Module: Carrying out Qualitative and Quantitative Analyses of the Fish, Seafood Products and Fish Habitat

1. Qualitative and quantitative analysis, general principles

The focus of this review is the traditional "wet chemistry" approach to analysis. Although the advanced instrumental analysis has become the main stream methodology in modern food laboratories, the traditional methods still have certain advantages. They are generally cheap, accurate and do not require expensive instrumentation.

In qualitative chemical analyses the intention is to specifically detect certain compound(s) in the sample. In many cases, just demonstration of the presence or absence of a substance is considered sufficient. Typically, different types of indicator reactions are applied, and the outcome of the assay is easily detectable, like color change, gas formation etc.

In quantitative analyses, not only the chemical identity of the components of the sample, but also their absolute or relative quantities are elucidated. They are usually based on the measurement of the consumption of some reagent or quantification of the reaction products. The final calculation of the amounts of analyses is performed according to the stoichiometric rules of the chemical reactions on which the determination is based.

1.1. The general chemical background[1]

1.1.1. Chemical equilibrium

When two or more chemical reactants start to react with each other, the production of reaction products simultaneously begins. Most reactions are to a certain degree reversible, meaning that the reaction products formed give rise to the original reactants. When the reaction is in equilibrium, the rate of the reaction equals the rate of the reverse reaction meaning that the concentrations of reactants and end products remain constant. From this basic situation the well-known general equilibrium equations are formulated:

Reaction: $aA + bB \leftrightarrow mM + nN$ (A and B are reactants, M and N their end products; a, b, m and n their molar quantities, respectively)

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[1] It is assumed that the student is familiar with atomic theory and concepts like ion, molecule and mole

Equilibrium constant: $K = \dfrac{[M]^m [N]^n}{[A]^b}$ $\qquad\qquad\qquad [M]^n$

If K > 1 the reaction proceeds almost totally, if K = 1 the amounts of reactants and end products are equal, if K < 1, reaction products are not formed in high quantities.

1.1.2. Acids and bases

Generally, a chemical that tends to release a proton (H^+) is an acid and a chemical that receive a proton is called a base. Acids have a tendency to form salts by catching a positively charged ion (usually a metal ion) to replace the released proton. Thus, for example, hydrochloric acid or HCl can react with Na^+ ions to form NaCl or common salt.

The amount of protons in the solution is the measure of its acidity, and is expressed as pH or the negative logarithm of the proton concentration. Water molecule can dissociate or split to form a proton (in the form a oxonium ion, H_3O^+) and a hydroxyl ion (OH^-). In pure water the concentration of both these ions is 10^{-7}, and thus the pH of pure water is 7, which means that water is neutral. Low pH means acidic, and pH > 7 alkaline conditions.

The dissociation constant or acid constant (K_a) for an acid is expressed as follows:

- Dissociation of an acid: $AH \leftrightarrow A^- + H^+$ (AH is the undissociated and A^- the dissociated acid)
- $K_a = \dfrac{[H^+][A^-]}{[AH]}$

With strong acids like the hydrochloric acid (HCl) the dissociation is practically complete, and the pH can be calculated assuming the total dissociation. Most acids are, however, weak and only partially dissociated, and for them the pH can be calculated by assuming that $[H^+]$ equals $[A^-]$, leading to the following expressions:

- $K_a = [X]^2 /[AH]$, where $[X] = [H^+] = [A^-]$

- $[X]^2 = K_a [AH]$

- $2 \log [X] = \log K_a + \log [AH]$

-   $-\log [X] = pH = - \frac{1}{2} \log K_a - \frac{1}{2} \log [AH]$

pH can drastically affect the chemical reactions, and in many occasions the aim is to keep the pH constant despite addition of acids or alkaline substances. For this purpose different buffer solutions based on a weak acids have been formulated. The pH of this type of solution is expressed by the Henderson-Hasselbach equation:

-   $pH = pK_a + \log [A] - \log [HA]$ $(pK_a = - \log K_a)$

Common, routinely used buffer solutions are often based on phosphoric acid and its salts or some organic acid liike acetic acid. When there is a need of a buffer solution with an alkaline pH, buffer solutions based on ammonia and ammonium chloride (NH_3 and NH_4Cl) are typically used.

1.1.3. Oxidation – Reduction

When an atom, ion or molecule receives an electron (e^-), it is reduced. When it loses an electron, it becomes oxidized. The oxidation-reduction status of a chemical profoundly affects its chemistry and reactions. With some inorganic ions the oxidation or reduction is reflected with characteristic colour changes. In general, some elements (and their atoms and ions) are considered as electronegative and have a strong tendency to attract electrons and get reduced, and in this process oxidize other atoms/ions.

