Modern methods of food analysis

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1. Introduction

The aim of food analysis is obtaining information, which process results information about the composition of food or food raw material sample. This obtaining information can be carried out on different levels. These levels can be the following: elemental, molecular, and structural. The level of the chemical elements (elemental) means that answer can be given to the question that what (qualitative analysis) and how much (quantitative analysis) can be found in the given sample. During the implementation of this task, range of the constituents in this experiment that can be carried out is fully known. Examining the chemical elements, is known that there are only 89 stable chemical elements on the Earth, however, altogether 118 known elements exist. Although, on the molecular level we are trying to answer what compounds and crystalline forms consist of the sample from the building elements. In this case however, the range of components that can be examined is unidentifiable both in practice and theory. In this task the number of possibilities are nearly infinite, currently 12 million compounds are known. From these circa 1.5 million are inorganic compounds, the rest are organic. The examination of the structure can mean arrangement of the molecules as well (e.g.: determining the order of the amino acids in a protein). The difficulty of the analytical task differs among levels. For example, an element, that technically cannot be further divided, can more easily be determined than a molecule or a structure, because in the analysis, during the sample preparation steps, the stability of the structure and the molecular composition are difficult to assure. While the quantitative change of the chemical elements can only be a result of loss or pollution, the structural and molecular transformations occur more easily. While the basis of the miscellaneous world of the organic compounds is represented by six elements only (C, H, N, O, S, P), on elemental level these compounds mean the determination of only six elements. In practice, the purpose of analytical tasks is usually to solve routine analyses. In many cases simplifications can be done more or less narrow down the analytical task to the examination of organic or inorganic groups of components. Beside of the aforementioned qualitative aspects the quantitative relations should also be taken into consideration, because the most suitable sample preparation and measuring methods for the implementation of the analytical tasks can only be chosen in the possession of this information. If this knowledge is not known the necessary information can only be acquired step by step thorough preliminary investigations, which is time and money consuming process. It must be mentioned that the process of the aforementioned acquiring information can be carried out only with adequate accuracy and precision if the sampling is representative enough. Figure 1 shows the magnitude of the error if any step of the analytical examination is not carried
out properly. If the representative sampling or the sample preparation is not carried out properly then the accuracy or the precision of the results cannot be corrected by instrumental measurements.

<table>
<thead>
<tr>
<th>Analytical steps</th>
<th>Estimation of errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defining the scientific problem</td>
<td>Producing of &quot;data cemetery&quot;</td>
</tr>
<tr>
<td>Discussion by experts</td>
<td></td>
</tr>
<tr>
<td>Benefit / Use calculation</td>
<td></td>
</tr>
<tr>
<td>Planning of analysis</td>
<td></td>
</tr>
<tr>
<td>Representaive sampling</td>
<td>up to 1000%</td>
</tr>
<tr>
<td>Sample preparation</td>
<td></td>
</tr>
<tr>
<td>I. Physical washing, drying, homogenization</td>
<td>100-300%</td>
</tr>
<tr>
<td>II. Chemical ashing, decomposition, enrichment, speciation</td>
<td></td>
</tr>
<tr>
<td>Instrumental measurement</td>
<td>up to 2-20%</td>
</tr>
<tr>
<td>Data evaluation, Solving the scientific problems</td>
<td>up to 50%</td>
</tr>
</tbody>
</table>

Figure 1 Simplified flowchart for inorganic environmental analysis, including a rough error estimation for the different analytical procedures (taken from Markert, 1993)

Figure 1 also shows the error of the instrumental analysis is the lowest during the analytical process. In this book however, our aim is to focus on the details of this point, for the analysis of the food or food raw material samples. Moreover it should also be pointed out that in this book, with regard to the importance of analytical practice, we only have the opportunity to discuss the most significant routine analytical methods.
2. Classifications of analytical methods

In case of the applied analytical methods at first we must decide whether the classical or the instrumental analytical method is better to apply. The classical analytical methods, in other words the wet-chemical methods, preceded the instrumental analytical methods by over a hundred years.

Classical analytical methods

In the first years of the analytical chemistry the majority of the analysis was done by dividing the components of the sample that should be examined. During this process precipitation, extraction or distillation was applied. Afterwards the divided components, meant to be used for qualitative analysis, were handled by other reagents with the help of which chemical reaction was used either results in coloured compound or changes of its boiling/freezing point or its solubility. Moreover, reactions which were applied leaded to variously perceptible gases (e.g.: odours) or changes in the compound’s optical characteristics or optical activity. When classical analytical method is chosen for the quantitative analysis of the components (to determine its relative or absolute concentration) gravimetric or volumetric method can be used. In gravimetric measurements, the determination of the components’ concentration in the given sample is led back to the changes in the mass of the examined analyte or to the mass of the precipitate that was formed with another component. In case of volumetric, also known as titrimetric methods, the component which is analysed, in form of a solution, must be reacted with the reagent, already being in the standard solution and after the reaction of all the amounts of reagent in the sample, from the loss of the amount of the standard solution (from the proportional value of the stoichiometric quantity), the concentration can be counted. All of these classical analytical methods, can be used to either for separating or defining these components, are still used in several laboratories nowadays; but the number of those, who generally use these methods is slowly decreasing due to the appearance of more developed and more conveniently applicable methods of instrumental analysis, these new methods are slowly, but surely superseding the aforementioned ones.

Instrumental analytical methods

At the beginning of the twentieth century scientists began to take more and more advantage of the different opportunities provided by the measured components’ physical correlations. With
the help of them they developed better and better instrumental analytical methods which they found solution for several problems of the classical analytical methods. Such physical characteristics are for example: conductivity, electrode potential, light absorption, light emission, fluorescence and the mass-charge ratio, which were started to be used for quantitative analysis. Furthermore, highly effective chromatographic and electrophoretic techniques were also used to substitute distillation, extraction or precipitation, applied to divide the mixture of components of food or food raw material samples with unusually complex matrix before the qualitative or quantitative determination. The aforementioned new methods, used for the separation and determination of different components, are called instrumental analytical methods. It also must be mentioned that lately the rapid development of the computer and electronics industry highly contributed to the improvement and spread of the modern instrumental analytical methods.
3. Performance characteristics of the analytical methods

From the performance characteristics of the analytical methods the most important ones are the selectivity, specificity, ruggedness, measurement range, linearity, detection limit, quantitation limit, accuracy and precision.

3.1. Selectivity and specificity

The selectivity of an analytical method, which is a quantitative concept, means what extend can the analytical method analyse the analyte in presence of other disturbing components in the sample matrix. In practice this means a method is selective if the measured signal is mainly originated from the analyte and other components of the matrix that contribute less to the measured response. If the analytical method is not selective enough the disturbing effect of the matrix can be decreased with the applied sample preparing method, so the measurement method will become more selective. The conductivity measurements, for example, applied to determine all of the salt content of the mineral waters, should not be called selective, however, an atomic spectrometry (e.g.: atomic absorption spectrometry) measurement is selective enough.

The method, which is completely selective for a certain component or a group of components, is called specific. That case is an ideal extremity of selectivity, which is called specificity. Although, as the matrix always influences the response of the measured component to a greater or lesser extent, it can be said that specific analytical methods do not exist.

3.2. Robustness/ruggedness

One of the most important characteristic of analytical methods is ruggedness. It is related to the instrument and environment as well.

The robustness/ruggedness of the analytical method (instrument robustness/ruggedness) is measured by changing the measurement’s environment on purpose (by the person conducting the measurements) and measuring the precision and accuracy. Afterwards the range of application of each parameter is determined where precision and accuracy do not significantly differ.

The robustness/ruggedness of the analytical method is determined by using the examined method in different laboratories where more or less different measurement circumstances are applied when circumstances (e.g.: different operator, tools, temperature, etc.). Then the
standard deviation of reproducibility of the measurements is determined (see later). From the values of the standard deviation it can be concluded whether the robustness/ruggedness of the examined analytical method is sufficient.

3.3. Range of measurement

The analytical method’s range of measurement is the concentration range of the components in which the given component can be examined with sufficient (established in standards) accuracy and precision. The method’s range of measurement is determined by the expected concentration of the measured sample’s components. The calibration range is determined in a way that the applied calibration points have to comprise and completely cover the concentration of the measured samples.

3.4. Linearity

Linearity of the calibration curve means between the concentration and the analytical response, in a given concentration range, the correlation is linear. This concentration range is called linear range of the calibration curve. The linear measuring range is determined by analysing standard samples. The fitted calibration equation is calculated with the method of the least square for the concentration response data pairs (Equation 1).

\[
y = m \cdot x + b
\]

(1)

where

- y: response
- m: slope
- x: concentration
- b: interception

On the Figure 2 m=4.87 and b=68.6.
Finally it is determined that in concentration range, regarded calibration is linear. Usually the correlation is regarded linear in the concentration range where the fitted and the measured value do not differ more than 10%. The maximum value of the linear measurement range is called LOL (limit of linearity).

3.5. Sensitivity

The sensitivity (S) is the slope of the calibration line (on Figure 2 for example S=m=4.87), in other words, the sensitivity is the response for the change in concentration within one unit. Generally speaking, sensitivity is the derivative of the analytical response according to the concentration (Equation 2). Therefore, if the calibration line is linear, the sensitivity of the measurement is the slope of the calibration line. In case of a curved calibration line the sensitivity cannot be given to the whole range but only to a given point on the line. Then sensitivity equals the slope of the tangent line drawn to the given point of the line ($\text{tg} \alpha$) (Figure 3) (Equation 2).

$$m = \text{tg} \alpha = S = \frac{dV}{dc} \quad (2)$$

where

S: sensitivity of the calibration line at the given point

V: response (y on the Figure 2)

c: concentration (x on Figure 2)
3.6. Detection limit

There are two ways of calculating in detection limit. According to one of them the detection limit of the analytical method (DL=detection limit or LOD=limit of detection) is the concentration value at the measurement which the response is clearly distinguishable from the background’s response. In other words it is the value of the concentration that belongs to the standard deviation of signal of blank sample times three (Equation 3).

\[ c_d = \frac{3 \cdot s_{\text{blank}}}{S} \]  

where

- \( c_d \): detection limit
- \( s_{\text{blank}} \): standard deviation of the blank sample’s response (the component that does not include the sample to be measured)
- \( S \): sensitivity

This mentioned latter is only valid if the blank value correction is applied before fitting the calibration curve. According to another view, which is more common one, if the blank value correction is not applied the theoretical detection limit (\( c_d \)) is that concentration of the given component to the response that belongs equals the sum of the blank sample’s response and three times the standard deviation of signal of blank sample (3\( \sigma \)) (\( \sigma = s_{\text{blank}} = \text{SD} \)) (Equation 4).

\[ R_d = R_b + 3 \cdot s_{\text{blank}} \]  

where
R_d: response belonging to the determination limit (c_d)

R_b: response of the blank sample

s_{blank}: standard deviation of the blank sample (the component that does not include the sample to be measured)

However, if the interception of the calibration curve is negligible the detection limit can be calculated according to the 3rd equation.

### 3.7. Quantitation Limit

The limit of quantitation (QL, LOQ) is the smallest concentration value which can be determined with acceptable accuracy and precision using a given method. The knowledge acquired in paragraph 3.6. Detection limit can be applied with calculating the quantitation limit, however, in practice, at the “acceptable” level in this case, instead of the 3σ applied in the detection limit equation 10σ is applied to calculate the quantitation equation (Equation 5).

\[ c_q = \frac{10 \cdot s_{blank}}{S} \]  

where

- \( c_q \): quantitation limit
- \( s_{blank} \): standard deviation of the blank sample’s response (the component that does not include the sample to be measured)
- \( S \): sensitivity

Taking into consideration what was discussed at the end of paragraph 3.6., if the interception of the calibration curve is not negligible the quantitation limit cannot be calculated with the 5th equation. In such case an equation should be used in which, similarly to the 4th equation (detection limit), and the quantitation limit of a given component are the concentration value to a response belongs that equals with the sum of the blank sample’s response and ten times the standard deviation of signal of blank sample (10σ) (Equation 6).

\[ R_q = R_b + 10 \cdot s_{blank} \]  

where

- \( R_q \): response belonging to the quantitation limit (c_q)
- \( R_b \): response of the blank sample
$s_{\text{blank}}$: standard deviation of the blank sample’s response (the component that does not include the sample to be measured)

Figure 4 illustrates the aforementioned and highlights the most important characteristics of a calibration line.

- **DL**: detection limit
- **LOD**: limit of detection
- **QL**: quantitation limit
- **LOQ**: limit of quantitation
- **LOL**: limit of linearity

![Calibration Curve Diagram](image)

**Figure 4 The most important characteristics of a calibration curve**

### 3.8. Accuracy

The accuracy of a method shows how close the analytical result is to the real value of the measured quantity. Its determination requires a material which measured component’s concentration is known, for example it can be performed with the analysis of a reliable reference material (CRM=certified reference material). If a reliable reference material is not available the value of accuracy can also be determined by adding the component to be measured to the sample, as known quantity of standard material, (an equivalent quantity (in the same magnitude) to the component’s concentration). Then the expected concentration difference can be calculated by measuring the value of concentration before and after the addition. This method is called spiking, which can be carried out before or after the sample preparation, so before the analytical measurement. In the first case the accuracy of the sample preparation and the
measurement’s will be evaluated simultaneously while in the second case conclusions can be drawn only about the accuracy of the analytical measurement.

Another common method for estimating the value of accuracy is comparing the results of other analytical measurements in the laboratory or calculating the accuracy of the examined analytical method by comparing collaborative studies (reference samples) of different laboratories. Accuracy expresses regular errors.

Of course, the result of a method is more accurate that less error occur. Whit determining the amount of accuracy the relative error related to the real value is given in percentages (%). For example: if the value of concentration is 10 mg/L and the average result of the analysis is 11 mg/L then the relative accuracy is 10%.

### 3.9. Precision, repeatability, reproducibility

**Precision**

The precision of a method is the measure of difference among the results of measurements carried out consecutively within a short period of time, which is usually expressed with standard deviation (Equation 7). This value can be calculated by standard deviation function (s) in Microsoft Excel according to the following:

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

(7)

where

- s: standard deviation
- x: measured values
- \(\bar{x}\): mean of measured values
- n: number of measurements

From the Equation 7 that is seen, by increasing the number of measurements the values of standard deviation can be decreased.

With evaluating experiments the relative standard deviation (s%) is given because it is easier to compare the values of standard deviation visually (Equation 8).
Figure 5 helps to understand the concept of accuracy and precision. Usually the ‘target shooting’ example is brought. The following four targets illustrate how the measured values (shootings) are located in the environment of the real values (centre of targets).

![Figure 5. Illustration of the precision and accuracy of a measuring method with the example of target shooting.](image)

**Repeatability**

The repeatability of measuring methods is a version of accuracy that means the closeness of results with repeating measurement of the same quantities within the same circumstances (measurement principle, measuring methods, operator, equipment, place, time, circumstances).

**Reproducibility**

The reproducibility of measurement results is a version of precision that means the closeness of results when there is at least one circumstance altered in the process of measurements (measurement principle, measuring methods, operator, equipment, place, time, circumstances).
4. Analytical methods of inorganic components

4.1. Calibration, standard addition, internal standard

4.1.1. Calibration method

The classical analytical methods are absolute methods of analysis (that is, they do not need the application of previous standard samples), although the majority of instrumental analytical methods (all of the methods detailed in this note) can be considered as relative methods. The latter means that in each case of analytical measurement of the samples has to be preceded by a calibration activity, during which, with the physical and physical-chemical characteristics of the component to be defined, the connection between the concentration of the component and the signal of the instrument is defined.

Thus, standard solutions with known concentration are made and analysed the exact same way, under the exact same circumstances, meaning that the connection that mentioned above and calibration curve are created which represent either the signal of the analytical instrument or the quantity that is directly derived from the signal compared to the concentration or the quantity of substance of the component to be measured. The calibration curve is usually created with the help of five to ten samples with known concentration.

During the calibration linear equation is not necessarily needed, but different degree polynomials (second, third) result in a more appropriately fitting calibration curve.

4.1.2. Standard addition method

If during the analytical measurement, any component that is present has an interfering effect of increasing or decreasing the signal, then a method of standard addition can be applied which can either eliminate or at least excessively decrease this interfering effect. The process of standard addition is also a possible calibration method. This method can be applied when a more accurate concentration is needed than what is possibly given by the matrix of the sample during normal (external) calibration. In this case numerous conditions are considered: the measured signal has to linearly change with the concentration and the amount of substance of the component to be measured; the slope (sensitivity) of the calibration curve cannot change with more than 20%; moreover it has to be free of spectral interferences. During the implementation of the process, at first the concentration and signal of the analyte are measured,
then around 50% (x), 100% (2x) and 150% (3x) (Figure 6) of the measured concentration of
the analyte are added to the sample solution and the signals of these solutions are also measured
with the instrument used during process. The obtained data pairs of concentration-signal
(response) are represented as shown in Figure 6, where the x-axis represents the element
contents added to the sample, while the y-axis represents the signals (responses) before and
after the addition. The value of the more accurate concentration compared to the normal method
of measurement is given by the intersection of the x-axis and the calibration linear.

![Figure 6 Calibration curve of a standard addition method](image)

**4.1.3. Internal standard method**

In fact, the internal standard method is not used as a separate calibration method. With the
application of this method, the number of errors resulted from the matrix of the sample or the
change of the parameters of the instrument, also known these changes as the drift of the
parameters of the instrument, can be decreased.

The process of the internal standard method is the following: a known and adequately large
amount (which are either not contained or a negligibly small amount of them are contained by
the sample used) of one or more of the substance or element (this is called the internal standard)
is added to each of the solutions (to the standard and sample solutions, also to the blank
solution).
The appropriate choice of measured element-internal standard pair is essential, since when it is chosen inadequately in extreme cases the direction of the result can even be reversed (poorer accuracy).

Summarising, when choosing the internal standard, is essential to take the similarity with the element into consideration, or else it does not correct in the appropriate degree, or in the worst scenario the modification will result poorer accuracy (that is into the wrong direction).

During the preparation of the calibration curve, the ratio of the signals of the element (to be measured) and the internal standard are represented on the y-axis and the concentration of the element (isotope) to be measured are represented on the x-axis (Figure 7). Hereafter the evaluation of the measurement and the results is carried out according to the principles specified in the Chapter 4.1.1.

![Figure 7 Calibration curve fitting applying internal standard](image)

**Figure 7 Calibration curve fitting applying internal standard**

### 4.1.4. Isotope-dilution process

The process of isotope-dilution that can possibly be applied in ICP-MS technics (detailed in the following), can be viewed as the mixture of standard addition and internal standard methods since the component (isotope) added has another mass number than the isotope used to measure the element to be measured.

In the sample the amount and proportion of the two isotopes of the element to be measured are defined, thereafter the known amount of one isotope is added to the sample. Since the quantity
added (masses), the concentrations and the ratios of the isotopes are known, also before and after the addition, the concentration of the element to be measured can be calculated.
4.2. Classifications of the optical spectroscopic methods

Two big groups of optical spectroscopic methods exist:

1. Atomic spectroscopic methods
2. Molecular spectroscopic methods.

There are several different ways to determine the quality and quantity of the components of food and food raw material samples. The UV-visible absorption molecular spectrometry is the most frequently used. Nowadays however, atomic spectrometry is the most wide-spread way of element analysis. Since the volume of this note limits mentioning of all methods of analysis, thus only atomic spectrometry techniques can be presented.

Strictly speaking, there are 3 different types of analytical measurement techniques in the group of atomic spectrometric methods (Table 1).

<table>
<thead>
<tr>
<th>Atomic spectroscopy methods</th>
<th>Theory of the method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Atomic emission method</td>
<td>emission of light</td>
</tr>
<tr>
<td>2. Atomic absorption method</td>
<td>absorption of light</td>
</tr>
<tr>
<td>3. Atomic fluorescence method</td>
<td>the absorption of light energy at one wavelength and its re-emission at another, usually longer wavelength emission of light</td>
</tr>
</tbody>
</table>

As the atomic spectrometry methods shown on Table 1, nearly covers the vast majority of frequent routine elemental measurement techniques, only ICP-MS is left out of the classification, which is also an elemental measurement technique, thus ICP-MS is divided into the same analytical group. As long as group of atom spectrometry methods is true, the analytical information is originated from the electron-excitation of free atoms or ions resulted atom- or ion-spectrum, which consist of relatively sharp lines; on the other hand in mass spectrometry, counting of the quantity of isotopes take places after the separation (according to mass/charge) of the isotopes to be analyzed. On the basis of the above consideration, Table 1 is logically completed with mass spectrometry in Table 2.
Table 2 Atomic spectroscopy methods, extended with mass spectrometry

<table>
<thead>
<tr>
<th>Atomic spectroscopy methods</th>
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<tr>
<td>1. Atomic emission method</td>
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<tr>
<td>3. Atomic fluorescence method</td>
<td>the absorption of light energy at one wavelength and its re-emission at another, usually longer wavelength emission of light</td>
</tr>
<tr>
<td>(Mass spectrometry method)</td>
<td>measurement of mass</td>
</tr>
</tbody>
</table>

In atomic emission spectrometry (AES) (Figure 8) thermal and electrical energy are used for the excitation of atoms and ions, which getting on a lower energy level and emit a ray of light, the wavelength which is characteristic for examined element and the concentration of the analysed element can be concluded from the relative intensity of the same light.

