteins

Numerous methods have been developed to measure protein content. The basic principles of these methods include the determinations of nitrogen, peptide bonds, aromatic amino acids, dye-binding capacity, ultraviolet absorptivity of proteins, and light scattering properties. In addition to factors such as sensitivity, accuracy, precision, speed, and cost of analysis, what is actually being measured must be considered in the selection of an appropriate method for a particular application.

Protein analysis is required when you want to know:

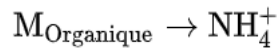
1. Total protein content
2. Content of a particular protein in a mixture
3. Protein content during isolation and purification of a protein
4. Nonprotein nitrogen
5. Amino acid composition
6. Nutritive value of a protein

III.3.1. Kjeldahl Method

III.3.1.1. Principle

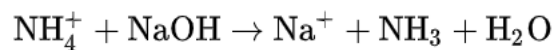
The first step is mineralization

The sample proteins are digested with high-temperature sulfuric acid (380°C) in the presence of catalysts (copper, sulfate and potassium sulfate). Total organic nitrogen is converted into ammonium salt (ammonium sulfate). The acidic pH allows the ammonium salt to appear in its acidic form as ammonium NH_4^+ :



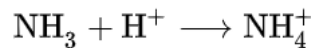
The second step is distillation

The digest is neutralized with a base (sodium hydroxide) to convert the ammonium into its volatile form, *ammonia*, before entraining it with steam during distillation:



The third step is quantification

Ammonia is collected in an excess solution of boric acid (H_3BO_3), which acts simply as a trap for ammonia (it does not react). The borate anions formed are titrated with a standardized solution of strong acid (HCl ou H_2SO_4) in the presence of a colored indicator and then converted into nitrogen in the sample:



The result of the analysis represents the crude protein content of the food, as nitrogen can also come from non-protein components (this method also measures nitrogen in ammonia and ammonium sulfate).

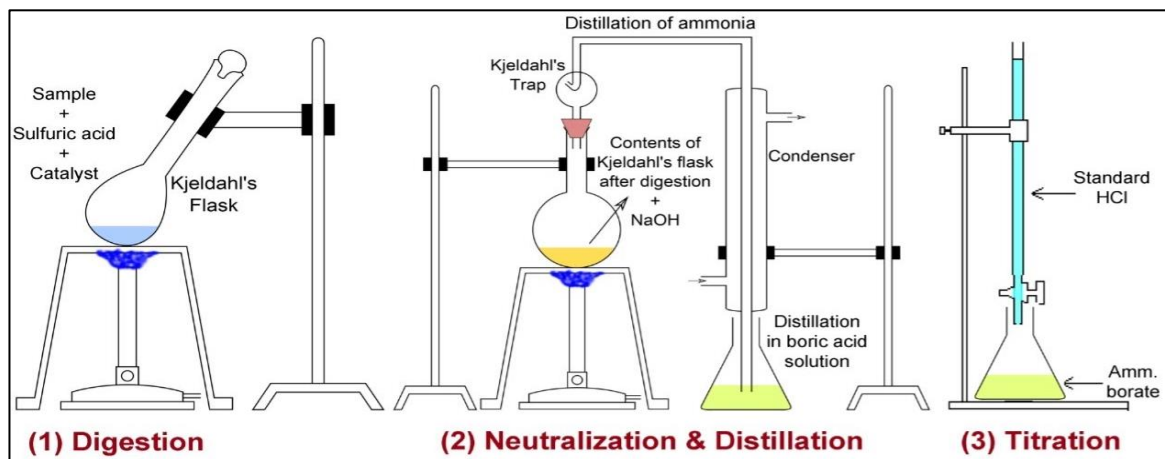


Figure 45. Principle of the Kjeldahl method

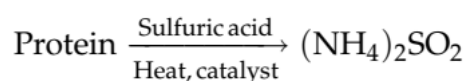
III.3.1.2. Procedure**III.3.1.2.1. Sample Preparation**

Solid foods are ground to pass a 20-mesh screen. Samples for analysis should be homogeneous. No other special preparations are required.

Rq : The "Mesh size" is the number of openings in one square inch of a screen.

III.3.1.2.2. Digestion

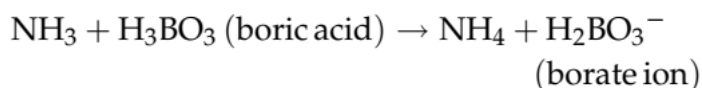
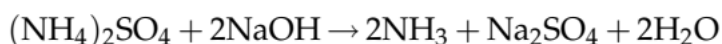
Place sample (accurately weighed) in a Kjeldahl flask. Add acid and catalyst; digest until clear to get complete breakdown of all organic matter. Nonvolatile ammonium sulfate is formed from the reaction of nitrogen and sulfuric acid.



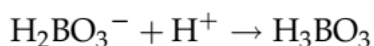
During digestion, protein nitrogen is liberated to form ammonium ions; sulfuric acid oxidizes organic matter and combines with ammonium formed; carbon and hydrogen elements are converted to carbon dioxide and water.

III.3.1.2.3. Neutralization and Distillation

The digest is diluted with water. Alkali-containing sodium thiosulfate (Helps release nitrogen) is added to neutralize the sulfuric acid. The ammonia formed is distilled into a boric acid solution containing the indicators methylene blue and methyl red (AOAC Method 991.20).

**III.3.1.2.4. Titration**

Borate anion (proportional to the amount of nitrogen) is titrated with standardized HCl

**III.3.1.2.5. Calculations**

Moles of HCl = moles of NH_3

= moles of N in the sample

A reagent blank should be run to subtract reagent nitrogen from the sample nitrogen.

$$\% \text{ N} = N_{\text{HCl}} \times \frac{\text{Corrected acid volume}}{\text{g of sample}} \times \frac{14\text{gN}}{\text{mol}} \times 100$$

where:

N_{HCl} = normality of HCl, in mol/1000 ml

Corrected acid vol. = (ml std. acid for sample) – (ml std. acid for blank)

14 = atomic weight of nitrogen

A factor is used to convert percent N to percent crude protein. Most proteins contain 16% N, so the conversion factor is 6.25 (100/16 = 6.25).

$$\frac{\% \text{ N}}{0,16} = \% \text{ protein} \quad \text{or} \quad \% \text{ N} \times 6,25 = \% \text{ protein}$$

Conversion factors for various foods are given in the following table:

Table 15. Nitrogen to protein conversion factors for various foods

	<i>Percent N in Protein</i>	<i>Factor</i>
Egg or meat	16.0	6.25
Milk	15.7	6.38
Wheat	18.76	5.33
Corn	17.70	5.65
Oat	18.66	5.36
Soybean	18.12	5.52
Rice	19.34	5.17



Figure 46. Kjeldahl apparatus

III.3.1.3. Applications

The technique has the following advantages:

- Applicable to all types of foods
- Inexpensive (if not using an automated system)
- Accurate; an official method for crude protein content

- Has been modified (micro Kjeldahl method) to measure microgram quantities of proteins

Disadvantages are:

- Measures total organic nitrogen, not just protein nitrogen
- Time consuming (at least 2 h to complete)
- Poorer precision than the biuret method
- Corrosive reagent

III.3.2. Dumas (Nitrogen Combustion) Method

Samples are combusted at high temperatures (700–1000°C) with a flow of pure oxygen. All carbon in the sample is converted to carbon dioxide during the flash combustion. Nitrogen-containing components produced include N₂ and nitrogen oxides.

The nitrogen oxides are reduced to nitrogen in a copper reduction column at a high temperature (600°C). The total nitrogen (including inorganic fraction, i.e., including nitrate and nitrite) released is carried by pure helium and quantitated by gas chromatography using a thermal conductivity detector (TCD). Dedicated traps remove water and carbon dioxide.

Ultra-high purity acetanilide and EDTA (ethylenediamine tetraacetate) may be used as the standards for the calibration of the nitrogen analyzer. The nitrogen determined is converted to protein content in the sample using a protein conversion factor.



Figure 47. The Dumas nitrogen analyzer.

III.3.2.1. Procedure

The combustion method is an alternative to the Kjeldahl method and is suitable for all types of food. Samples (approximately 100–500 mg) are weighed into a tin capsule and introduced to a combustion reactor in automated equipment. The nitrogen released is measured by a built-in gas chromatograph.

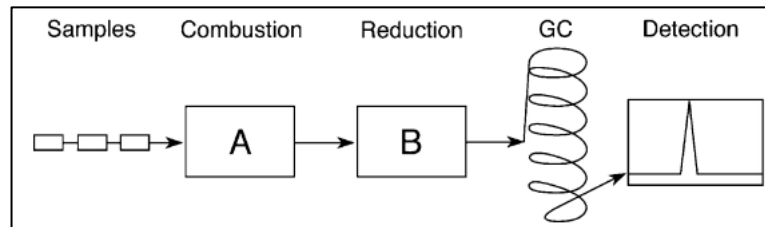


Figure 48. General components of a Dumas nitrogen analyzer..

Advantages:

- Requires no hazardous chemicals.
- Can be accomplished in 3 min.
- Recent automated instruments can analyze up to 150 samples without attention.

Disadvantages:

- Expensive equipment is required.
- Measures total organic nitrogen, not just protein nitrogen.

III.3.3. Infrared Spectroscopy

Infrared spectroscopy measures the absorption of radiation (near- or mid-infrared regions) by functional groups of food molecules. For proteins and peptides, various mid-infrared bands (6.47 μm) and near-infrared (NIR) bands (e.g., 3300–3500 nm; 2080–2220 nm; 1560–1670 nm) characteristic of the peptide bond can be used to estimate the protein content of a food.

By irradiating a sample with a wavelength of infrared light specific for the constituent to be measured, it is possible to predict the concentration of that constituent by measuring the energy that is reflected or transmitted by the sample (which is inversely proportional to the energy absorbed).

III.3.3.1. Application

Near-infrared spectroscopy is applicable to a wide range of food products (e.g., grains; cereal) (AOAC Method 997.06). Instruments are expensive and they must be calibrated properly. However, samples can be analyzed rapidly (30 s to 2 min) by analysts with minimal training

III.3.4. Biuret Method

A violet-purplish color is produced when cupric ions are complexed with peptide bonds (substances containing at least two peptide bonds, i.e., biuret, large peptides, and all proteins) under alkaline conditions. The absorbance of the color produced is read at 540 nm. The color intensity (absorbance) is proportional to the protein content of the sample

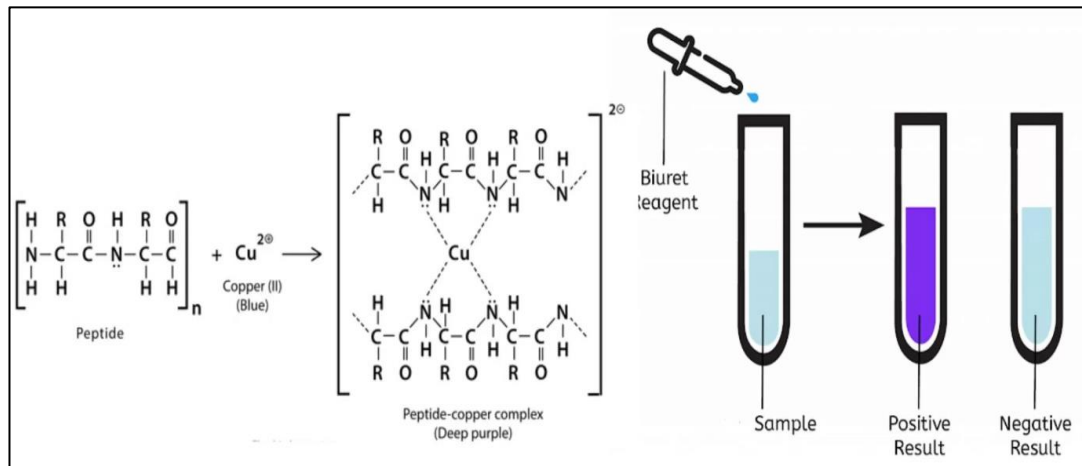


Figure 49. Biuret reaction

III.3.4.1. Procedure

A 5-ml biuret reagent is mixed with a 1-ml portion of protein solution (1–10 mg protein/ml). The reagent includes copper sulfate, NaOH, and potassium sodium tartrate, which is used to stabilize the cupric ion in the alkaline solution. After the reaction mix is allowed to stand at room temperature for 15 or 30 min, the absorbance is read at 540 nm against a reagent blank.

Filtration or centrifugation before reading absorbance is required if the reaction mixture is not clear. A standard curve of concentration versus absorbance is constructed using bovine serum albumin (BSA).

III.3.4.2. Application

The biuret method has been used to determine proteins in cereals and soybeans. The advantages of this technique are:

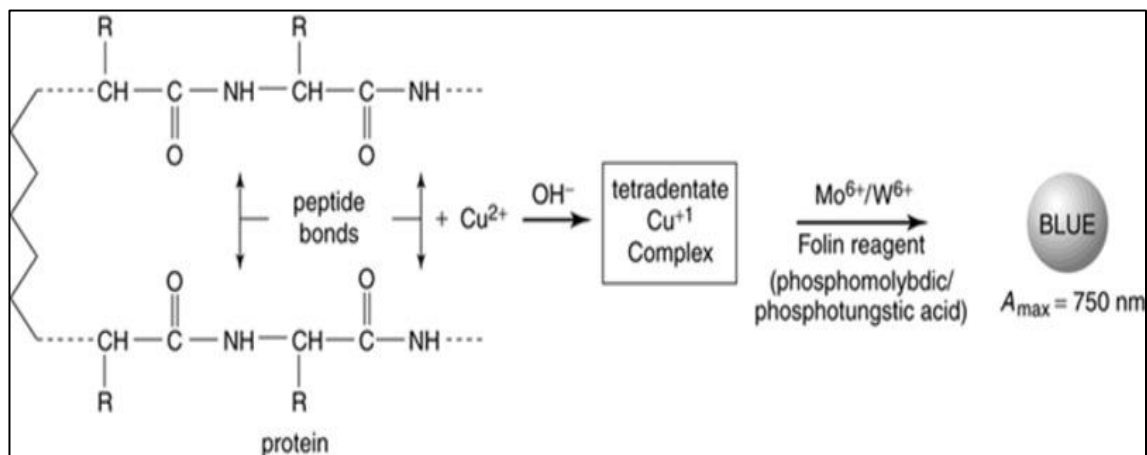
- Simplest method for analysis of proteins, less expensive than the Kjeldahl method; rapid (less than 30 min);
- Color deviations are encountered less frequently than with Lowry, ultraviolet (UV) absorption, or turbidimetric methods;
- Very few substances other than proteins in foods interfere with the biuret reaction;
- Does not detect nitrogen from nonpeptide or nonprotein sources.

Disadvantages:

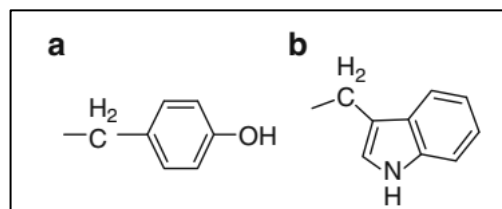
- Not very sensitive as compared to the Lowry method; requires at least 2-4 mg protein for assay;
- Absorbance could be contributed from bile pigments if present;
- High concentration of ammonium salts interfere with the reaction;
- Color varies with different proteins; gelatin gives a pinkish-purple color;
- Opalescence could occur in the final solution if high levels of lipid or carbohydrate are present;
- Not an absolute method: color must be standardized against known protein (e.g., BSA) or against the Kjeldahl nitrogen method.

III.3.5. Lowry Method

The Lowry method combines the biuret reaction with the reduction of the Folin–Ciocalteu phenol reagent (phosphomolybdic-phosphotungstic acid) by tyrosine and tryptophan residues in the proteins. The bluish color developed is read at 750 nm (high sensitivity for low protein concentration) or 500 nm (low sensitivity for high protein concentration)



A



B

Figure 50. A) Principle of the Lowry technique, B) Side chains of the amino acids (a) tyrosine and (b) tryptophane.

III.3.5.1. Procedure

The following procedure is based on the modified procedure of Hartree:

- Proteins to be analyzed are diluted to an appropriate range (20–100 µg) and a K Na Tartrate- Na_2CO_3 solution is added after cooling and incubated at room temperature for 10 min;
- CuSO_4 -K Na Tartrate-NaOH solution is added after cooling and incubated at room temperature for 10 min;
- Freshly prepared Folin reagent is added and then the reaction mixture is mixed and incubated at 50°C for 10 min;
- Absorbance is read at 650 nm. A standard curve of BSA is carefully constructed for estimating protein concentration of the unknown.

III.3.5.2. Applications

Despite its simplicity and sensitivity, the Lowry method is not used in food until the proteins are extracted from the food mixture.

Advantages:

- Very sensitive: 50–100 times more sensitive than biuret method, 10–20 times more sensitive than 280-nm UV absorption method;
- Less affected by turbidity of the sample;
- More specific than most other methods, relatively simple; can be done in 1–1.5 h.