As movement of electrons, oxidation and reduction are associated with electrochemical phenomena. When two pieces of metal having a different electronegativity are both immersed in a solution that can conduct an electric current, and the metal pieces are further connected with an external conductive wire, an electric circuit is formed, with an electromotoric force generated by the difference in electronegativity. The simple example of this kind of arrangement is the so called Daniell's cell (Figure 1):
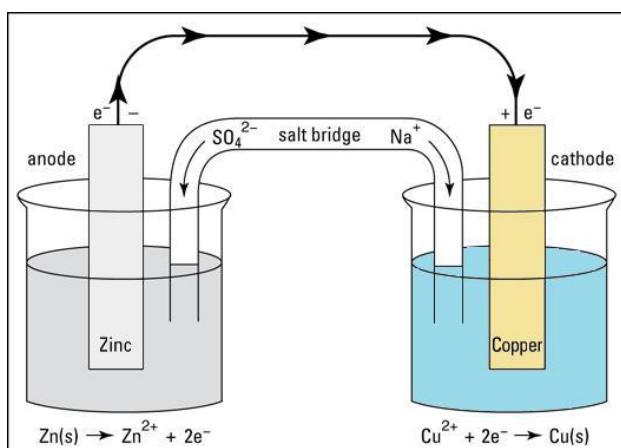
In the example present in the Figure, the two metals – the electrodes - are Zinc and Copper, both immersed in the respective salt solutions ($ZnSO_4$ and $CuSO_4$), the beakers connected by a salt bridge (moist paper impregnated by Na_sSO_4), and the metal slabs by an external wire. Copper is more electronegative than Zink, and thus the flow of electrons is from zink to copper, meaning that copper is in this reaction called as cathode and zink as anode. In the process Cu^{+2} ions get reduced to metallic copper by receiving two electrons, while metallic zink gets oxidized losing electrons and forming Z^{2+} ions. The associated electromotive forces can be calculated and also experimentally determined.

Also, elementary hydrogen, H_2, can participate in oxidation-reduction reactions. Protons, or H^+ ions catch electrons from less electronegative elements, like most metals, oxidizing them. On the other hand, ions of metals like copper (Cu), mercury (Hg), silver (Ag) will readily oxidize elementary hydrogen to protons. It has been agreed that the electrode potential of reaction $2 H^+ + 2 e^- \rightarrow H_2$ is 0, and acts as a reference point for the electrode potentials for other oxidation-reduction systems. Thus in a Daniell's cell consisting of a hydrogen electrode and a metal electrode the potential of the former is zero and that of the metal electrode either positive or negative in comparison (Figure 2). Tabulated values for different oxidation-reduction pairs are available in most advanced chemistry textbooks.
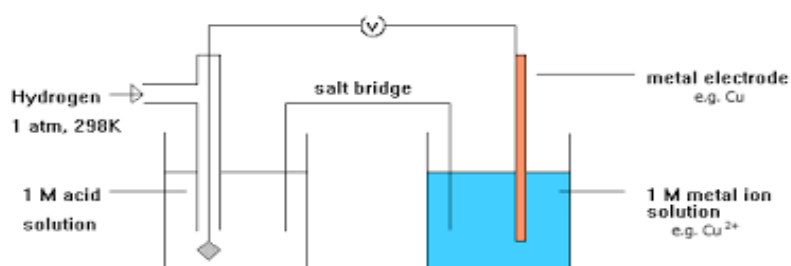
1.1.4. Solubility of ionic compounds in water

Generally dissociated charged ions are soluble in water, while compounds that do not have a net charge tend to form solid precipitates. The solubility of a salt can be expressed by the following equation, using Lead iodide as an example:

PbI_2 (s) $\leftrightarrow$ $Pb^{+2}_{(aq)}$ + 2 $I^{-1}_{(aq)}$; (s) = solid, (aq) = dissolved in water
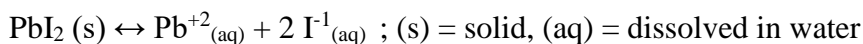
$K_{sp} = [Pb^{+2}_{(aq)}] [I^-_{(aq)}]^2 = 7.9$ x 10^{-9}

Another parameter important for processes related solubility/insolubility is the ionic product, which has actually the same formula as K_{sp}:

Ionic product of $PbI_2 = [Pb^{+2}] [I^{-1}]^2$

Now, if we mix two soluble compounds, lead nitrate $Pb(NO_3)^2$ and potassium iodide in water, so that both reach the final concentration of 0.1 M, we get into a situation, where the concentrations of Pb^{+2} and I^{-1} reach both 0.1 M, and their ionic product = [0.1] $[0.1]^2$ or 0.001 or 1 x 10^{-3}. This is much larger than the Ksp of PbI_2, meaning that PbI_2 rapidly precipitates. In general, when K_{sp} is less than the ionic product, the ions precipitate as an insoluble compound, if K_{sp} is larger than the ionic product, then the ions remain in the solution.

2. Facilities, equipment, reagents and solutions

A laboratory should have sufficient space for the intended personnel, be well ventilated and naturally have the necessary electricity, gas and tap water. Dry but well ventilated storage room for chemicals and preferably a separate storage room for samples as well as office space are also needed.

The basic equipment for an analytical laboratory include accurate balances and analytical balances, water distillation and deionization system, refrigerators, fume cupboards, bench top and microcentrifuges, laboratory ovens, hot plates and pH meters.

For the actual analytical work at least the following types of labware are needed:
- Volumetric cylinders

- Funnels
- Büchner funnels
- Decanters
- Volumetric flasks
- Erlenmeyer flasks
- Suction flasks
- Crucibles
- Pipettes (both glass pipettes and automated pipettes)
- Rubber bulbs
- Desiccators
- Burettes

2.1. The calibration of volumetric vessels

Usually the quality of modern volumetric vessels is high and one can trust their readings. However, every now and then it is good to calibrate at least the most critical equipment, in particular the automated pipettes, which may lose their accuracy in prolonged use. This should preferably be done at the temperature of 20 °C. The procedure is to pipette a standard nominal volume of distilled water, eject it from the pipette on a tared vessel and weigh the dispensed water recording the weight. This is repeated ten times. From the recorded weights one can calculate the exact volume pipetted at each time by using the tabulated density values of water at ambient temperatures, and statistically evaluate, whether the variation is within the acceptable limits. Pipette manufacturers usually provide instructions for the calibration.