In atomic absorption spectrometry (AAS) (Figure 9) the analysed element is transformed into free ground state atoms with energy transfer (in a flame or graphite furnace). Through this atomic vapour a light with the wavelength characteristic for the element that is directed through and the decrease of the intensity of light is measured. The wavelength of the used light determines the quality of the analysed material, while the relative decrease of the intensity of light determines the relative and absolute quantity of the element.

![Figure 8 Theory of atomic emission spectrometers](image-url)
In atomic fluorescence spectrometry (AFS) (Figure 10), similar to AAS, free ground state atoms are created. These atoms are radiated with characteristic wavelength of the light for the measured element, but here this radiation is used for the excitation of the measured atoms (instead of light absorption), then the relative intensity of the fluorescent light that is emitted by the excited atoms is measured, which is proportional to the concentration of the measured element. The wavelength of the radiated fluorescent light determines the quality of the measured element.

In the field of elemental analytical measurements, inductively coupled plasma mass spectrometry (ICP-MS) was introduced 30 years ago, which is one of the most sensitive method nowadays. In ICP-MS the ions of the measured element (isotope) are produced, and when directed into the mass spectrometer, the ions are separated in the magnetic or electrostatic field according to mass/charge (m/z). The mass-charge ratio of the isotope is typical for the quality of the element, while relative intensity of the produced ion beam is proportional to the relative or absolute quantity of the measured element.
4.3. Atomic absorption spectrometry (FAAS)

4.3.1. Theory of the atomic absorption spectrometry

As mentioned in the Chapter 4.2., in the atomic absorption spectrometry (AAS), or more specifically in the flame atomic absorption spectrometry (FAAS), energy has to be transferred to the element which is going to be examined. In this case, the flame ensures the energy, which produces the free atoms in ground state, through these a light, having the same wavelength as the examined element, is released and the loss of the light intensity is measured. The loss degree of the light intensity is called the absorbance (A), which can be defined as the following: the absorbance (A) is the decimal logarithm of the quotient of the ratio of the $I_0$ (the intensity of light before absorption) measured on the given $\lambda$ wavelength and the $I$ (the intensity of light after absorption) measured after passing through the atomic vapour (Equation 9).

$$A = \lg \frac{I_0}{I} \quad (9)$$

where

- $A$: absorbance
- $I_0$: original intensity of light before absorption
- $I$: intensity of light source after absorption

The measurement of the absorbance can be used for quantitative determination while applying the following equation (10). The absorbance is directly proportional to the concentration of the examined element and to the length in which the absorbance is realized. Equation 10 is also known as the Lambert-Beer principle (Bouguer-Lambert-Beer principle).

$$A = a \cdot c \cdot l \quad (10)$$

where

- $A$: absorbance
- $a$: absorption coefficient
- $c$: concentration of the atoms to be determined in atomizer cell
- $l$: optical length, in this case the width of flame or graphite tube

The sensitivity of the method of measurement increases in direct proportion to the magnitude of $A$. Enlarging $A$ can be achieved with enlarging the numbers of multiplication (between normal values). Since in Equation 10 the absorption coefficient is almost constant, and the
extent of this cannot be influenced. The thickness of the layer of the absorber (l) can be modified between given limits (5-10 cm) through changing the geometric arrangement of the atomizer system. The extent of the absorbance can be influenced in the highest degree by changing the concentration free ground state atoms in the atomizer cell. The increase of the ability of detection of atomic absorption methods can be reached with creating a higher atomic concentration in the light beam, during the atomization of a sample with given concentration. It also has to be noted that the atom stays in this excited state for a short period of time, for approximately $10^{-9}$ seconds and it jumps back to its main orbit.

4.3.2. **Structure and operation of the atomic absorption spectrometers**

The schematic structure of the atomic absorption spectrometer can be found on Figure 12. Since the atomic absorption spectrometer is based on light absorption, the AAS system needs a light source in order to function. The light of the light source goes through the atomizer cell and pass through the optical system (the wavelengths of light are dispersed), where the separation of the line of the given wavelength of the given element happens. The analyte line then reaches the detector, where the measurement of the intensity of light happens. The control of the atomic absorption spectrometers, the collection and evaluation of data are done entirely by the computer.

![Figure 12 Units of atomic absorption spectrometers](image)

4.3.3. **Light sources**

The atomic absorption equipment needs a light source which emits the same wavelength light what the free ground state atom to be measured can absorb. This condition can be satisfied in
two different ways. The first option is the application of the hollow cathode lamp (HCL), the linewidth of 0.01 to 0.001 nm. The other options are the production of the intensity of light with the same linewidth, being in the range of 200 to 800 nm, with the application of light of an intensive continuous high pressure xenon arc lamp. Since the radiation of this is continuous, the properly narrow linewidth in this case can be reached with the help of a high resolution Echelle grating spectrometer. In both cases the properly narrow wavelength, or better said range of wavelength, can be produced for measuring the intensity of light before \( I_0 \) and after \( I \) the absorption.

The available hollow cathode lamps nowadays are enclosed to cylindrical glass tubes, and their diameters are 3 to 6 cm. The filling gas in these is noble gas with a pressure of 100 to 500 Pa (0.001 to 0.005 bar). Argon and neon are applied in most cases. The hollow cathode lamp (Figure 13, Photo 1 and 2) is made of glass, but a quartz window is used at the end of the lamp, where the light leaves the hollow cathode lamp, if the wavelength of the element to be analysed is in the ultraviolet range. The anode of the lamp is made of a tungsten wire, while the cylindrical hole of cathode is made of the same material (if it is possible to be mechanically processed) as the element to be analysed or the element is built into the cathode. The inner diameter of the cathode is 2 to 5 mm. In AAS techniques mostly single element lamps are used, but lamps with more elements are also available. The multi-element lamps contain 2 to 6 elements. Since the price of these multi-element lamps is higher (even twice as much), and the lifetime is rather shorter, the use of these is only worth if they are used for relatively short periods of time with each elements. The theory of AAS techniques means that the analysis of each different element needs a separate lamp. The potential in case of the hollow cathode lamps is 100 to 300 volt with filament current of 5 to 20 mA, with a lifetime of a 1000 to 2000 hours of operation.

Figure 13 Hollow cathode lamp
The wavelength of the line of several elements falls in the range of ultraviolet (around 200 nm or less). These elements are for example arsenic, zinc, mercury, cadmium and selenium. In this range, the intensity of light of the hollow cathode lamps is small, because the molecular oxygen of the air absorbs most of the emitted light. Electrodeless Discharge Lamps (EDL) have been developed for this problem. The most important part of the EDL lamps is a vial made of enclosed quartz (2 to 3 cm length and 1 cm in diameter). The volatile compound of the element in the vial, for example its halide, is excited in a low pressure (100 Pa) noble gas space with a high frequency generator (27.12 MHz), which results in the intensity of the emitted light being around 6 to 10 times as strong as the normal hollow cathode lamp.

### 4.3.4. Sample introduction

In the atomic spectrometry methods it is possible to analyse gas, liquid or solid samples as well, although mostly liquids are analysed. Therefore if the analysed sample is not a liquid, a sample preparation method is applied where an aqueous solution is prepared. These are the different digestion and extraction methods. The application of aqueous solution is recommended, because of the homogeneity and usability of it and it is also important that the aqueous solution sample can be introduced into the atomizing cell with constant volume flow rate, in a controllable and reproducible way. Moreover calibration standards can be prepared to these more easily, furthermore the automatic measurement of liquid samples can be solved with automatic sampler changers. Thereby this results more comfortable handling of the analytical equipment, moreover it results more favourable schedule for the operator.
In atomic absorption spectrometry there are several different ways of sample introduction, in case of food samples the nebulization methods are the most wide-spread. When the solutions are nebulized, micro sized drops are created from the liquids. Usually pneumatic nebulizers, more infrequently ultrasonic nebulizers and very rarely hydraulic high pressure nebulizers are used. In almost all cases, while using the pneumatic nebulizer, the oxidant gas (usually air) is also used as nebulization gas. The different nebulization technics are detailed in the Chapter 4.4.4.

In case of liquid nebulization, two most important parameters are the sample introduction flow rate and the nebulization efficiency. The sample introduction flow rate is the volume of the solution introduced into the nebulizer under a given period of time, mostly given in cm³/min (mL/min), which is usually 4 to 6 cm³/min in the case of FAAS.

The nebulization efficiency shows that how many percent from the nebulized solution reaches the atomizing space, in case of flame atomic absorption spectrometry how much reaches the flame. When using the flames, the efficiency of the pneumatic nebulizers is usually between 5 to 10%.

According to Hungarian and International scientific literature, only the fraction under approximately 10 μm of the nebulized drops reaches the flame. The quantity of this fraction, in case of pneumatic nebulizers, can be defined with the help of the Nukiyama-Tanasawa empirical relationship:

$$d_s = \frac{585}{v_{g}-v_{l}} \cdot \left( \frac{\gamma}{\rho} \right)^{0.5} + 597 \cdot \left( \frac{\eta}{(\gamma \rho)^{0.5}} \right)^{0.45} \cdot \left( \frac{1000 F_l}{F_g} \right)^{1.5}$$  \hspace{1cm} (11)

where

- $d_s$: Sauter mean diameter of droplets (ratio of volume/surface of droplets)
- $v_g$: linear flow rate of nebulizer gas
- $v_{l}$: linear flow rate of nebulized liquid
- $\gamma$: surface tension of sample solution
- $\rho$: density of sample solution
- $\eta$: viscosity of sample solution
- $F_l$: volumetric flow rate of sample solution
- $F_g$: volumetric flow rate of nebulizer gas
In all of the analytical technics, thus in atomic absorption technics also, one of the most important expectation to maintain all of the operational parameters as constant as possible, for example the sample uptake flow rate and the nebulization efficiency to be constant. In the Nukiyama-Tanasawa equation (11), the fact that the detailed parameters (the surface tension, density, viscosity and flow rate of the measured samples) are already taken into consideration. One of the most important expectations is to carry out and to ensure almost the same analogy of the calibration standards and prepared sample solutions (in other words: matrix matching).

**4.3.5. Atomizer space**

The atomic absorption spectrometry measurements can be classified in 3 different groups:

1.) Flame atomic absorption spectrometry (FAAS)
2.) Graphite furnace atomic absorption spectrometry (GF-AAS)
3.) Hydride generation and cold vapour atomic absorption spectrometry (HG-AAS and CV-AAS)

The sample introduction part of the hydride generation atomic absorption spectrometry and the cold vapour atomic absorption spectrometry is hydride and cold vapour generation is detailed in the Chapter 4.4.4.2.

**4.3.5.1. The flame as atomizer**

The sample introduction technique detailed in Chapter 4.3.4. provides the sample introduction into the atomizer cell of the flame atomic absorption spectrometer, where preparation of the free ground state atoms happens and be measured. This process, or the more important steps of transformation of the wet aerosol getting from the nebulizer into the flame, is shown in Figure 14.

The size of the aerosol particle shown on the bottom of Figure 14 is below 10 μm, which is called wet aerosol, because it is in a hydrated state in case of an aqueous solution (in case of other solvent, solvated state). The desolvation of wet aerosol starts already with entering the burner. The main reason for this is that the operating temperature of the slot burner of the acetylene-air flame used in atomic absorption measurements that is between 110 and 120°C.
When the vaporization of the aerosol particles takes place, the solid dry aerosol particles are either melted and then evaporated or directly transformed from solid state into gas state that is sublimated. The gas molecules are created into free ground state atoms as a consequence of the process of homolytic decomposition. Those who deal with atomic absorption spectrometry try to enlarge this fraction as much as it is possible, because the characteristic radiation from the hollow cathode lamp, and the free ground state atoms (neutral) can absorb. Thanks to this, the analytical measurement can be performed because there are different particles in various states in the flame, such as free ground state atoms (which is important), not free atoms (molecules), excited atoms, ions and excited ions, thus it is really important to choose a compromised group of parameters, with the largest proportion of free ground state atoms that can be created for light absorption. For example if the temperature of the flame is not high enough, the given element cannot be atomized and stays in form of metal-oxide or metal-hydroxide. Conversely if the temperature of the flame is too high, the ionization or excitation after the atomization step is facilitated, with which the proportion of free ground state atoms decrease.
Table 3 Flames and their parameters applied in atomic absorption spectrometry

<table>
<thead>
<tr>
<th>combustible gas</th>
<th>oxidant gas</th>
<th>reaction heat (kJ)</th>
<th>combustion temperature (K)</th>
<th>combustion rate (cm/s)</th>
<th>flame temperature (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetylene (C₂H₂)</td>
<td>air</td>
<td>1260</td>
<td>620</td>
<td>160</td>
<td>2500</td>
</tr>
<tr>
<td>acetylene (C₂H₂)</td>
<td>dinitrogen-oxide (N₂O)</td>
<td>1680</td>
<td>160</td>
<td>3000</td>
<td></td>
</tr>
<tr>
<td>hydrogen (H₂)</td>
<td>air</td>
<td>250</td>
<td>800</td>
<td>310</td>
<td>2300</td>
</tr>
<tr>
<td>hydrogen (H₂)</td>
<td>oxygen (O₂)</td>
<td>250</td>
<td>720</td>
<td>1400</td>
<td>3000</td>
</tr>
<tr>
<td>propane (C₃H₈)</td>
<td>air</td>
<td>2220</td>
<td>510</td>
<td>80</td>
<td>2200</td>
</tr>
<tr>
<td>butane (C₄H₁₀)</td>
<td>air</td>
<td>2890</td>
<td>490</td>
<td>80</td>
<td>2200</td>
</tr>
</tbody>
</table>

In the last 60 years of atomic absorption spectrometry, mainly acetylene-air flame was used, which has the same temperature and chemical composition day by day, so it can be reproduced. A combustible and an oxidant gas are required for the burn, and the ratio of them can be controlled with rotameters. In atomic absorption spectrometry different compositional gases have been used. The most common ones are: firstly the acetylene-air, secondly the acetylene-dinitrogen-oxide. The others are so rarely used in practice that they are insignificant. 3000 K temperature (Table 3) of the acetylene-dinitrogen-oxide flame is able to atomize several elements.

Figure 15 shows which elements can be analysed with flame atomic absorption spectrometry, and which type of flame is recommended to result in the largest proportion of free ground state atoms.

Of course in order to be able to judge, which flame is the most appropriate in practice, not only the temperature of the flame is important, but also numerous other parameters must be known like the geometry of the burner head and the flame, the dispersion of the temperature, the quality of the gas mixture of the flame, moreover the ratio and the volumetric flow rates of the applied gas mixture, the different particles occurring in the flame and their spatial distribution, the
chemical characteristic of the flame (reducing or oxidizing). The burner head and the acetylene-air flame occurring during the measurement can be seen on Figure 16.
On both sides of Figure 17, it can be seen that which units have been built up the structure of the atomic absorption spectrometer, as well as the different physical processes that are taking place. On the left side the hollow cathode lamp emits characteristic lights from which the line with the most intense wavelength is chosen, which can be seen on the right side of the figure. In the following step, the sample is nebulized into the atomizing space, which in case of flame atomic absorption is a flame and in case of a graphite furnace atomic absorption is a graphite furnace. The free ground state atoms are formed in the atomic source absorb of the light, which leads to the light attenuation in proportion to their concentration in the solution. For simplification the light absorption on the figure is only shown on the most intense line, but since hollow cathode lamps emit numerous lines per element (possibly thousands), each line of the elements absorbs the similar proportion of light. Then with the help of the optical system, the physically most intense (most sensitive) line is chosen. Knowing the intensity of this line before \((I_0)\) and after \((I)\) the absorption, and the absorbance \((A)\) can be formed, according to the Lambert-Beer principle (Equation 10) that is in direct proportion to the concentration of the analyte \((c)\). The atomic absorption method is a relative measuring method, meaning that the instrument must be calibrated with standard solutions before the measurement. Then the absorbance resulted from the measurement of the solution with unknown concentration is substituted in the fitted calibration curve and the absorbance that is correspond to the concentration (unknown).
Figure 17 Units of atomic absorption spectrometers and measurement process based on light absorption

4.3.5.2. The graphite furnace as atomizer

Using a flame is not the only option to create free ground state atoms, but the temperature (2500 to 3000 K, as in the flames) and thermal energy require the process shown on Figure 14 that can be reached in a graphite tube as well. The energy in case of a graphite tube is electricity. The graphite furnace is a 20 to 30 mm long with an inner diameter of 5 to 8 mm graphite tube placed horizontally (Figure 18). On the upper part of the graphite tube, lengthwise in the middle, 5 to 50 μL volume sample can be introduced through a hole with a diameter of 1 to 1.5 mm. The heating of the tube mostly happens in transversely direction, which is better than doing in a longitudinally-heated furnace. In case of transversely-heated furnace, the whole length of the graphite cuvette is heated evenly. Around the bottom of the graphite tube, argon gas is introduced to protect the graphite tube, which eventually surrounds the graphite tube inside and out, protecting it from the oxygen of air. The solution that should be determined is either put
directly into the graphite tube (Figure 18) or into a so-called L’vov platform (Photo 3) which indeed is putting into the bottom of the graphite tube. The L’vov platform is formed in a way that it can possibly be put inside the bottom of a graphite tube (Photo 3). With the application of the L’vov platform, the temperature of inside the atomizing space is adequately high to form the most completed atomization. Temperature delay must be used for this purpose, which can be realized with the L’vov platform. It is a pyrolytic graphite flat piece with a small edge, so both analytically and taking of its lifetime into consideration, and the circumstances are more favourable. This statement applies for pyrolysed graphite tubes (Photo 4) as well with the application of which the penetration of the solutions into the wall of the graphite tube can be prevented. The lifetime of a graphite tube is approximately 200 to 1000 heating. This number is defined by both the composition of the sample to be examined and the applied maximal temperature.

Figure 18 Graphite tube applied in a graphite furnace atomic absorption spectrometer

Photo 3
L’vov platform

Photo 4
Pirolized graphite tube

After dropping the liquid sample into the graphite tube, a heating programme is applied, the temperature steps are shown in Figure 19.
Figure 19 Heating program of graphite tube

After dropping the sample the first step is drying (105 to 150°C). The sample is evaporated during this process. The solvent (mainly water) evaporates, the solid parts stay on the inside wall of the graphite tube. The temperature of drying must be adjusted to the boiling point of the solvent. If the temperature is higher than the boiling point of the solvent, the boiling sample splashes around and smears in the tube. If the temperature is too low, the sample does not dry.

The second step is ashing (300 to 1500°C). Ashing and thermal decomposition takes place here, during all matrix material mainly organic materials decomposes and transforms into inorganic material. Thereby the sample is transformed and it contains volatile, inorganic components. Then in the following step, during atomization a possibly, least interfering effects can be reached. The temperature level should be as high to allow the accompanying material to leave the graphite tube, but the whole quantity of the element to be analysed while staying inside.

The third step is atomization (1200 to 2700°C). This step consists evaporation of all the solid materials, then forming the atomic vapour (free ground state atoms). The optimal temperature should be chosen, taking into consideration the thermal characteristics of the element to be examined.

In the following heating step (2000 to 2800°C) the graphite tube is cleaned out and all the remaining products are removed from the tube. Thereby, the tube is prepared to measure the next sample (avoiding the memory effect).
The last step of the heating process is cooling the graphite tube down. The graphite tube is cooled down to approximately room temperature, in order of the next sample to be dropped into it.

4.3.5.3. Comparison of detection limits of the FAAS and the GF-AAS

The graphite furnace atomic absorption spectrometers (GF-AAS) are suitable for determining an approximately three magnitudes that have smaller concentration, than the flame atomic absorption spectrometers (FAAS). This can be explained with the following:

- The light beam of the hollow cathode lamp, both in a graphite tube and a flame, passes through only a narrow channel. However, the time while the free ground state atoms flow in this channel is relatively short and the duration of staying of them is only $10^{-5}$ to $10^{-4}$ sec (Figure 16), the duration of staying of the free ground state atoms is limited to seconds. This effect increases significantly the size of the absorbance (Equation 10) in the case of GF-AAS comparatively to the FAAS.

- As the graphite tube (Figure 18, Photo 4) represents a smaller atomizing space (20 to 30 mms in length, with an inner diameter of 5 to 8 mm) is opposed to a narrow (depth: 1 to 3 cm) flame on a wide burner (width: 5 to 10 cm) (Figure 20), then the nebulized sample is diluted in the flame and then in the graphite tube. Thus the graphite tube has advantage in this point.