Disadvantages:

For the following reasons, the Lowry procedure requires careful standardization for particular applications:

- Color varies with different proteins to a greater extent than the biuret method;
- Color is not strictly proportional to protein concentration;
- The reaction is interfered with to varying degrees by sucrose, lipids, phosphate buffers, monosaccharides, and hexoamines;
- High concentrations of reducing sugars, ammonium sulfate, and sulfhydryl compounds interfere with the reaction.

III.3.6. Bradford dye-binding method

When Coomassie Brilliant Blue G-250 binds to protein, the dye changes color from reddish to bluish, and the absorption maximum of the dye is shifted from 465 to 595 nm. The change in the absorbance at 595 nm is proportional to the protein concentration of the sample.

The Bradford relies on the amphoteric nature of proteins: when the protein containing solution is acidified to a pH less than the isoelectric point of the protein(s) of interest, the dye added binds electrostatically and its absorbance spectrum changes relatively to the unbound dye.

III.3.6.1. Procedure

- Coomassie Brilliant Blue G-250 is dissolved in 95% ethanol and acidified with 85% phosphoric acid;
- Samples containing proteins (1–100 $\mu\text{g/ml}$) and standard BSA solutions are mixed with the Bradford reagent;
- Absorbance at 595 nm is read against a reagent blank. Protein concentration in the sample is estimated from the BSA standard curve.

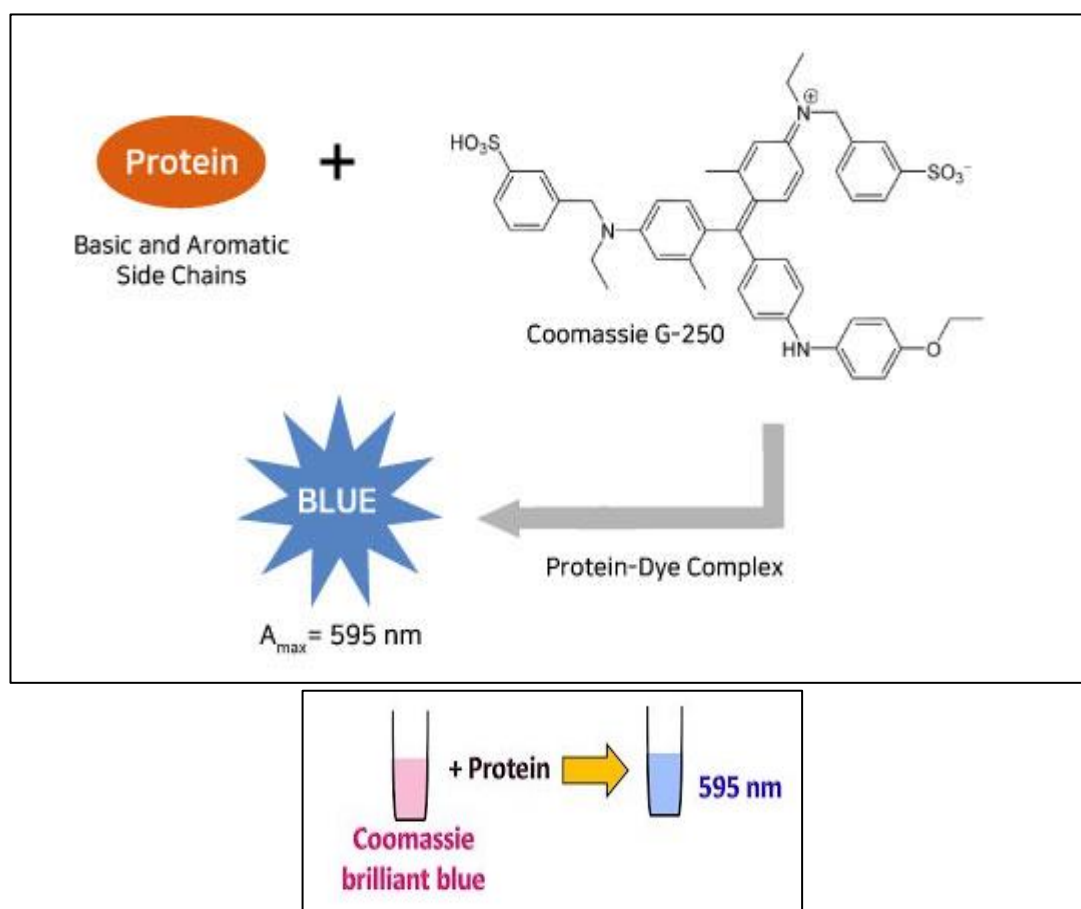


Figure 51. Principle of the Bradford method

III.3.6.2. Applications

Due to its rapidity, sensitivity, and fewer interferences than the Lowry method, the Bradford method has been used widely for the analysis of low concentrations of proteins and enzymes in their purification and characterizations. It has also been successfully used to determine the protein content of potato tubers.

Advantages:

- Rapid; reaction can be completed in 2 min, reproducible and sensitive; several fold more sensitive than the Lowry method;
- No interference from ammonium sulfate, polyphenols, carbohydrates such as sucrose, or cations such as K^+ , Na^+ , and Mg^{+2} ;
- Measures protein or peptides with molecular mass approximately equal to or greater than 4000 Da

Disadvantages:

- Interfered with by both nonionic and ionic detergents, such as Triton X-100 and sodium dodecyl sulfate. However, errors due to small amounts (0.1%) of these detergents can be corrected using proper controls.
- The protein–dye complex can bind to quartz cuvettes. The analyst must use glass or plastic cuvettes.
- Color varies with different types of proteins. The standard protein must be selected carefully.

III.3.7. Ultraviolet 280 nm Absorption Method

Proteins show strong absorption in the region at ultraviolet (UV) 280 nm, primarily due to tryptophan and tyrosine residues in the proteins. Because the content of tryptophan and tyrosine in proteins from each food source is fairly constant, the absorbance at 280 nm could be used to estimate the concentration of proteins, using Beer's law.

Since each protein has a unique aromatic amino acid composition, the extinction coefficient (E_{280}) or molar absorptivity (E_m) must be determined for individual proteins for protein content estimation.

III.3.7.1. Procedure

Proteins are solubilized in buffer or alkali and the absorbance of protein solution is read at 280 nm against a reagent blank. Protein concentration is calculated according to the equation

$$A = a \times b \times c$$

A = absorbance, a = absorptivity, b = cell or cuvette path length, c = concentration

III.3.7.2. Applications

This technique is better applied in a purified protein system or to proteins that have been extracted in alkali or denaturing agents such as 8 M urea.

Advantages:

- Rapid and relatively sensitive; At 280 nm, 100 µg or more protein is required; several times more sensitive than the biuret method;
- No interference from ammonium sulfate and other buffer salts;
- Nondestructive; samples can be used for other analyses after protein determination.

Disadvantages:

- Nucleic acids also absorb at 280 nm;
- Aromatic amino acid contents in the proteins from various food sources differ considerably;
- The solution must be clear and colorless. Turbidity due to particulates in the solution will increase absorbance falsely;
- A relatively pure system is required to use this method.

III.4. Carbohydrates

III.4.1. Sample preparation

Sample preparation is related to the specific raw material, ingredient, or food product being analyzed and the specific carbohydrate being determined, because carbohydrates have such a wide range of solubilities. However, some generalities can be presented.

- For most foods, the first step is drying, which also can be used to determine moisture content. For other than beverages, drying is done by placing a weighed amount of material in a vacuum oven and drying to constant weight at 55°C and 1 mm Hg pressure;
- Then, the material is ground to a fine powder, and lipids are extracted using 19:1 vol/vol chloroform–methanol in a Soxhlet extractor. Prior extraction of lipids makes extraction of carbohydrates easier and more complete.

However, other sample preparation schemes may be required. For example, the AOAC International method calls for removal of fats by extraction with petroleum ether (hexane) rather than the method described above.

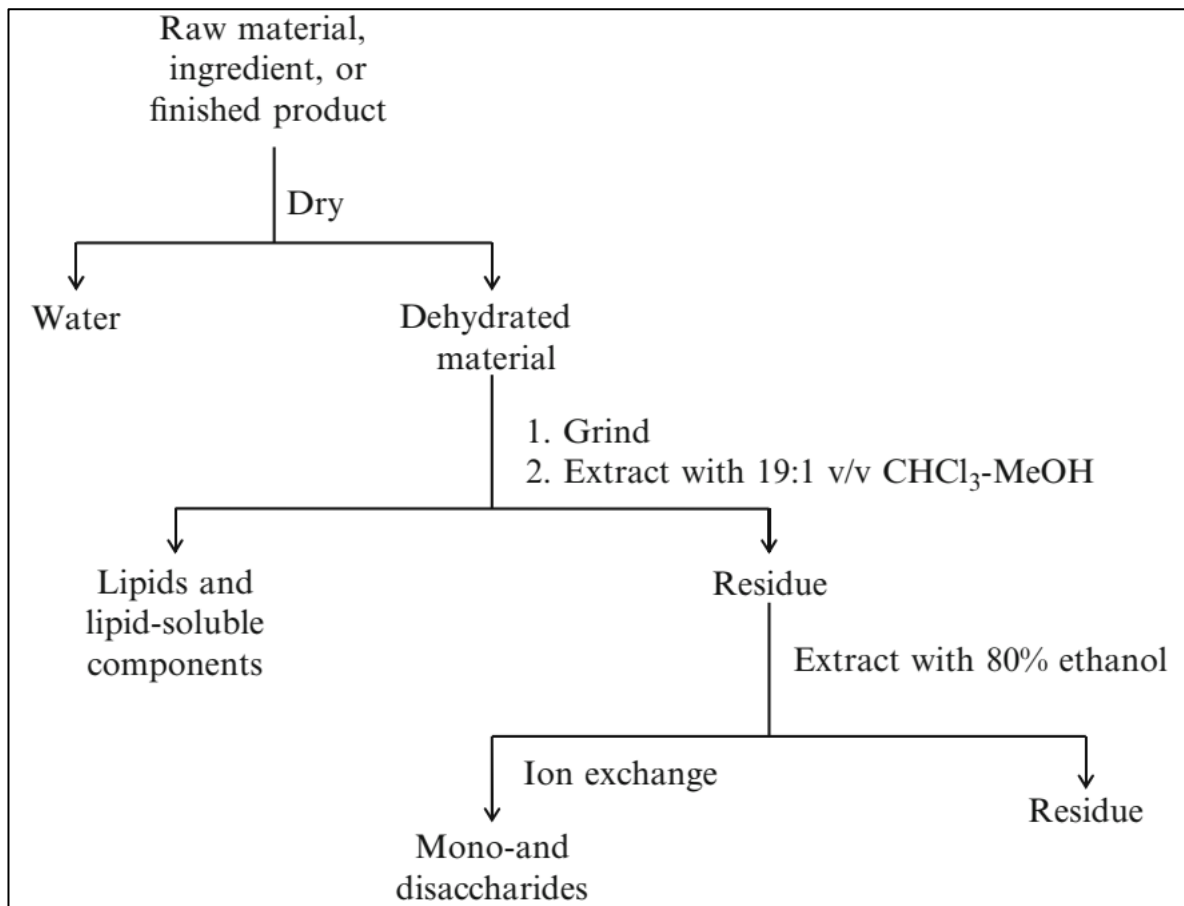


Figure 52. Flow diagram for sample preparation and extraction of mono- and disaccharides.

III.4.2. Mono- and oligosaccharides

III.4.2.1. Extraction

Food raw materials and products and some ingredients are complex, heterogeneous, biological materials. Thus, it is quite likely that they may contain substances that interfere with measurement of the mono- and oligosaccharides present, especially if a spectrophotometric method is used.

Interference may arise either:

- from compounds that absorb light of the same wavelength used for the carbohydrate analysis;
- from insoluble, colloidal material that scatters light, since light scattering will be measured as absorbance
- Also, the aldehydo or keto group of the sugar can react with other components, especially amino groups of proteins, a reaction (the nonenzymatic browning or Maillard reaction) that simultaneously produces color and destroys the sugar.

Thus, for determination of any mono- (glucose, fructose), di- (sucrose, lactose, maltose), tri- (raffinose), tetra- (stachyose), or other oligo- (maltodextrins) saccharides present, the dried, lipid-free sample is extracted with hot 80% ethanol (final concentration) in the presence of precipitated calcium carbonate to neutralize any acidity (AOAC Method 922.02, 925.05). This extraction is quite specific because almost all polysaccharides and proteins (as well as other polymers) are insoluble in 80% hot ethanol:

- Extraction is done by a batch process. Refluxing for 1 h, cooling, and filtering is standard practice. (A Soxhlet apparatus cannot be used because aqueous ethanol undergoes azeotropic distillation as 95% ethanol.)
- Extraction should be done at least twice to check for and ensure completeness of extraction.
- If the foodstuff or food product is particularly acidic (a low-pH fruit), a neutralization with precipitated calcium carbonate is routinely carried out before extraction to prevent the hydrolysis of acid-labile sucrose.

III.4.2.2. Purification

Since mono- and oligosaccharides are neutral, charged contaminants present in the 80% ethanol extract (ashes, pigments, organic acids, free amino acids, and low molecular weight peptides) can be removed using ion exchange techniques. Cleaning the ethanol extracts following the (AOAC 931.02C) method:

- Place a 50-ml aliquot of the ethanol extract in a 250-ml Erlenmeyer flask. Add 3 g of anion-exchange resin (hydroxide form) and 2 g of cation-exchange resin (acid form);
- Let it stand for 2 h with occasional swirling;
- The aqueous alcohol of the ethanol extract is removed under reduced pressure using a rotary evaporator and a temperature of 45–50°C;
- The residue is dissolved in a known, measured amount of water. Filtration should not be required, but should be used if necessary.

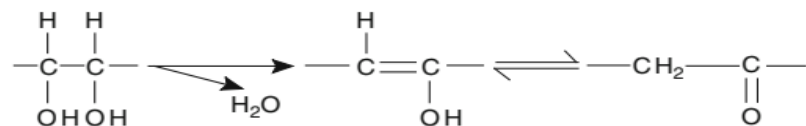
III.4.2.3. Techniques

III.4.2.3.1. Total carbohydrate: Phenol-Sulfuric acid method

Beyond its utility as a quantitative test, the phenol-sulfuric acid procedure is often used as a qualitative analysis for the presence of carbohydrates. Neither sorbitol nor any other alditol (polyol, polyhydroxyalcohol) gives a positive test.

III.4.2.3.1.1. Principle and characteristics

Carbohydrates are destroyed by strong acids and/or high temperatures to produce colored compounds whose intensity is determined by a spectrophotometer. Under these conditions, a series of complex reactions takes place, beginning with a simple dehydration reaction:



Continued heating in the presence of acid produces various furan derivatives, which then condense with themselves, as well as with various phenolic compounds (phenol) or various aromatic amines (aniline and *o*-toluidine) to produce brown and black substances..

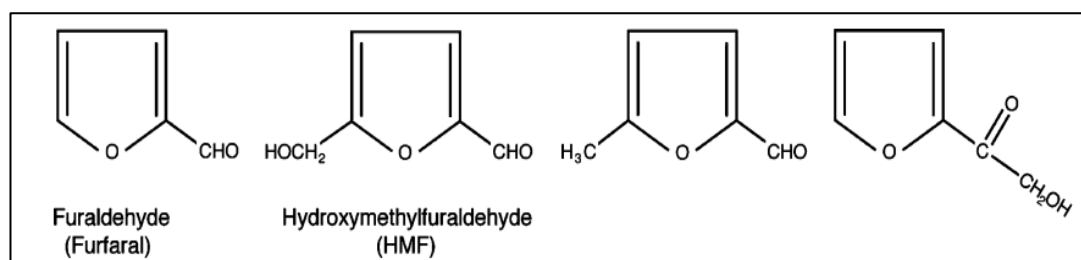


Figure 53. Furan products that could arise from, in order, pentoses and hexuronic acids, hexoses, 6-deoxyhexoses, and ketohexoses.

In practice, the most commonly used condensation is with phenol itself (AOAC method 44.1.30). This method is simple, rapid, sensitive, accurate, specific to carbohydrates, and widely applied. The reagents are inexpensive, readily available, and stable.

Furthermore, all classes of sugars, including sugar derivatives and oligo- and polysaccharides, can be determined using the phenol-sulfuric acid method (releasing monosaccharides through hot acid hydrolysis). A stable color is produced, and the results are reproducible. Under appropriate conditions, the phenol-sulfuric acid method is accurate to $\pm 2\%$.

The extent of reaction is, in part, a function of the structure of the sugar. Therefore, a standard curve of D-glucose is most often used. In any analysis, the concentrations used to construct the standard curve must cover the sample concentrations and extend beyond them (all sample concentrations must fall within the standard concentration range), and both must be within the limits reported for the method's sensitivity.