2.2. Standards and working solutions

In analytical chemistry there are several specific reagents or that are used in titrations and precipitations or which specifically react with some analyte. They are usually called primary standards, and are used to prepare standard solutions.

For the preparation of a carefully weighed amount of the primary standard is placed into a volumetric flask, which is the filled up to the desired volume. The amount of standard is calculated to give the desired concentration in the resulting standard solution. The water or buffer solution that is used

should be added stepwise ensuring that the standard is completely dissolved, before filling the flask up to the final volume.

Solid primary standards should be as pure and dry as possible. Generally the standard is dried in an oven (appr 110 °C, for 2 h) and stored in an desiccator. The procedure can be repeated until the weight of the standard does not change anymore after the drying step.

Solutions of strong acids and bases can be directly diluted into the desired concentration taking into account the initial concentrations declared by the manufacturer. The safety aspect of mixing water directly with strong acids should always be considered. For example, concentrated sulphuric acid should be diluted by adding first water into the volumetric flask and then gradually (and with continuous stirring) the calculated amount of acid to avoid the violent splashing of the mixture.

With weak acids one can pipette a measured amount of the acid solution into the volumetric flask and then add water to the desired volume. For example, to prepare 500 ml of 0.5 M working solution of 99.5 % (w/v) acetic acid, one has first to transform the percentage to molarity. Because the 99.5 % acetic acid has a density of 1.051 kg/l, the weight of the acetic acid in one 1 dm^3 is 0,995 x 1051 g or 1045.75 g. Because the molecular weight of acetic acid is 60.05, the molar concentration of the acetic acid is 1045.75/60.05 or 17.04 M. This concentration has to be diluted by 34.08 to get 1 M solution. The volume that should be pipetted into the volumetric flask is thus 500 ml/34.08 or 14.7 ml.

3. Sample preparation for qualitative and quantitative analysis

The validity of an analytical approach – whether qualitative or quantitative – critically depends on the quality of sample collection and sampling. Indeed, sampling errors often influence the outcome of the study more than the experimental errors during the analysis itself.

Factors that may induce qualitative and quantitative changes to the sample include dusting and dust formation with dry samples, evaporization/volatilization, reactions with the sample and container and contaminations from the equipment and facilities. In the following chapters some aspects that should be taken into account specifically when sampling fish and seafood and fish habitats are briefly outlined.

3.1. Fish samples

Most fish and seafood samples naturally are moist and have a high water content. Evaporization of moisture can affect the relative quantities of different analytes and affect also the biochemical parameters of the sample. Also, the post mortem changes in fish may affect the composition of the tissue and the extractability of different analytes. Consequently, the samples should be stored in chemically inert, closed containers (such as screw cap plastic tubes or closed plastic boxes) and in cold conditions (on ice or in the refrigerator). For prolonged storage deep freezing is recommended.

The size of the sample depends of the nature of analysis. For the proximate analysis of the major food components (proteins, lipids, carbohydrates, ash) few grams are sufficient, while the analysis of some environmental contaminants present in very small quantities (µg per g or even less) may require samples of several kg for accurate testing.

Generally, if the fish material to be analyzed represents small fish (anchovis, fingerlings etc) whole animals can be used as a representative sample. For larger fish special samples for different purposes may be required (such as muscle, gills, liver, skin, gonads etc), and a sufficient knowledge of fish anatomy is naturally required while sampling.

When the samples are processed for the analysis care must be taken to remove or wash away any external material such as dirt, mud or, in the case of crustaceans or clams remove the shells. However, in this process care must be taken not to affect the sample composition by, for example, excessive washing.

Often the first step of the actual preparation of the sample for the analytical procedures is the homogenization or grinding the sample. For fresh samples consisting of soft tissues this can be done by mechanical choppers, blenders and mixers. Dry and in some cases deep frozen samples can be ground with pestle and mortar. Again, the possibility of chemical changes by, for example, enzymatic reactions should be taken into account. Generally, the temperature should be kept low during this step, but sometimes boiling of samples in water-methanol or water-ethanol mixtures can be recommended.

3.1.1. Fish products

Sampling of processed fish products (salted, dried, smoked, canned etc) depends very much of the type of product. However, the general principle of avoiding any procedures that might affect the composition of the sample either during the sampling or the storage apply. For canned or packed

products it is usually best to keep the product in the original container until the analysis but applying either refrigerator or freezer temperatures during the storage.

3.2. Liquid samples

In addition to solid samples, samples of water and other liquids often need to be analyzed. In the handling and storage of liquid samples one has to take into account their chemical stability, possible microbial degradation or spoilage and their physico-chemical properties, such as volatility. It may be necessary to store the samples frozen, or to apply various pretreatments like filtration, autoclaving, or other procedures to ensure that the quality and composition of the sample until the actual analysis. Plastic tubes are not recommended for water samples, because plastic may absorb components from the sample or react with them. This may also happen in glass bottles and tubes, and if the sample is to be extracted with some organic solvent at later stage, it may be advisable to add the solvent beforehand into the sample vessel.

Sometimes chemicals like $HgCl_2$ or sulphuric acid can be added into water samples to improve their chemical stability. Generally, storage at either + 4 °C or at -20 °C is recommended.

3.3. Bottom sediments

Environmental samples related to fishing and fish farming include also bottom sediments. In collecting these types of samples it is often critical not to mix the different layers in the sediment. Again, plastic containers are not recommended, but dry samples should be stored in aluminium foil and moist ones in glass tubes or bottles.

3.4. Atmosphaeric samples

Particulate material present in the atmosphere of, for example, fish farming facility or industrial plant, can be collected by collecting them on glass fibre or membrane filters with the aid of an adjustable pump allowing the measurement of the air volume passed per unit of time.