- The efficiency of sample introduction in the GF-AAS is approximately 100%, since almost all 100% of the injected sample during atomization stays inside the tube (Figure 21). However, thanks to the nebulization efficiency, only at most 10% of the nebulized sample reaches the flame (Figure 22).

- Regarding the previous point of comparison, it has to be mentioned, that in case of an FAAS the 10% of a few mL sample (generally 4 to 6 ml) is nebulized into the atomizer. By comparison, in case of a GF-AAS approximately 100% of the sample stays out of 5 to 50 μL. This fact ensures a bigger proportion of sample fraction in case of a flame.
Figure 20 Nebulizer-burner system of a FAAS equipment

Figure 21 Efficiency of sample introduction into a graphite tube is approximately 100%

4.3.6. Optical system

Single and double beam atomic absorption spectrometers

The various manufacturers offer atomic absorption spectrometers with single and double beam, moreover single- and multichannel atomic absorption spectrometers as well. The units and layout of the single beam atomic absorption spectrometer are shown on Figure 22, and the double beam on Figure 23.
In case of the single beam (Figure 22), the light from the hollow cathode lamp passes over the atomizing space directly through the optical system, then reaches the detector. \( I_0 \) intensity of light is measured before introduction of the sample into the atomizing space and the equipment is reset to this value (as zero value), and comparing the intensity of light after the absorption (I) to the initial one is done. However, if the intensity of light of the lamp changes (e.g. due to any electrical fluctuation), by this means the result is loaded with error, because it is compared to the initial (original) intensity of light. This error can be eliminated with the double beam instrument (Figure 23), which a chopper is applied, and the initial and later intensities (before and after absorption) of light are measured at once and the difference of these two results in the weakening of light. The rotation of the chopper (Figure 23) allows the measurement of both intensities alternately 50 to 100 times per second. When the hole of the chopper is met with the light of HCL, then the light can pass above or through the atomizing space (flame or graphite tube) and the absorption of light takes place. When the light beam reaches the mirror part of the chopper, the light reaches the detector with the help of the mirrors without light absorption.
While the previous figures show the schematic units of AAS equipment, Photo 5 and 6 both show real FAAS instruments.

![Photo 5 FAAS equipment (Carl Zeiss Inc.)](image1)

![Photo 6 FAAS equipment (Perkin-Elmer Inc.)](image2)

**Single- and multichannel atomic absorption spectrometers**

As mentioned, there are also single- and multichannel atomic absorption instruments. This classification suggests how many elements can be measured simultaneously. Nowadays the available AAS instruments are mostly single channel instruments, because of the economic considerations. This means AAS instrument can analyse only one element at a time with the application of one lamp.

Because of taking place of the analysis on one wavelength that is shown on the right side of Figure 17, possibly the most intensive wavelength of light should be chosen out of the light of HCL. This can be performed with the optical system (monochromator). The word of monochromator consists words of mono, meaning single, and chromos, the Greek word for
colour which literally means ‘one colour’ and has one wavelength. In selection of wavelength an optical grating, a filter or a prism can play the main function. The filter can be an absorbance-filter or an interference-filter. The optical grating is the most modern and most frequently used one. The used reflection grating nowadays consists of numerous (70 to 4800 grooves/mm) parallel, equidistant grooves that have the same shape. The light beam enters to the optical grating where the wavelengths of light are dispersed. The more numerous the grooves, the better its ability of light resolution. The given wavelength of light is reflected from the optical grating after the resolution of light, in one direction not only one given wavelength of light is reflected, but its multiples (n·λ) also, which are called spectral orders.

The continuous source AAS (CS-AAS) was introduced 10 years ago in the market, which uses a xenonarc lamp. Its range of wavelength is 185 to 190 nm. The light is dispersed with Echelle optics (shown in 4.4.5. in detail), where the appropriate resolution is reached by the application of the 50 to 150th spectral orders. The degree of resolution in the Echelle optics is reached with a prism and a grating simultaneously. In the high resolution AAS instrument (HR-AAS), a solid state detector (CCD=Charge Coupled Device) is applied to detect the intensity of light in the commercial instrument. The CCD detector of the device uses 200 to 588 pixels for analytical purposes. Thanks to the Echelle optics and the continuous source, different pixels can be irradiated at once and the result of the measurement can be read. Hereby this enables multichannel AAS measurements.

**Background correction methods**

In atomic absorption measurement, the weakening of light is not only resulted by the absorption of light, but light scattering as well. (Remark: if you put your hand before a lamp, the light weakens, but it cannot be called absorption.) In atomic absorption spectrometry such a significant interfering effect can be observed if the sample solution is measured with a flame that has a high concentration or the graphite tube is used in it. In this case, the solid particles getting into the light path can also result in the weakening of light, which is detected with the HR-CS-AAS as a virtual light absorption. The net light absorption is calculated when any kind of background correction is used for eliminating of optical interfering effect.

Methods of background correction:

- Two line method
- Deuterium lamp background correction
- Zeeman background correction
- Smith-Hieftje background correction

In case of the two line method, sample is measured on 2 lines. One of them is the analyte line of the measured element, the second one is another, which is close, but not origins from the element.

Since the phenomenon of light scattering depends on the wavelength, background correction should be used mainly in the ultraviolet wavelength range. The deuterium lamp is one of the most suitable way of background correction. The D$_2$ lamp provides a constant spectrum in a range of 180 to 400 nm. A tungsten lamp can solve the same in a higher spectrum range. The difference between the measured absorbance values made by the HCL and deuterium lamp separately (practically at the same time) results the net absorption of light.

![Figure 24 Schematic diagram of atomic absorption spectrometer applying deuterium lamp background correction](image)

The Zeeman method is based on the Zeeman effect, which the measured line symmetrically splits in the magnetic field (2 lines appear on the left and right side of the measured line). When the magnetic field is turned on, only light scattering happens, but when it is on, absorption happens as well. The net intensity is given by the difference of the two measurements.

The Smith-Hieftje method is based on the self-absorption, or also known as on the basis of line-reversion. This effect is well-known in emission spectrum analysis. By using this method, HCL lamp is first heated with normal current, then with a high one. In this case the difference of two
measurements is also calculated. This is only possible in case of those elements and during the measurements, when complete line-reversion happens.

4.3.7. Detector

The task of the detector is to transform the intensity (or absorption) of light into electrical signal. In AAS techniques, a photo multiplier tube (PMT) is usually used. The photomultiplier tube consists of a photocathode, an anode and dynodes between the two previous. If light reaches the photocathode, electrons emerge it.

The emerged electrons from the photocathode, collide into the dynode closest, then 2 to 5 times as much secondary electrons emerge to the following dynode. This process continues until a measurable signal forms.

As previously mentioned solid state detectors are integrated in the most recently developed AAS instruments, the charge coupled (CCD) and charge injection devices (CID) must be mentioned.

4.3.8. Interfering effects in AAS

The atomic absorption spectrometry was introduced as a free of interfering effects method. However, when later turned out, though there are only few spectral interfering effects, there are several other ones.

Interfering effects in flame AAS

The interfering effects were earlier categorised into spectral, physical, gas phase and condensed phase chemical ones. Although nowadays, according to the International Union of Pure and Applied Chemistry (IUPAC), only two groups are distinguished: spectral and non-spectral interfering effects.

Spectral interfering effects

Spectral interfering effect is experienced when the absorbed radiation of the component, furthermore the emitted radiation of the component to be analysed cannot be distinguished from other radiations or weakening of light.
Line-coincidence in atomic absorption spectrometry is uncommon. The probability of the spectra is formed in the flame or graphite tube when atomization gets close, though the probability of becoming coincidence of the spectra with the absorption line of the measured element, gets higher. Choosing another wavelength or using either the Zeeman or the Smith-Hieftje background correction can eliminate or decrease these problems. The interfering effect that has been caused by light scattering can be eliminated with use of the above specified background methods (Chapter 4.3.6).

**Non-spectral interfering effects**

Non-spectral interfering effects are the ones that have an effect on the formation of free atoms. This step of the formation of free atoms is affected and decides what group is taken into. The non-spectral interfering groups are the following:

- **Transport interference**: affecting the quantity of the sample that are appearing at the height of measurement during a given period of time. These effects affect the flow rate of sample introduction and nebulization efficiency, as well as the effects affecting the evaporated (desolvated) fraction.
- **Interference of evaporation of dissolved material**: affecting the evaporation of the examined element, such as the ones resulting from the fluctuation of the speed of the evaporation of dry aerosol particles.
- **Gas phase interference**: caused by changes in the dissociated or ionized part in the gas phase of the examined material.
- **Interference of spatial distribution**: appearing when the change in the concentration of the interfering material affects the speed of mass-flow or the distribution of mass flow of the examined material.
- **Interference of redistribution of ionized particles**: as long as proportion of the concentration of ions varies in the different sizes of droplets.

**4.3.9. Compensation methods for interferences**

Regarding the frequency and extent of the interfering effects that mentioned above, the most important are the non-spectral ones, more precisely the interference of evaporation of dissolved material and the gas phase interferences. There are different ways of decrease that eliminate the errors caused by the non-spectral interfering effects. The most frequent ones are:
- Standard addition method: detailed in Chapter 4.1.2.
- Simulation technique: the characteristics of the standard solution are likened to the characteristics of the sample solution, resulting the similarity of the interfering effects in both (in other words: matrix matching).
- Buffer-additional technique: a spectrochemical buffer is added both to the samples and to the calibration solutions in order for the measurement that is less sensitive for changing in the concentration of interfering elements. These are the spectrochemical buffers, which are the following:
  - Releasing additive, forming a compound with the interfering agent to decrease its interfering effects on the evaporation of the measured element. For example when calcium is measured, the added lanthanum-chloride eliminates the interfering effects of the alkaline-phosphates.
  - Protective additive, which forms a chemical compound with the element to be measured, in order to preclude the effect of interfering material. For example, the different complex-forming ligands usually interfere the analysis of transition metals. Added calcium-cyanide can counteract these, because it forms the most stable complex with transition metals.
  - Ionisation buffer, which increases the concentration of free electrons in the flame gases to press back the extent of ionization and to stabilize the degree of ionization.
  - Evaporation of promoted buffer, which increases the volatility of the accompanying components or forms a more volatile component than the measured one.
  - Saturation additive, it means if the interfering agent is added in high concentration, it sets the constant level of interference.
- Elimination of interference in the graphite furnace with the application of matrix modifiers: The atomization processes in the graphite tube result more favourable circumstances and to eliminate or at least decrease the interference, which are mainly condensed and gas phase types. Different additives that are used for this purpose are: ammonium-nitrate (copper analysis), nickel-salts (arsenic, selenium and tellurium analysis) and ammonium-hydrogen-phosphate (cadmium analysis), as well as molybdenum compounds, potassium-dichromate and magnesium-nitrates as matrix modifiers. Furthermore, the mixture of palladium-salts and magnesium-nitrates is the so-called universal matrix modifiers.
4.4. Inductively coupled plasma optical emission spectrometry

4.4.1. Historical overview of inductively coupled plasma

The first observation of atom emission happened in Glasgow in 1752, and is connected to Thomas Melville. According to him, the flame was produced by the mixture of alcohol and sea salts that emits a bright yellow light. In absence of salts, yellow light is not experienced. If Thomas Melville had not died a year later, the conquest of spectrochemical methods would have started earlier.

In 1776 Alessandro Volta used a spark device for chemical analysis. According to his experiments, he established the use of different solids result in different-coloured flames.

At the end of 18th and beginning of 19th century, Fraunhofer and other researchers compared the emission spectra of the flames and sparks to the spectra of Sun and planets. In 1826 Talbot reported an experiment which had observed the colouring of flames by a variety of salts.

In 1859 Kirchhoff and Bunsen assumed that the sharp spectrum-lines of the flames are not assigned to molecules, except atoms. This was the first time that the nature of spectra tried to be interpreted. Between 1860 and 1864 Kirchhoff and Bunsen elaborated emission spectroscopy methods that led to the discovery of 4 elements: caesium, rubidium, thallium and indium.

Until the middle of 20th century, arc and spark excitation were used as the best analytical equipments. Unfortunately in case of non-conductors the preparation was so hard and time-consuming that they were mainly appropriate to examine solid material. 5 to 10% of standard deviation of the results was accepted while arc and spark excitation emission techniques were mainly used for the analysis of solid metal samples, flame spectrometry was used for analysis of elements with low excitation energies such as alkali elements. It should be noted that historically the arc and spark excitation was the first multielemental determination which mainly spark excitation is the most wide-spread in metal industry nowadays.

From the 1960s the application of arc and spark atomic emission significantly decreased, because the atomic absorption spectrometry (AAS) started to gain ground gradually. The AAS method has fewer interfering effects compared to the previous ones and the measurement can be carried out more precisely (0.2 to 2%). Its huge disadvantage is a mono-element method,
opposed to the simultaneous multi-element emission methods. To prepare solid samples, an appropriate method of dissolution or destruction is required.

The GF-AAS had a lot of interfering effects after its introduction, and its reproducibility fell far short of the common values in AAS. However, after elaborating and applying the deuterium and Zeeman background correction, the mentioned disadvantages of the GF-AAS decreased, while the detection limit improved more as well.

Later, both flame and graphite furnace atomic absorption spectrometry became wide-spread for examining elements. The AAS instruments are only suitable for mono-element examination, with a few exceptions. Their range of analytical determination is only two or three magnitudes, so the samples must be diluted in several cases.

In 1964 Stanley Greenfield was the first to report a new type of radiation source in atomic emission spectrometry and the atmospheric pressure inductively coupled plasma (ICP). In their work, the advantages of inductively coupled plasma optical emission spectrometry (ICP-OES) were compared with arc, spark and flame radiation sources:

- The new type of radiation source provides great stability so the disadvantageous interfering effects can be eliminated.
- The ICP-OES method can determine more elements from the periodic system, than traditional chemical flames.
- Its detection capability is far better than the above methods.
- Operating the plasma is much easier than handling traditional arc and spark methods especially in case of solution and liquid samples.
- Creation of plasma does not require an electrode, so it is uncontaminated and can be realized with a really low interfering background.

The breakthrough of the ICP-OES method in the determination of the concentration of elements did not happen immediately, because it provided the analysts with only a few more opportunities. Nevertheless Velmer Fassel optimized and refined the parameters of the ICP-OES based on works of Greenfield. He looked up and eliminated the possible sources of interference in the instrument and created a new type of plasma torch. By the beginning of the 70s’ thanks to the research of Fassel, the performance of the ICP-OES method had gone beyond the others. The optimized equipment had a favourable range of detection, which was freedom
from interferences, furthermore enabled the use of a wider calibration range. The first modern ICP-OES became available in 1974.

Nowadays the ICP-OES method has been become the most frequently applied analytical technique in area of routine elemental analytics. Besides, in the domestic and international practice, the routine laboratories apply increasingly inductively coupled plasma mass spectrometer (ICP-MS) and inductively coupled plasma optical emission spectrometer (ICP-OES). By the application of the above analytical techniques the vast majority of the inorganic elements in food and food raw materials can be determined by using excellent detection limits (µg/dm³). The ICP-MS is capable to analyse the different isotopes of the elements. Unfortunately the purchase price and the maintenance costs of the ICP-MS limit its Hungarian spreading.

4.4.2. Theoretical basics of the ICP-OES measurement

The ICP instruments elaborated in 70’s and are suitable for routine determination of about 70 elements. The plasma torch was developed by Fassal has been used in plasma emission spectrometry up to today. The plasma torch consists of 3 concentric quartz (or other suitable materials) tubes, as is seen in Figure 25. The torch is surrounded by a cylindrical induction coil with 2-4 hollow copper Turns, which is connected to the radio frequency (RF) generator. The RF generator of commercially available ICPs is either on 27 (27.12 MHz) or 40 MHz (40.68 MHz), however 56 and 78 MHz ICPs were prepared by experimental character. To ignite the plasma a Tesla-, or piezoelectric spark is applied to the argon gas that is caused some free electrons that be stripped from their argon atoms. Then the energy from the RF generator (so called forward power) is passed from the copper coil to the above free electrons for forming high energy electrons. The high speed electrons collide with the argon atoms are stripping off more free electrons in order to produce a high temperature plasma condition. This process is inductive coupling.

The ICP instruments are usually running on argon gas, but there are also plasmas that are operated by helium. These plasmas are not wide-spread due to economic reasons.

As is seen in Figure 25, the cooling or plasma gas flows between the outer and intermediate tubes. From its double name, it has a double function:
- cooling gas: it cools down the space between the plasma and the outer tube. When it is not operated properly, the outer tube will melt,
- plasma gas: maintains the function of the plasma (the only gas, without it, is creating plasma is impossible).

The auxiliary gas flows between the intermediate and the inner tube with two functions:
- with proper gas flow rate, it lifts the plasma and resulting increased lifetime because the end of the torch does not melt,
- stabilizes the operation of the plasma to result more stable plasma.

The nebulizer or sample gas (actually different gases in some instruments) flows in the inner tube, from its double name, it has a double function:
- nebulizer gas: nebulizes the liquid sample and forming aerosol.
- sample gas: transports the part of the nebulized sample which already consists of fine enough aerosol particles.

Figure 25 Schematic diagram of a torch (radial)

Several ICP-OES instruments use flushing gas between the plasma and the spectrometer that the task of them are excluding the oxygen molecule because the intensity of emission lines with a wavelength of less than 200 nm (exactly 194 nm) significantly decreases because the oxygen
in air absorbs significant part of intensities of emission lines in this wavelength range. E.g. sensitive lines of arsenic, mercury, sulphur and phosphorus can be found in this range. The flow rate of this flushing gas is usually less than 0.5 L/min.

There are so many equipments which use either a shear gas or a cone similar to the cones that are used in the ICP-MS technique, even though these cones have larger inner diameter hole. On the one hand, they stop oxygen of the air to absorb, on the other hand they also eliminate the self-absorption of the atoms in the colder parts of the plasma.

The typical flow rates of the above gases are:

- cooling gas (plasma gas): 10-20 L/min.
- auxiliary gas: 0.5-2 L/min.
- sample gas (nebulizer gas): 0.8-2 L/min.
- shear gas: 30-40 L/min.

As it is shown in Figure 26, the plasma can be divided into: a lower part, responsible for preheating the measured aerosol, and the temperature of the radiation zone that increases going upward. The terminology is shown in Figure 26. The induction region is the hottest part with 10000 K temperature. The copper coil transfers the energy into this region, from where it spreads further and the most part of the white light (argon continuum) appears here as well. For analytical purpose, the above part of the plasma core is applied, because of its excellent characteristics (Figure 26).

![Figure 26 Temperature regions of the intermediate path and different zones of the plasma](image)
Although there are numerous international publications on the emerging excitation of atoms and ions in the inductively coupled plasma, the exact description of these processes in the plasma are still unknown.

As already mentioned, the needed energy for ionization and excitation is provided by radiofrequency energy (RF) (so called forward power). Generally in the ICP techniques 1000 to 1600 W of the forward power is applied. The RF energy transfer can be divided to 4 steps:

1. The load of the RF energy into the outer part of the plasma.
2. Energy is passed into the inner part of the plasma. The components that should be analysed are found here.
3. Partial atomization and ionization of the aerosol
4. Excitation of the atoms and ions.

Emission spectrum only occurs if the atoms/ions absorbs enough energy and namely they get into an excitation state. Excitation means the whole process by means of which the measured material emits the characteristic radiation. The sample to be measured goes through the following processes, until it reaches the emission of the typical light (Figure 27):

![Figure 27 Excitation process of the nebulized sample](image)

As is seen in Figure 27, the sample reaches the reaction chamber in form of aerosol and solvent evaporates that measured sample gets into gas state then the molecules fall into free atoms and
depending on their ionization energies which they are ionized. The generated atoms/ions get into an excitation state with energy absorption then they reach a lower energy level with emission of energy by means of radiation of a characteristic light. The radiated light can be an atomic (emitted by excited atoms) or an ionic line (emitted by excited ions).

The intensity of the emitted light shows the quantity of the measured element in the sample. The intensity of the emitted light (I) and the concentration (c) of the measured element in the steam chamber is expressed by the Scheibe-Lomakin equation (12) used in the emission spectroscopy:

\[ I = K \cdot c^m \]  \hspace{1cm} (12)

K and m are constants in Equation 12 and they depend on the experimental conditions. The equation seems simple but as is seen in Figure 27, several physical and chemical processes are carried out until the emission of light from the sample is happening what is necessary for the quantitative analysis. These processes are obviously not independent from both the concentration of the measured elements and the outcome of one another. Consequently, K and m are not constants in fact so their values cannot be determined. K includes the excitation and other parameters and m as an exponential factor refers to the absorption of the emitted light in the lower temperature steam chamber.