In any analysis, the concentrations used to construct the standard curve must span the sample concentrations and beyond (i.e., all sample concentrations must fall within the limits of

the standard concentrations), and both must be within the limits reported for sensitivity of the method. If not, dilutions should be used.

N.B :

Ideally, the standard curve will be prepared using mixtures of the same sugars present in the same ratio as they are found in the unknown. If this is not possible, for example, if a pure preparation of the sugar being measured is not available, or if more than one sugar is present either as free sugars in unknown proportions or as constituent units of oligo- or polysaccharides or mixtures of them, D-glucose is used to prepare the standard curve.

III.4.2.3.1.2. Procédure

- A clear, aqueous solution of carbohydrate(s) is transferred using a pipette into a small tube. A blank of water also is prepared;
- An aqueous solution of phenol is added, and the contents are mixed;
- Concentrated sulfuric acid is added rapidly to the tube so that the stream produces good mixing and the tube is agitated (Adding the sulfuric acid to the water produces considerable heat.). A yellow-orange color results;
- Absorbance is measured at 490 nm;
- The average absorbance of the blanks is subtracted, and the amount of sugar is determined by reference to a standard curve.

III.4.3. Total Reducing Sugar : Somogyi–Nelson Method

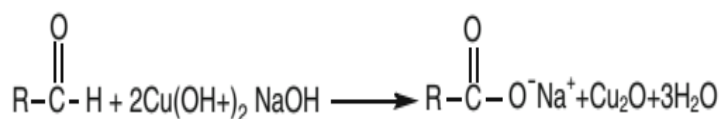
Oxidation is a loss of electrons; reduction is a gain of electrons. Reducing sugars are those sugars that have an aldehydo group (aldoses) that act as a reducing agent by giving up electrons to an oxidizing agent, which is reduced by receiving the electrons. Oxidation of the aldehydo group produces a carboxylic acid group.

A ketone group cannot be oxidized into a carboxylic acid group, and as a result, ketoses are not considered reducing sugars. However, under the used alkaline conditions, ketoses isomerize into aldoses, and therefore, they are measured as weak reducing sugars. The response is weaker with ketoses, so a standard curve made with D-fructose as one of the sugars in the sugar mixture should be used if it is present.

III.4.3.1. Principle

The Somogyi–Nelson (*or Nelson-Somogyi*) method is based on the reduction of Cu(II) ions to Cu(I) ions by reducing sugars. The Cu(I) ions then reduce an arsenomolybdate complex, prepared by reacting ammonium molybdate $[(NH_4)_6Mo_7O_{24}]$ and sodium arsenate (Na_2HAsO_7) in

sulfuric acid. Reduction of the arsenomolybdate complex produces an intense, stable blue color that is measured spectrophotometrically. This reaction is not stoichiometric and must be used with a standard curve of the sugar(s) being determined or D-glucose.



This method can be used in combination with enzymic methods for determination of oligo- and polysaccharides. In enzymic methods, specific hydrolases are used to convert the oligo- or polysaccharide into its constituent monosaccharide or repeating oligosaccharide units, which are measured using a reducing sugar method.

III.4.3.2. Procedure

- A solution of copper(II) sulfate and an alkaline buffer are added by pipettes to a solution of reducing sugars(s) and a water blank;
- The resulting solution is heated in a boiling water bath;
- A reagent prepared by mixing solutions of acidic ammonium molybdate and sodium arsenate is added;
- After mixing, dilution, and remixing, absorbance is measured at 520 nm;
- After subtracting the absorbance of the reagent blank, the A₂₅₀ is converted into glucose equivalents using a standard plot of micrograms of glucose vs. absorbance.

Remark

The dinitrosalicylic acid method will measure reducing sugars naturally occurring in foods or released by enzymes, but is not much used. In this reaction, 3,5-dinitrosalicylate is reduced to the reddish monoamine derivative :

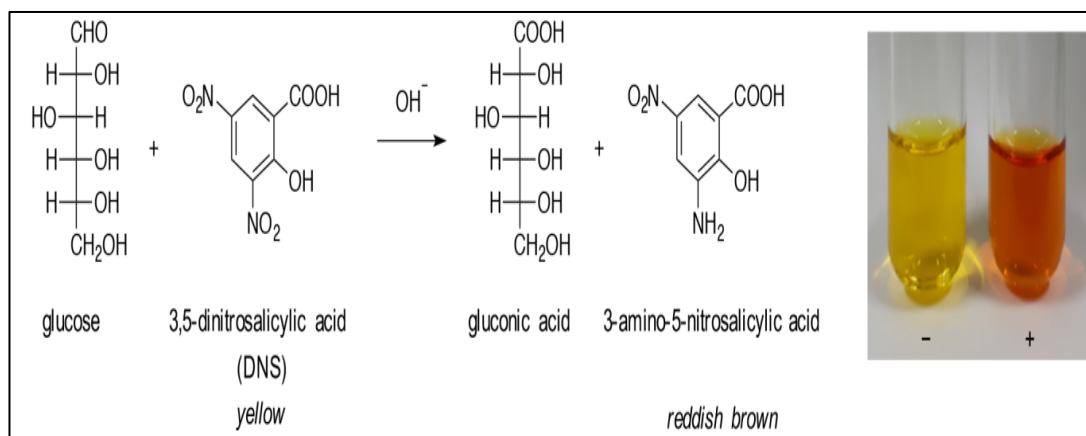


Figure 54. Reaction scheme with DNS and image of DNS reacted solution.

III.4.4. Specific analysis of mono- and oligosaccharides

III.4.4.1. High-performance liquid chromatography

HPLC is the method of choice for analysis of mono- and oligosaccharides or complex mixtures of both and can be used for analysis of polysaccharides after hydrolysis. HPLC gives both qualitative analysis (identification of the carbohydrate) and, with peak integration, quantitative analysis. HPLC analysis is rapid, can tolerate a wide range of sample concentrations, and provides a high degree of precision and accuracy, as it does not require any prior derivatization of carbohydrates, unlike GC of sugars.

III.4.4.2. Anion-exchange chromatography (AE-HPLC)

Carbohydrates have pK_a values in the pH range 12–14 and are, therefore, very weak acids. In a solution of high pH, some carbohydrate hydroxyl groups are ionized, allowing sugars to be separated on columns of anion-exchange resins. The general elution sequence is sugar alcohols (alditols), monosaccharides, disaccharides, and higher oligosaccharides.

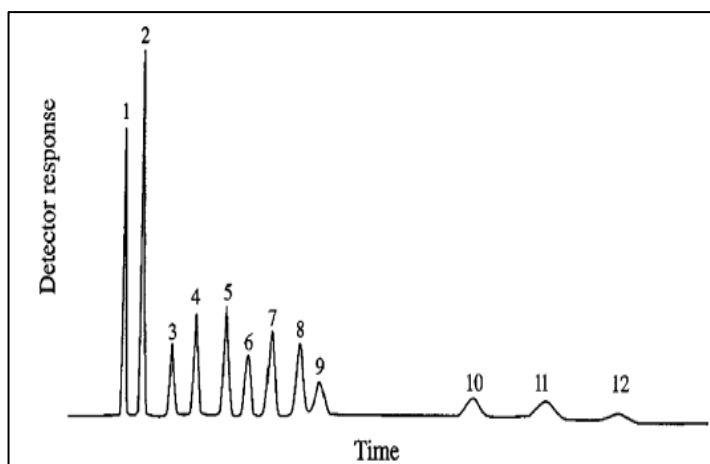


Figure 55. Chromatogram of some common monosaccharides, disaccharides, alditols, and the trisaccharide raffinose separated by anion-exchange chromatography: 1, glycerol; 2, erythritol; 3, L-rhamnose; 4, D-glucitol (sorbitol); 5, mannitol; 6, L-arabinose; 7, D-glucose; 8, Dgalactose; 9, lactose; 10, sucrose; 11, raffinose; 12, maltose.

III.4.4.3. Normal-phase chromatography

Normal phase chromatography is a widely used HPLC method for carbohydrate analysis; employs a polar stationary phase with elution using an increasingly polar mobile phase. Stationary phases known as amine-bonded phases, formed by a silica gel incorporated with amine groups, are commonly utilized with acetonitrile-water (50 to 85% acetonitrile) as the eluent, and they are effective in carbohydrate separations. The elution order is as follows: monosaccharides and sugar alcohols, followed by disaccharides and oligosaccharides.

III.4.4.4. Cation-exchange chromatography

Microparticulate spheres of sulfonated resin are used for cation exchange stationary phases, loaded with Ca^{2+} , Pb^{2+} , or Ag^{+} as metal counter-ions depending on the type of separation desired. The mobile phase used is water plus varying amounts (typically <40%) of an organic solvent such as acetonitrile and/or methanol. These columns normally are operated at elevated temperatures ($>80^{\circ}\text{C}$) to increase column efficiency by increasing the mass transfer rate between the stationary and mobile phases which effects peak narrowing and improved resolution.

Carbohydrate elution from cation-exchange resins takes place in the order of decreasing molecular weight: oligosaccharides with a degree of polymerization ($\text{DP}>3$), trisaccharides, disaccharides, monosaccharides, and alditols. The real strength of this stationary phase is in the separation of individual monosaccharides.

III.4.4.5. Reversed-phase chromatography

The stationary phase is hydrophobic, and the mobile phase is largely water. The hydrophobic stationary phase is made by reacting silica gel with a reagent that adds alkyl chains, such as an 18-carbon-atom alkyl chain (a C18 column) or a phenyl group (a phenyl column). Reversed phase chromatography has been used for separation of mono-, di-, and trisaccharides by groups.

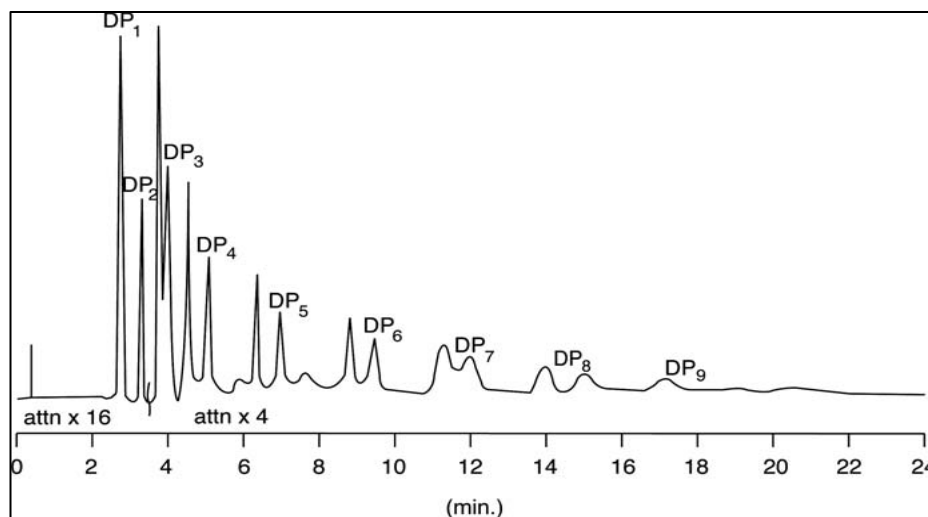


Figure 56. High-performance reverse-phase liquid chromatogram of maltodextrins (DP 1-9).

III.4.4.6. Gas Chromatography

Like HPLC, provides both qualitative and quantitative analysis of carbohydrates. For GC, sugars must be converted into volatile derivatives. The most commonly used derivatives are the

alditol peracetates and aldonic acid pertrimethylsilyl ethers from uronic acids. A flame ionization detector is the detector of choice for peracetylated carbohydrate derivatives.

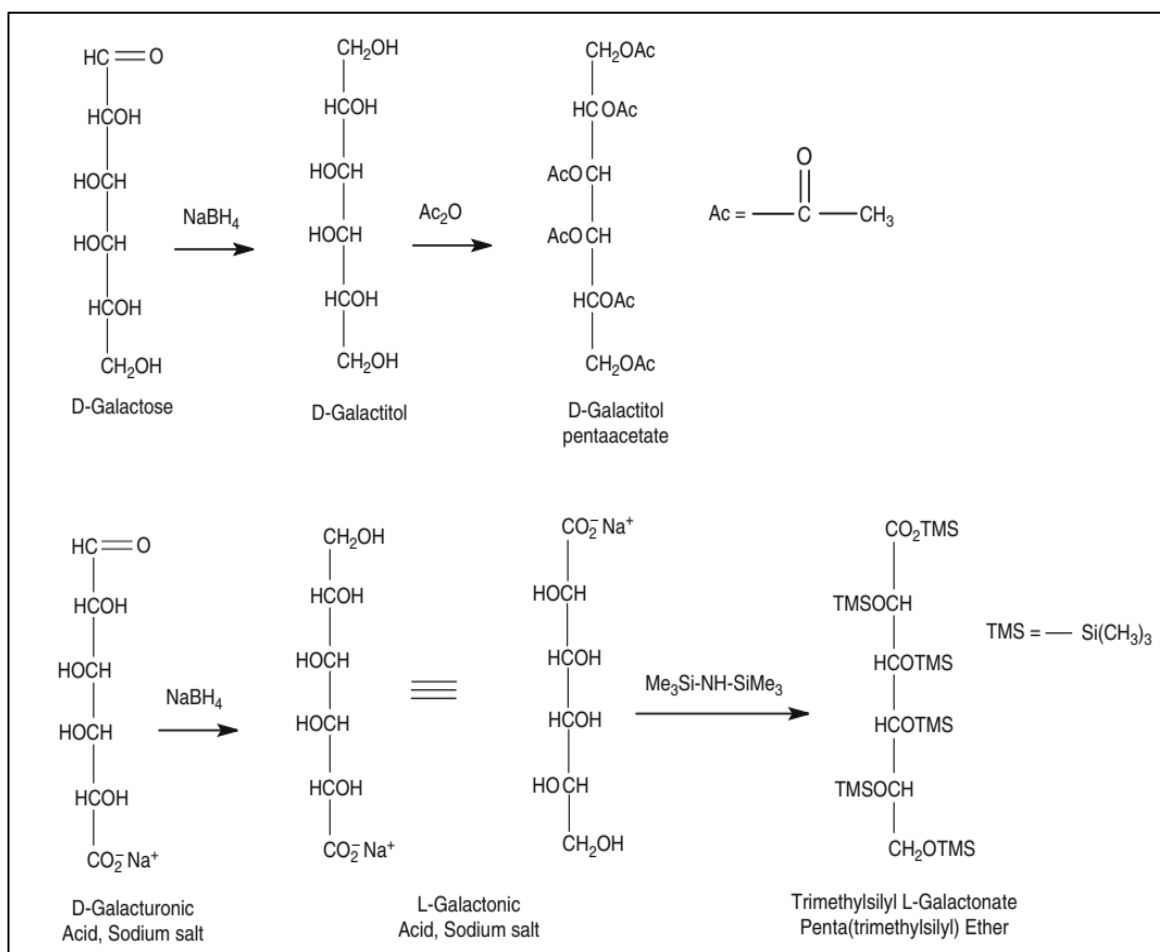


Figure 57. Modification of D-galactose and D-galacturonic acid in preparation for gas chromatography

Carbohydrate gas chromatography involves two preparation steps:

- Reduction of aldehyde groups to primary alcohol groups
- Conversion of the reduced sugar into a volatile peracetylate ester or a pertrimethylsilyl ether derivative.

Of course, for the analysis to be successful, each of these steps must be complete at 100% (stoichiometric).

III.4.5. Neutral sugars: Outline of Procedure

III.4.5.1. Reduction to alditols

Neutral sugars from the 80% ethanol extract or from hydrolysis of a polysaccharide are reduced with an excess of sodium or potassium borohydride dissolved in dilute ammonium hydroxide solution.

III.4.5.2. Acetylation of alditols

Acetic anhydride and 1-methylimidazole (as a catalyst) are added. After 10 min at room temperature, water and dichloromethane are added. The dichloromethane layer is washed with water and evaporated to dryness. The residue of alditol peracetates is dissolved in a polar organic solvent (usually acetone) for chromatography

III.4.5.3. GC of alditol peracetates

Alditol acetates may be chromatographed isothermally and identified by their retention times relative to that of inositol hexaacetate, inositol being added as an internal standard prior to acetylation. It is wise to run standards of the alditol peracetates of the sugars being determined with inositol hexaacetate as an internal standard.

Chapter IV: Micronutrients measurement

IV.1. Vitamins

IV.1.1. Importance of Analysis

Accurate food composition information is required to determine dietary intakes to assess diet adequacy and improve human nutrition worldwide. From the consumer and industry points of view, reliable assay methods are required to ensure accuracy of food labeling. This chapter provides an overview of techniques for analysis of the vitamin content of food and some of the problems associated with these techniques. Please note that the sections below on bioassay, microbiological, and chemical methods are not comprehensive, but rather just give examples of each type of analysis.