Atmospheric volatile compounds can be collected by using different adsorbents like activated charcoal or resins like Tenax. From these the adsorbed substances can be released by so called thermic desorption or heating the adsorbent. Alternatively, organic solvent extraction can be used for this purpose. During the storage of these types of samples care should be taken to avoid the loss of volatiles, and the samples should be kept in closed containers at refrigerator temperatures or at -20 °C.

3.5. Pretreatment of samples before the analysis

Foods, including fish and seafood, are complex matrices containing proteins, lipids (fats and oils), fibre and minerals. Many of these component may affect and interfere with the actual analysis, and therefore several pretreatments are often required. Removal of proteins and lipids are the most common of these procedures

3.5.1 Deproteinization of the samples

The removal of proteins, which usually form 15 – 20 % of the fish muscle, is often a critical step in analysis. This can be done by several methods. A homogenate can be centrifuged and filtered to remove the insoluble proteins. Soluble proteins can be precipitated and removed from the resulting supernatant/filtrate by acid treatment using, for example perchloric acid or trichloroacetic acid followed by centrifugation and/or filtration. Alternatively, solvents like acetonitrile can be used for protein precipitation. In selecting the analytical procedures, one has to take into consideration the possible interference of the precipitation agents in the procedure.

3.5.2. Removal of fats and oils

Fats and oils are not water soluble, and therefore are present either bound to tissues or as dispersed micelles in aqueous homogenates. They are soluble in non-polar solvents, and therefore extraction of the supernatant/filtrate obtained after centrifugation and filtration by a solvent like hexane using a separation funnel, can be performed.

4. Analytical methodology

Central methodologies in traditional analytical chemistry include gravimetric and volumetric techniques. They require a certain knowledge of the reactions of inorganic ions and of the fundamental aspect of acid-base theory and the pH concept. On the other hand, no complicated instrumentation is needed. Accurate and well calibrated pipettes and balances, clean glassware (decanters, Erlenmayer flasks, test tubes), pH meters, refrigerators, and laboratory ovens form the basic repertoire of the laboratory.

4.1. Gravimetric methods and their applications in food analysis

The most common form of gravimetric methods applied in analytical laboratories are based on the precipitation. On the basis of the chemical properties of the intended analyte, another reagent is selected that forms an insoluble precipitate, when allowed to react with the analyte in aqueous solution. The formed precipitate can be then collected, dried, weighed and the amount of the analyte calculated.

Another approach of gravimetry is burning, extraction or vaporization of the sample and weighing the remaining substance(s). This is actually a common technique in food analysis to determine the so called ash content of the sample. As pointed out in chapter 4.1.1. This approach is actually widely used in analytical laboratories.

Gravimetric analyses can be used both for qualitative and quantitative purposes. For example, demonstration of halides (Cl^-, I^- an Br^-) by precipitation by silver nitrate $AgNO_3$ from a solution acidified by nitric acid leads to formation of white, yellow and cream-coloured precipitates, respectively. These precipitates can be then used for quantitative calculations of the halide content in the sample.

4.1.1. Examples of gravimetric food analysis, analysis of proximates

In proximate analysis the major components of foods are analyzed. These include moisture or water content,) ash or mineral matter, crude fat, crude protein, true protein, non-proteinous nitrogen, crude fibre, carbohydrates, calcium, potassium, sodium and phosphate. Many of these analyses are gravimetric, as outlined below.

a. Moisture content

Moisture determination is a simple gravimetric analysis, in which an accurately weighed amount of food (usually ground or minced) is incubated at 100 °C overnight. The sample is cooled in a desiccator and then weighed. The moisture percentage is calculated from the formula $[(W_1 - W_2)/ W_1] \times 100$, where W_1 is the original weight, and W_2 the weight after the heat treatment. It should be noted that, depending of the nature of the sample, there might be also other volatile compounds than water, and this should be taken into account, when evaluating the result.

b. Ash content

The term "ash" covers the mineral components of a foodstuff that remains after ignation or complete oxidation of a food sample. In the traditional dry ashing a food sample is burned in a muffle furnace at 500 – 600 °C. As the result carbon and nitrogen are volatilized as CO_2 and N_2, and the minerals   are turned mainly respective oxides, sulphates, phosphates, chlorides and silicates.

In wet ashing the sample is treated with strong acids oxidizing agents, such as nitric acid, perchloric acid or mixture of sulphuric acid and hydrogen peroxide to solubilize the minerals. This is mainly done as a preliminary step for a quantitative analysis of certain compounds that
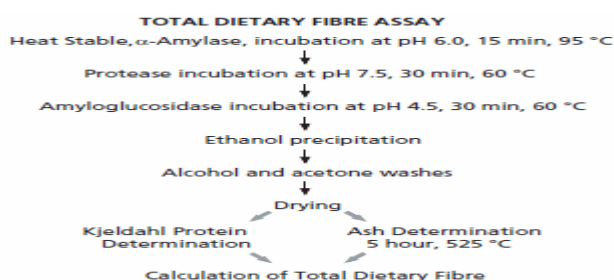
might be volatilized during dry ashing (Fe, Cu, Zn, P). The use of perchloric acid entails an explosion risk and requires extra precautions.

c. Crude fat

Gravimetric determination of crude fat of a food sample is based on the solubility of fat in organic solvents like diethyl ether, chloroform and the like. In the basic procedure the dried food sample is ground, and sometimes treated with acid to break down the proteins, followed by solvent extraction – often using the Xoxhlet apparatus (Figure). After extraction the solvent is evaporated and the remaining fat residue weighed.

d. Crude fibre

Fibre is a generic name for complex insoluble polymeric compounds (cellulose, lignin, chitin etc) that can be present in the food. There are no actual tests directly to measure the fibre content. Usually the estimate is based on the process of elimination. Fats and bulk proteins are removed (the latter by a treatment of protein degrading enzymes, proteases). Starch and other soluble polysaccharides are removed by enzymatic treatment. The insoluble fibre is then precipitated by alcohol or acetone, filtered, dried and weighed. Often the remaining protein and ash are analyzed and their contents subtracted from the weight of the insoluble residue in order to get a very accurate value (Figure 3 below).