In the thermal balanced system with an absolute temperature of T the Boltzmann-distribution can be used to calculate the density (n(p)) of the particles being on the atomic level of p:

\[ n(p) = n_a \cdot \left[ \frac{g_p}{Z_a(T)} \right] \cdot e^{\left( \frac{-E_p}{kT} \right)} \]  \hspace{1cm} (13)

where

- \( n_a \): total concentration of the a atoms
- \( g_p \): statistical weight of level p
- \( Z_a(T) \): distribution function of the a atoms
- \( E_p \): excitation energy of level p
- k: Boltzmann-constant (k=1.380622 \cdot 10^{23} \text{ J/K})
- T: absolute temperature

The temperature by which n(p) value is determined, is called excitation temperature (Tg).
According to the classic Rutherford-Bohr model and the wave theory, electrons have discrete energy values in the electron shells of the atom. Energy absorption or emission is only possible in the form of discrete quantities of energy. The atoms/ions have higher level of energy than the ground state that are in excited states. The excited particles are unstable and they can become more stable with emitting energy. One possible way to release energy that the particles in the excited state transfer their excess energy to not excited atoms or ions during collisions. In this case, the energy is converted to heat. The other possible way is to release energy that is utilized in emission techniques for the determination of element concentration: the excited particles release the absorbed energy in the form of electromagnetic radiation.

The energy changing between the energy levels of \( p \) and \( q \) by emission of light quantum is directly proportional to the frequency of the light (thus it is inversely proportional to the wavelength of the light). This relation is shown by the Planck equation (14).

\[
E_q - E_p = \Delta E = h \cdot \nu = h \cdot \frac{c}{\lambda}
\]  

(14)

where

- \( E_p \): energy level of the original state (before the absorption) (J)
- \( E_q \): energy level of the final state (after the absorption) (J)
- \( \Delta E \): energy difference between two levels (before and after the absorption) (J)
- \( h \): Planck’s constant (6.626196·10\(^{-34}\) J·s)
- \( \nu \): frequency of the electromagnetic radiation (Hz=1/s)
- \( c \): the speed of light (in air 2.997925 \(10^8\) m/s)
- \( \lambda \): wavelength of light (nm)

As each element has given energy levels, and therefore energy transitions, thus the wavelength of the emitted light (Equation 14) is characteristic for its quality, the intensity of the emitted light is proportional to the concentration according to Equation 12.

### 4.4.3. Major units of an ICP-OES equipment

The major units of the ICP-OES equipment (Figure 28): argon gas, peristaltic pump, sample introduction unit (e.g. nebulizer), radio frequency generator, torch (radiation source), optical system, detector and computer. Physically larger ICP instruments were manufactured (Photo 7) earlier because measurement with proper optical resolution could only be ensured in the large size, furthermore the operation and control were supplied by large electronic units and panels.
However, smaller electronic units and panels are produced recently, moreover in smaller size of ICP-OES (desktop) instruments, they are also able to produce adequately high enough resolutions (Photo 8).

The structure of the applied gases and the torch has already been detailed in Chapter 4.4.2.

A peristaltic pump and a nebulizer are needed to introduce the liquid samples into the plasma. A peristaltic pump can be seen on the left side of Photo 9 while on the right side of Photo 9 the operator of the ICP sets the clamping strength of the clamps of head from the back side of the same peristaltic pump. The flow rate of the delivered liquid sample can be set by using different inner diameter of the pump-tube and by changing the rotational speed of the peristaltic pump (100 to 1000 rot./min=rpm). The colour coding of the pump-tubes represents size of their inner diameters.

Figure 28 Major units of an ICP-OES equipment
Photo 7 ICP-OES equipment (Perkin-Elmer, Inc.)

Photo 8 ICP-OES equipment (Thermo Fisher Scientific, Inc.)
4.4.4. Sample introduction techniques in the ICP-OES

4.4.4.1. Nebulization method

As already mentioned in Chapter 4.3.4., in atomic spectrometry is possible to analyse solid, liquid and gas state of food and food raw material samples, at the same time due to the aspects of accuracy, precision and automatization (favourable utilization of measurement duration) that liquid samples are the most frequently preferred. The same applies for ICP-OES techniques where the application of some nebulization, within that, mainly the use of a kind of pneumatic nebulizer is applied. Nebulizer systems consist of two basic units (in the middle of Figure 28): the nebulizer and the nebulizer chamber are connected to it. Three methods of nebulization technique are differentiated according to their operational principles (Table 4): pneumatic, ultrasonic and hydraulic high-pressure nebulization.

The nebulizers used in ICP are similar to the ones in FAAS but as mentioned in Chapter 4.3.4, the sample introduction flow rate (mL/min) and nebulization efficiency (%) are two most important parameters during nebulization of liquid samples. The values of these two parameters using 8 to 10 L/min air flow in AAS technique that are usually 4 to 6 mL/min and 5 to 10%, however the values of the same parameters applying ICP techniques and 1 mL/min flow rate of nebulization gas (argon) are approximately only 1 to 2 mL/min and 1%.
Table 4 Nebulization sample introduction techniques

1. Nebulization sample introduction
   1.1. Pneumatic nebulizer
       Concentric nebulizer
       Microconcentric nebulizer
       Cross-flow nebulizer
       Babington-nebulizer
       V-groove nebulizer
       Conespray nebulizer
       GMK nebulizer
       „Mist” (Burgener) nebulizers
   1.2. Ultrasonic nebulizer
   1.3. Hydraulic high pressure nebulizer

**Pneumatic nebulization**

Concentric nebulizers (Figure 29) are the most frequently used of the pneumatic ones (Table 4). The argon gas flowing around the 200 to 400 μm liquid-capillary according to the Bernouille equation that causes the suction effect of the liquid, thus resulting continuous nebulization of the liquid. The nebulization efficiency of the concentric nebulizers is usually between 1 to maximum 2% and the flow rate of the sample introduction is around 1 mL/min.

![Figure 29 Concentric nebulizer](image)

If these are manufactured on a smaller scale, it is called a microconcentric nebulizer (Figure 30). The smaller diameter (approx. 100 μm) of the liquid capillary results smaller size of the aerosol
particles, reaching the efficiency of sample introduction of 10 to 20%. The microconcentric nebulizers need a liquid flow rate of 10 to 100 μL/min.

The cross-flow nebulizers (Figure 31) can be characterized by similar values as the concentric nebulizers. Their salt-tolerance may be better, so they can tolerate slightly higher quantity of dissolved solid material.

The next are the Babington-nebulizers (Figure 32), then the so-called Babington-family (Figure 32 to 34). Babington-nebulizer is not available nowadays, but historically it is needed to discuss, that is the following nebulizers named as modified Babington-nebulizers. So, the operation of the Babington-nebulizer is theoretically the following: there is a hole on the glass ball, in front of which only the accidentally flowing liquid that is nebulized, so neither above data can be explained.
The V-groove nebulizer (Figure 33) needs at least 1 mL/min sample flow rate and their efficiency is more favourable than those of the concentric nebulizers (more than 1% even 2%). Opposed to the Babington-nebulizer, where the flowing liquid before the argon gas output orifice is accidentally nebulized, in the V-groove nebulizer, a V-formed groove developed and the liquid flowing downwards is nebulized by the argon gas. The liquid sample output orifice is larger, than the argon gas output orifice, as shown on Figure 33. This is why the nebulizer is suitable for nebulizing solutions with higher (20%) salt content. On the other hand, biological and similar samples can be nebulized effectively without clogging. Even suspensions can be nebulized with this type of nebulizer.
Conespray (Figure 34) and GMK nebulizers (Figure 35) are modified Babington-nebulizers as well. The liquid sample is ‘forced’ to flow before the output orifice of the argon gas. Here the flow must be about 1 mL/min and the nebulization efficiency 1 to 2%.

Different Burgener-product nebulizers (Photo 10 and 11) have been developed in recent years. The products of Burgener, as the leader of the firm, cover most of the possibility of parameters of the nebulizers. Depending on the exact type of nebulizers from his firm (OpalMist, VeeSpray, SeaSpray, MicroMist, MiraMist, Conikal, DuraMist, AriMist), the flow rate of sample introduction changes from 0.05 to 2.5 mL/min and their nebulization efficiency is between 1 to 10%. They are also able to nebulize a solution containing HF or even 30% of solid material. In operation and layout from the above nebulizers a few of them are corresponded to V-groove, because they also have a smaller (for argon gas) and a bigger orifice (for the sample).
(Figure 36). According to the type, only a little bit of clogging occurs. It can be also declared that among of the pneumatic nebulizers with the same flow rate of sample introduction, they have one of the smallest scale of clogging (e.g. MiraMist nebulizer). Their material is either glass, PEEK or PFA.

![MiraMist nebulizer](image1)

**Photo 10 MiraMist nebulizer**

![DuraMist nebulizer](image2)

**Photo 11 DuraMist nebulizer**

**Figure 36 Schematic diagram of a MiraMist nebulizer**

### Ultrasonic nebulization

As the above it can be seen, the pneumatic nebulizer has low nebulization efficiency. The analyst is trying continuously to develop all of the classical and instrumental analytical steps with the utilization of analytical possibilities to extent the concentration range to the lower detection limits. One of the possibilities is increment of the nebulization efficiency. It is supported by the following two nebulization techniques (Table 4), namely the ultrasonic (USN=Ultrasonic Nebulization) and the hydraulic high pressure nebulization (HHPN). Figure 37 shows the units of the USN and Photo 12 and its actual appearance with the piezoelectric crystal being the principal unit. When electricity is connected it starts to vibrate on a high
frequency (>500 kHz). When one solution is flowing continuously to the surface of the crystal (transducer) flow rate (0.5 to 2.5 mL/min), as a consequence of vibration of the crystal, the sample is nebulized into an aerosol. Because of these aerosol drops being smaller in diameter, than in pneumatic nebulizers and the efficiency increases up to 10 to 20%.

As the nebulization is carried out by the vibration of the crystal, the formed aerosol needs a sample gas to get into the plasma. The flow rate of the sample gas usually changes from 0.5 to 1.5 mL/min. Since this method is ten times more efficient than the pneumatic nebulizers, however the capacity of the plasma cannot stand this quantity of the liquid, thus the plasma would go out. To avoid this, desolvation (removal of the solvent) must be done before the sample reaching the plasma.

The desolvation unit can remove the current solvent from the aerosol, which is usually water. The wet aerosol is directed through a heated glass tube in which the temperature is between 120 to 160°C. The solvent is transformed to gas state in this unit then the major part of the solvent is condensed in the cooled part and the temperature of which is between -20 and +10°C. Ultimately the solvent does not reach the plasma in a relatively great quantity (it is even less than with the pneumatic nebulizers), thereby the cooling down of the plasma is smaller (beneficial), while the massflow of the measured element in the nebulized aerosol becomes ten times more than before (with the pneumatic nebulizers). The detection limits can be on average
ten times as beneficial this way, depending on the element (the exact values of the improvement vary on the range of 2 to 100).

![Ultrasonic nebulizer](image)

Photo 12 Ultrasonic nebulizer (Cetac Technologies, Inc.)

![Schematic diagram](image)

Figure 38 Schematic diagram of hydraulic high pressure nebulizer

**Hydraulic high pressure nebulization**

The highest nebulization efficiency is achieved by hydraulic high pressure nebulization (HHPN) (Figure 38) from the nebulization techniques, which was introduced in 1988. The exact values vary from 40 to 50% in case of aqueous solutions and in case of samples with organic solvent, they can even reach 90%. Basically the sample is pressed through a 10 to 30 μm
diameter hole on a pressure of 40 to 200 bar, resulting in a liquid thread. If this liquid thread is collided with an impact bead (a glass ball with a diameter of 6 to 8 mm), the major part of the solution transforms to tiny aerosol droplets. Photo 13 shows a home-made HHPN device and Photo 14 shows its nebulizer chamber and the formation of the fine aerosol.

At the bottom of Photo 13 and the top of Figure 38 is shown that high pressure liquid chromatography (HPLC) pump presses the liquid sample into the system. The 100 to 1000 μl volume of the sample is injected into the continuously flowing carrier liquid with a sample injector. The actual nebulizer is a 2 mm wide industrial diamond or a flat ruby piece situated in a metallic socket. Inside of it there is a conical hole narrowing towards the front of nebulizer. Usually the diameter of thinner part of the hole is as mentioned above (10 to 30 μm).

Comparing the ultrasonic nebulizer with the hydraulic high pressure nebulizer (HHPN), the HHPN also does not require a nebulization gas. In order to get the aerosol into the plasma,
sample gas is needed. Since in the HHPN system the sample introduction efficiency is even higher than in the ultrasonic nebulization, which would go out the plasma, so it requires even more desolvation of aerosol, which can be carried out by the desolvation unit. The wet aerosol is directed through a heated glass tube when the temperature is 120 to 300°C (mostly 140°C is enough). In this unit the solvent is transformed to gas and the dissolved salt particles stay solid. Most of the solvent is condensed in two-step cooler unit. First, in a normal Liebig cooler (liquid cooler) and then in the second step even -40°C cooler (Peltier cooler) can be used. Most of the organic solvents can even be condensed. Organic solvents are frequently used in speciation analyses. The aerosol getting into the plasma is practically almost dry after cooling. The massflow of the analyte can be increased comparatively to the USN with the application of HHPN method, when more favourable (smaller) detection limits can be achieved than with ultrasonic nebulizer.

4.4.4.2. Other sample introduction methods

The nebulization sample introduction techniques in ICP-OES or ICP-MS instruments, that previously detailed in Chapter 4.4.4.1., cover mostly the possible elemental analyses of food and food raw material samples. However there are other sample introduction techniques depending on the analytical purpose of the sample, the concentrations of the components to be determined, the state of the sample, the amount of the sample and the other physical and chemical properties of the sample.

Table 5 Sample introduction techniques

1. Nebulization sample introduction
2. Hydride generational sample introduction
3. Graphite furnace sample introduction
4. Direct insertion sample introduction
5. Direct injection nebulization
6. Ablation sample introduction
   6.1. Arc ablation sample introduction
   6.2. Spark ablation sample introduction
   6.3. Laser ablation sample introduction
7. Slurry technics (solid samples in suspension form)
Table 5 shows those sample introduction methods that can be generally applied for analysis of element contents in different types of samples by large instrumental analytical techniques.

**Hydride generation sample introduction**

In examination of food samples by atomic spectrometry methods, after the most frequently used nebulizer techniques, the hydride generation (HG) is the second most frequently used sample introduction technique (Figure 39). Hydrides can be generated in lesser or greater degree by the following elements: As, Bi, Ge, Pb, Sb, Se, Sn and Te. Besides in case of the examination of mercury, the same conditions and reactants are needed, however not a hydride but a gas state element mercury is produced. Therefore it is called cold vapour (CV) technique. These elements form hydrides according to the following reaction equation:

\[
\begin{align*}
\text{As(III)} & + \text{NaBH}_4 + \text{H}^+ \\
\text{Se(IV)} & \\
\text{Hg(II)} & \rightarrow \text{AsH}_3 \\
& \rightarrow \text{SeH}_4 \\
& \rightarrow \text{Hg(g)}
\end{align*}
\]  

(15)

Those formed hydrides are in gas state and the efficiency and speed of formation of hydrides depend on the concentration of the applied acid and the sodium borohydride, moreover the oxidation state of the hydride-forming element also. Hydride-generation has two advantageous. The first one: since the formed hydrides are gases, they can be introduced into the plasma practically almost entirely when are separated from the sample solution. The second one: as a result of hydride generation, they can be separated from the complicated matrix, as the complicated matrix is highly detrimental to the sample measurement. As, Se and Hg elements can be analysed reaching 100 or 1000 times better (smaller) detection limits.

So, gases that are formed according to Equation 15 are separated in the gas-liquid separator which is seen in the right side of Figure 39. They are also separated from the matrix and transported into the ICP with an argon gas flow.
Graphite furnace sample introduction

Solid or liquid samples is electrothermally vaporized in a small graphite tube just like in the graphite furnace atomic absorption technique (Figure 40). The formed molecule steams are directed into the plasma. Each physical process takes place in high temperature plasma (Figure 26) what is needed for the light emission of the analyte (Figure 27).

Direct insertion sample introduction

Direct insertion of sample introduction method is not at all wide spread in the atomic spectrometry. The method can be used for analysis of food samples when sample preparation is skipped, but then the analysis of sample is measured directly. This is mainly recommended for solid samples. As shown on Figure 41 there is a graphite rod in the middle of the plasma torch to which the sample is placed before getting into the plasma. Then the sample is pushed into the 6000 to 10000 K of high temperature plasma where all the physical processes take place (Figure 27) what is needed for the light emission of the analyte.
Direct injection nebulization

In the area of ICP measurements there is also such sample introduction method where the liquid sample is directly introduced into the plasma through a microconcentric nebulizer (without any nebulizer chamber), applying high pressure liquid chromatography pump. The efficiency of sample introduction by using the direct injection nebulizer (Figure 42) is 100%. This technique
was successful when the elements had significant memory effects like iodine, mercury and boron.

Figure 42 Direct injection nebulization

Ablation sample introduction

The ablation (vaporization) sample introduction, in general, can be applied with the application of arc, spark or laser beam. Since the application of arc and spark nebulization methods need conductive solid samples, therefore they are not suitable for the analysis of food samples. Arc and spark nebulizations are mostly used for fast measurement of metal alloys (Figure 43).

Figure 43 Laser ablation sample introduction equipment
The ablation (vaporization) sample introduction can be performed with a laser beam. The advantage of the laser is that it can be focused well. This advantage opens the door to perform such microanalysis of the surface which was not executable beforehand. In some cases it is advantageous, in some not so, only a small amount (10 to 100 μg) of substance is vaporized without destruction.

**Slurry technics**

The slurry technique (nebulization of solid material in the form of suspension) is a possible sample introduction method for non-destruction analysis of solid samples. In this case such pneumatic nebulizer and nebulization chamber must be used which is able to nebulize larger particles as well. V-groove and MiraMist nebulizers can be used along with a Scott-type nebulizer chamber. The solid samples must be grinded to make a properly textured, homogeneous suspension, meaning that their particle size is less than 5 μm. Grinding after the material has been frozen with liquid nitrogen that is the best execution nowadays. The suspension is continuously homogenized in an ultrasonic cleaner or with a magnetic mixer during nebulization.

**4.4.3. Nebulizer chamber**

The aerosol particles are directed from the pneumatic nebulizers through a nebulizer (mist) chamber. The chamber has double functions. On the one hand, it allows the particles into the plasma that the diameter of it is only smaller than around 10 μm and on the other hand also decreases the pulsation of the massflow of the nebulized particles, thus stabilize the operation of the plasma. Three different types of nebulizer chambers have become wide-spread (Table 6) in ICP techniques. The Scott-type double pass and cyclonic system types are used in ICP-OES and the conical single passes with impact bead also, mostly cooled and chambers are used in ICP-MS. The quantity of the solvent (e.g. water, but mainly volatile organic) can be reduced with the condensation of the formed solvent steams, decreasing both load of the plasma and the quantity of the formed hydroxides/oxides. The drops of liquid which are too large to get into the plasma, they are collected at the bottom of the chamber, form a liquid block in order to stop the leaving of the argon gas from the system in this way.
Table 6 Different types of nebulizer chamber

1. Scott-type
   - single pass
   - double pass
2. Conical single pass
3. Cyclonic system
   - single pass
   - double pass

The cyclonic (Figure 44) and the conical (Figure 45) chambers can be made of borosilicate glass or also some kinds of plastic which are suitable for analysis of smaller salt-content samples. However the Scott-type nebulizer chambers (Figure 46) can be made of borosilicate glass, moreover acid and base resistant material (Ryton polymer) also.

Figure 44 Cyclonic single pass spray chamber

Figure 45 Conical single pass nebulizer chamber
4.4.5. Optical systems

Both the radial and axial view of the plasma (Figure 47) is possible in ICP-OES. In axial view of the plasma, the light emission occurring in the core of the plasma can be used for analysis where the temperature of the plasma is around 8000 K.

In radial view 6000 K temperature part of the plasma is used for analysis. The hotter the plasma, the larger the ratio of the formed atoms and ions furthermore the more effective the excitation of atoms and ions. Due to this, the possible intensity of light is around ten times more in axial view causing the detection limits to be ten times as better (smaller) in the radial. If in higher salt content samples that the relatively small concentration of the measured element is analysed, in case of axial view, ten times larger element interferences occur comparatively to the radial view plasma. If we want to avoid the larger interference effects from the elements, moreover the measured concentrations allow the usage of radial view plasma that is recommended.
There are three plus one different optical systems in the inductively coupled plasma optical emission spectrometry (Table 7). The monochromator is the simplest one, which is flexible, but slow method. Flexible, because any measureable element by ICP, furthermore any wavelength line of the element, between 160 to 850 nm range, can be analysed. It can regard as slow, because its operation is sequent, meaning that it can only measure the given lines of an element line by line.