IV.1.2. Extraction Methods

Vitamin assays in most instances involve the extraction of a vitamin from its biological matrix prior to analysis. This generally includes one or several of the following treatments: heat, acid, alkali, solvents, and enzymes. In general, extraction procedures are specific for each vitamin and designed to stabilize the vitamin.

In some instances, some procedures are applicable to the combined extraction of more than one vitamin (thiamin and riboflavin, some of the fat-soluble vitamins). Typical extraction procedures are as follows:

- Ascorbic acid: Cold extraction with metaphosphoric acid/acetic acid;
- Vitamin B1 and B2: Boiling or autoclaving in acid plus enzyme treatment;
- Niacin: Autoclaving in acid (nongrain products) or alkali (grain products);
- Folate: Enzyme extraction with α -amylase, protease and γ -glutamyl hydrolase (conjugase)
- Vitamins A, E, or D: Organic solvent extraction, saponification, and re-extraction with organic solvents. For unstable vitamins such as these, antioxidants are routinely added to inhibit oxidation.
- Analysis of fat-soluble vitamins may require saponification, generally either overnight at room temperature or by refluxing at 70°C. In the latter case, an air-cooled reflux vessel provides excellent control of conditions conducive to oxidation.



Figure 58. Reflux vessel useful for saponification.

IV.1.3. Dosage Methods

Vitamin assays can be classified as follows:

1. Bioassays involving humans and animals;
2. Microbiological assays making use of protozoan organisms, bacteria, and yeast;
3. Physicochemical assays that include spectrophotometric, fluorometric, chromatographic, enzymatic, immunological, and radiometric methods.

IV.1.3.1. Bioassay Methods : Vitamin D Dosage

The biological assays are limited in their use as routine analysis, to cases where no satisfactory alternative method is available. Outside of vitamin bioavailability studies, bioassays at the present are used only for the analysis of vitamins B12 and D.

For the latter, it is the reference standard method of analysis of food materials (AOAC Method 936.14), known as the line test, based on bone calcification. Because the determination of vitamin D involves deficiency studies as well as sacrificing the test organisms, it is limited to animals rather than humans as test organisms.

VITAMIN D BIOASSAY PROCEDURE	
Sample Preparation	
AOAC International provides specific instructions for preparation of various matrices for the bioassay. In some cases, saponification is used.	
Depletion Period	
Rats are suitable for depletion at age ≤ 30 days with body weight of ≥ 44 g but ≤ 60 g. A rachitogenic diet is fed for 18–25 days.	
Assay Period	
The assay period is the interval of life of the rat between the last day of the depletion period and the eighth or eleventh day thereafter. Feeding protocols are specified. During the assay, depleted rats are fed known and unknown amounts of vitamin D from standards and samples, respectively.	
Potency of Sample	
Vitamin D in the sample is determined by the line test from staining of the proximal end of the tibia or distal end of the radius or ulna.	

Figure 59. The bioassay of vitamin D by the line test, AOAC Method 936.14, 45.3.01.

IV.1.3.2. Microbiological Assays

Microbiological assays are limited to the analysis of water-soluble vitamins. The methods are very sensitive and specific for each vitamin, but they are somewhat time consuming, and strict adherence to the analytical protocol is critical for accurate results. All microbiological assays can use microtiter plates (96-well) in place of test tubes. Microplate usage results in significant savings in media and glassware, as well as labor.

I.3.2.1. Principle

In microbiological assays the growth of a certain microorganism (bacteria, yeast, or protozoans) in an extract of a vitamin-containing sample is compared against the growth of this microorganism in the presence of known quantities of that vitamin.

Growth can be measured in terms of turbidity, acid production, gravimetry, or by respiration. With bacteria and yeast, turbidimetry is the most commonly employed system, in which clear sample and standard extracts vs. turbid ones, are essential.

With regard to incubation time, turbidity measurement is also a less time-consuming method. The microorganisms are specified by ATCCTM numbers and are available from the American Type Culture Collection (ATCCTM).

IV.1.3.2.2. Niacin (AOAC method 944.13, 45.2.04)

A stock culture of *Lactobacillus plantarum* ATCCTM 8014 needs to be prepared and maintained by inoculating the freeze-dried culture on Bacto Lactobacilli agar followed by incubation at 37°C for 24 h prior to sample and standard inoculation.

In general, growth is measured by turbidity or acidimetry if obtaining a clear sample extract before inoculation is not possible. However, it should be kept in mind that an extended incubation period of 72 h is necessary for acidimetry.

NIACIN MICROBIOLOGICAL ASSAY PROCEDURE Test Sample Preparation Weigh out a sufficient amount of sample to contain ≤ 5.0 mg niacin/ml, add volume of 1 N H₂SO₄ equal in ml to $\geq 10\times$ dry weight of test portion in g, macerate, autoclave 30 min at 121–123°C, and cool. If dissolved protein is not present, adjust mixture to pH 6.8 with NaOH solution, dilute with deionized H₂O to volume (ca. 0.1–0.4 μg niacin/ml), mix, and filter. Assay Tube Preparation In at least duplicate use 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ml test sample filtrate and make up the difference to 5.0 ml with deionized H₂O, then add 5.0 ml of DifcoTM Niacin Assay Medium to each tube, autoclave 10 min at 121–123°C, and cool. Niacin Standard Preparation Prepare assay tubes in at least duplicate using 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 ml standard working solution (0.1–0.4 μg niacin/ml), make up difference to 5.0 ml with deionized H₂O, then add 5.0 ml of DifcoTM Niacin Assay Medium and treat identically as the sample tubes. Inoculation and Incubation Prepare inoculum using <i>Lactobacillus plantarum</i> ATCCTM 8014 in DifcoTM Lactobacilli Broth AOAC. Add one drop of inoculum to each tube, cover tubes, and then incubate at 37°C for 16–24 hr; that is, until maximum turbidity is obtained as demonstrated by lack of significant change during a 2-hr additional incubation period in tubes containing the highest concentration of niacin. Determination Measure %T at any specific wavelength between 540 and 660 nm. Set transmittance to 100% with the inoculated blank sample. Prepare a standard concentration-response curve by plotting %T readings for each level of standard solution used against amount of reference standard contained in respective tubes. Determine the amount of niacin for each level of the test solution by interpolation from the standard curve.
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Figure 60. The microbiological assay of niacin, AOAC Method 944.13, 45.2.04.

IV.1.3.2.3. Folate (Vitamin B₉)

Folate is the general term including folic acid (pteroylglutamate, PteGln) and poly- γ -glutamyl conjugates with the biological activity of folic acid. Because of the presence of multiple forms in food products and its instability (labile to oxidation, light, thermal losses, and leaching when foods are processed), folate presents a rather difficult analytical problem.

IV.1.3.2.3.1. Principle

Folate in the sample, protected from oxidation and photochemical degradation by reducing agents including ascorbic acid, is extracted with a buffer at 100°C (boiling water bath). The extract is then digested with α -amylase and protease (to free macromolecularly bound folates) and conjugase to cleave poly- γ -glutamyl folates to PteGln3 or lower.) Growth response of the assay microorganism « *Lactobacillus casei* (spp. *rhannosus*) ATCC™ 7469 » is measured by percent transmittance depending on folate concentration. Results are reported as micrograms of vitamin per 100 g or per serving.

FOLATE MICROBIOLOGICAL ASSAY PROCEDURE Sample Preparation To 1.2–2.0 g of sample, add 50 ml of specified buffer, homogenize, and proceed to digestion step. (Note: High fat samples should be extracted with hexane, and all samples should be protected from light and air.) Trienzyme Digestion Boil samples for 5 min and cool to room temperature. Digest each sample with specified α-amylase, protease, and conjugase. Deactivate enzymes by boiling for 5 min. Cool tubes, filter, and dilute an appropriate aliquot to a final concentration of <i>ca.</i> 0.15 ng/ml. Preparation of Standard Curve and Blank Tubes Construct an 8-point standard curve using a working standard solution of folate. Add 5 ml of <i>Lactobacillus casei</i> (spp. <i>rhannosus</i>) ATCC™ 7469 assay medium to each tube. Prepare an uninoculated blank and an inoculated blank to zero the spectrophotometer, and an enzyme blank to determine the contribution of the enzymes to microbial growth. Assay Folic acid is assayed by the growth of <i>Lactobacillus casei</i> (spp. <i>rhannosus</i>) ATCC™ 7469 according to AOAC International (2). Prepared tubes of samples, standard curve, inoculated and uninoculated blanks, and enzyme blank are autoclaved at 121–123°C for 5 min and then inoculated with one drop of the prepared inoculum per tube. After tubes have been incubated at 37°C for 20–24 hr, the growth response is measured by percent transmittance at $\lambda = 550$ nm.

Figure 61. Analysis of folate in cereals and cereal products or other foods using *Lactobacillus casei* (spp. *rhannosus*) ATCC™ 7469 and a trienzyme extraction procedure

IV.1.3.3. Chemical Methods

IV.1.3.3.1. Vitamin A

Vitamin A is sensitive to ultraviolet (UV) light, air (and any prooxidants, for that matter), high temperatures, and moisture. Therefore, steps include using low actinic glassware, nitrogen, and/or vacuum, as well as avoiding excessively high temperatures. All work must be performed

in subdued artificial light, while the addition of an antioxidant (pyrogallol) at the onset of the procedure is highly recommended.

High-performance liquid chromatographic (HPLC) methods are considered the only acceptable methods to provide accurate food measurements of vitamin A activity.

The test sample is saponified with ethanolic KOH, vitamin A (retinol) is extracted into organic solvent and then concentrated. Vitamin A isomers – all-trans-retinol and 13-cis-retinol – levels are determined by HPLC on a silica column.

IV.1.3.3.2. Vitamin E (Tocopherols and Tocotrienols)

Vitamin E is present in foods as eight different compounds: all are 6-hydroxychromans. The vitamin E family is comprised of α -, β -, γ -, and δ -tocopherol, characterized by a saturated side chain of three isoprenoid units and the corresponding unsaturated tocotrienols (α -, β -, γ -, and δ -).

Details follow of vitamin E analysis in food products using HPLC:

1. General food products. The sample is saponified under reflux, extracted with hexane, and injected onto a normal phase HPLC column connected to a fluorescence detector, Ex λ = 290 nm, Em λ = 330 nm (Ex, excitation; Em, emission);
2. Margarine and vegetable oil spreads. The sample is dissolved in hexane, anhydrous MgSO_4 is added to remove water, and the filtered extracts are assayed by HPLC
3. Oils. Oil is dissolved in hexane and injected directly onto the HPLC column

Since Vitamin E is subject to oxidation, saponification is completed under reflux, in the presence of the antioxidant, pyrogallol, with the reaction vessel protected from light. Vitamin E is quantitated by external standards from peak area by linear regression.

VITAMIN E HPLC ANALYSIS PROCEDURE

Sample Preparation

- General food products:** Add 10 ml of 6% (w/v) pyrogallol in 95% ethanol to sample, mix, and flush with N₂. Heat at 70°C for 10 min with sonication. Add 2 ml of 60% (w/v) KOH solution, mix, and flush with N₂. Digest for 30 min at 70°C. Sonicate 5 min. Cool to room temperature, and add sodium chloride and deionized H₂O. Extract 3X with hexane (containing 0.1% BHT). Combine hexane extracts. Add 0.5 g of anhydrous MgSO₄ and mix. Filter through a Millipore filtration apparatus (0.45 µm). Dilute to volume with hexane. Inject sample into HPLC.
- Margarine and vegetable oil spreads:** Add 40 ml of hexane (containing 0.1% BHT) to a 10-g sample and mix. Add 3 g of anhydrous MgSO₄, mix, let stand ≥2 hr. Filter and dilute combined filtrate to volume with hexane (0.1% BHT). Inject sample into HPLC.

Chromatography Parameters

Column	Hibar® LiChrosorb Si 60 (4 mm × 250 mm, 5-µm particle size) and LiChromCART® 4-4 guard column packed with LiChrospher® Si 60 (5 µm)
Mobile Phase	Isocratic, 0.85% (v/v) 2-propanol in hexane
Injection Volume	20 µl
Flow	1 ml/min
Detector	Fluorescence, $E_x\lambda = 290\text{ nm}$, $E_m\lambda = 330\text{ nm}$
(Note: Determine recovery for each food product.)	

Figure 62. Analysis of vitamin E in food products using HPLC.

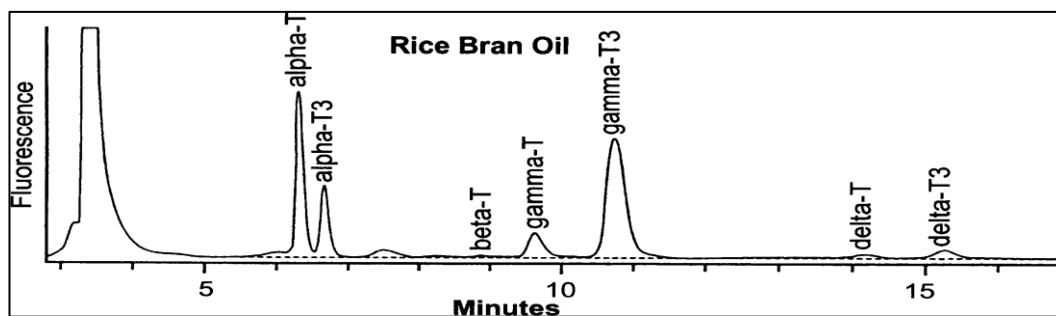


Figure 63. Chromatogram of rice bran oil showing tocopherols and tocotrienols.

IV.1.3.3.3. Vitamin C : 2,6-Dichloroindophenol titrimetric method (AOAC 967.21, 45.1.14)

The vitamin (L-ascorbic acid and L-dehydroascorbic acid) is very susceptible to oxidative deterioration, which is enhanced by high pH and the presence of ferric and cupric ions. For these reasons, the entire analytical procedure needs to be performed at low pH and, if necessary, in the presence of a chelating agent.

Mild oxidation of ascorbic acid results in the formation of dehydroascorbic acid, which is also biologically active and is reconvertible to ascorbic acid by treatment with reducing agents such as β -mercaptoethanol and dithiothreitol.

L-ascorbic acid is oxidized to L-dehydroascorbic acid by the oxidation–reduction indicator dye, 2,6-dichloroindophenol. At the endpoint, excess unreduced dye appears rosepink in acid solution.

Procedure

In the presence of significant amounts of ferrous Fe, cuprous Cu, and stannous Sn ions in the biological matrix to be analyzed, it is advisable to include a chelating agent such as ethylenediaminetetraacetic acid (EDTA) with the extraction to avoid overestimation of the ascorbic acid content. The light but distinct rose-pink endpoint should last more than 5 s to be valid.

With colored samples such as red beets or heavily browned products, the endpoint is impossible to detect by human eyes. In such cases it, therefore, needs to be determined by observing the change of transmittance using a spectrophotometer with the wavelength set at 545 nm.

VITAMIN C ASSAY PROCEDURE 2,6-DICHLOROINDOPHENOL TITRATION	
Sample Preparation	
Weigh and extract by homogenizing test sample in metaphosphoric acid-acetic acid solution (<i>i.e.</i> , 15 g of HPO_3 and 40 ml of HOAc in 500 ml of deionized H_2O). Filter (and/or centrifuge) sample extract, and dilute appropriately to a final concentration of 10–100 mg of ascorbic acid/100 ml.	
Standard Preparation	
Weigh 50 mg of USP L-ascorbic acid reference standard and dilute to 50 ml with HPO_3 - HOAc extracting solution.	
Titration	
Titrate three replicates each of the standard (<i>i.e.</i> , to determine the concentration of the indophenol solution as mg ascorbic acid equivalents to 1.0 ml of reagent), test sample, and blank with the indophenol reagent (<i>i.e.</i> , prepared by dissolving 50 mg of 2,6-dichloroindophenol sodium salt and 42 mg of NaHCO_3 to 200 ml with deionized H_2O) to a light but distinctive rose pink endpoint lasting ≥ 5 sec.	

Figure 64. Analysis of vitamin C by the AOAC Method 967.21, 45.1.14

IV.1.3.3.4. Thiamin (Vitamin B1) in foods, thiochrome fluorometric procedure (AOAC Method 942.23)

Following extraction, enzymatic hydrolysis of thiamin's phosphate esters and chromatographic cleanup (purification), this method is based on the fluorescence measurement of the oxidized form of thiamin, thiochrome, in the test solution compared to that from an oxidized thiamin standard solution.

THIAMIN ANALYSIS BY THIOCHROME PROCEDURE**Sample Preparation**

Weigh out sample containing thiamin, add volume of 0.1 M HCl equal in ml to $\geq 10X$ dry weight of test portion in g, mix, autoclave for 30 min at 121–123°C, then cool. Dilute with 0.1 M HCl to measured volume containing *ca.* 0.2–5 µg thiamin/ml.