TOTAL DIETARY FIBRE ASSAY
Heat Stable, α-Amylase, incubation at pH 6.0, 15 min, 95 °C
↓
Protease incubation at pH 7.5, 30 min, 60 °C
↓
Amyloglucosidase incubation at pH 4.5, 30 min, 60 °C
↓
Ethanol precipitation
↓
Alcohol and acetone washes
↓
Drying
Kjeldahl Protein Determination   Ash Determination 5 hour, 525 °C
Calculation of Total Dietary Fibre

4.2.2. Example of gravimetric analysis based on precipitation: Calcium content of a water sample

The amount of Ca^{2+} ions in water is associated with its "hardness" or the decreased efficiency of detergents used washings and the tendency to form chalky deposits. The soluble Ca^{2+} ion can be quantitatively precipitated by carbonte ions (CO_3^{-2}) as insoluble $CaCO_3$.

The method is straightforward. Into an appr. 20 ml of water to be analyzed an excess of 0.5 M sodium carbonate ($Na2CO_3$) solution is added, the precipitation is allowed to form. It is subsequently washed a couple of times with pure water and then harvested using a Bühner funnel. The precipitate is dried at 50 °C and repeatedly weighed, till all moisture has evaporated.

The reaction equation is:

$$[Ca^{2+}_{(aq)}] + [CO_3^{-2}_{(aq)}] \leftrightarrow [CaCO_{(s)}]$$
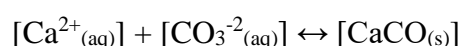
The molecular weight of Ca is 40,08 and that of CO_3^{-2} 60. Thus, the amount of Ca in the precipitated in the amount m of $CaCO_3$ is 2m/3.

4.3. Volumetric (titrimetric) methods and their applications in food analysis

The volumetric methods are based on known specific reactions of the analytic with some analytical reagent. The volume of the reagent used in the reaction is recorded and this value is used to evaluate the amount of the analytic. At the end point of titration equivalent amounts of the titration reagent and the analytic have reacted.

In the standard experimental set up there is a measured volume of the liquid containing the unknown amount of the analytic and a sufficient amount of titration solution containing an accurate concentration of the titration reagent (which should react with the analytic). An essential tool is burette (Figure 4), with which one can accurately add the titration reagent to the analyte solution and simultaneously measure its consumption.

The titration solution is gradually added to the analytic solution from the burette mixing continuously the titration vessel, until the end point, marked by an easily observable change

(color, appearance, solubility etc) in the analytic solution, is reached. From the volume of the titration solution used, one can calculate the concentration of the analytic.

If the titration reagent is A and the analytic B, and the general reaction equation $nA + mB \rightarrow$ products, and C_1 is the molar concentration of A, V_1 its volume consumed during the titrations, C_2 the (unknown) molar concentration of B and V_2 its volume in the titration, then

$(C_1 \times V_1)/n = (C_2 \times V_2)/m$

$C_2 = \dfrac{(C_1 \times V_1)\ m}{V_2 \times n}$

The following types of titrations are commonly used:

- Acid-base titrations
- Complex metric titrations
- Precipitation titrations
- Oxidation-reduction titrations
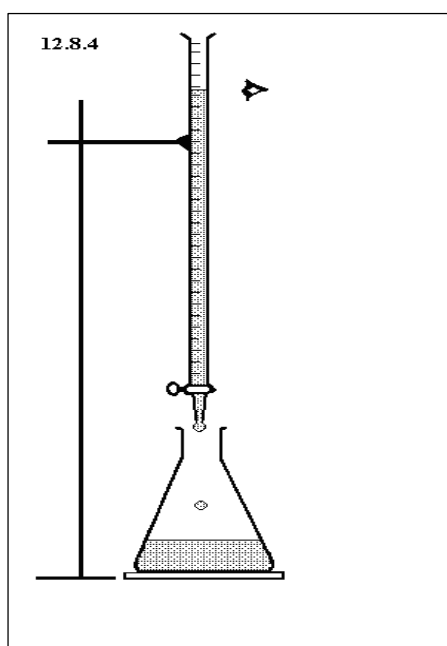
Of these the acid-base titrations are the most common and generally used in order to accurately determine the concentrations of acid(s) and bases in the solution. The other titration types are useful for some specific analyses.

4.3.1. Acid-base titrations

These titrations are based on the general acid-base neutralization reaction:

$$H^+_{(aq)} + OH^{-1} \rightarrow H_2O$$

For the neutralization to occur it is necessary to mix amounts of acids and bases that contain equivalent amounts of H^+ and OH^{-1} ions. For example, 2 mol NaOH and 2 mol H_2SO_4 would be equivalent in this respect.

As pointed out in 1.1.2. Acid can be classified as either strong or weak depending on their tendency to release protons. Likewise, bases can be classified as strong or weak depending on their tendency to receive protons. Thus, the acid base-titrations can lead to different situations:

- strong acid and strong base
- strong acid and weak base
- weak acid and strong base
- weak acid and weak base (not very common)

For the best results the titration solution should be either strong acid or base. It should also be taken into account that after the neutralization only in the first option (strong acid and strong base) the pH is strictly neutral. In other combinations, the salts that are formed during the neutralization affect the final pH of the solution.