The examination of the polychromator is fast, but inflexible, because a lot of elements or lines can be measured (usually maximum 60 elements or lines with different wavelengths) simultaneously. Its inflexibility is explained by the fact that only those lines can be measured which are already set up in the factory during manufacturing. The additional type that was mentioned above: when purchasing an ICP-OES instrument, you can choose to have one with a combined optical system (a mono- and a polychromator together) which can be flexible and fast also at the same time. However this is naturally more costly.

Nowadays the cost-effective solution can be the ICP-OES with Echelle optics. The operator can choose the elements and their wavelengths practically day by day by applying the ICP-OES. So purchasing this equipment results a fast and flexible method also at the same time.

**Table 7 Optical systems**

1. Monochromator (flexible, but slow)
   - Czerny-Turner mount monochromator
   - Ebert mount monochromator
2. Polychromator (fast, but inflexible)
   - Paschen-Runge mount polychromator
   - Rowland-circle polychromator
3. Solid-state detector (flexible and fast)

The ICP-OES instruments with monochromator use either a Czerny-Turner or an Ebert mount monochromators.
The collimating mirror makes parallel beams from the white light entering through the entrance slit, and the light is directed towards the optical (diffraction) grating. The white light is then dispersed into components (Figure 49).

The wavelength of the measured line of the element is chosen with rotating the optical grating with a stepper motor and the selected wavelength that is directed to the exit slit by another collimating mirror. The wavelength (or more specifically the narrow wavelength range) on which the light emission of the analysed element can be measured and is chosen with the use of the optical grating and the exit slit at the same time. In the Paschen-Runge mount polychromator all of the optical units (entrance slit, optical grating, exit slits) are arranged on an arc, which is called a Rowland circle (Figure 50). More wavelengths can be measured with it simultaneously, causing relatively fast measurement.
The third possible layout of optical systems is Echelle optics (Figure 51). Recently the manufacturers sell such modern inductively coupled plasma optical emissions spectrometers which on the one hand are capable of using radial and axial view plasma as well (the computer controlled mirrors seen in Figure 51 choose the view) and on the other hand analysis of a lot of wavelengths is also possible simultaneously, on selectable mode.

In the Echelle optics the light utilization of the radiation reflected from the optical grating is really good, thus the appropriate resolution of the light is reached by the application of 50 to 150<sup>th</sup> spectral orders for the analysis of the elements, where the intensity of the light is still good enough. When the optical units resolve the light, the spectrum slices are fallen in a row and two-dimensional spectrum that reaches the detector. This means the rows of the different wavelengths of spectra appear on the 2x2.5 cm area of CCD or CID detector is in an increasing spectral order. The smallest measurable wavelength appears on the top left corner and wavelengths grow from left to right and downwards as well. The solid state detectors in the ICP-OES instruments are either the charge coupled device (CCD) or the charge injection device (CID) detector. The Echelle type ICP-OES equipments are able to examine at least 250 different emission lines (a narrow wavelength range) simultaneously. As 72-74 elements can be determined simultaneously by ICP-OES at the same time, thus in extreme cases it is possible to measure small, medium and high concentrations at the same time with an ICP-OES
instrument because a very sensitive, a moderately sensitive and an even less sensitive line can be applied at the same time.

![Echelle optical mount](image)

**Figure 51 Echelle optical mount**

### 4.4.6. Detectors

The inductively coupled plasma optical emission spectrometers with monochromator and polychromator usually use a photomultiplier tube (PMT) (Figure 52) in the ultraviolet and the visible range of the light. As is seen from the figure, given wavelength \((h \cdot \nu)\) of light enters the photo-sensitive photocathode and this releases electrons from the surface of the electrode, which electrons are accelerated by the effect of difference in the controllable potentials of the dynodes and they collide into the dynodes. Electrons can leave the dynode 4 to 6 times more (multiplied) and this whole process continues on the other dynodes as well. The most modern instruments use semiconductor (solid state) sensors, CCD or CID detectors. 160 to 850 nm analytical wavelength range can be covered with one of the above or two independent detectors. In case of two independent detectors, the analysis of the UV and visible light are carried out one after another in time of two steps in the same measurement.
4.4.7. Interfering effects and correcting for interferences in ICP-OES

The ICP-OES was introduced (in 1964) as a method which is freedom from interferences. Later, after thorough examinations it appeared, the results of analysis are affected by numerous interference effects. There are 4 main types of interference effects in the ICP-OES techniques:

1.) Physical interfering effects:

   a. The effects depending on the physical characteristics of the nebulized solution (density, viscosity, surface tension, volatility, temperature etc.).

   b. The matrix effects depending on the quality and quantity of the elements in the solution (decrease of the temperature of the plasma).

   c. The effects depending on the structural characteristics of the equipment (type and size of the nebulizers, the structure of the nebulizer chambers, the construction of the torch, the temperature of the induction coil and etc.)

2.) Spectral interfering effects:

   a. Line coincidence, which can be originated not only from the element content of the solution introduced into the plasma, but the argon or the silicon also dissolved from the torch.
b. Band coincidence, which can largely change the background of the measured element. In many cases it is caused by the emission of the undissociated molecules (N₂, C₂, CN, CH, OH etc.) in the plasma.

c. Line broadening, among which the Doppler broadening that is ten times more than the effect of all the others together.

d. Background shift, which can be caused by either the scattered light or the continuous radiation originated by the ion-electron recombination of the elements (Ca, Mg, K etc.) in the solution with a relatively high concentration.

3.) The effect of the easily ionisable elements, which is caused by the high concentration of the elements with small ionization potential (K, Na, Cs).

4.) The memory effect, which occurs when a standard or sample solution enters the equipment in high concentration.

These interfering effects can have a hazardous effect for the activity of the practical analysts. However, in case of routine measurements, most of them can be eliminated by taking into considerations of a few simple rules.

The interfering effect caused by the difference of the measured sample and the calibration standard solutions in density, viscosity, surface tension and volatility, moreover temperature (1a.) can largely be eliminated if the sample solutions and standard solutions contain the same acid, in the same concentration applied for the sample preparation. Moreover the solutions should be stored in the same place or on almost the same temperature before the examination.

The best elimination of the 1b. interfering effects can be performed if the quality and the quantity of the elements are used in standard solutions for calibration are similar with type of the measured sample.

The 1c. interfering effects do not play a significant role from the other physical interfering effects, for as much as the structural units (e.g. the nebulizer and the torch) of the equipment are not changed during the measurements.

Although manufacturers usually select the installed analytical wavelengths of the ICP-OES but before it is assembled according to the known line coincidence (2a.) and the known band coincidence (2b.) interfering effects of the matrix. In the course of selection of the best lines they take the element contents (which are in high concentration in the sample, moreover always present argon (argon gas) and silicon (torch)) into consideration – thus they can eliminate
several line and band coincidence effects – before the installation of the instrument. In spite of the above line selection method the spectral interfering effects are the most difficult problems for practical analysts by using ICP-OES. The interference calibration also is known as interfering element calibration that is able to decrease the interfering effects of elements which do not have too large concentrations. The interference calibration and the actual concentration of the elements can be calculated with control and evaluation computer of recently manufactured ICP-OES spectrometers by setting up the proper number of iterations. Most ICP-OES instruments usually contain a database where the possible line coincidences are indicated and this helps predicting the interfering ions. The degree of the interfering effects are largely determined by the characteristics of the ICP-OES equipment. Therefore in case of introduction of a new analytical method, the interference calibration of the interfering elements in relatively high concentration is needed to perform depending on the element content to be analysed. When the spectral interference is too large, in the modern ICP-OES instruments there is a possibility to select another line for analysis of the element which line does not have interference effect in the case of the given type of sample.

The phenomenon of the line broadening (2c.) is observed when the calibration line bends (similar to the saturation effect). This can be eliminated or decreased with choosing the proper amplifications and voltages of the PMTs, or to establish the proper dynamic range of measurement of the calibration curve. Moreover a proper calibration activity (e.g. proper polynomial curve fitting) promotes to reach the more accurate measurement result.

The background shift (2d.) among the spectral interfering effects can also be eliminated or decreased automatically with the application of ICP-OES. Background shift can be eliminated by subtracting the background measurement (intensity) of the immediate environment of the line from the gross signal (response). There are 3 types of background measurement: left-, right- and both-sides background correction. The proper side of background correction can properly eliminate the interfering effect of background shift. The interfering emission can be observed on the left side (smaller wavelength than the analytical line), on the right side (higher wavelength than the analytical line) of the analytical line which can origin from undissociated molecules (e.g. N₂, C₂, CN, CH, OH, etc.) or numerous interfering emission from other elements. Knowing the above the place of the background correction is determined.

With the inductively coupled plasma as a source of radiation numerous researchers dealt with the effect of the easily ionisable elements (Li, Na, K) (3.). On the one side this examination was
the continuation of investigation of the physical processes taking place in the flame of the atomic absorption instruments and on the other side the examination was performed in order to examining sea water with high salt content. These effects can be avoided if the alkali and alkali earth element content of the standard solutions are similar to the concentrations of the same elements in the sample.

In the atomic spectrometry methods, a memory effect can be experienced in the nebulizer system or e.g. on the cones of the ICP-MS instruments if the nebulized solution contains the examined element in a relatively high concentration. The memory effect can be eliminated by increasing the washing duration of the washing solution applied between the solutions, moreover diluting the sample, furthermore choosing a more appropriate nebulizer system which results smaller memory effect.
4.5. Inductively coupled plasma mass spectrometry

4.5.1. The theory of inductively coupled plasma mass spectrometry

In 1975 Alan Gray in Luton, England published the first paper about investigations of direct current (DC) arcplasma, who determined isotope ratios by using mass spectrometry detection. Three years later Alan Gray and Velmer Fassel started to research together about which research work they published the first common paper, along with other co-authors in 1980 that paper dealt about this research on inductively coupled plasma mass spectrometry (ICP-MS). It needed three more years for the first commercial ICP-MS instrument to be introduced.

The inductively coupled plasma mass spectrometry – issuing from its name – has 2 main parts. The first of them is the inductively coupled plasma, and the second one is the mass spectrometer which carries out the separation and the detection. What about the inductively coupled plasma optical emission spectrometry (ICP-OES) is already detailed in the previous chapter, that knowledge which can be used in this section in large part also, we do not consider to repeat again in this chapter. As the both techniques (ICP-OES and ICP-MS) have practically the same first part (inductively coupled plasma=ICP), thus the knowledge of the ICP-OES till the end of the plasma is the same more or less, what is needed to discuss the knowledge of ICP-MS (Figure 28).

In ICP-MS measurements, the plasma is not already a light source but ionsource. Of course the processes is shown in Figure 27, carry out in the plasma of the ICP-MS but in this system the light radiation is only a side process. The most important task of the plasma of the ICP-MS to produce ions – if it is possible these should be single positive ions – which are later separable and detectable by the mass spectrometer. The formed cations are separated and detected according to mass/charge (m/z), the unit of measurement which is amu (atomic mass unit).

4.5.2. Major units of an ICP-MS equipment

For introducing the major parts of the ICP-MS equipment, Figure 28 can be used with a slight difference: instead of the optics, spectrometer and detector of the ICP-OES, there is the mass spectrometer in ICP-MS containing the ionoptics, analyzer and detector (Figure 53, 54). The cones (sampler and skimmer) after the ionsource (plasma) are essentials for the mass spectrometer, however the collision and reaction cell before the analyzer are not absolutely necessary (not all of the ICP-MS equipment contain it). Reaching the high vacuum, which
pressure can be even $10^{-9}$ to $10^{-10}$ bar, is also necessary for the normal operation of the mass spectrometer. This is performed with a two step pressure reduction. Of course there is $1$ bar pressure in the outer environment of the instrument, $10^{-3}$ bar between the sampler and skimmer cones and $10^{-9}$ to $10^{-10}$ bar where there are the ionoptics, the quadrupol and the detector (Figure 55). This vacuum is performed with the help of a rotary and at least one turbomolecular pump (Figure 54). The high vacuum should be provided in order to avoid the secondary collisions (harmful by-reaction) of the particles (ions and molecular-ions). The ionoptics first deflects the ion beam and focuses the ions to the entrance slit of the analyzer.

Figure 53 Schematic diagram of mass spectrometer part of ICP-MS (from above)

Figure 54 Units of mass spectrometer (side view)
4.5.3. The plasma, as an ionsource

The plasma part of the ICP instrument is placed horizontally, like as in the axial view ICP-OES (Photo 15). The plasma can function as an ionsource, because with the ionization of the argon (the first ionization energy of argon is 15.76 eV) the plasma represents such a potential, in which most of the elements (more than 50) are able to be ionized in almost 90% or even higher degree on such potential. This is because of the ionization potential of the major part of the elements is smaller than that of argon, so the temperature (6000 to 8000 K) of the plasma ensures the ionization of the elements.

Photo 15 Sampler cone, induction coil and torch

The degree of the thermal ionization of the atoms getting to the plasma can be described by the Saha equation (Equation 16).
\[
\frac{n_{i+1}}{n_i} = \frac{(2\pi m k T)^{\frac{3}{2}}}{n_e h^3} \cdot \frac{2g_{i+1}}{g_i} \cdot e^{\left(\frac{-V}{kT}\right)}
\]

(16)

where

\(n_{i+1}\): the density of atoms in \((i+1)^{\text{st}}\) state of ionization

\(n_i\): the density of atoms in \(i^{\text{th}}\) state of ionization

\(m_e\): the mass of an electron \((9.1095 \cdot 10^{-31} \text{ kg})\) (1836th part of the mass of a proton)

\(k\): the Boltzmann-constant \((k=1.380622 \cdot 10^{23} \text{ J/K})\)

\(T\): the absolute (electron) temperature

\(n_e\): the electron density

\(h\): Planck's constant \((6.626196 \cdot 10^{-34} \text{ J·s})\)

\(g_{i+1}\): the statistical weight of \((i+1)^{\text{st}}\) ion

\(g_i\): the statistical weight of \(i^{\text{th}}\) ion

\(V\): the ionization potential

Ions from plasma get to the mass spectrometer through the nickel sampler cone (Photo 16) and platinum skimmer cone (Photo 17). These cones must be made of conductive metals because the cones need intensive water cooling. So these are mainly made of nickel and sometimes platinum, aluminium or copper. The diameter of the hole in the sampler cone is around 1 mm (usually between 0.8 and 1.2 mm). The second cone which is skimmer cone has a smaller hole with a diameter of around 0.4 to 0.8 mm.

When the samples which need to be examined with the ICP-MS have a higher salt content, the salt deposit on the colder skimmer cone is usually bigger (Photo 18), the cone can even be clogged by it while any visible salt deposit on the hotter cone does not exist.

Photo 16 Nickel sampler cone
4.5.4. Types of analyzers

In analytics there are different analyzers used for various analytical purposes, however only three mass spectrometers are available in element analytics which practically mean four different types of analyzers.

<table>
<thead>
<tr>
<th>Table 8 Types of mass spectrometers</th>
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<tbody>
<tr>
<td>1. Quadrupole (Q)</td>
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<tr>
<td>2. Time of flight (TOF)</td>
</tr>
<tr>
<td>3. High resolution (HR), sector field (SF)</td>
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The quadrupole is the most frequently applied analyzer for separating and detecting of the ions of the ion beam in research and routine analyses of elements in food and food raw materials. High resolution mass spectrometers are used to lesser extent, furthermore the application of time of flight analyzer is even more infrequent.

**High resolution (sector field) ICP-MS**

The sector field, or high resolution, ICP-MS instruments are shortened in both Hungarian and International literature to HR-ICP-MS (High Resolution Inductively Coupled Plasma Mass Spectrometry or ICP-HR-MS) and SF-ICP-MS (Sector Field Inductively Coupled Plasma Mass Spectrometry or ICP-SF-MS) acronyms.
The sector field term comes from the fact that 2 analyzers are used in it: the first magnetic and the second electrostatic analyzer (Figure 56).

In case of magnetic analyzer, the single positive ions are accelerated in a high voltage region, thus they are going to have equal kinetic energies. The ions enter the magnetic field through the entrance slit which ions are deflected according to their mass/charge (m/z) ratio. The cations are separated according to this ratio and the cations with smaller mass move on a smaller circular than the bigger ones.

The second analyzer is the electrostatic analyzer which consists of a positive (outer curve plate) and a negative (inner curve plate) electrodes. Ions with different speed and the same m/z value leave the magnetic analyzer and get to the electrostatic analyzer through a slit. The electrostatic analyzer separates and focuses the ions according to their kinetic energy which ions finally collide into the detector. With this arrangement the best (highest) resolution is 10000, moreover this HR-ICP-MS equipment has additional two selectable resolutions, one of them 4000 (intermediate resolution) and another 300 (low resolution). As the resolution is selected by the width of the entrance and exit slits, therefore with increasing of the resolution the amount of ions getting into the detector decreases. Hereby the sensitivity of the detector decrease to tenth to a hundredth. With applying a higher resolution, the detection limits worsen (increase) significantly.
The resolution (R) in high resolution mass analyzers is usually defined with the definition of the so-called 10% valley, which says two neighbouring peaks are considered to be separated if the height of the valley between two peaks is no more than 10% of their height. The resolution (R) is the capability of a mass spectrometer to differentiate between masses of the neighbouring peaks (cations). The resolution R is calculated according to Equation 17 where according to Figure 57: m is the mass of the given ion and Δm is the difference between the masses of two cations. In quadrupole instruments the resolution is measured at 50% of the peak height (Figure 58).

\[ R = \frac{m}{\Delta m} \]  

(17)

where

- R: resolution
- m: the mass of ion
- Δm: difference of masses of two neighbouring cations

Figure 57 Calculation of resolution in the case of high resolution (sector field) ICP-MS systems

Figure 58 Calculation of resolution in the case of quadrupole ICP-MS systems
Time of flight analyzer

From the plasma the ions get to the injector plate through the cone, where applying acceleration voltage of the injector plate, cations with identical kinetic energy are produced. The cations with different m/z ratio created in the ionsource are accelerated with an electric field of force in the acceleration region, then the ions hit the detector through the flight tube, which is under vacuum (Figure 59). As all ions with the same kinetic energy get to the flight region at the same time, their time of flight to the detector depends on their masses. The speed of lower mass ions with the same charge (lower m/z) is higher, so they reach the detector on shorter duration. The heaviest ions reach the detector at 50 μs at most, only after this duration new ions can be introduced to the system. This is a source for trouble in case of an ICP plasma because it is a continuous ionsource.

Mostly orthogonal layout is applied in ICP-TOF-MS instruments in which the accelerating voltage pulse is applied perpendicularly to the flying direction of the ion beam. Until all the ions reach the detector, a new accelerating voltage pulse is not started. So the continuous ion beam from the plasma is decomposed into ion packets. Thus the ICP-TOF-MS equipment can analyse around 30000 complete spectra per second. In the orthogonal layout the ions penetrate to the reflectron, until their kinetic energy is zero and they start to accelerate backwards (are reflected back) while they are refocused and then hit the detector. The ion mirror (the so-called reflectron) is composed of a series of charged flat.

Figure 59 Schematic diagram of orthogonal ICP-TOF-MS equipment
For as much as the resolution of the ICP-TOF-MS instrument is calculated on the basis of definition of 10% valley, its resolution is in 450 to 500 range and in 1200 to 1300 range with the 50% calculation.

**Quadrupole analyzer**

In the quadrupole analyzer (issuing from its name) has 4 parallel metals (e.g. molybdenum) rods to separate cations according to their m/z value. The rods are maximum 40 cm long. Direct current (DC) and alternating high voltage (AC) is applied on two-two opposite pairs of the rods (Figure 60). The ions with a given m/z value are going to resonate with the alternating voltage of the opposite rods and they move toward the detector on the route of the sine curve. Those ions which have a higher or lower m/z values and those have continually larger amplitude movement (those cannot resonate), finally fall out of the ionchannel. The same phenomenon happens on the other pair of rods as well. The resulting form of the sine-movements of the ions between previous 2-2 rods is a spiral trajectory towards the detector. The mass/charge value can be determined approx. 1 amu mass range with changing the level of voltage that wants to be detected. These ions are going to hit to the detector.

![Figure 60 Schematic diagram of quadrupole analyzer](image)

The quadrupole mass spectrometer usually allows a detection between 2 to 260 amu mass range. As the quadrupole ICP-MS instruments can approximately measure units of mass, therefore
they have a low resolution \((R=m/\Delta m=300)\). It is possible to apply a relatively higher resolution but in this case 0.5 amu range can be separated from each other.

Since the analysis with the quadrupole mass spectrometer (both peak jumping method on the given masses of selected isotopes and the scanning method of mass ranges) is rapid, so the ICP-MS method is capable to analyse a large number of elements or isotopes that are relatively short period of time, thus considering the sample measurement duration by ICP-MS, the performance (number of samples and elements) is comparable with inductively coupled plasma optical emission spectrometry which is also simultaneous and multielement method.