Enzyme Hydrolysis

Take aliquot containing *ca.* 10–25 µg thiamin, dilute to *ca.* 65 ml with 0.1 M HCl and adjust pH to 4.0–4.5 with *ca.* 5-ml 2 M CH₃COONa. Add 5 ml of enzyme solution, mix, incubate for 3 hr at 45–50°C. Cool, adjust to *ca.* pH 3.5, dilute to 100 ml with deionized H₂O, and filter.

Sample Extract Cleanup

Apply an aliquot of the test sample extract containing *ca.* 5 µg thiamin to a specified ion-exchange resin column, and wash column with 3X 5-ml portions of almost boiling water. Then elute thiamin from the resin with 5X 4.0 to 4.5-ml portions of almost boiling acid-KCl solution. Collect the eluate in a 25-ml volumetric flask and dilute to volume with acid-KCl solution. Treat standards identically.

Oxidation of Thiamin to Thiochrome

To a test tube, add 1.5 g of NaCl and 5 ml of the thiamin · HCl standard solution (1 µg/ml). Add 3 ml of oxidizing reagent [*i.e.*, basic K₃Fe(CN)₆], swirl contents, then add 13 ml of isobutanol, shake vigorously, and centrifuge. Repeat steps for the standard blank but instead replace the oxidizing reagent with 3 ml of 15% (w/v) NaOH. Decant the isobutanol extracts (*i.e.*, the standard and blank) into fluorescence reading tubes, and measure at $E_x \lambda = 365 \text{ nm}$ and $E_m \lambda = 435 \text{ nm}$. Treat the test solution identically, and record the fluorescence intensity of the test sample and blank.

Figure 65. Analysis of thiamin (vitamin B1) by the thiochrome fluorometric procedure, AOAC Method 942.2.3, 45.1.05

Thiochrome is light sensitive, the analytical steps following the oxidation must be performed under subdued light. Given that Thiamin is sensitive to heat, especially at alkaline pH, the analytical steps beginning with the oxidation of thiamin through to the fluorescence measurement must be carried out rapidly and precisely according to the instructions.

The enzymatic treatment and subsequent chromatographic cleanup may not be necessary with certain matrices, such as vitamin concentrates that contain nonphosphorylated thiamin and no significant amounts of substances that could interfere with the determination

IV.1.3.3.5. Riboflavin (Vitamin B2) in Foods and Vitamin Preparations, Fluorometric Method (AOAC Method 970.65, 45.1.08)

Following extraction, cleanup, and compensation for the presence of interfering substances, riboflavin is determined fluorometrically

Due to the extreme sensitivity of the vitamin to UV radiation, all operations need to be conducted under subdued light. The analyst also needs to be aware that exact adherence to the permanganate oxidation process is essential for reliable results.

In spite of the fact that riboflavin is classified as a water-soluble vitamin, it does not readily dissolve in water. When preparing the standard solution, the analyst must pay special attention and ensure that the riboflavin is completely dissolved

RIBOFLAVIN ASSAY PROCEDURE BY FLUORESCENCE Sample Preparation Weigh out homogenized sample, add volume of 0.1 M HCl equal in ml to $\geq 10X$ dry weight of test portion in g; the resulting solution must contain ≤ 0.1 mg riboflavin/ml. Mix contents, autoclave for 30 min at 121–123°C and then cool. Precipitate interfering substances by adjusting pH to 6.0–6.5 with dilute NaOH immediately followed by a pH readjustment to 4.5 with dilute HCl. Dilute with deionized H₂O to <i>ca.</i> 0.1 µg of riboflavin/ml, and filter. Oxidation of Interfering Materials Oxidize as follows: Transfer 10 ml of test filtrate to each of four tubes. To two of these tubes, add 1.0 ml of deionized H₂O, and to the remaining ones add 1.0 ml of a standard solution (<i>i.e.</i>, 1 µg/ml of riboflavin). Then to each tube, one at a time, add 1.0 ml of glacial HOAc followed by 0.5 ml of 4% (w/v) KMnO₄. Allow the mixture to stand for 2 min, and then add 0.5 ml of 3% (v/v) H₂O₂. Shake vigorously until excess O₂ is expelled. Measurement of Fluorescence Measure fluorescence at $E_x \lambda = 440$ nm and $E_m \lambda = 565$ nm. First read test samples containing 1 ml of added standard riboflavin solution, and then samples containing 1 ml of deionized H₂O. Add, with mixing, 20 mg of Na₂S₂O₄ to two of the tubes, and measure the minimum fluorescence within 5 sec.
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Figure 66. Analysis of riboflavin (vitamin B2) by fluorescence, AOAC Method 970.65, 45.1.08

IV.2. Minerals

Calcium, phosphorus, sodium, potassium, magnesium, chlorine, and sulfur make up the dietary macro minerals, those minerals required at more than 100 mg/day by the adult. An additional ten minerals are required in milli- or microgram quantities per day and are referred to as trace minerals. These include iron, iodine, zinc, copper, chromium, manganese, molybdenum, fluoride, selenium, and silica.

There is also a group of minerals called ultra-trace minerals, including vanadium, tin, nickel, arsenic, and boron, that are being investigated for possible biological function, but that currently do not have clearly defined biochemical roles.

Some mineral elements have been documented to be toxic to the body and should, therefore, be avoided in the diet. These include lead, mercury, cadmium, and aluminum. Essential minerals such as fluoride and selenium also are known to be harmful if consumed in

excessive quantities, even though they do have beneficial biochemical functions at proper dietary levels.

IV.2.1. Basic considerations

IV.2.1.1. Nature of analyses

Separation of minerals from the food matrix is often specific, such as complexometric titrations or precipitation titrations. In these cases of specific separation, nonspecific measurements such as volume of titrant are made and are later converted to mass of mineral based on fundamental stoichiometric relationships.

In other cases, nonspecific separation procedures such as ashing or acid extraction, require that a specific measurement be made as provided by colorimetry, ion-selective electrodes (ISE), atomic absorption spectroscopy, or inductively coupled plasma-atomic emission spectroscopy

IV.2.1.2. Sample Preparation

Sample preparation is generally required to ensure a well-mixed and representative sample ready for the procedure to follow:

- Contamination during sample preparation, during comminution (grinding or chopping) and mixing using metallic instruments can add significant mineral to samples. This step should be performed using nonmetallic instruments or instruments not composed of the sample mineral;
- Glassware used in sample preparation and analysis should be scrupulously cleaned using acid washes and triple rinsed in the purest water;
- Solvents, including water, can contain significant quantities of minerals and require the use of the purest reagents available. When the cost of ultrapure reagents may be prohibitive, the alternative is to always work with a reagent blank. A reagent blank is the same as that used in the sample but without any of the material being analyzed. It represents the sum of the mineral contamination in the reagents, and is then subtracted from the sample values to more accurately quantify the mineral.

IV.2.1.3. Interferences

Factors such as pH, sample matrix, temperature, and other analytical conditions and reagents can interfere with the ability of an analytical method to quantify a mineral.

Often there are specific interfering substances that must be removed or suppressed for accurate analysis using either of the two most common approaches:

- Isolate the sample mineral;
- Remove interfering minerals, using selective precipitations or ion exchange resins: boil water to remove carbonates that interfere with several traditional methods of mineral analysis.

If other interferences are suspected, it is a common practice to develop the standard curve using sample mineral dissolved in a background matrix containing interfering elements known to be in the food sample.

Example: if a food sample is to be analyzed for calcium content, a background matrix solution of the known levels of sodium, potassium, magnesium, and phosphorus should be used to prepare the calcium standards for developing the standard curve.

Alternatively, the standard curve can be developed using a series of sample mineral spikes added to the food sample. A spike is a small volume of a concentrated standard that is added to the sample without appreciably changing the overall composition of the sample, except for the mineral of interest. Thus, measurements of both the standards and the sample are made in the presence of the same background. If the spikes are added before implementation of the analysis protocol, possible effects of incomplete extractions, sample mineral degradation, and other losses are integrated into the standard curve.

IV.2.2. Methods

IV.2.2.1. EDTA complexometric titration

EDTA complexometric titration is suitable for determining calcium in the ash of fruits and vegetables (AOAC Method 968.31).

IV.2.2.1.1. Principles

The hexadentate ligand **ethylenediaminetetraacetate** (EDTA) forms stable 1:1 complexes with numerous mineral ions, providing them a broad application in mineral analysis. Stability of mineral–EDTA complexes generally increases with valence of the ion, although there is significant variation among ions of similar valence due to their coordination chemistry:

- Endpoints are detected using mineral chelators that have coordination constants lower than EDTA (less affinity for mineral ions) and that produce different colors in each of their complexed and free states. Calmagite and Eriochrome Black T (EBT) are such indicators that change from blue to pink when they complex with calcium or magnesium. The endpoint of a complexometric EDTA titration using either Calmagite or EBT as the indicator is detected as the color changes from pink to blue;

- The pH affects a complexometric EDTA titration in several ways and must be controlled for best performance. As the pH decreases, the chelating sites of EDTA become protonated, thereby reducing its effective concentration. EDTA complexometric titration of calcium and magnesium is specified at $\text{pH } 10 \pm 0.1$ using an ammonia buffer.

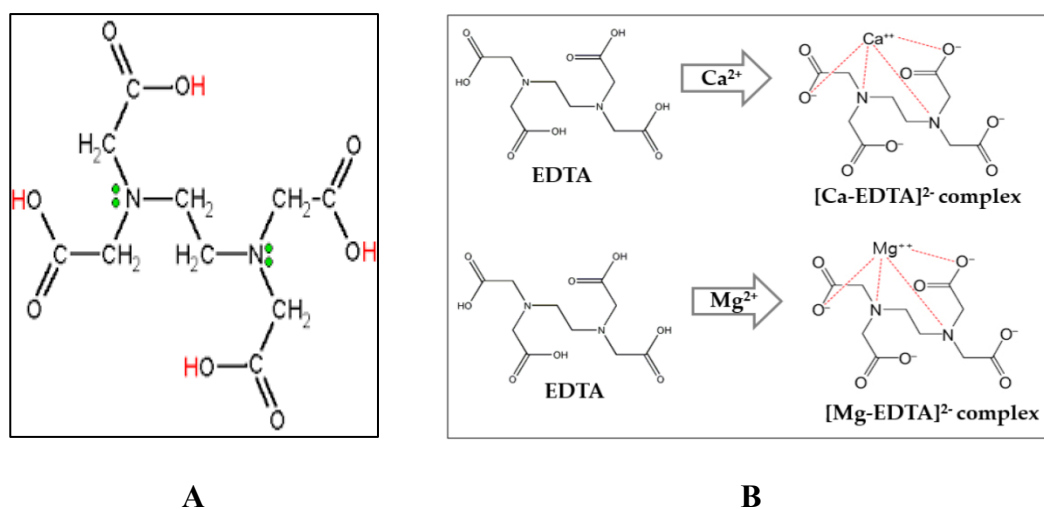


Figure 67: A) Structure of EDTA, B) Chelation of calcium and magnesium by EDTA.

IV.2.2.1.2. Procedure

The calcium– Calmagite complex is not stable, and calcium alone cannot be titrated using the Calmagite indicator. However, Calmagite becomes an effective indicator for calcium titration if we include in the buffer solution a small amount of neutral magnesium salt and enough EDTA to bind all magnesium.

Upon mixing sample into the buffer solution, calcium in the sample replaces the magnesium bound to EDTA. The free magnesium binds to Calmagite, and the pink magnesium– Calmagite complex persists until all calcium in the sample has been titrated with EDTA. The first excess of EDTA removes magnesium from Calmagite and produces a blue endpoint.

IV.2.2.2. Precipitation titration: Mohr method

Precipitation titration methods are well suited for any foods that may be high in chlorides. An automatic titration system commonly used to rapidly measure the salt content of potato chips is simply doing a Mohr titration.

IV.2.2.2.1. Principle

When at least one product of a titration reaction is an insoluble precipitate, it is referred to as precipitation titrimetry. The Mohr method for chloride determination is a direct or forward

titration method, based on the formation of an orange-colored solid, silver chromate, after silver from silver nitrate has complexed with all the available chloride:

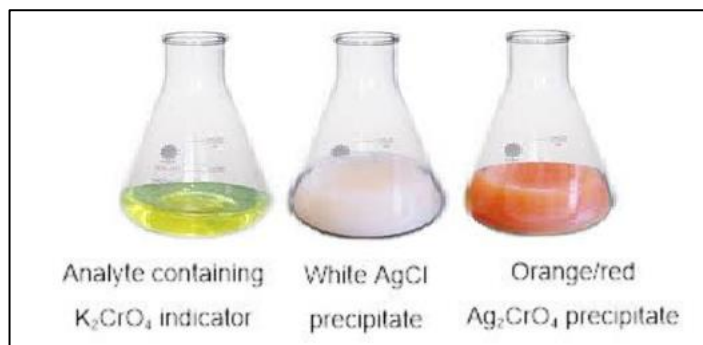
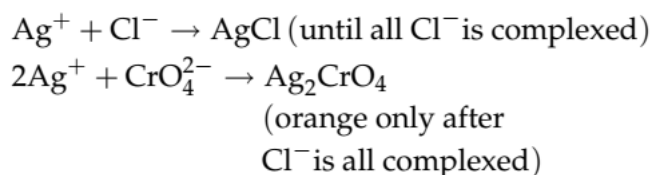


Figure 68. Color change during the titration of Cl^- using the Mohr method.

IV.2.3. Colorimetric methods

Colorimetry is used for the detection and quantification of a wide variety of minerals in food, and it is often a viable alternative to atomic absorption spectroscopy. Colorimetric methods generally are very specific, robust and often immune to matrix effects and to the presence of other minerals.

IV.2.3.1. Iron dosage

Chromogens are chemicals that, upon reaction with the compound of interest, form a colored product that can be quantified by absorption of light at a specified wavelength (562 nm for Fe). Generally, concentration of mineral is determined from a standard curve developed during the analysis

Samples generally must be ashed or treated in some other manner to isolate and/or release the minerals from organic complexes that would otherwise inhibit their reactivity with the chromogen:

- The mineral of interest must be solubilized from a dry ash and subsequently handled in a manner that prevents its precipitation;
- The soluble mineral may need to be treated (e.g., reduced or oxidized) to ensure that all mineral is in a form that reacts with the chromogen.

Another interesting aspect of this method of interest to the food scientist is the fact that the ferrozine reagent only reacts with ferrous iron (Fe^{2+}), and not ferric (Fe^{3+}). The addition of ascorbic acid in the second to last step is necessary to convert all ionic iron to the detectable ferrous form. Repeating the procedure with and without ascorbic acid allows determination of total and ferrous ionic iron, respectively. Ferric iron is calculated by difference.

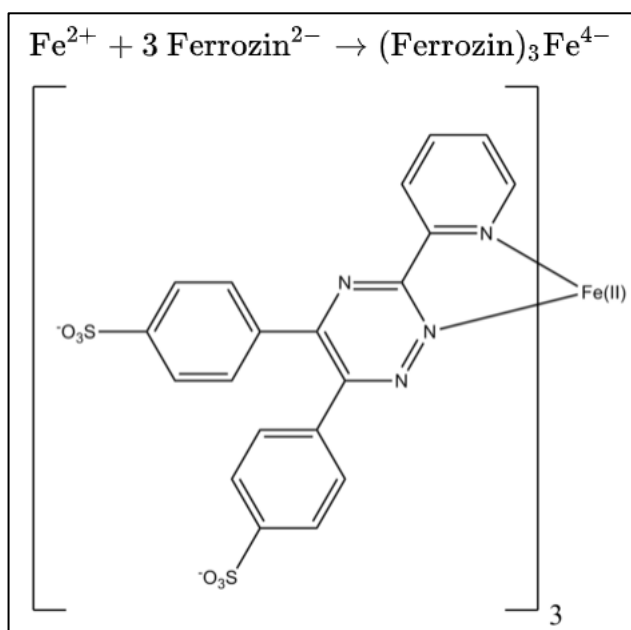


Figure 69. Ferrozine-Fe(II) complex

IV.2.4. Ion-Selective Sensor (ISE)

Many electrodes have been developed for the selective measurement of various cations and anions, such as bromide, calcium, chloride, fluoride, potassium, sodium, and sulfide.

Despite inherent limitations of electrode design and construction, electrode performance is affected by:

- The presence of interfering ions, often those having size and charge similar to the ion of interest. Relative response of ISE to interfering ions may be expressed as selectivity coefficients;
- Most ISEs operate over a broad pH range, although pH may need to be controlled for best performance;
- Because the ISE responds to ionic activity, it is important that the activity coefficient relating ion activity to ion concentration ($A = \gamma C$), be kept constant in samples and calibration standards;

- It is important to keep the electrode and solutions at a constant temperature (effect on potentials), and allowing sufficient time for all samples and standards to equilibrate to this temperature (25°C is one of the internationally accepted temperatures for electrochemical measurements);
- Solutions should be gently stirred during the measurement to attain rapid equilibrium and to minimize concentration gradients at the sensor surface;
- Finally, it is important to allow sufficient time for the electrode to stabilize before taking a reading. Minimum response times for ISEs fall in the range of 20 s to 1 min.