When one plots the pH of the solution of analytic as a function of the titration solution added, one notices that the change in the pH occurs very sharply around the equivalence point (Figure 5)
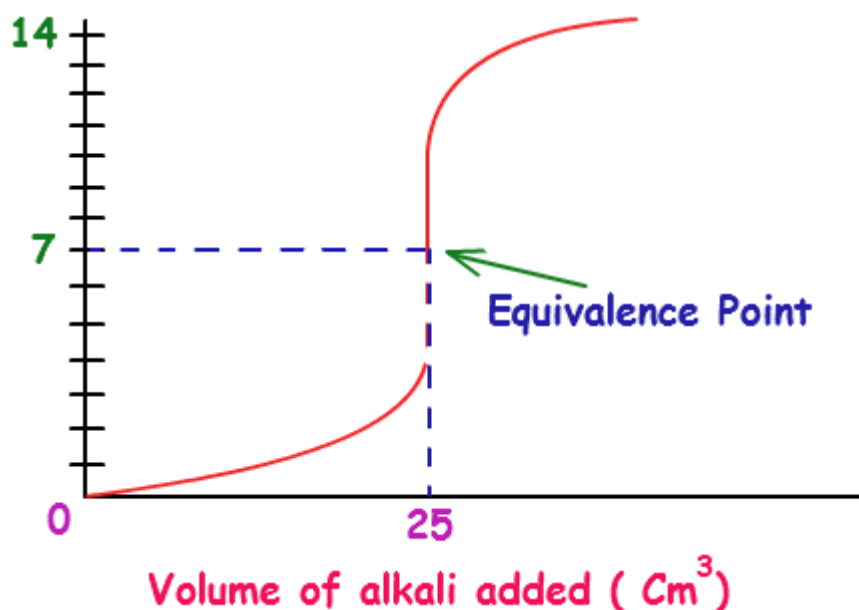
Different indicator compounds are used for the observation of the titration end point. They typically change color at a certain pH range, and this change indicates that the equivalence point has been reached. Phenolphthalein, changing from colorless to pink in the solution, when pH reaches 8.2, is one of the common indicators in strong acid, strong base indicators.

In a typical titration a few drops of phenolphthalein solution is added into the analyte solution (strong acid), and titration by a NaOH solution of known molarity, is started. The NaOH is added in small quantities following the development of the color. When the first indications of color change have observed, the titration should continue at even slower pace and with smaller NaOH additions in order to avoid the over titration and missing the equivalence point.

There are several other pH indicators that have their colour change threshold at different pH values, and their combinations can be used to follow the development of pH during the titration and to accurately define the titration end points.

The Kjeldahl method for the determination of the total nitrogen in foods, is one of the examples of the applications of acid-base titrations in food analysis.

For the analysis the food sample is first digested by boiling in sulphuric acid (338 °C or higher): In this process the nitrogen is reduced to NH_4^+. After the complete digestion, the reaction mixture is made alkaline by a large excess of NaOH, leading to the liberation of gaseous NH_3. This is led into a receiver containing a known amount of hydrochloric acid (HCl), which reacts with NH_3:

$NH_3 + HCl \rightarrow NH_4Cl$

The remaining, unreacted HCl, is then titrated with standard NaOH and from the consumption of NaOH one can calculate the amount of NH_3 produced during the digestion/neutralization.

The total nitrogen content gives an indication of the protein content of the sample. For meat and fish the conversion factor is 6.25 meaning that the total nitrogen in the sample multiplied by 6.25 is a reasonable estimate of the amount of protein.

4.3.2. Complex metric titrations

Divalent metal cations can form very stable complexes with organic compounds having free electron pairs. These types of compounds are called ligands or chelating agents, and they have many uses and applications in chemistry. By far the most common chelating agent is ethylenediaminetetraacetic acid or EDTA. The typical complex with EDTA and Ca^{+2} is shown in Figure 7
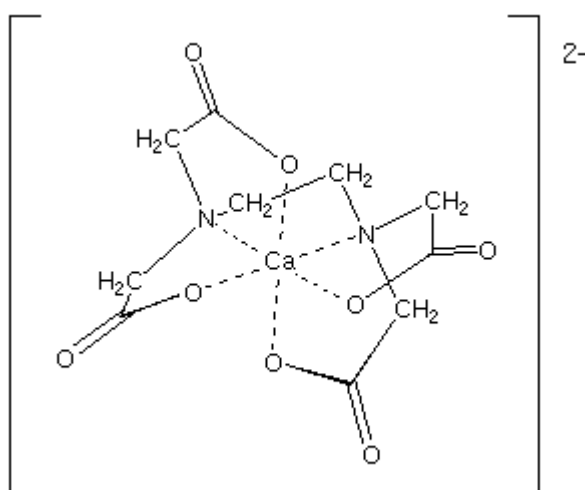
Free metal ions can be determined from water samples by complex metric titration. As in acid-base titrations, suitable indicators – in this case chemicals forming colored complexes with free metal ions - are required. Eriochrome black, xylenol orange are common indicators used, both forming a red complex with most metal ions.