Three different analytical modes of the quadrupole mass spectrometer are also possible to apply according to the handling of the plasma and the temperature of the plasma. These are the following: normal mode, hot plasma and cool (cold) plasma. With using the cool and hot plasma, a shielded (screened) plasma is needed to apply. To apply shielded plasma a nickel ring is needed for inserting between the outer quartz tube of the torch and the induction coil. Besides if the flow rate of the sample gas is increased, moreover the forward power is significantly decreased (to 500 to 700 W value) then the temperature of the plasma is reduced by 2000 to 3000 K, so a cool plasma is created. In this case regarding Saha equation (16), the elements having lower ionization energies (mainly alkaline and alkaline earth metals) can be measured with 100 to 1000 times favourable (smaller) detection limits. With using the nickel ring (shielded plasma) both a better ionisation efficiency can be reached, moreover with the significant decrease of the temperature the interferences of the molecular-ions formed in the plasma are significantly decreased. If the nickel ring is used with the normal plasma parameters, hot plasma mode is produced. Thus the background intensities are not changed though the intensities of the signals (responses) that increase significantly. Thus generally the sensitivities of the measurement methods are increased around twice of before. Summarizing the above, application of nickel ring has positive effects in both cool and hot plasma mode. It also needs to be highlighted that this rule only applies for a group of elements.

4.5.5.3 Interfering effects and correcting for interferences in ICP-MS

As it was mentioned repeatedly before, ICP-MS has the same characteristics in several cases as ICP-OES, because in this method the source of the analytical signal is an inductively coupled plasma. Therefore the common interfering effects that mentioned in Chapter 4.5.7. do not need
There are four main types of the interfering effects appearing in both ICP-OES and ICP-MS techniques:

1.) Physical interferences (a., b. and c. types)
2.) Spectral interferences
3.) Effect of easily ionisable elements
4.) Memory interferences

The 1., 3. and 4. interfering effects appear in both techniques and their elimination or reduction can be performed by the same methods. Of course the spectral interferences appear in both techniques as well while these mean optical interfering effects in ICP-OES, however in ICP-MS these spectral interferences are derived from the problem of mass measurement. In the latter technique, spectral interferences can be caused by any cation (consisting of one or more atoms), the m/z value of which is approximately same as the target mass needed for our examination. Accordingly elemental isobar and molecular isobar interferences can be distinguished.

Elemental isobar interference is when an interfering single-elemental ion appears with the same m/z value as the mass of the given isotope of the examined element. The cation of the interfering element can be single, double or triple positive charged.

The interfering effects of single positive cations occur in the case of analysis of titanium, chromium or vanadium if the concentration of the other two mentioned cations is at an interfering level. The m/z values of these are following: $^{50}\text{Ti}^+ = 49.9447921$; $^{50}\text{Cr}^+ = 49.9460464$ and $^{50}\text{V}^+ = 49.9471609$. More examples for the same phenomenon are $^{87}\text{Sr}^+ = 86.9088841$ and $^{87}\text{Rb}^+ = 86.909187$. Double positive cation interference occurs on $^{44}\text{Ca}^+$ isotope if $^{88}\text{Sr}^{2+}$ is present in an interfering concentration. Another problem is presence of $^{135}\text{Ba}^{3+}$ during measuring the $^{45}\text{Sc}^+$ isotope.

The above mentioned elemental isobar interfering effects can be eliminated or decreased with the following procedures:

- If there is another isotope (the natural abundance of which allows the examination) of the element to be examined, that isotope should be used for analysis.
- With a high resolution ICP-MS instrument, if the resolution is calculated from difference of exact masses according to Equation 17, is smaller than 10000. The any
interfering effects of single positive cations mentioned above cannot be eliminated with this method.

- Using the method of mathematical correction, if the extent of the interference can be calculated with analysis of another isotope of the interfering element and the analytical result of the analyte is corrected with this value.

- The formation of multiple charged cations can be prevented if the temperature of the plasma is set up to a lower level, with choosing a lower forward power. The use of cool plasma must be mentioned here as well.

Molecular isobar interference is when the interference is caused by polyatomic ions. These polyatomic particles are either called molecules or adducts. These can be: dimer, trimer or tetramer (Table 9). These atoms can be derived from the components of the measured solution (e.g. if the Ca, Na or Cl are in high concentration), or from the sample matrix (e.g. H or O), or from the plasma gas (Ar) or from the air surrounding the instrument (e.g. N or O) as well, which can diffuse to the plasma. The interfering adducts are resulted by the connection (reaction) of these interfering atoms.

**Table 9 Some interfering adducts**

<table>
<thead>
<tr>
<th>The isotope of analyte element</th>
<th>Mass (amu)</th>
<th>Interfering particles with single positive charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>24</td>
<td>CC</td>
</tr>
<tr>
<td>Si</td>
<td>28</td>
<td>N₂</td>
</tr>
<tr>
<td>S</td>
<td>32</td>
<td>O₂</td>
</tr>
<tr>
<td>K</td>
<td>39</td>
<td>ArH</td>
</tr>
<tr>
<td>V</td>
<td>51</td>
<td>ClO</td>
</tr>
<tr>
<td>Cr</td>
<td>52</td>
<td>ArC</td>
</tr>
<tr>
<td>Fe</td>
<td>56</td>
<td>ArO</td>
</tr>
<tr>
<td>Cu</td>
<td>63</td>
<td>ArNa</td>
</tr>
<tr>
<td>Zn</td>
<td>66</td>
<td>ArMg</td>
</tr>
<tr>
<td>As</td>
<td>75</td>
<td>ArCl</td>
</tr>
<tr>
<td>Se</td>
<td>80</td>
<td>ArAr</td>
</tr>
<tr>
<td>Se</td>
<td>82</td>
<td>ArHArH</td>
</tr>
</tbody>
</table>
The molecular isobar interfering effects mentioned above can be eliminated or decrease with the following methods:

- If there is another isotope (the natural abundance of which allows the examination) of the element to be examined, that isotope should be used for analysis.
- In case of a high resolution ICP-MS instrument, if the resolution is calculated with difference of exact masses according to Equation 17 is smaller than 10000. By application of HR-ICP-MS, all the interfering effects of the interfering cations can be separated from the isotope of the measured element in Table 9, so these can be examined without interference, at least without the above interfering effects. However by using high resolution mode, the detection limits of the method are increased (worse) to 10 or 100 times compared to the low resolution.
- Using the method of mathematical correction, if the extent of the interference can be calculated with the analysis of another isotope of the interfering element and the analytical result of the analyte is corrected with this value.
- By using of desolvation system, the quantity of matrix of measured sample can be reduced, thus the interference of the sample solvent (e.g. of water or acid) can be decreased.
- Usage of cool plasma decreases the formation of several molecular-ions.
- Using such quadrupole mass spectrometer that has a collision and/or reaction cell.

**Operation of collision/reaction cells**

The collision cell technology (CCT) (Figure 53 and 61) is such a hexapole, octapole or quadrupole system (depending on the manufacturer) with the use of which, most of the molecules, getting from the inductively coupled plasma to the mass spectrometer, can be eliminated from the ion beam. The CCT unit is placed after the cones (Figure 53), into which collision and reaction gases are introduced which eliminate the vast majority of interfering polyatomic species through different mechanisms. Mainly H₂, He, NH₃, CH₄ or mixing these (e.g. 7-8% H₂ + 92-93% He, 1% NH₃ + 99% He), or other gases (e.g. CO or water vapour) can be used as collision and reaction gases. Actually for another purpose, even O₂ can be introduced through the CCT valve.
The efficiency of the elimination of molecular-ions of the CCT unit is 99.99% which means only one particle out of 10000 remains in an unchanged form.

There is $^{80}\text{Se}$ isotope in last but one row in Table 9, the natural abundance of which among selenium isotopes is the highest (around 50%). Therefore using this selenium isotope results the best (smallest) detection limit for selenium. However, this is only true if this applied isotope does not have significant spectral interfering effects. As shown in Table 9 and Figure 62, there is significant molecular-ion interference ($^{40}\text{Ar}^{40}\text{Ar}^+$) on $^{80}\text{Se}$. On selenium isotope ($^{80}\text{Se}^+$), here $^{40}\text{Ar}^{40}\text{Ar}^+$ (polyatomic particle) interference can be eliminated/decreased with 4 different known mechanisms:

- collisional dissociation
  \[ \text{ArAr}^+ + \text{He} = \text{Ar} + \text{Ar}^+ + \text{He} \]

- chemical reaction
  \[ \text{ArAr}^+ + \text{H}_2 = \text{ArH} + \text{ArH}^+ \]

- charge transfer
  \[ \text{ArAr}^+ + \text{H} = \text{ArAr} + \text{H}^+ \]

- collisional retardation/energy filtering
  \[ \text{ArAr}^{++} + \text{He} = \text{ArAr}^+ + \text{He}^+ \]
Figure 62 Selenium analysis with and without hydrogen, as the reaction gas

In case of collisional dissociation, the helium gas (the collision gas) meets a dimer particle, collides with it and strikes it to a neutral atom and a 40 m/z value ion which do not cause interference on the $^{80}$Se$^+$ isotope. The neutral atoms/particles are eliminated by the pumps creating vacuum.

In case of chemical reaction the molecular-ion reacts with hydrogen (the reaction gas). The result of this reaction is similar to the previous one but created particles have one amu higher m/z values.

The argon-argon dimer reacts with the hydrogen atom and electron transition (charge transfer) takes place. The quadrupole analyzer cannot resonate with 80 mass number molecule formed at the end of the reaction, as it is a neutral particle. This is the reason why it stops in the being of an interference.

The kinetic energy discrimination (KED) is the fourth mechanism. As $^{40}$Ar$^{40}$Ar$^+$ dimer is a larger particle than $^{80}$Se$^+$ particle, the collision cross-section of the argon-argon dimer is much larger than that of the selenium cation. Consequently polyatomic particles collide with the gas molecules of the collision and reaction gas much more often than the single-atom cations. Thanks to the more frequent collisions with larger particles, the kinetic energy of the larger particles significantly decreases. If a potential barrier is set up along the ion beam, the larger particles cannot be longer pass this barrier, while smaller ones which almost keep their kinetic energy can pass this potential barrier.
According to Figure 62, the efficiency of the CCT can even visually be acknowledged: if a CCT gas is not applied for the analysis, this causes an extraordinary large argon-argon dimer background (red peak) on mass number of 80. Conversely if collision/reaction cell is applied in quadrupole mass spectrometry, this interfering background can be decreased to its 10000th part thanks to the 99.99% efficiency of the method. As mentioned above, in the sector field (high resolution) mass spectrometer with the application of high resolution (R=10000), peaks of $^{40}\text{Ar}^{40}\text{Ar}^+$ dimer and $^{80}\text{Se}^+$ cations can be separated. Thus selenium ($^{80}\text{Se}^+$) can be analysed without interference. Although troubling drawbacks appear: the detection limits of the method are increased (worsen) with one or two magnitudes (tenth to a hundredth) and the prices of purchase and operation are 2 to 2.5 times of their previous value. The detection limits of the quadrupole instrument does not change significantly by the application of CCT mode.
5. Chromatography

Chromatography is a useful separation method in the field of food analysis, and has a great impact in analytical chemistry. The author of this part of book during his 40 years research work used the chromatographic methods on many parts of food analysis, mainly on the field of all protein and amino acid analysis by ion exchange (IEC) and high performance liquid chromatography, and fatty acid and other volatile compounds by gas chromatography (GC), so that in this chapter of the book, after the basic principle of chromatography, the liquid- and gas chromatography will be discussed.
5.1. History of the chromatography

The first two men who used chromatography for separation of the different materials were Day American geologist and Tsvet Russian botanist in the late nineteenth and early twentieth century. Day developed procedures for fractionating crude petroleum by passing it through Fuller’s earth, and Tsvet used a column packed with chalk to separate leaf pigments into coloured bands. Tsvet was the first who recognized and interpreted the chromatographic processes and named the method chromatography. Nowadays modern chromatographic techniques, automated systems are widely used for food analysis, characterization, quality control of raw food materials and food products in most analytical laboratory in the world.
5.2. Extraction during chromatography

There are some physical procedures which are very important in the chromatographic separation methods. These are the extractions, which are the base of chromatographic procedure. Extraction refers to the transfer of a solute from one liquid phase to another, which process can be categorized as batch, continuous, or counter-current extraction. During batch extraction the solute is extracted from one solvent by shaking it with a second, immiscible solvent, in the course of this procedure the solute are partitions, or distributes between the two phases. When equilibrium reached, the partition coefficient, $K$, is a constant (Equation 17):

$$K = \frac{\text{Concentration in solute in phase 1}}{\text{Concentration in solute in phase 2}}$$

Continuous extraction is more efficient than batch separation, but requires special apparatus, in which the solvent is recycled so that the solid is repeatedly extracted with fresh solvent. Good example for this procedure is fat content determination by a Soxhlet extractor, where the materials are extracted from the solid. Counter current extraction refers to a serial extraction process, when two or more solutes are used for separation of the different components with different partition coefficients between two immiscible liquid phases.
5.3. Chromatography

5.3.1. Terminology of the chromatography

Chromatography is a general term that is applied for a wide variety of separation techniques based on the partitioning or distribution of a sample (solute) between a moving or mobile phase and a fixed or stationary phase. The relative interaction of a solute with these two phases is described by the partition (K) or distribution (D) coefficient, which is the ratio of concentration of solute in stationary phase to concentration of solute in mobile phase. The mobile phase may be either a gas (gas chromatography, GC), liquid (liquid chromatography, LC) or a supercritical fluid (supercritical fluid chromatography, SFC), the stationary phase may be a liquid or a solid material.

The chromatographic procedures can be subdivided according to the various techniques applied, or to the physicochemical principles play role in the separation. The chromatographic procedures according to the various mobile phases can be divided into three parts: Gas chromatography (GC), supercritical fluid chromatography (SFC) and liquid chromatography (LC). According to the mobile phase, GC can be divided into two subclasses: Gas-liquid (GLC) and gas-solid (GSC) chromatography. The liquid chromatography can be divided into paper chromatography (PC), which is a liquid-liquid chromatography, into column liquid chromatography which may be is liquid-liquid or liquid-solid chromatography, and into thin layer chromatography (TLC), which may be is liquid-liquid or liquid-solid chromatography. According to the polarity of mobile and stationery phase there are reversed-phases chromatography, where the mobile phase is more polar than the stationary phase which is nonpolar liquid or solid, and normal-phase chromatography, where the mobile phase is less polar liquid, the stationary phase is more polar liquid or solid.

5.3.2. Gas chromatography

Gas chromatography is a column chromatography technique, where the mobile phase is gas and the stationary phase is either an immobilized liquid or a solid packed in a closed tube. GC is useful for separation of thermally stable volatile components of a mixture (for example fatty acid methyl esters). During the gas–liquid GC the sample is vaporized and injected into the head of the column. By using a controlled temperature gradient, the sample is transported
through the column by the mobile phase, which usually is an inert gas. The volatile components then are separated based on boiling point, molecular size, and polarity.

5.3.3. Liquid chromatography

The following liquid chromatography techniques are applied in food analysis: Planar chromatography (paper chromatography and thin layer chromatography (TLC), and column liquid chromatography, which involve a liquid mobile phase and either a solid or a liquid stationary phase. At liquid chromatography the physical form of the stationary phase is quite different, and the separation of the components is based on their physicochemical interactions with two phases.

5.3.3.1. Paper chromatography

At paper chromatography (partition chromatography) the stationary phase and the mobile phase are both liquid. The paper generally serves as a support for the liquid stationary phase. The sample is applied as a small spot or streak one and the half, two cm from the edge of a strip or square of filter paper, which then is allowed to dry. The dry strip is suspended in a closed container in which the atmosphere is saturated with the developing solvent (mobile phase), and the paper chromatogram that is developed. When the front solvent travel the length of the paper, the strip is removed from the developing chamber and the separated zones are detected. The stationary phase in paper partition chromatography is in most cases is water.

In the case of complex sample mixtures, two-dimensional techniques are used. In this case, the sample is spotted in one corner of a square sheet of paper, and one solvent is used to develop the paper in one direction. The chromatogram is then dried, turned 90°, and developed again, using a second solvent of different property (mainly polarity). To improve the resolution sometimes exchange papers is used in paper chromatography. After the chromatographic procedure, the components of the analysed material are characterized by their relative mobility (R_f-value) (Equation 18).

\[
R_f = \frac{\text{Distance moved by component}}{\text{Distance moved by solvent}}
\]  

(18)

R_f value is relatively constant, the value of it is influenced by many factors (stationary phase, layer thickness, humidity, temperature, and distance from the front).
5.3.3.2. Thin-layer chromatography

Thin-layer chromatography (TLC) has been largely replaced paper chromatography because it is faster, more sensitive, and more reproducible. The resolution in TLC is greater because the particles on the plate are smaller and more regular than paper fibres. The advantages of thin layer chromatography are the high sample throughput, the low cost, the possibility to analyze several samples and standards simultaneously, minimal sample preparation is necessary, and the plate may be stored for later identification and quantification.

TLC is applied in many fields, including environmental, clinical, forensic, pharmaceutical, food, flavours, and cosmetics. Within the food industry, TLC may be used for quality control and fast identification of poisoning materials from food. For example, corn and peanuts are tested for aflatoxins/mycotoxins prior to their processing into corn meal and peanut butter.

5.3.3.2.1. General procedures of the thin layer chromatography

TLC utilizes a thin (approx. 250 μm thick) layer of sorbent or stationary phase bound to an inert support in a planar configuration. The support is often a glass plate, plastic sheets and aluminium foil. Precoated plates, of different layer thicknesses, are commercially available in a wide variety of sorbents. Four frequently used TLC sorbents are silica gel, alumina, diatomaceous earth, and cellulose. Modified silicas for TLC may contain polar or nonpolar groups, so both normal and reversed-phase thin-layer separations may be carried out. The most commonly used sorbents at TLC are silica gel which is useful for separation of steroids, amino acids, alcohols hydrocarbons, lipids, mycotoxins, bile acids, vitamins and alkaloids by adsorption, silica gel RP which is used for fatty acids, vitamins, steroids, hormones, carotenoids by reversed phase LC, cellulose, kiselguhr, which are used for carbohydrates, sugars, alcohols, amino acids, carboxylic acids, fatty acids y partition, aluminium oxide which is used for amines, alcohols, steroids, lipids, mycotoxins, bile acids, vitamins, alkaloids by adsorption, cellulose derivatized by polyethyleneamin for nucleic acids, nucleotids, nucleosids, purines, pyrimidines by ion exchange and magnesium silicates for steroids, pesticides, lipids and alkaloids by adsorption.

High-performance thin-layer chromatography (HPTLC) simply refers to TLC performed using plates coated with smaller and more uniform particles. This permits better separations in shorter
times. If TLC adsorption be performed, the sorbent is first activated by drying for a specified time and temperature.

Sample (in carrier solvent) is applied as a spot or 1–2 cm streak from one end of the plate. After evaporation of the carrier solvent, the TLC plate is placed in a closed developing chamber with the end of the plate that is nearest spot in the solvent at the bottom of the chamber. Traditionally, solvent migrates up to the plate (ascending development) by capillary action and sample components are separated.

After the TLC plate that has been removed from the chamber and solvent has been allowed to evaporate, the separated bands are made visible or detected by other means. Specific chemical reactions (derivatization), which may be carried out either before or after chromatography, often are used for this purpose.

Common physical detection methods include the measurement of absorbed or emitted electromagnetic radiation by means of autoradiography and the measurement of β-radiation from radioactively labelled compounds. Biological methods or biochemical inhibition tests can be used to detect toxicologically active substances.

Quantitative evaluation of thin-layer chromatograms may be performed in situ directly on the layer by using a densitometer or after scraping zone off the plate, eluting compound from the sorbent, and analyzing the resultant solution.

5.3.3.2.2. Factors affecting thin-layer separations

Separation can occur by adsorption, partition, ion-exchange, size-exclusion, or multiple mechanisms. Solvents for TLC separations are selected for specific chemical characteristics and solvent strength (a measure of interaction between solvent and sorbent. It can be stated, that higher solvent strength, greater Rf value of the solute, and Rf value of 0.3–0.7 are typical. Mobile phases have been developed for the separation of various compound classes on the different sorbents. Other factors must be considered when performing planar chromatography is type of developing chamber that is used, like vapour phase conditions (saturated vs. unsaturated), development mode and development distance.
Column liquid chromatography

Column chromatography is the most useful method of separating compounds in a mixture. Fractionation of compounds occurs as a result of differential migration through a closed tube of stationary phase, and the separated compounds can be monitored while the separation is in progress. In liquid column chromatography, the mobile phase is liquid and the stationary phase can be either solid or liquid supported by an inert solid.