IV.2.4.1. Electrode calibration and determination of concentration

In using an ISE, ion concentration can be determined using either a calibration curve, standard addition, or endpoint titration.

IV.2.4.1.1. Calibration curve

Calibration curve allows a large number of samples to be measured rapidly. Upon analysis of a test sample, the observed electrode potential is used to determine ion concentration by referring to the calibration curve. Total ionic strength and the concentration of interfering ions are especially important factors limiting selective detection of low levels of ions (Note the nonlinear region of the curve at the lowest concentrations).

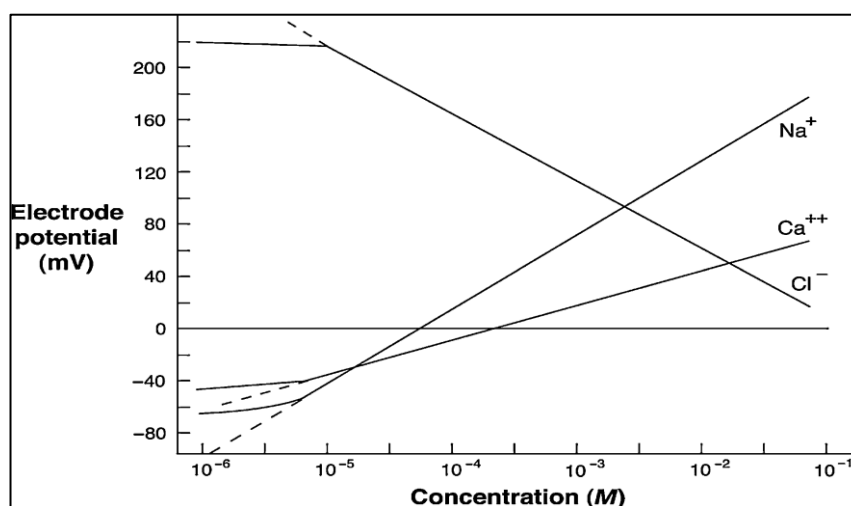


Figure 70. Examples of ion-selective electrode calibration curves for ions important in foods.

IV.2.4.1.2. The standard addition method

This method is of great value when only a few samples are to be measured and time does not permit the development of a calibration curve. It also eliminates complex and unknown

background effects that cannot be replicated when developing a calibration curve using standards:

- The ISE is immersed in the sample and the resulting voltage is recorded (E_{sample});
- An aliquot, or spike, containing a known amount of the measured species is added to the sample, and a second measurement of electrode potential is determined (E_{spike});
- Concentration of active species in the original sample is determined from the absolute difference in the voltage readings ($\Delta E = |E_{\text{spike}} - E_{\text{sample}}|$) according to the following relationship algebraically derived from the Nernst equations:

$$C_O = \frac{C_\Delta}{(10^{\Delta E/S} - 1)}$$

Where :

C_O = original concentration of sample ion (mol/L), C_Δ = change in sample ion concentration when spike was added (mol/L), ΔE = difference in potential between the two readings (V), $S = 0.059/\text{number of electrons in the half reaction of the sample ion}$

IV.2.4.1.3. Detection of endpoints of titrations

This is done by using species that form a precipitate or strong complex with the sample ion. If an ISE is selected that detects

- titrant species, a T-type titration curve results from the large increase in titrant activity detected at the equivalence point;
- the sample ion, an S-type titration curve results from the removal of sample ion activity at the equivalence point.

In either case, sample concentration is calculated from titrant volume to reach the equivalence point and the stoichiometric relationship between titrant species and sample ion.

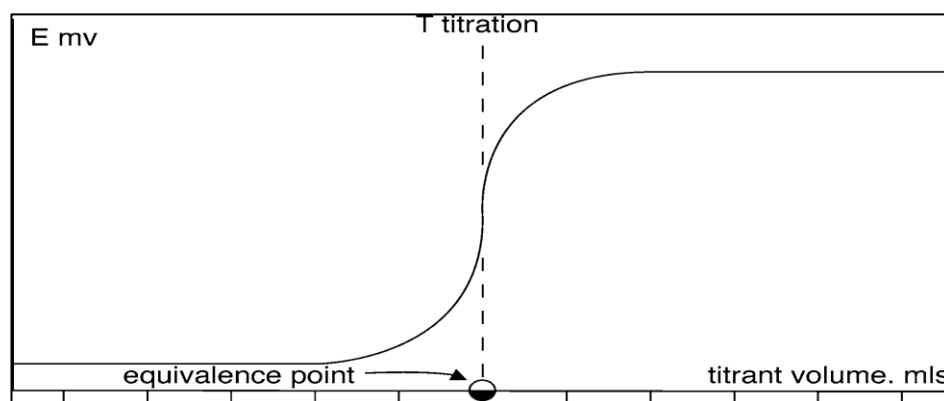


Figure 71. A typical T-type titration.

IV.3. Provitamin A (carotenoids) and polyphenols

IV.3.1. Methods for quantifying carotenoid pigments

All classes of carotenoids (hydrocarbons: carotenes and lycopenes, and oxygenated xanthophylls) are lipophilic compounds and are therefore soluble in organic solvents. They are moderately heat-stable and are prone to color loss through oxidation. Additionally, they can be easily isomerized by heat, acid, or light:

- Procedures for extracting carotenoids from tissues use organic solvents that need to penetrate a hydrophilic matrix. Hexane-acetone mixtures are commonly used for this purpose. Numerous chromatographic procedures, including HPLC, have been developed to separate carotenoids;
- Since their color ranges from yellow to red, the detection wavelengths typically range from about 430 to 480 nm. Higher wavelengths are generally used for certain xanthophylls to avoid chlorophyll interferences;
- Specific analytical challenges arise when carotenoid esters, cis/trans isomers, and optical isomers need to be separated and identified.

IV.3.2. Polyphenols

The chemical nature of plant phenolic compounds varies from simple substances to highly polymerized ones, including variable amounts of phenolic acids, phenylpropanoids, anthocyanins, and tannins. Thus, possible interactions of phenolic compounds with carbohydrates, proteins, and other plant components also contribute to the complexity.

Consequently, phenolic extracts from plant materials always consist of a mixture of different classes soluble in the solvent system used. Additional steps are necessary to eliminate unwanted phenolic and non-phenolic substances.

The extraction of polyphenols is influenced by:

- The nature of the solvent: methanol, ethanol, acidified methanol, acetone, water, and their combinations are commonly used solvents;
- The extraction period: Extraction times ranging from 1 minute to 24 hours have been reported. Longer extraction times increase the risk of phenolic compound oxidation unless reducing agents are added to the solvent system;
- The sample-solvent ratio (R): Ratios of 1:5 to 1:10 are very common.

IV.3.2.1. Determination of Total Phenols: Folin–Ciocalteu Technique

This is the most widely used procedure for quantifying total phenolic compounds in plant materials. The reduction of phosphomolybdic-phosphotungstic acid (Folin-Ciocalteu reagent) by the electrons of phenolic compounds forms a blue complex in an alkaline solution, which is detected at 760 nm. The phenolic content is calculated in equivalents of the standard.

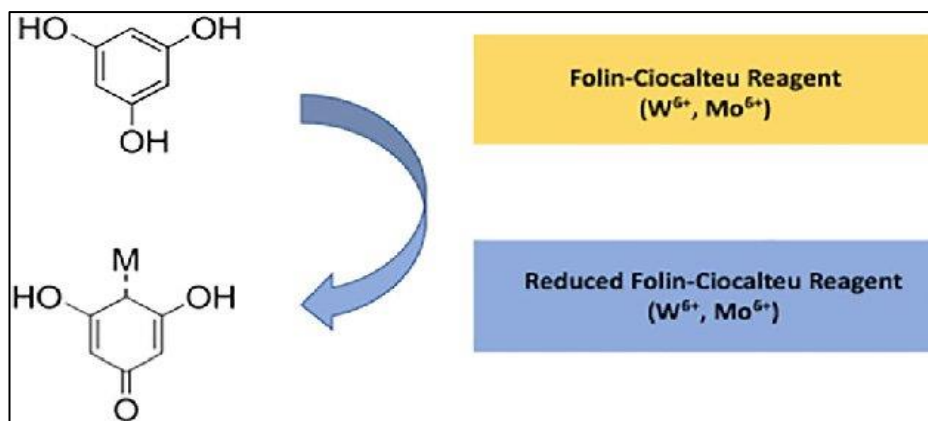


Figure 72. Reduction of the Folin-Ciocalteu reagent caused by the oxidation of phenolic compounds.

This method lacks sensitivity, and its precision is questionable due to the interference of other substrates (soluble sugars, proteins, thiols, ascorbic acid, Fe²⁺, Mn²⁺, I⁻ et SO³⁺) that can react with F-C reagents, leading to inaccurate values.

Chapter V: Aromas

V.1. Extraction

V.1.1. Headspace SPME (Headspace Solid Phase Microextraction)

The SPME is the most widely used aroma extraction technique today. It employs a coated fused silica fiber mounted inside a device resembling a syringe. SPME fibers can be coated with an absorbing material (volatile substances adhere to the fiber's surface) or an adsorbing material (volatile substances are trapped in the pores of the stationary phase), or a combination of both.

Although the fiber can be directly immersed in a liquid sample for aroma extraction, it is more appropriate to obtain an extract from the headspace above the food sample because non-volatile substances present in the food, especially lipids, can contaminate the fiber and render it unsuitable for use.

During SPME extraction, there is a balance between the sample, headspace, and the fiber for all volatile compounds in the sample. By minimizing the headspace volume, the amount of material absorbed/adsorbed on the fiber can be maximized. Other means of increasing sensitivity include adding salt and agitating the sample.



Figure 73. Solid Phase Microextraction (SPME) showing the fiber in the raised and lowered positions.

One of the sampling methods involves both the extraction and GC-MS steps:

- Immediately before extraction, the vials are transferred to an incubator where the sample is heated to a desired temperature for a defined period;
- After pre-incubation, the SPME syringe needle penetrates the septum of the screw cap, and the fiber is lowered for extraction;
- Before and during extraction, a motor in the incubator shakes the sample vials, increasing the sample surface area and facilitating the extraction process;

- After extraction, the fiber is retracted and then desorbed in the gas chromatograph injection port, which contains a specific narrow-bore coating for SPME.

V.1.2. Solvent-assisted flavor evaporation (SAFE)

The SAFE is widely regarded as providing the most representative aroma extracts from the foods they originate from. It has evolved from high-vacuum transfer (HVT) techniques, where volatile substances are separated from non-volatile substances in a vacuum system by transferring them from a relatively high-temperature zone to a very low-temperature zone.

SAFE is more efficient and allows better thermal control and a more compact arrangement of glassware compared to HVT, resulting in higher yields of polar and high-boiling-point compounds.

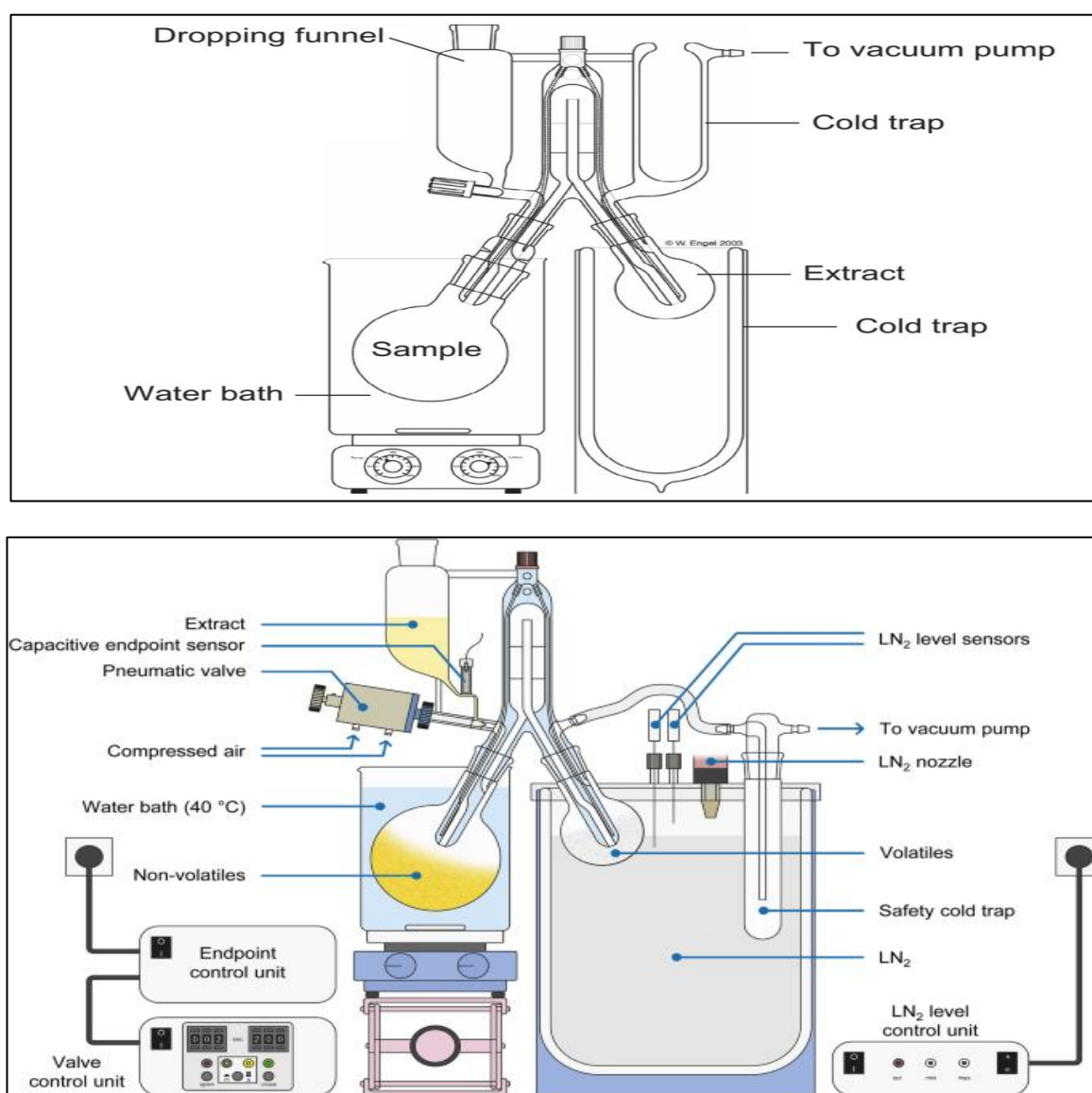


Figure 74. Solvent-Assisted Flavor Evaporation (SAFE)

In a typical SAFE procedure:

- An extract of the food is prepared by shaking it with 2 to 3 volumes of solvent at room temperature. After extraction, the solvent is decanted from the sample and filtered;
- Diethyl ether is widely used as the extraction solvent, although dichloromethane is also an option (more effective for dissolving more polar substances);
- If a large amount of solvent has been used in the extraction process, it may be necessary to concentrate the extract by fractional distillation before conducting SAFE;
- The extract moves from the dropping funnel into the distillation chamber of the SAFE, which is under high vacuum (10^{-4} et 10^{-5} mbar) and heated between 20 and 30°C;
- The volatile matter from the extract immediately evaporates and condenses on the walls of the collection vessel submerged in liquid nitrogen. Non-volatile substances remain in the distillation chamber;
- The entire extraction process takes about 45 minutes. After extraction, the condensate is removed from the collection vessel and concentrated for analysis using a Claisen-Vigreux column, resulting in a final volume of approximately 1 mL. Additional concentration can be achieved by bubbling a stream of nitrogen over the extract.

V.1.3. Solid-phase extraction (SPE)

SPE is a widely used analytical technique for cleaning non-volatile organic compounds in liquid samples. It is a one-step extraction and concentration procedure that yields a sample suitable for analysis, typically by high-performance liquid chromatography (HPLC):

- A sorbent material (e.g., styrene-divinylbenzene polymer) in a small plastic or glass cartridge is conditioned with a solvent;
- The liquid sample is then loaded onto the sorbent; the compounds of interest are retained on the sorbent as the liquid passes through (e.g., dichloromethane, ethyl acetate), and the sorbent is subsequently dried;
- There are then two different ways to proceed:
 - ✓ The sorbent can be washed to remove impurities, followed by elution of the compounds of interest with a second solvent;
 - ✓ Alternatively, the compounds of interest could be eluted directly, leaving impurities on the sorbent.