In the simplest case the water sample is mixed with the indicator, the pH adjusted to the level that is optimal for the color reaction with the indicator, and the resulting colored solution is titrated with a solution containing a known concentration of EDTA. If the indicator used was eriochrome black, the change of colour from red to blue shows the equivalence point. Often an

acid-base indicator is added to the reaction mixture to improve the detection of the color change. For example, methyl red is yellow at alkaline pH suitable for the titration of magnesium ions with eriochrome black as indicator. At the equivalence point the color turns green, and the change is easy to detect, when also methyl red is present.

In some cases, when for example the analytic forms a precipitate in the absence of EDTA, the so called back titration must be performed. In this procedure an excess of EDTA is added to the analytic solution, and the remaining free EDTA is titrated using a solution containing a known concentration of ions of another metal. The metal in the titration solution must have a lower affinity to EDTA than the analytic. Thus, for example, Ni^{2+} can be assessed by back titration with Zn^{2+}.

The determination of the total concentration of Ca^{2+} and Mg^{2+} ions in water is one of the common complex metric titrations. Sometimes the water sample must be pretreated with ascorbic acid cyanide to prevent minor metal ions such as Fe^{3+}/Fe^{2+} from interfering with the analysis. The pH is adjusted at 10 using ammonia-based buffer, and eriochrome black is used as the indicator (with the addition of methyl red).

4.3.3. Precipitation titrations

Precipitation titration utilizes the solubility equilibria familiar from the gravimetric analyses. In this case the end point of the titration is the formation of the insoluble precipitate. In the practical performance one has to take into account that the precipitation reactions can be slow, particularly near the equivalence point. However, precipitation titrations can be used for some specific analytical purposes, such as to determine the amount of salt (NaCl) in a food sample (Mohr's titration).

For the titration salt is extracted from the sample using warm/hot water. The titration solution is silver nitrate ($AgNO_3$), and the titration is based on the formation of insoluble silver chloride (AgCl) precipitate. In order to help the accurate determination of the equivalent point, potassium chromate (K_2CrO_4) is used as an indicator, because the almost instantaneous formation of red insoluble Ag_2CrO_4, when excess free Ag^+ ions are available after all the Cl^- ions have reacted with Ag^+. The titration has to be performed in slightly alkaline solutions.

4.3.4. Oxidation-reduction titrations

In oxidation-reduction titration an oxidizing agent can be used to titrate the concentration of an reducing agent or vice versa. For example, permanganate ion (MnO_4^-) is a common oxidizer. In

acidic conditions the deep purple Mn^{-7} is reduced to colorless Mn^{+2} in the presence of some oxidizable substance. In the titration, the solution remains colorless, as long as the reaction is incomplete, but after the equivalence point the excess permanganate gives the solution a characteristic color.

There are also several oxidation-reduction indicators, such as diphenylamine or methyl blue, which change color according to their oxidation reduction status. This reaction is supposed to occur after the equivalence point of the titration, and therefore the indicator must be selected so that the reaction with the titration agent and the analytic is chemically the most favorable one, and all the other oxidation-reduction reactions, including those with the indicator, are only secondary.

Among the oxidation reduction titrations the ones utilizing iodine are particularly useful for many purposes. Iodine (I_2), forms a characteristic deep blue color with starch, and therefore soluble starch is a common indicator in iodometric/iodimetric[2] titrations based on the oxidation-reduction balance of iodine between the elementary iodine and reduced iodide ion (I^-). The reduction of I_2 to $2I^-$ in the presence of starch causes the deep blue colour to disappear.
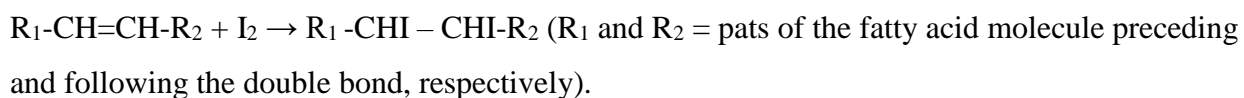
In the practical iodometric titrations, the standard is usually the so called triiodide solution. I_2 is poorly soluble in water, but the solubility is enhanced by the presence of I^- ions according to the equation:

$I_{2\ (aq)} + I^- \rightarrow I_3^-$ (triiodide)
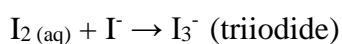
In practice triiodide solutions are prepared by mixing an excess of KI solution with I_2.

Iodometry has several applications in food analysis, such as the analysis of ascorbic acid or of the harmful hydroperoxides that are formed as oxidation products in fats and oils. The hydroperoxides oxidize the I^- to I_2, which can be titrated using starch as the indicator.

Iodine titration is also used to estimate the amount of unsaturated fatty acids, because the tendency of iodine to get reduced and bound by the carbon - carbon double bonds, as shown below:

R_1-CH=CH-R_2 + I_2 → R_1 -CHI − CHI-R_2 (R_1 and R_2 = pats of the fatty acid molecule preceding and following the double bond, respectively).

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[2] When the analytic is titrated with iodine the procedure is called iodimetry, when the formation of iodine is followed by titration with I^-, the titration is called iodometry.

Because of this reaction, the Iodine number is one of the classical parameters that are used to characterize an oil.

5. Statistical treatment of the analytical results

There are many factor that can cause variations and errors in the analytical work. Sampling, accuracy of weighing and measuring, purity of the chemical etc, are typical factors that affect the accuracy the results. In order to get some indication of the correctness of the analytical result obtained, some elementary statistical approaches are usually applied.

When aiming at quantitative accuracy, several samples should be tested and the tests repeated, if possible. As a rule of thumb it is often suggested to take at least three representative samples from your material and make at least three replicate analyses per sample. The results of the replicate samples give an indication of the variation inherent in the sampling while the replicate results obtained from each sample indicate the variation inherent in the analytical procedure.