First stationary phase (resin, gel, or packing material) must be prepared by the analyst. The stationary phase often must be hydrated or preswelled in the mobile phase, when the prepared stationary phase is packed into a column. Longer and narrower columns usually enhance resolution and separation of the investigated materials. Adsorption columns may be either dry or wet packed; the most common technique is the wet packing which involves making slurry of the adsorbent with the solvent and pouring this into the column. As the sorbent settles, excess solvent is drained off and additional slurry is added. This process is repeated until the desired bed height obtained. If the packing solvent is different from the initial eluting solvent, the column must be thoroughly washed (equilibrated) with the starting mobile phase. The classical low-pressure chromatography utilizes only gravity flow or a peristaltic pump to maintain a flow of mobile phase (eluent or eluting solvent) through the column.

The flow rate is governed by the hydrostatic pressure, measured as the distance between the level of liquid in the reservoir and the level of the column outlet. The process of passing the mobile phase through the column is called elution; the portion that emerges from the outlet end of the column is sometimes called the eluate or effluent.

Elution may be isocratic, when the composition of the mobile-phase is constant, or gradient when the composition of the mobile-phase is changing, for example increasing solvent strength or pH, during elution in order to enhance resolution and decrease analysis time.

The column eluate may be directed through a detector and then into tubes, changed at intervals by a fraction collector. The detector response, in the form of an electrical signal, may be recorded, which creates the chromatogram, using either a chart recorder or a computerized software, and used for qualitative or quantitative analysis.
5.3.3.4. Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) refers to chromatography that is performed above the critical pressure (P_c) and critical temperature (T_c) of the mobile phase. A supercritical fluid (or compressed gas) is neither a liquid nor a typical gas. The combination of P_c and T_c is known as the critical point. A supercritical fluid can be formed from a conventional gas by increasing the pressure or from a conventional liquid by raising the temperature.

Carbon dioxide frequently is used as a mobile phase for SFC, because it is not a good solvent for polar and high molecular-weight compounds. Other supercritical fluids are nitrous oxide, trifluoromethane, sulphur hexafluoride, pentane and ammonia. The high diffusivity and low viscosity of supercritical fluids mean decreased analysis times and improved resolution compared to LC.

SFC offers a wide ranges of selectivity adjustment, by changes in pressure and temperature as well as changes in mobile phase composition and the stationary phase. SFC makes possible separation of nonvolatile, thermally labile compounds that are not amenable to GC. SFC can be performed by using either packed columns or capillaries, and has used primarily for nonpolar compounds. Fats, oils, and other lipids are compounds which SFC is increasingly applied.
5.4. Physicochemical principles of chromatographic separation

Several physicochemical principles are involved in chromatography mechanisms which are adsorption, when the solute absorbed on the surface of stationary phase, ion exchange, when mobile anions held near cations that are covalently attached to stationary phase, or at anion exchange resin, only anions can be attracted to the resin, partition, when the solute dissolved in the liquid phase coated surface of solid support, molecular exclusion, when large molecules are excluded, small molecules are included, and affinity, when one kind of molecule in the mixture is attached to molecule which is covalently bound to stationary phase, and all other molecules are washed through the stationary phase.

5.4.1. Adsorption (liquid-solid) chromatography

The stationary phase is a finely divided solid to maximize the surface area of the adsorbent. The stationary phase (adsorbent) is chosen to permit differential interaction with the components of the sample to be separated. The intermolecular forces which are responsible for chromatographic adsorption include the following: Van der Waals forces, electrostatic forces, hydrogen bonds and hydrophobic interactions. Binding sites with greater affinities, the most active sites, tend to be populated first, so that additional solutes are less firmly bound. The net result is that adsorption in a concentration-dependent process, and the adsorption coefficient is not a constant.

Classic adsorption chromatography utilizes slightly acidic silica, slightly basic alumina, nonpolar charcoal, or a few other materials as the stationary phase. The elution order of compounds from these adsorptive stationary phases can often be predicted on the basis of their relative polarities. The increasing polarity order is the following: fluorocarbons, saturated hydrocarbons, olefins, aromatics, halogenated compounds, ethers, nitro compounds, esters, ketones, aldehydes, alcohols, amines, amides, carboxylic acids. Compounds with the most polar functional groups are retained most strongly on polar adsorbents and are eluted last, and nonpolar solutes are eluted first.

A proposed model to explain the mechanism of liquid–solid chromatography is that solute and solvent molecules are competing for active sites on the adsorbent. As relative adsorption of the mobile phase increases, adsorption of the solute must decrease. Solvents can be rated in order
of their strength of adsorption on a particular adsorbent, such as silica. Such a solvent strength (or polarity) scale is called an eluotropic series.

The eluotropic series for alumina is the following: pentane, isooctane, cyclohexane, carbon tetrachloride, xylene, toluene, benzene, ethyl ether, chloroform, methylene chloride, tetrahydrofuran, acetone, ethyl acetate, aniline, acetonitrile, propanol, ethanol, methanol, acetic acid. Once an adsorbent is chosen, solvents can be selected from the eluotropic series for that adsorbent. Mobile phase polarity can be increased until elution of the compound(s) of interest has achieved.

Adsorption chromatography separates aromatic or aliphatic nonpolar compounds, based primarily on the type and number of functional groups present. It has been used for the analysis of fat-soluble vitamins, and it is used as a batch procedure for removal of impurities from samples prior to other analyses.

5.4.2. Partition (liquid-liquid) chromatography

At partition chromatography solutes are partitioned between two liquid phases according to their partition coefficients, hence is named partition chromatography. More polar of two liquids is held stationary on the inert support and the less polar solvent is used to elute the sample components; this is the normal-phase chromatography. Using a nonpolar stationary phase and a polar mobile phase that has come to be known as reversed-phase chromatography.

Polar hydrophilic substances, such as amino acids, carbohydrates, and water-soluble plant pigments, are separable by normal-phase partition chromatography. Lipophilic compounds, such as lipids and fat-soluble pigments, and polyphenols may be resolved with reversed-phase systems.

5.4.2.1. Coated supports

The stationary phase for partition chromatography consists of a liquid coating on a solid matrix. The solid support should be as inert as possible and have a large surface area in order to maximize the amount of liquid held. Silica, starch, cellulose powder, and glass beads are capable of holding a thin film of water, which serves as the stationary phase. Materials that are prepared for adsorption chromatography must be activated by drying them to remove surface
water. The liquid stationary phase is often stripped off, which problem can be overcome by chemically bonding the stationary phase to the support material.

5.4.2.2. Bonded supports

The liquid stationary phase may be covalently attached to a support by a chemical reaction, and the bonded phases are very popular for HPLC use. Widely used is reversed-phase HPLC a nonpolar bonded stationary phase, silica covered with C8 or C18 groups, and a polar solvent, water–acetonitrile.

5.4.3. Ion exchange chromatography

Ion exchange is a separation/purification process occurring naturally, for example, in soils and is utilized in water softeners and deionizers. Three types of separation may be achieved: ionic from nonionic, cationic from anionic, mixtures of similarly charged species.

Ion-exchange chromatography may be viewed as a type of adsorption chromatography in which interactions between solute and stationary phase are primarily electrostatic in nature. The ion exchanger stationary phase contains fixed functional groups that are either negatively or positively charged, and the exchangeable counterions preserve charge neutrality. A sample ion can exchange with the counterion to become the partner of the fixed charge.

Cation exchangers contain covalently bound negatively charged functional groups, anion exchangers contain bound positively charged groups. The strongly acidic sulfonic acid moieties $\text{SO}_3^-$ of “strong”-cation exchangers are completely ionized at all pH values above 2. Strongly basic quaternary amine groups $\text{RNR}^+_3$ on “strong”-anion exchangers are ionized at all pH values below 10. “Weak”-cation exchangers contain weakly acidic carboxylic acid functional groups, $\text{RCO}_2^-$, their exchange capacity varies considerably between ca. pH 4 and 10. Weakly basic anion exchangers possess primary, secondary, or tertiary amine residues, $\text{R} - \text{NHR}^+_2$, which are deprotonated in moderately basic solution, thereby losing their positive charge and the ability to bind anions.

One way of eluting solutes bound is an ion-exchange medium to change the mobile-phase pH. A second way to elute bound solutes is to increase the ionic strength (use NaCl) of the mobile phase, to weaken the electrostatic interactions.
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Chromatographic separations by ion exchange are based upon differences in affinity of the exchangers for the ions to be separated. The factors that govern selectivity of an exchanger for a particular ion include the ionic valence, radius, and concentration; the nature of the exchanger, and the composition and pH of the mobile phase. Synthetic ion exchangers are cross-linked polyelectrolytes, they may be inorganic or organic compounds. Polystyrene, made by cross-linking styrene with divinyl benzene (DVB), may be modified to produce either anion- or cation-exchange resins.

The extent of cross-linking controls the rigidity and porosity of the resin, which, in turn, determines its optimal use. Lightly cross-linked resins permit rapid equilibration of solute, but particles swell in water, thereby decreasing charge density and selectivity of the resin for different ions. More highly cross-linked resins exhibit less swelling, higher exchange capacity, and selectivity, but longer equilibration times. The small pore size, high charge density, and inherent hydrophobicity of the older ion-exchange resins have limited their use to small molecules [molecular weight (MW)<500].

Ion exchangers based on polysaccharides, such as cellulose, dextran, or agarose, have proven very useful for the separation and purification of large molecules, such as proteins and nucleic acids. These materials, called gels, are much softer than polystyrene resins, and thus may be derivatized with strong or with weak acidic or basic groups via OH moieties on the polysaccharide backbone. They have much larger pore sizes and lower charge densities than the older synthetic resins.

Food-related applications of ion-exchange chromatography: separation of amino acids, sugars, alkaloids, and proteins. Fractionation of amino acids in protein hydrolyzates was initially carried out by ion-exchange chromatography. Automation of this process led to the development of commercially produced amino acid analyzers. Many drugs, fatty acids, and the acids of fruit, being ionizable compounds, may be chromatographed in the ion-exchange mode.

5.4.4. Size-exclusion chromatography

Size-exclusion chromatography (SEC), also known as molecular exclusion, gel permeation (GPC), and gel-filtration chromatography (GFC). It is widely used in the biological sciences for the resolution of macromolecules, such as proteins and carbohydrates.
In the ideal SEC system, molecules are separated solely on basis of their size; no interaction occurs between solutes and the stationary phase. The stationary phase in SEC consists of a column packing material that contains pores comparable in size to the molecules to be fractionated. Components that are too large to enter the pores, travel with the mobile phase in the interstitial space, between particles, outside the pores, so that the largest molecules are eluted first from an SEC column.

As solute dimensions decrease, approaching those of the packing pores, molecules begin to diffuse into the packing particles and, consequently, are slowed down. Solutes of low molecular weight that have free access to all the available pore volume. The behaviour of a molecule in a size-exclusion column may be characterized by available partition coefficient. The value of $K_{av}$ (available partition coefficient) defines the proportion of pores that can be occupied by that molecule.

A size-exclusion column is calibrated with a series of solutes of known molecular weight. An estimate of molecular weight (or size) of the unknown is made by interpolation of the calibration curve. The relationship between $K_{av}$ and log molecular weight of globular proteins is linear, for this the molecular weight of the unknown protein can be determined after the protein that was chromatographed on a special column like Sephadex G-150 Superfine.

Column packing materials for SEC can be divided into two groups: semirigid, hydrophobic media, and soft and hydrophilic gels. SEC can be used, directly, to fractionate mixtures or, indirectly, to obtain information about a dissolved species. SEC is used to determine the molecular weight distribution of natural and synthetic polymers, such as dextrans and gelatin preparations.

### 5.4.5. Affinity chromatography

Affinity chromatography is unique in that separation is based on the specific, reversible interaction between a solute molecule and a ligand immobilized on the chromatographic stationary phase. Affinity chromatography usually involves immobilized biological materials as the stationary phase. These ligands can be antibodies, enzyme inhibitors, lectins, or other molecules that selectively and reversibly bind to complementary analyte molecules in the sample.
The steps of bioselective affinity chromatography: The support presents the immobilized ligand to the analyte to be isolated. The analyte makes contact with the ligand and attaches itself. The analyte is recovered by the introduction of an eluent, which dissociates the complex holding the analyte to the ligand. The support is regenerated, ready for the next isolation.

A ligand, chosen based on its specificity and strength of interaction with the molecule to be isolated, is immobilized on a suitable support material. As the sample is passed through this column, molecules that are complementary to the bound ligand are adsorbed while other sample components are eluted. Bound analyte is subsequently eluted via a change in the mobile-phase composition. After reequilibration with the initial mobile phase, the stationary phase is ready to be used again. The ideal support for affinity chromatography should be a porous, stable, high-surface-area material that does not adsorb anything itself.

Affinity ligands are usually attached to the support or matrix by covalent bond formation, and optimum reaction conditions often must be found empirically. Immobilization generally consists of two steps: activation and coupling. During the activation step, a reagent reacts with functional groups on the support, such as hydroxyl moieties, to produce an activated matrix. After removal of excess reagent, the ligand is coupled to the activated matrix. When small molecules are immobilized, a spacer arm, containing at least four to six methylene groups, is used to hold the ligand away from the support surface, enabling it to reach into the binding site of the analyte.

Ligands for affinity chromatography may be either specific or general. Specific ligands, such as antibodies, bind only one particular solute. General ligands, such as nucleotide analogs and lectins, bind to certain classes of solutes. Bound solutes then can be separated as a group or individually, depending upon the elution technique used.

In addition to protein purification, affinity chromatography may be used to separate supramolecular structures such as cells, organelles, and viruses; concentrate dilute protein solutions; investigate binding mechanisms. Affinity chromatography has been useful especially in the separation and purification of enzymes and glycoproteins. Carbohydrate-derivatized adsorbents are used to isolate specific lectins.
5.5. Analysis of chromatographic peaks

As separation is achieved and chromatographic peaks are obtained, qualitative as well as quantitative analysis can be carried out. The analyst begins by evaluating what is known about the sample and defines the goals of the separation. How many components need to be resolved? What degree of resolution is needed? Is qualitative or quantitative information needed? Molecular weight (or molecular weight range), polarity, and ionic character of the sample will guide the choice of chromatographic separation mechanism.

By choosing a separation mode for the sample at hand, one must select an appropriate stationary phase, elution conditions, and a detection method. Trial experimental conditions may be based on the results of a literature search, the analyst’s previous experience with similar samples, or general recommendations from chromatography experts.

Isocratic elution is the most simple and widely used technique which solvent composition and flow rate are held constant. Gradient elution involves reproducibly varying mobile phase composition or flow rate (flow programming) during the LC analysis. Gradient elution is used when sample components possess a wide range of polarities, so that an isocratic mobile phase does not elute all components within a reasonable time. The change may be continuous or stepwise. Gradients of increasing ionic strength are extremely valuable in ion-exchange chromatography. It is commonly used for desorbing large molecules, such as proteins, which can undergo multiple-site interaction with a stationary phase. Increasing the “strength” of the mobile phase, shortens the analysis time.

Once an initial separation is achieved, the analyst can proceed to optimize resolution. This generally involves manipulation of mobile phase variables, including the nature and percentage of organic components, pH, ionic strength, nature and concentration of additives, flow rate, and temperature.

5.5.1. Chromatographic resolution

The main goal of chromatography is to segregate components of a sample into separate bands or peaks as they migrate through the column. A chromatographic peak is defined by several parameters including retention time, peak width, and peak height. The volume from an LC column is called the retention volume, $V_R$. The associated time is the retention time, $t_R$. Shifts in retention time and changes in peak width greatly influence chromatographic resolution.
The resolution of two peaks from each other is related to the separation factor, $\alpha$. Values for $\alpha$ depend on temperature, the stationary phase, and mobile phase that is used. Resolution is defined:

$$R_S = \frac{2\Delta t}{w_2 + w_1}$$  \hspace{1cm} (19)

where

- $R_S$ = resolution,
- $\Delta t$ = difference between retention times of peaks 1 and 2,
- $w_2$ = width of peak 2 at baseline,
- $w_1$ = width of peak 1 at baseline.

Chromatographic resolution is a function of column efficiency, selectivity, and the capacity factor.

**Column efficiency**

An efficient column keeps the bands from spreading and gives narrow peaks.

**Column selectivity**

Chromatographic resolution depends on column selectivity as well as efficiency. Column selectivity refers to the distance, or relative separation between two peaks. Good selectivity is more important for a given separation than high efficiency.

**Column capacity factor**

The capacity or retention factor, $k'$ is a measurement of amount of time a chromatographed species (solute) spends in/on the stationary phase relative to the mobile phase. Small values of $k'$ indicate little retention, and components will be eluted close to the solvent front, resulting in poor separations. Large values of $k'$ result in improved separation but also can lead to broad peaks and long analysis times. On a practical basis, $k'$ values within the range of 1–15 are generally used.
5.5.2. Qualitative analysis

Comparing $V_R$ or $t_R$ with that chromatographed standards under identical conditions often enables one to identify an unknown compound. When it is necessary to compare chromatograms that are obtained from two different systems or columns, it is better to compare adjusted retention time, $t'_R$. Different compounds may have identical retention times. Even if the retention time of an unknown and a standard are equivalent, two compounds might not be identical.

5.5.3. Quantitative analysis

Quantification involves measuring peak height, area, or mass and comparing these data with those for standards of known concentration. Nearly all chromatography systems now use data analysis software, which recognizes the start, maximum, and end of each chromatographic peak, even when not fully resolved from other peaks. These values are used to determine retention times and peak areas. At the end of each run, a report is generated that lists these data and postrun calculations, such as relative peak areas, areas as percentages of the total area, and relative retention times. Data from external or internal standards can be used to calculate analyte concentrations.

For having quantified sample peaks, first must compare these data with appropriate standards of known concentration to determine sample concentrations. Comparisons may be by means of external or internal standards. Comparison of peak height, area, or mass of unknown samples with standards that are injected separately (i.e., external standards) is common practice. Standard solutions covering the desired concentration range, preferably diluted from one stock solution, are chromatographed, and the appropriate data, peak height, area, or mass, plotted against concentration to obtain a standard curve. An identical volume of sample is then chromatographed, and height, area, or mass of the sample peak is used to determine sample concentration via the standard curve. This absolute calibration method requires precise analytical technique and requires detector sensitivity that be constant from day to day if the calibration curve is valid for remain.

Use of the internal standard, relative or indirect, method can minimize errors due to sample preparation, apparatus, and operator technique. In this technique, a compound is utilized that is structurally related to, but is eluted independently from compounds of interest in the sample to
be analyzed. First preparing is a set of standard solutions containing varying concentrations of the compound(s) of interest. Each of these solutions is made to contain a known and constant amount of the internal standard. These standard solutions are chromatographed, and peak height, area, or mass is measured. Ratios of peak height, area, or mass (compound of interest/internal standard) are calculated and plotted against concentration to obtain calibration curves.

Next, a known amount of internal standard is added to the unknown sample, and the sample is chromatographed. Peak height, area, or mass ratios are calculated and used to read the concentration of each relevant component from the appropriate calibration curve. The advantages of using internal standards are injection volumes that are not needed be accurately measured and the detector response is not needed to be remain constantly since any changes will not alter ratios. The main disadvantage is finding a standard that does not interfere chromatographically with components of interest in the sample.
5.6. Summary of first part of chromatography

Chromatography is a separation method based on the partitioning of a solute between a mobile phase and a stationary phase. The mobile phase may be liquid, gas, or a supercritical fluid. The stationary phase may be an immobilized liquid or a solid, in either a planar or column form. Based on the physicochemical characteristics of the analyte, and the availability of instrumentation, a chromatographic system is chosen to separate, identify, and quantify the analyte. Chromatographic modes include adsorption, partition, ion exchange, size exclusion, and affinity chromatography. Factors should be considered while developing a separation include mobile phase variables, strength, pH, temperature, and flow rate, and column efficiency, selectivity, and capacity. Following detection, a chromatogram provides both qualitative and quantitative information via retention time and peak height area data.
5.7. The most frequently used chromatographic methods in the practice

5.7.1. High-performance liquid chromatography

Originally, high-performance liquid chromatography (HPLC) was the acronym for high-pressure liquid chromatography, reflecting the high operating pressures generated by early columns. By the late 1970s, high-performance liquid chromatography had become the preferred term, emphasizing the effective separations achieved. HPLC can be applied to the analysis of any compound with solubility in a liquid that can be used as the mobile phase.

Although most frequently employed as an analytical technique, HPLC also may be used in the preparative mode. There are many advantages of HPLC over traditional low pressure column liquid chromatography: Speed, because many analyses can be accomplished in 30 min or less, a wide variety of stationary phases, improved resolution and greater sensitivity, because various detectors can be employed, and easy sample recovery, because of less eluent volume to remove.