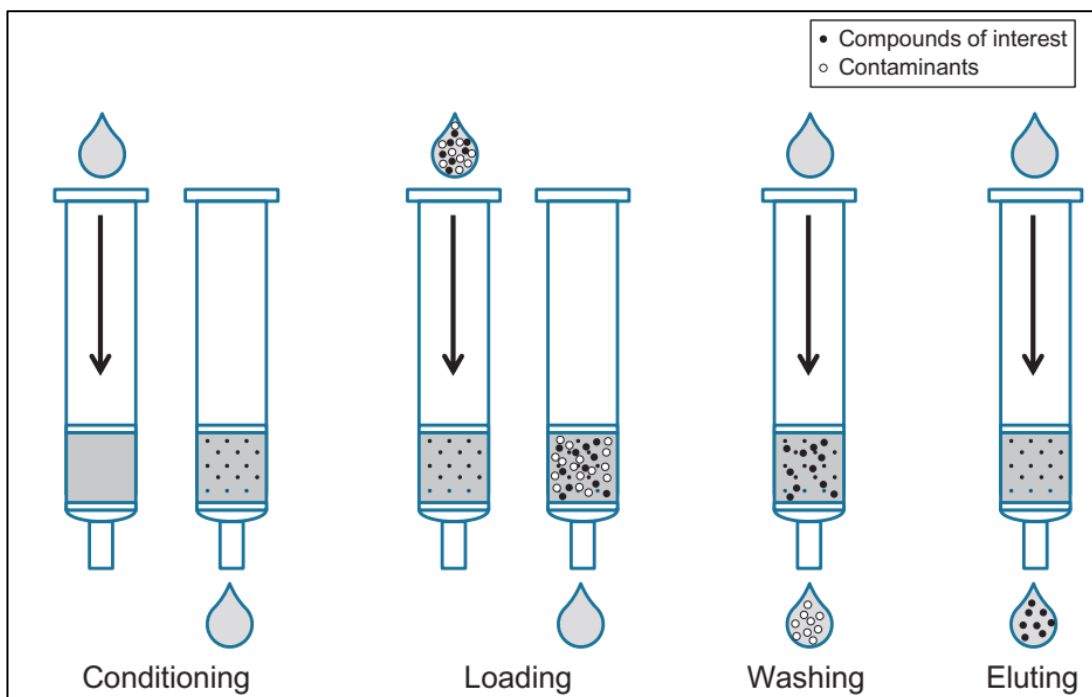


Figure 75. Solid-Phase Extraction of Aromatic Compounds.

V.2. Analysis of Aromas

There are two common ways to establish relationships between a sample's odor and a chemical compound:

- One involves finding statistical associations between data obtained from sensory analysis and GC-MS analysis for the specific sample or volatile compounds;
- The other involves using trained panelists sniffing at GC-MS ports to detect and identify volatile compounds that are also identified by sensors and computer programs (GC-MS Olfactometry).

Furthermore, an odorous compound can be identified through direct relationships established by comparing the sample's odor to a number of volatile compounds with a similar smell, requiring knowledge of odorous volatile compounds.

V.2.1. Gas chromatography–Olfactometry or GC-sniffing

The GC-sniffing, is essential for determining which aromatic compounds are likely to contribute to the aroma of a food and forms the basis of the sensomic approach. After the appropriate extraction of aromatic compounds, the extract's components are separated by gas chromatography. As they elute from the column, they are assessed by trained individuals who describe the aroma and estimate its intensity.

Note: The sensomic approach is the procedure that identifies and quantifies key odorants in a food matrix, thus defining their sensory impact on the overall aroma of the food.

GCO is an important analytical tool for flavorists, perfumers, and food scientists to understand the roles of volatile compounds in complex aromas. The chromatographic part of this system involves sampling, extraction, separation, and instrumental detection of volatiles. The human aspect focuses on subjective detection, quality, intensity, and temporal dynamics of volatiles.

Despite the chromatographic technicalities, GCO techniques can be classified based on their procedure and the obtained data. These techniques include dilution analysis, intensity evaluation, time-intensity evaluation, and frequency of detection methods:

V.2.1.1. Dilution analysis methods

There are two major variants: Aroma Extract Dilution Analysis (AEDA) and CHARM analysis. They determine the potency of individual volatiles by serially diluting extracts until the volatiles are no longer detectable by subjects:

V.2.1.1.1. Aroma extract dilution analysis « AEDA »

This method involves evaluating an aroma extract using GC-O to identify all odor compounds present in the extract. Subsequently, GC-O is repeated on a series of dilutions until only the most potent aromatic compounds are detected. The compounds persisting through the final dilutions are deemed to be those that contribute to the aroma of the food.

It uses flavor dilution factors (FD values), the ratio between concentrations of volatiles in the initial extract and the final concentration of volatiles detected by subjects, to determine the potency of volatiles. The obtained aromagrams are plots between FD values (y-axis) and the Kovats index (related to retention time) (x-axis).

Aroma dilution factors

The relative aroma dilution factor (FD) is defined as the number of times the original extract can be diluted before the aroma is lost by GC-O. For example, in a series of dilutions where the original extract is serially diluted on a 1:1 basis, compounds:

- losing their aroma after the first dilution have an FD factor of 2;
- losing their aroma after the second dilution have an FD factor of 4;
- and those that persist for an additional dilution have an FD factor of 8.

It obviously depends on the concentration of the original extract, but FD factors of 1024 or 2048 are not uncommon, and serial dilutions based on a 1:2 dilution can be used to reduce the number of required GC-O evaluations (FD factors become 3, 9, 27, etc.).

In practice, typically only one or two trained evaluators are used for AEDA. However, it is currently recommended, especially when a wide range of odorants is present, to use more evaluators and fewer dilutions for better efficiency (minimizing standard deviation with the minimum number of GC-O cycles). Dilutions of 1:10 and a larger pool of assessors is recommended.

V.2.1.1.2. CHARM Analysis (combined hedonic of aromatic response measurement)

Based on the same principle, this analysis determines the potency of volatiles and expresses it in CHARM values (c). The CHARM value integrates the duration over which an odor is perceived and distinguishes CHARM analysis from AEDA. The CHARM aromagram is the graph between CHARM values (y-axis) and the retention index (x-axis).

Conceptually, the CHARM value is the ratio of the amount of odorant compound eluted at a specific retention index to the threshold of the compound in the same mixture:

Odor thresholds

A threshold concentration is defined as the concentration at which an individual first perceives the stimulus. For aroma, this can be either:

1. A detection threshold: the point at which the individual can sense an aroma,
2. A recognition threshold: the point at which an individual can recognize the aroma.

Table 16. Threshold odor concentrations of odorous substances perceived during normal inhalation.

Compound	Threshold concentration (molecules/cc)
Allyl mercaptan	6×10^7
Sec. butyl mercaptan	1×10^8
Isopropyl mercaptan	1×10^8
Isobutyl mercaptan	4×10^8
Tert. butyl mercaptan	6×10^8
Thiophenol	8×10^8
Ethyl mercaptan	1×10^9
1,3-Xylen-4-ol	2×10^{12}
μ -Xylene	2×10^{12}
Acetone	6×10^{13}

Because individual aroma thresholds can vary by several orders of magnitude, odor thresholds are normally determined for a number of assessors, and the value quoted is the concentration at which half the assessors can perceive the aroma. Many odor thresholds are

quoted in the literature, but care must be taken to ascertain whether these have been determined in oil, water or air, or by GC-O.

Odor-activity values

Once the odor threshold of a compound and its concentration in the extract have been determined, this allows calculation of the odor-activity value (OAV). (Note that these two concentrations must be in the same units.)

The OAV is defined as the concentration of the aroma compound divided by its odor threshold, so an $OAV > 1$ indicates that the compound is present above its threshold value and is likely to contribute to the aroma profile.

However, OAVs can be misleading since, for many aroma compounds, the perceived intensity is not proportional to the concentration, and there is a decelerating relationship as the concentration increases. In other words, a doubling of an OAV that is several orders of magnitude above the threshold, may have little impact on the perceived aroma whereas, a doubling of the OAV around threshold could have a major impact.

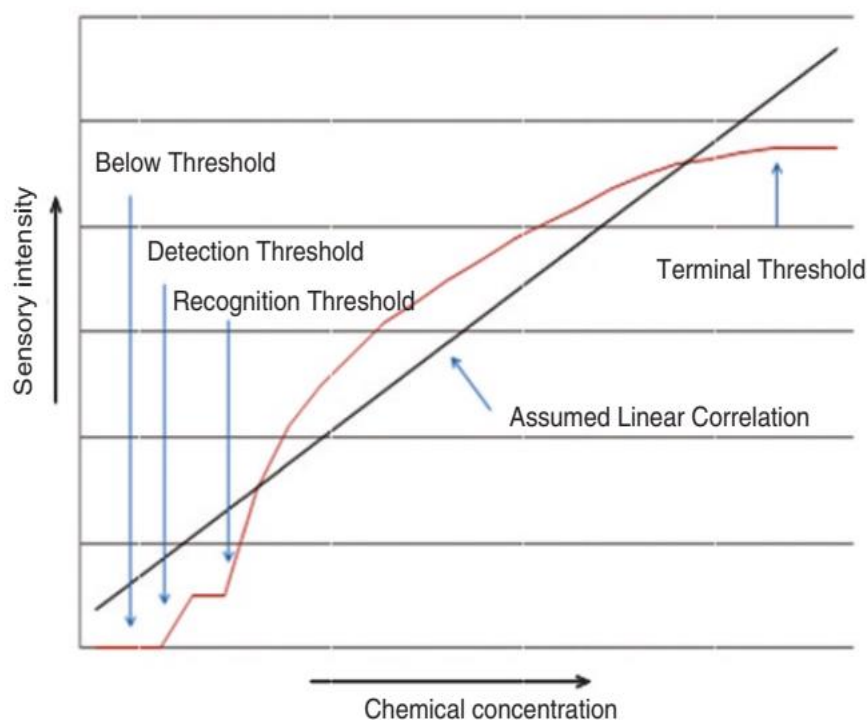


Figure 76. Schematic diagram of the potential relationships between chemical concentration and sensory intensity.

V.2.1.2. Methods of time-intensity evaluation

These reliable methods lead to more precise data than AEDA. The advantage of time-intensity methods is obtaining a complete chromatogram in a single chromatographic pass;

however, the drawback lies in individual differences in the use of scales, requiring training and complex data processing before results can be meaningfully interpreted.

V.2.1.2.1. Odor-Specific Magnitude Estimation (OSME)

Developed at the Oregon State University, this method utilizes only one representative extract, and multiple highly qualified subjects must assess the intensity of eluted volatiles from the first perception until the aromas dissipate. They then describe the aromas using a 16-point intensity scale.

Since the responses collected from OSME are time-intensive, various parameters of time-intensity curves, such as maximum intensity, duration, and area under the odor peak, can be used to present the perception of volatiles.

V.2.1.2.2. Finger span cross-matching method (FSCM)

This variant of the time-intensity method differs from OSME in the scoring procedure. Here, cross-modality matching called "Finger Span" is used, whereas OSME employs direct scaling on a 16-point scale.

Note

Cross-modal matching refers to the ability to recognize objects presented in two different sensory modalities. For example, a photo of a familiar person could be identified from a recording of their voice.

V.2.1.3. Intensity evaluation method

These techniques are abbreviated versions of time-intensity methods. Intensity evaluation data are similar to the intensity peaks obtained from the time-intensity method, but there is no temporal component involved in the responses.

This technique shares the same advantages as time-intensity methods and also suffers from the same disadvantages. However, the evaluation task in the latter technique is easier than that in the time-intensity method.

V.2.1.4. Detection frequency method/Global olfactometry analysis

These techniques do not use direct scaling as required by time-intensity and intensity evaluation methods, nor do they employ serial dilution techniques. The method utilizes a representative extract and instructs subjects (at least nine subjects) to smell the eluted volatile substances. Subsequently, subjects indicate the presence of the volatiles. The number of subjects

detecting the volatiles is counted as the detection frequency and represents the intensity of the volatiles.

This method is faster than other methods, but it does not provide the actual intensity as time-intensity and intensity evaluation methods do, as it does not determine the threshold concentration of volatiles. This method is not useful when volatile substances are detected by all subjects; on the other hand, concentrations of volatiles are well above the thresholds.

V.2.1.5. Response interval methods

These methods utilize subjects' detection capabilities, but instead of recording the detection frequency, they record the duration over which a volatile is detected. These methods suffer when eluted peaks of volatiles are not symmetrical or flattened, and they depend on chromatographic conditions rather than the actual psychophysical properties of the volatiles.

V.2.2. Recombinates

The FD factor and the odor-activity value (OAV) give different, but complementary, information. However, the only way to ascertain the role of individual compounds in a food is to reconstruct the aroma in a bland but representative base to produce a recombine. The aroma of the recombine is usually assessed by a sensory panel and compared to the original extract. If a good match is obtained, this is a good indication that all of the compounds contributing to the aroma have been identified. If not, the hunt continues for the missing components.

V.2.2.1. Omission tests

By systematic removal of each compound from the recombine, those compounds that truly have an impact on the aroma profile can be determined. Likewise, the impact of fractional changes in concentration can be assessed to give an idea of the tolerance of the aroma to small changes in the concentration of each volatile. In practical terms, this exercise indicates which of the volatile components are worth targeting for flavor optimisation.

V.2.2.2. Character impact compounds

The volatile profile of most foods contains many odor-active compounds but very few of these actually give character to the food. A food may contain hundreds of odor-active compounds, many of which impart distinctive generic notes to the food, while other existing aromas appear to be unrelated to it.

Only a few compounds confer the characteristic aroma and are called "*character impact compounds*", as without them, the food would be unrecognizable. It is also worth noting,

however, that not all foods have compounds with a character impact; their unique character may be attributed to the combination of aromatic compounds.

Table 17. List of character impact compounds in common foods

Compound	Characteristic aroma
<i>Vegetables</i>	
Methional (55) 2-Methoxy-3-isobutylpyrazine Hexanal (<i>E,Z</i>)-2,6-Nonadienal 1-Octen-3-ol (6) 5-Methylthiopentanenitrile (76) 2,4-Dithiapentane (57)	Potato Bell pepper Green beans Cucumber Mushroom Broccoli, cabbage Truffle oil
<i>Fruits</i>	
4-(4-Hydroxyphenyl)butan-2-one (raspberry ketone) (11) 3-Methylbutyl acetate (15) Allyl hexanoate γ -Decalactone (4-decanolide) (17)	Raspberry Pear drops Pineapple Peach
<i>Spices and herbs</i>	
Thymol Eugenol L-Carvone (<i>E</i>)-2-Undecenal (–)-Menthol (27)	Thyme Clove Caraway Coriander Mint
<i>Cereals/grains</i>	
2-Acetyl-1-pyrroline (46) 6-Acetyl-1,2,3,4-tetrahydropyridine (48) 2-Methylimino-3-butanone (43)	Cooked rice, popcorn Bread crust Corn tortilla

V.2.3. Electronic Nose

With advancements in sensor and computer technologies, scientists are able to develop instruments that mimic the olfactory system, known as electronic noses (e-noses). Current electronic noses use sensor arrays to detect chemicals and respond with distinctive patterns to different chemicals, much like receptors behave in olfactory and gustatory systems. The "brain" of e-noses is a computer with pattern recognition programs.

Although the sensors and brains of e-noses are not as sophisticated as human sensory systems, these instruments work well for limited functions. The performance of electronic noses depends on the types of detection sensors used in the manufacturing of the instruments. These sensors are components that signal in the presence of chemicals in a reversible real-time manner (shortest possible response time). The sensors are not destroyed or altered by the act of detection.

There are three types of sensors commonly used, and their sensitivity ranges from parts per billion (ppb) to parts per trillion (ppt), depending on the sensor types:

1. Metal Oxide Semiconductor Sensors: Detect changes in electrical conductivity caused by the deposition of compounds on the sensor surface;
2. Conductive Polymer Sensors: Detect changes in electrical conductivity caused by the swelling of polymers covering the sensor surface. Swelling increases the resistance within the sensor system;
3. Quartz Microbalance Sensors (detect ppt in 10 seconds): Operate differently as the deposition of compounds on the sensor causes changes in oscillation frequency. Different compounds alter the frequency to varying degrees. A reference frequency is usually defined before use, and deviations from the reference frequency are the responses;
4. Fluorescent Sensors: Emit light (visible or non-visible) with distinctive patterns when sensors come into contact with chemicals.