The results should be given at a reasonable accuracy. For example, the number of decimals used in the analytical values should reflect the accuracy that can be expected using the instrumentation that has been available. It is not meaningful to calculate a theoretical value with four decimals, when the measuring methods allow only one or two.

The most elementary statistical parameter id the mean. It is simply the sum of the results of each individual test divided by the number of tests. So, if one did n number of analyses of one sample, the mean of these values is:

$$\overline{x} = \frac{(x_1 + x_2 + \text{........} + x_n)}{n} = \frac{\sum\limits_{i=1}^{n} x_i}{n},$$

(Σ = symbol indicating the sum of all the values of a series, in this case from x_1 to x_n)

In order to obtain information of the variability of the results, one should estimate, how much the individual results differ from this mean. This is done by calculating the variance (σ^2) of the results. For this each individual result is subtracted from the mean and the result is squared. The squared values are summed and the result is divided by n. The square root of σ^2 is often called the standard deviation (SD). The corresponding formulae, after some mathematical simplification are:

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Population Variance

$$\sigma^2 = \frac{\sum x^2 - \frac{(\sum x)^2}{N}}{N}$$

Population Standard Deviation

$$\sigma = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{N}}{N}}$$

$\sum x^2$ = the sum of the squares of all the values of x; $(\sum x)^2$ = the square of the sum of all the values of x, $N = n$

For example, if a sample was titrated five times, and the amounts of acid calculated were the following: 0.10 M, 0.11 M, 0.09M, 0.12 M and 0.10 M, the mean is 0,104. The variance would have been: (0.0419 – 0.000216)/5=0.0079, and the SD =0.089. By using reasonable decimals, one could express the result as mean ± SD, or 0.10 ± 0.09 M, indicating that each individual measurement deviated from the mean on the average by 0.09M. The mean and the SD should both end at the same decimal place.

In the formulae applied above are somewhat simplified, and assume that we have actually all the analytical data at our disposal. If there would have been only this sample in the world and there would have been material for just five titrations, and our results thus represent the whole population of all the possible titrations of that sample, then we would have been justified in this assumption. However, in the real life, although you take samples from five fish they do not normally represent the only five specimen of that particular fish species in the world, but these five fishes represent a sample of the whole population of millions of this fish species. In this case it is more prudent to speak of sample variance (S^2) and sample standard deviation (S). These are calculated using values (n-1) (four, instead of five, in our example) instead of n in the denominators of the equations as shown below:

Sample Variance

$$s^2 = \frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}$$

Sample Standard Deviation

$$s = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

The n -1 value is called "degrees of freedom", and they represent the number of independent data points in our calculations. If we, namely calculate the mean, we can always calculate the nth value after knowing the mean value and the n-1 values.

The calculation of S^2 and S is nowadays programmed in most function calculators and can easily be performed by programs like excel.

The standard deviation is one of the parameters of the Gaussian curve (figure 8). Gaussian curve shows the distribution of values in a typical series of observations, the majority grouping in the middle of the bell-shaped curve.
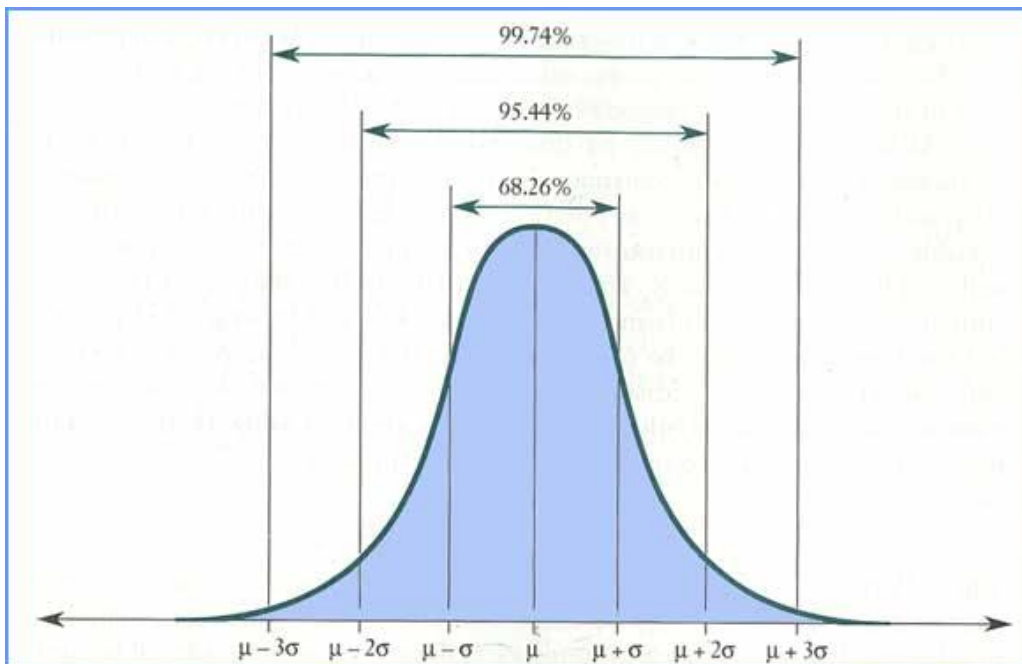
From the curve it can be seen that appr. 70 % of the data points should be within ± σ of the mean value (μ), and more than 99% of the data points within ± 3σ from the value of μ.

If our S, the estimate of the standard deviation is very large in comparison to the our mean value, then this indicates that in our sample has a large distribution of values, and it is probably not quite representative to the actual population.

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[3] A vewry comprehensive but not a very accurate book containing many errors