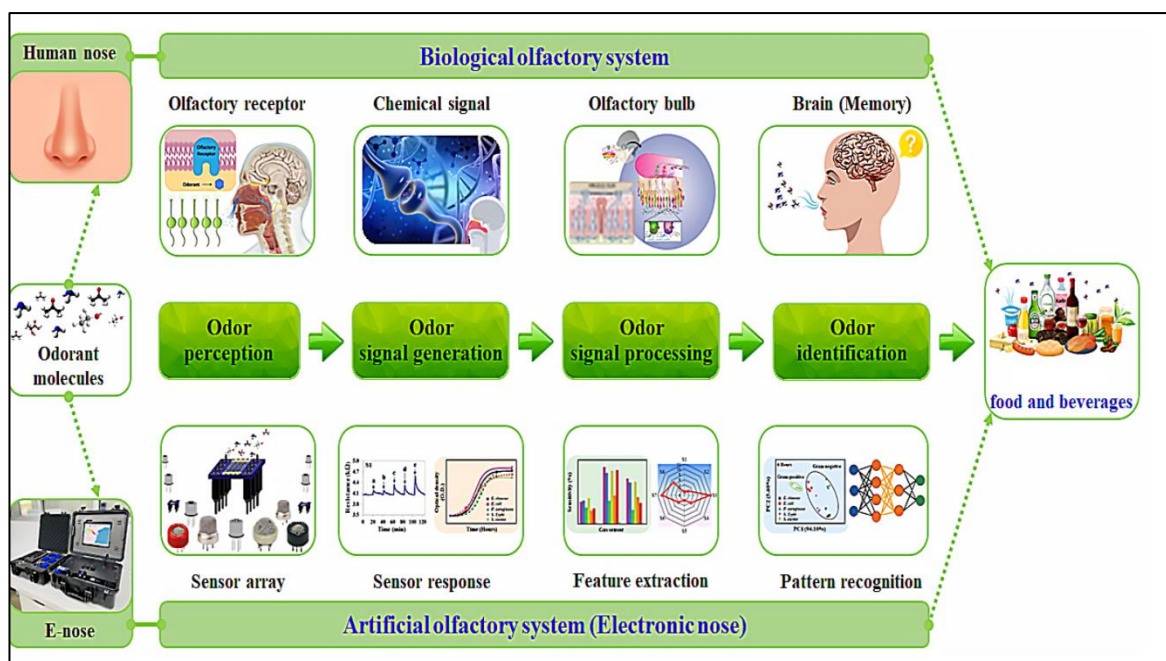


Figure 77. Principle of the Electronic Nose

The basic principle of each new electronic nose is standardization to detect, recognize, and respond to the pattern of a stimulus. Consequently, this pattern becomes a characteristic "digital fingerprint" of the stimulus, which is stored in a computer.

Sensor arrays can be manipulated to be highly specific and sensitive to a particular compound, which is an attractive property of electronic noses compared to the human nose. Electronic noses require a powerful capacity for selectivity and discrimination to respond to

olfactory interactions resulting from the perception of new aromas by changing the concentration of an odorant and/or by mixing an odorant.

In the realm of food, electronic noses have been used to detect impurities in raw materials, determine the freshness of foods, distinguish between vine-ripened tomatoes and green-picked tomatoes, identify foods from different treatments, and detect solvent residues in food packaging.

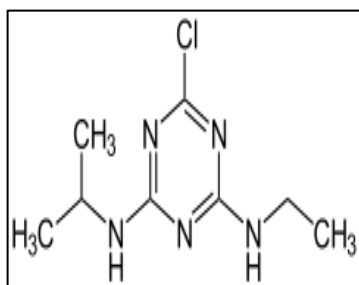
Chapter VI: Pesticide residue analysis

A pesticide is any substance or a mixture of substances formulated to destroy or control any organism that competes with humans for food, destroys property, or spreads disease.

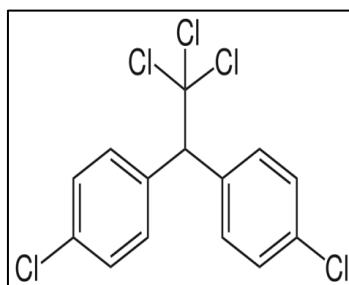
“Pests” include weeds, microorganisms (e.g., fungi or bacteria), insects, and even mammals. Currently, more than 800 active substances, belonging to different chemical groups, are formulated in pesticide products (~500 registered pesticide products).

The main classes of pesticides include:

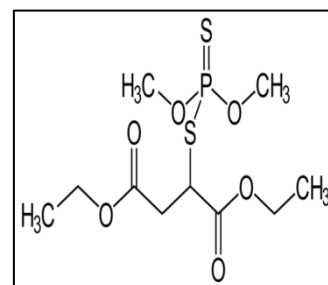
- Herbicides: for weed control, triazine (atrazine);
- Insecticides : organochloride (OC) (dichlorodiphenyltrichloroethane, DDT); organophosphates (OP) (malathion, dimethoate, omethoate), and methylcarbamates (aldicarb);
- Fungicides : phthalimide (Captan) ;
- Other types of pesticides may include acaricides, molluscicides, nematocides, pheromones, plant growth regulators, repellents, and rodenticides.



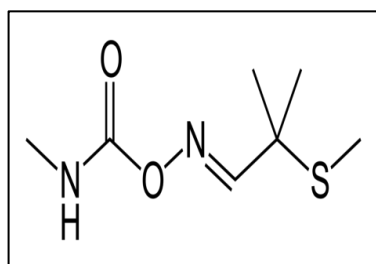
Atrazine



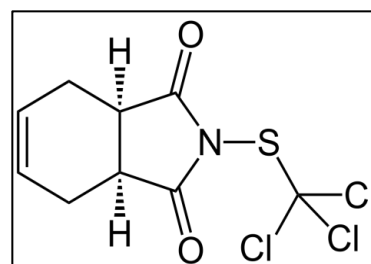
DDT



Malathion



Aldicarb



Captan

Figure 78. Chemical structure of certain pesticides.

If pesticides were not applied, an estimated one-third of the crop production would be lost. However, pesticides may have adverse effects on human health, including, but not limited to, cancer, acute neurologic toxicity, chronic neurodevelopment impairment, and dysfunction of the immune, reproductive, and endocrine systems.

VI.1. Types of analytical methods

Several factors make the analysis of pesticide residues in food a complex task: the complexity of the food matrix, the significantly greater abundance of matrix components relative to that of the target pesticides, the possibility of having pesticide levels as low as pictogram or femtogram amounts, and the considerable differences in the physical and chemical properties of pesticides.

Analytical methods employed for the analysis of pesticides can be categorized into

- Single residue methods (SRM): SRMs are designed to measure a single analyte and, often, its toxic metabolites and transformation products. The majority of SRMs have been developed for purposes of registration (homologation) and setting tolerance levels or for investigating the metabolism and environmental fate of a specific pesticide;
- Multiple-residue methods (MRM): For purposes of monitoring quality and safety, given the large number of pesticides and the considerable differences in their physical and chemical characteristics, MRMs are much preferred. Pesticides can be acidic, basic, or neutral, polar or nonpolar, and volatiles or nonvolatiles. Therefore, a MRM that can determine various pesticides in a single run is the most efficient analytical approach.

VI.2. Analytical techniques used for the detection, identification, and/or quantification

The identification and quantification in MRM currently used by the FDA are based on GC and HPLC analysis.

VI.2.1. Preparation of sample

Prior to chromatographic analysis, sampling, extraction, and fractionation/cleanup in MRMs are optimized to ensure an efficient transfer of most if not all of the pesticide residues present in the sample matrix to the organic phase. To achieve efficient transfer of as much of the pesticide residues as possible from the sample matrix into the organic phase, partitioning using water-miscible solvent is performed.

- To achieve an efficient transfer of pesticide residues from the sample to the organic phase, separation is carried out using a water-miscible solvent;
- This is followed by partitioning with nonpolar solvent that is miscible with the polar solvent, yet immiscible with water;
- After the extraction step, a cleanup step is required to minimize matrix effects and maximize sensitivity and selectivity. Cleanup techniques involve using adsorption

columns, in particular Florisil, alumina and silica gels, with solvent mixtures of low polarity for elution.

Commercial kits are available for pesticide cleanup; some examples are provided in the top half of table 18:

Table 18. Example commercial test kits for pesticide residue analysis

<i>Assay Type</i>	<i>Example Matrices</i>	<i>Pesticide</i>	<i>Commercial Source</i>
Cleanup kits			
Solid phase extraction – chromatography	Fruits and vegetables, including those with fats, waxes, and pigments	Multiclass, multiresidue pesticides	QuEChERS® (Agilent Technologies, Inc., 5301 Stevens Creek Blvd, Santa Clara, CA)
Solid phase extraction – chromatography	A variety of food commodities including fruits and vegetables	Multiclass, multiresidue pesticides	DisQuE® Dispersive Sample Preparation Kit (Waters Corporation, 34 Maple Street, Milford, MA)
Enzyme inhibition test kits			
Insect esterase activity with color change	Water. Feeds, grains, fruits, tubers, leafy vegetables, <i>Brassica</i> vegetables, dairy products, and meats	Organophosphates (malathion, diazinon, parathion, etc.) and metabolites, <i>N</i> -methylcarbamates (aldicarb, carbofuran, etc.)	Cidelite® (Charm Sciences, Inc., 659 Andover Street, Lawrence, MA)
Insect cholinesterase activity with color change	General	General	Agri-Screen Ticket® (Neogen Corporation, 620 Leshar Place, Lansing, MI)

VI.2.2. Biochemical Techniques

VI.2.2.1. Enzyme inhibition assays (test kits)

These assays are mostly used as screening methods, with limited sensitivity and selectivity. Their principle is based on the inhibition of a certain enzyme, essential for vital functions in insects, by pesticides present in the sample:

- If no pesticides are present, the enzyme will be active and act on the substrate to cause a change in color;
- If no change in color occurs, then the test is positive, and further confirmation can be performed following more sophisticated analysis, such as HPLC and GC, to identify and quantify the specific pesticides present.

VI.2.2.2. Immunoassays

These tests can be tailored to particular purposes ranging from simple screening tests (field-portable) to quantitative laboratory tests. Immunoassays are simple and sensitive, have

high throughput, and are cost effective as compared with other conventional methods. Additionally, extensive cleanup of extracts is not necessary, unless crossreactivity exists. Therefore, this technique can be very useful for monitoring programs when there are a large number of samples.

Immunoassays, of which ELISA represents nearly 90%, can be either class- or compound-specific with different applications.

- The antibodies for ELISA testing of acylodien insecticides and triazine herbicides are class-specific and have broad reactivity, enabling the detection of closely related compounds.

For this type of assay, the hapten moiety of the hapten–protein conjugate is common for a group of chemicals;

- Immunoassays for atrazine, chlorpyrifos, and flufenoxuron are compound specific, with hapten–protein conjugate designed to preserve the unique portion of the target analyte.

Therefore, these compound-specific assays can be applied in quantitative analyses. However, in some instances there is the possibility of under- or overestimation of the true pesticide concentrations due to matrix interference and cross-reactivity.

VI.2.3. Chromatographic Techniques

VI.2.3.1. Thin-layer chromatography (TLC)

Because of its low resolving capacity, low precision, and limited detection relative to GC and HPLC, it is not used as a quantitative method, but mainly for screening purposes or as a semiquantitative method that precedes more accurate detection and quantification.

An example application is the detection and estimation of pesticides (OP and carbamate insecticides) that inhibit insect enzymes such as cholinesterases. Once a crude extract is separated by TLC, the plate is sprayed with a solution containing the enzyme(s), followed by a solution containing a specific substrate, which releases a colored product upon hydrolysis. The lack of color change indicates enzyme inhibition, due to the presence of pesticide residues, and the zone of inhibition is proportional to the quantity of pesticide present.

VI.2.3.2. Gas Chromatography GC

In general, GC is the preferred method for the determination of volatile and thermally stable pesticides, such as the OC and OP classes. Choice of columns and detectors is made based on the nature of the pesticides:

- With fused silica capillary columns, a large number of pesticides with similar physical and chemical properties can be separated and detected;
- 5% diphenyl, 95% dimethylpolysiloxane stationary phase columns are commonly used in MRMs;
- Element-selective detectors are often used. Flame photometric detector (FPD) is suited for the detection of P-containing compounds and is widely used for the detection of OP pesticides in various crops, without extensive cleanup required;
- For the determination of OC, the electron capture detector is used extensively due to its high sensitivity to organic halogen compounds;
- Coupling of fused capillary columns to the highly specific and sensitive MS (mass spectrometry) detection enhances not only the confirmation process, but also the quantitative determination

Exp : Rapid analysis of 98 pesticides in fruit-based baby foods (at levels ≥ 0.01 mg/kg) was achieved using a GC/TOF-MS technique.

VI.2.3.3. High-Performance Liquid Chromatography (HPLC)

Development of HPLC analysis for the separation and detection of pesticides became a necessity as the number of pesticides with poor volatility, relatively high polarity, and thermal instability increased. Classes such as *N*-methyl carbamate (NMC), urea herbicides, benzoylurea insecticides, and benzimidazole fungicides are typically analyzed by HPLC. These compounds are often analyzed by:

- Reversed-phase chromatography with C18 or C8 columns and aqueous mobile phase;
- Followed by UV absorption, UV diode array, fluorescence, or MS detection;
- These detectors might not be specific enough to resolve spectral differences, which are often too small. Utilization of MS detection widened the scope of HPLC analysis of pesticides. LC-MS is becoming one of the most powerful techniques for the analysis of polar, ionic, and thermally labile pesticides.

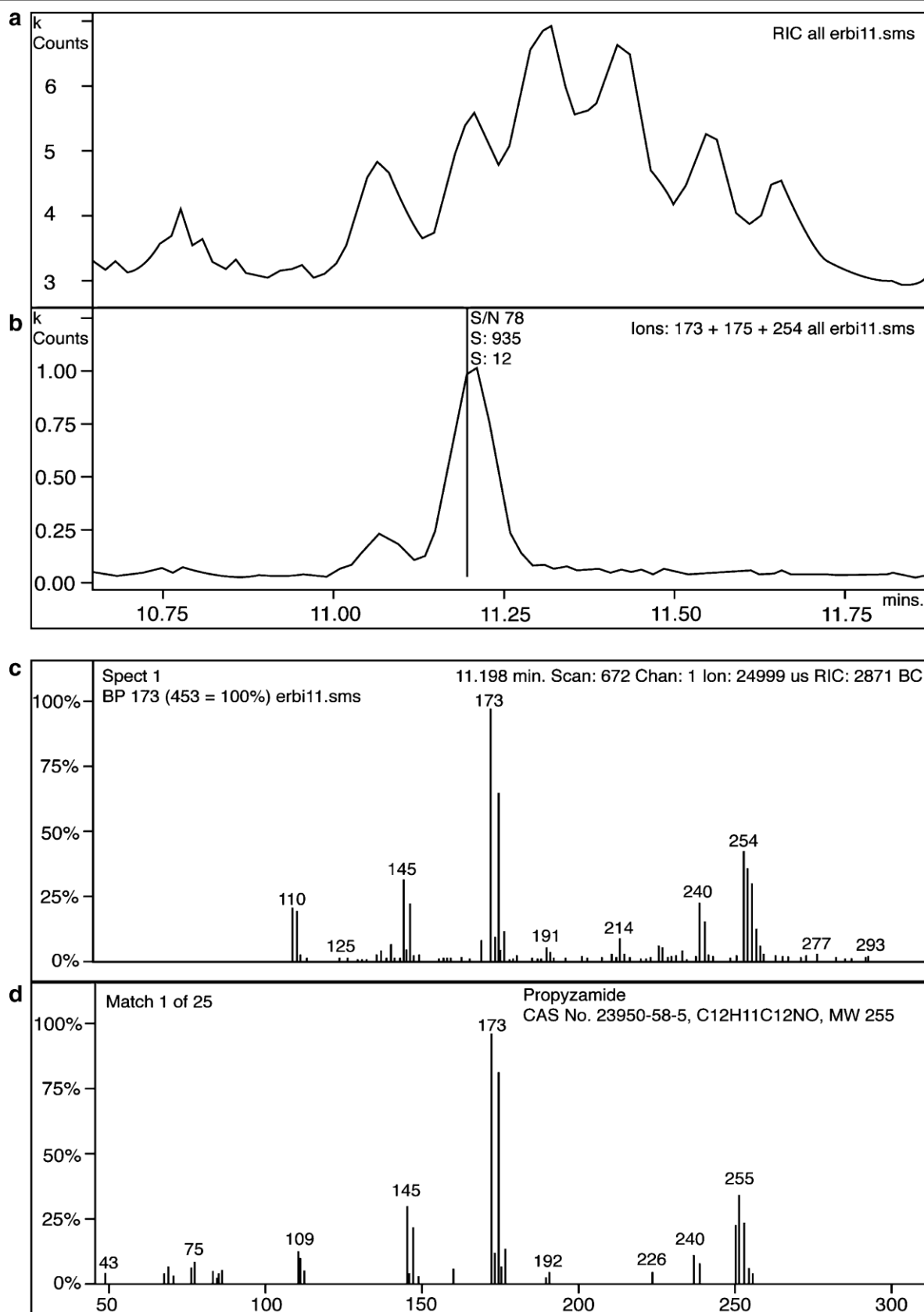


Figure 79. Detection of propyzamide using GC-ITD MS. (a) Total ion chromatogram from retention time 10–12 min of an apricot sample fortified with 10 ng/g propyzamide. (b) Extracted ion chromatograms of the most characteristic propyzamide ions. (c) Mass spectrum of the eluate at 11.19 min. (d) NIST library spectrum of propyzamide.

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