5.7.1.1. Components of an HPLC system

The main components of this system are pump, injector, column, detector, and data evaluation system also important that are the mobile phase (eluent) reservoirs, and a fraction collector, which is used if further analysis of separated components is needed. Tube connecting, tube fittings, and the materials out of so-called components are constructed also influence system performance and lifetime. Column(s) and detector may be thermostatted, for operation at elevated temperature.

5.7.1.1.1. Pumps

The HPLC pump delivers the mobile phase through the system, typically at a flow rate of 0.4–1 ml/min, in a controlled, accurate, and precise manner. The majority of pumps currently used in HPLC are reciprocating, piston-type pumps. One disadvantage of reciprocating pumps is that they produce a pulsating flow, requiring the addition of pulse dampers to suppress fluctuations.

Gradient elution systems for HPLC are used to vary the mobile phase concentration during the run, by mixing mobile phase from two or more reservoirs. This is accomplished with low-pressure mixing which mobile phase components are mixed before entering the high-pressure pump, or high-pressure mixing that two or more independent, programmable pumps are used.
Gradient HPLC is extremely important for the effective elution of all components of a sample and optimal resolution. It is routinely applied to all modes of HPLC except size-exclusion chromatography.

All HPLC pumps contain moving parts such as check valves and pistons, and are quite sensitive to dust and particulate matter in the liquid being pumped. Degassing HPLC eluents, by the application of a vacuum or by sparging with helium, also is recommended to prevent the problems caused by air bubbles in a pump or detector.

5.7.1.1.2. Injector

The role of the injector is placing the sample into the flowing mobile phase for introduction onto the column. Virtually all HPLC systems use valve injectors, which separate sample introduction from the high-pressure eluent system. With the injection valve in the load position, the sample is loaded into an external, fixed-volume loop using a syringe. Meanwhile eluent flows directly from the pump to the column at high pressure.

In the case of valve-type injector, the valve allows the sample loop to be isolated from the pump eluent stream (load position). The valve allows the sample loop to be positioned in it (inject position) when the valve is rotated to the inject position, the loop becomes part of the eluent flow stream and sample is carried onto the column.

Changing the loop allows different volumes to be injected. Injection volumes of 10–100 μl are typical, both larger (1–10 ml) and smaller (≤2 μl) sample volumes can be loaded. Automated sample injectors, or autosamplers, may be used to store and inject large numbers of samples. Samples are placed in uniform-size vials, sealed with a septum, and held in a possibly refrigerated tray. A computer actuated needle penetrates the septum to withdraw solution from the vial, and a mechanically or pneumatically operated valve introduces it onto the column.

Autosamplers can reduce the tedium and labour costs associated with routine HPLC analyses. Because samples may remain unattended for 12–24 h prior to automatic injection, sample stability is a limiting factor for using this accessory.
5.7.1.1.3. Column

5.7.1.1.3.1. Column hardware

An HPLC column is usually constructed of stainless steel tubing with terminators that allows it to be connected between the injector and detector of the system. Many types and sizes of columns are commercially available, ranging from 5 cm × 50 cm or larger preparative columns down to wall-coated capillary columns.

Precolumns. Auxiliary columns that precede the analytical HPLC column are termed precolumns. Short (≤5 cm) expendable columns, called guard columns, often are used to protect the analytical column from strongly adsorbed sample components.

Analytical columns. The most commonly used analytical HPLC columns are 10, 15, or 25 cm long with an internal diameter of 4.6 or 5 mm. Short (3 cm) columns, packed with ≤3 μm particles, are gaining popularity for fast separations; for example, in method development or process monitoring. The use of columns with smaller internal diameters (<0.5–2.0 mm), including wall-coated capillary columns, has increased. The advantages of using smaller diameter columns include a decreased consumption of mobile phase, an increased peak concentration, increased resolution, and the ability to couple HPLC with mass spectrometry.

A capillary column is a narrow-bore open tubular column, that the inner surface is coated with a thin layer of stationary phase. Because of the extremely high operating pressures of these systems, they are often referred to as ultra-HPLC (UHPLC).

5.7.1.1.3.2. HPLC column packing materials

A packing material serves, first of all, to form the chromatographic bed; in most modes of chromatography the column packing material serves both support and the stationary phase. Requirements for HPLC column packing materials are good chemical stability, sufficient mechanical strength to withstand pressure generated during use, and the availability of a well-defined particle size, with a narrow particle size distribution. Two materials that meet the above criteria are porous silica and synthetic organic resins.

Silica-based column packings. Porous silica can be prepared in a wide range of particle and pore sizes, with a narrow particle size distribution. Bonded phases are made by covalently bonding hydrocarbon moieties to −OH groups (silanols) on the surface of silica particles. Often
the silica is reacted with an organochlorosilane, where the substituents may be are halides or methyl groups. The nature of substituents determines whether the resulting bonded phase will exhibit normal-phase, reversed-phase, or ion-exchange chromatographic behaviour. The main disadvantage is that the silica skeleton slowly dissolves in aqueous solutions, and the rate of this process becomes prohibitive at pH<2 and >8.

A pellicular packing material is made by depositing a thin layer or coating onto the surface of an inert, usually nonporous, microparticulate core. Core material may be either inorganic, such as silica, or organic, such as poly(styrene-divinylbenzene) or latex.

**Porous polymeric column packing.** Synthetic organic resins offer the advantages of good chemical stability and the possibility to vary interactive properties through direct chemical modification. Microporous or gel-type resins are comprised of crosslinked copolymers which the apparent porosity, evident only when the gel is in its swollen state.

Macroporous resins are highly crosslinked and consist of a network microspheric gel beads that are joined together to form a larger bead.

### 5.7.1.1.3.3. Detector

A detector translates sample concentration changes in the HPLC column effluent into electrical signals. The most widely used HPLC detectors are based on ultraviolet-visible (UV-VIS) and fluorescence spectrophotometry, refractive index determination, and electrochemical analysis. Mass spectrometry also can be applied to the detection of analytes in HPLC eluents. More than one type of HPLC detector may be used in series, to provide increased specificity and sensitivity for multiple types of analytes.

**UV-VIS absorption detectors.** UV-VIS absorption detector can measure the absorption of radiation by chromophore-containing compounds. The three main types of UV-VIS absorption detectors are fixed according to wavelength, variable-wavelength, and diode array spectrophotometers. At fixed wavelength a filter is used to isolate a single emission line (e.g., at 450 nm) from a source such as a mercury lamp. In the case of variable-wavelength detector deuterium and tungsten lamps serve as sources of ultraviolet and visible radiation. Wavelength selection is provided by a monochromator. At diode array spectrophotometric detectors all the light from a deuterium lamp is spread out into a spectrum. To provide the full absorption
spectrum from 200 to 700 nm every 0.1 s, which may enable the components of a mixture to be identified.

**Fluorescence detectors.** Some organic compounds can re-emit a portion of absorbed UV-VIS radiation at a longer wavelength (lower energy). This is known as fluorescence, and measurement of the emitted light provides another useful detection method. Fluorescence detection is both selective and very sensitive, providing up to 1000-fold lower detection limits then for the same compound in absorbance spectrophotometry.

**Refractive index detectors.** Refractive index (RI) detectors measure changes in the RI of the mobile phase due to dissolved analytes. RI detectors are less sensitive than other types and cannot be used with gradient elution.

**Electrochemical detectors.** Electroanalytical methods used for HPLC detection are based either on electrochemical oxidation–reduction of the analyte or on changes in conductivity of the eluent.

**Coupled analytical techniques.** HPLC with mass spectrometric detection (LC–MS), the liquid mobile phase that affect the vacuum in the MS. Commercial interfaces allow solvent to be evaporated so that only analyte is carried to the spectrometer. LC–MS applications are expanding to nearly every class of relatively low molecular weight compounds, including bioactives and contaminants.

**Chemical reactions.** Detection sensitivity or specificity may be is enhanced by converting the analyte to a chemical derivative with different or additional characteristics. An appropriate reagent can be added to the sample prior to injection (precolumn derivatization) or combined with column effluent before it enters the detector (postcolumn derivatization). Automated amino acid analyzers utilize postcolumn derivatization, usually with ninhydrin. Precolumn derivatization of amino acids with o-phthalaldehyde or similar reagents permits are highly sensitive HPLC determination of amino acids using fluorescence detection. Fractions may be collected after passing through the detector and aliquots of each fraction analyzed by various means, including chemical/colorimetric assays, such as the Lowry protein assay.
5.7.1.3.4. Data stations systems

Data stations and software packages are nearly ubiquitous with modern HPLC, all come with very powerful tools for sample identification and quantitation. As an HPLC analysis progresses, the data from the HPLC detector(s) are digitized and saved to the hard drive of a dedicated computer. Then the operator can manipulate the data, by assigning and integrating the peaks, and then print out plots and tables for further assessment.

Retention times can be calculated relatively to an internal standard in pesticide residue analysis, the results compared to a stored database of standards that the software automatically accesses when the analysis is complete.

The software packages also include all the parameters that are needed to run the HPLC, including start and stop, injection of the sample, and developing the gradient via control of the proportioning pump systems. When combined with an autosampler, the data station can carry out the entire operation, on hundreds of samples.

5.7.1.2. Normal phase HPLC

5.7.1.2.1. Stationary and mobile phases

In normal-phase HPLC, the stationary phase is a polar adsorbant, such as bare silica or silica to which polar nonionic functional groups - hydroxyl, nitro, cyano (nitrile), or amino - have been chemically attached. These bonded phases are moderately polar and the surface is more uniform, resulting in better peak shapes. The mobile phase for this mode consists of a nonpolar solvent, such as hexane which is added a more polar modifier, such as methylene chloride, to control solvent strength and selectivity. Weak solvents increase retention, strong solvents decrease retention.

5.7.1.2.2. Applications of normal-phase HPLC

Normal-phase HPLC was used for the analysis of fat-soluble vitamins, reverse phase is currently applied more frequently for these analyses. Normal phase is used for the analyses of biologically active polyphenols, for the analysis of relatively polar vitamins, such as vitamins A, D, E, and K, natural carotenoid pigments, which impart both colour and health benefits to
foods. Highly hydrophilic species, such as carbohydrates may be resolved by normal-phase chromatography.

Some examples for applications of HPLC in the analysis of various food constituents are: mono- and oligosaccharides can be analysed by ion exchange, normal or reversed phase chromatography by electrochemical or refractive index detection or postcolumn analysis, amino acids by ion exchange or reversed phase chromatography by post- or precolumn derivatization, and mycotoxins by reversed phase chromatography by fluorescence detection.

5.7.1.3. Reversed phase HPLC

5.7.1.3.1. Stationary and mobile phases

More than 70% of all HPLC separations are carried out in the reversed-phase mode, utilizes a nonpolar stationary phase and a polar mobile phase. Octadecylsilyl (ODS) bonded phases, with an octadecyl (C18) chain [–(CH2)17CH3], are the most popular reversed-phase packing materials, and shorter chain hydrocarbons, octyl (C8) or butyl (C4) [−(CH2)4CH3] or phenyl groups are also used.

Reversed-phase HPLC utilizes polar mobile phases, usually water is mixed with methanol, acetonitrile, or tetrahydrofuran. Solutes are retained due to hydrophobic interactions with the nonpolar stationary phase and are eluted in order of increasing hydrophobicity or decreasing polarity.

5.7.1.3.2. Applications of reversed-phase HPLC

Reversed-phase HPLC mode most is used for analysis of plant proteins, and cereal proteins are routinely analyzed by this method. Both water- and fat-soluble vitamins can be analyzed by reversed-phase HPLC. Reversed-phase ion-pair HPLC can be used to resolve carbohydrates on C18 bonded-phase columns, the constituents of soft drinks, caffeine, aspartame, can be rapidly separated. Reversed-phase HPLC has been applied for the analysis of lipids, antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), phenolic flavour compounds and pigments that are easily analyzed.

When analysing of vitamin B6 compounds by reversed-phase HPLC with fluorescence detection, the order of the elution of the different compounds is following: PL, pyridoxal; PLP, pyridoxal phosphate; PM, pyridoxamine; PMP, pyridoxamine phosphate; PN, pyridoxine; PNG, pyridoxine β-D-glucoside. At reversed-phase HPLC separation of α-carotene (AC) and
β-carotene (BC) isomers in fresh and canned carrots using a 5 mm C$_{30}$ stationary phase, the elution order of the peaks is: 13-cis AC; unidentified cis AC; 13-cis AC; 15-cis BC; unidentified cis AC; 13-cis BC; all-trans AC; 9-cis AC; 9, all-trans BC and 9-cis BC.

5.7.1.4. Ion exchange chromatography

5.7.1.4.1. Stationary and mobile phases

Packing materials are usually functionalized organic resins, such as sulfonated or aminated poly(styrene-divinylbenzene). Macroporous resins are most effective for HPLC columns due to their rigidity and permanent pore structure. The mobile phase in ion-exchange HPLC is usually an aqueous buffer; solute retention is controlled by changing mobile phase ionic strength and/or pH. Gradient elution (gradually increasing ionic strength) is frequently employed.

5.7.1.4.2. Ion chromatography

Ion chromatography is a high-performance ion-exchange chromatograph using a relatively low-capacity stationary phase and a conductivity detector. All ions conduct an electric current; measurement of electrical conductivity is an obvious way to detect ionic species. Ion chromatography can be used to determine inorganic anions and cations, transition metals, organic acids, amines, phenols, surfactants, and sugars. Some specific examination of ion chromatography: determination of organic and inorganic ions in milk; organic acids in coffee extract and wine; chlorine in infant formula; trace metals, phosphates, and sulphites in foods. A typical application of the ion chromatography is the determination of the organic and inorganic anions in coffee. The elution order of the ten anions is: acetate, glycolate, quinate, formate, chloride, tartrate, oxalate, fumarate, phosphate and citrate. During the analysis an IonPac AS5A column ( Dionex) using a sodium hydroxide gradient and suppressed conductivity detection were used.

5.7.1.4.3. Ion exchange chromatography of carbohydrates and proteins

Both cation- and anionexchange stationary phases have been applied to HPLC of carbohydrates. One common application is the determination of oligosaccharide distributions in corn syrups and other starch hydrolysates. Amino acids have been resolved on polymeric ion exchangers
for more than 50 years. Ion exchange is one of the most effective modes for HPLC of proteins and, for the fractionation of peptides.

A good example for using anion exchange, the analysis of iso-amylase-treated waxy corn starch. The enzyme debranches the amylopectin, and the chromatogram represents the branch chain-length distribution, from four sugars in length and up. The analysis was performed with anionexchange HPLC, with a pulsed amperometric detector.

5.7.1.5. Size-exclusion chromatography

Size-exclusion chromatography (SEC) fractionates first solutes solely on the basis of size, with larger molecules eluting.

5.7.1.5.1. Column packings and mobile phases

Hydrophilic packings, for use with water-soluble samples and aqueous mobile phases, may be surface-modified silica or methacrylate resins. Poly(styrene-divinylbenzene) resins are useful for nonaqueous size-exclusion chromatography of synthetic polymers.

5.7.1.5.2. Applications of high performance SEC

Hydrophilic polymeric size-exclusion packings are used for the rapid determination of average molecular weight and molecular weight range of polysaccharides. Molecular weight distribution can be determined directly from high performance size-exclusion chromatography.

5.7.1.6. Affinity chromatography

Affinity chromatography is based on the principle that the molecules for being purified can form a selective but reversible interaction with another molecular species that has been immobilized on a chromatographic support.

5.7.1.7. Summary of HPLC

HPLC is a chromatographic technique of great versatility and analytical power. A basic HPLC system consists of a pump, injector, column, detector, and data system. The pump delivers mobile phase through the system. An injector allows sample to be placed into the flowing mobile phase for introduction onto the column. The HPLC column consists of stainless steel or
polymer hardware filled with a separation packing material. Various auxiliary columns, particularly guard columns, may be used prior to the analytical column. Detectors used in HPLC include UV-Vis absorption, fluorescence, RI, electrochemical, as well as coupled analytical systems, such as a mass spectrometer. Detection sensitivity or specificity sometimes can be enhanced by chemical derivatization of the analyte. Computer-controlled data station systems offer data collection and processing capabilities.

The column packing materials may be categorized as silica-based (porous silica, bonded phases, pellicular packings) or polymeric (microporous, macroporous, or pellicular/nonporous). The success of silica-based bonded phases has expanded the applications of normal-phase and reversed-phase modes of separation in HPLC. Separations also are achieved with ion-exchange, size-exclusion, and affinity chromatography. HPLC is widely used for the analysis of small molecules and ions, such as sugars, vitamins, and amino acids, and is applied to the separation and purification of macromolecules, such as proteins and polysaccharides.

5.7.2. Gas chromatography

The first publication on gas chromatography (GC) was in 1952, the first commercial instruments were manufactured in 1956. GC has been used for the determination of fatty acids, triglycerides, cholesterol and other sterols, gases, solvent analysis, water, alcohols, and simple sugars, as well as oligosaccharides, amino acids and peptides, vitamins, pesticides, herbicides, food additives, antioxidants, nitrosamines, polychlorinated biphenyls, drugs, flavour compounds, and many more.

GC is ideally suited for the analysis of thermally stable volatile substances. Substances that do not meet these requirements, sugars, oligosaccharides, amino acids, peptides, and vitamins, are more suited to analysis by a technique such as high-performance liquid chromatography.

5.7.2.1. Sample preparation for gas chromatography

One cannot directly inject a food product into a GC without some sample preparation generally. The high temperatures of the injection port will result in the degradation of nonvolatile constituents and create a number of false GC peaks. One must generally do some types of sample preparation, component isolation, and concentration prior to GC analysis.
Foods may undergo changes during sample storage and preparation. Many foods contain active enzyme systems that will alter the composition of the food product. Inactivation of enzyme systems via high-temperature-short-time thermal processing, sample storage under frozen conditions, drying the sample, or homogenization with alcohol may be is necessary.

Chemical reactions often will result in the formation of volatiles that will give false peaks on the GC again. The sample must be maintained under conditions such that degradation that does not occur.

5.7.2.1.1. Isolation of solutes from food

If one were analyzed the triglyceride bound of fatty acids in a food, would first have to extract the lipids, free fatty acids; mono-, di-, and triglycerides; sterols; fat-soluble vitamins from the food by solvent extraction, then isolate only the triglyceride fraction by adsorption chromatography on silica. The isolated triglycerides then would have to be treated to first hydrolyze of fatty acids from the triglycerides and subsequently to form esters to improve gas chromatographic properties. Two latter steps might be accomplished in one reaction by transesterification.

The analysis of volatiles in foods, alcohols, flavours or off-flavours, may be is a simpler task. These materials may be are isolated by headspace analysis, distillation, preparative chromatography, simple solvent extraction, or some combinations of these basic methods. The used procedure will depend on the food matrix as well as the compounds to be analyzed. The used isolation procedure is critical in determining the obtained results. An improper choice or poor technique at this step negates the best gas chromatographic analysis of the isolated solutes.

Headspace methods

One of the simplest methods of isolating volatile compounds from foods is direct injection of the headspace vapours above a food product. Direct headspace sampling has been used extensively when rapid analysis is necessary and major component analysis is satisfactory. The headspace of the sample is taken by using a gastight syringe and then injected directly into the GC. Only volatiles present in the headspace at concentrations that are greater than $10^{-7}$ g/l headspace would be at detectable levels.
Dynamic headspace sampling or purge and trap has found wide usage in recent years. This concentration method may involve simply passing large volumes of headspace vapours through a cryogenic trap or, alternatively, more complicated extraction and/or adsorption trap. A simple cryogenic trap will collect headspace vapours irrespective of compound polarity and boiling point. Adsorbent traps offer the advantages of providing a water-free volatile isolate (trap material typically has little affinity for water) and are readily automated.

**Distillation methods**

Distillation processes are quite effective at isolating volatile compounds from foods for GC analysis. Product moisture or outside steam is used to heat and codistill the volatiles from a food product.

**Solvent extraction**

Solvent extraction is often the preferred method for the recovery of volatiles from foods. Recovery of volatiles will depend upon solvent choice and the solubility of the solutes being extracted. Solvent extraction typically involves the use of an organic solvent.

**Solid-phase extraction**

A liquid sample is passed through a column filled with chromatographic packing or a Teflon\textsuperscript{R} filter disk, that has the chromatographic packing embedded in it. The chromatographic packing may be is any of a number of different materials. When a sample is passed through the cartridge or filter, solutes that have an affinity for the chromatographic phase will be retained on the phase while those with little or no affinity will pass through. The phase is next rinsed with water, perhaps a weak and then a stronger solvent. The strong eluent is chosen such that it will remove the solutes of interest.

Solid-phase extraction has numerous advantages: less solvent is required, speed, less glassware is needed, better precision and accuracy, minimal solvent evaporation for further analysis, and it is readily automated.
**Direct injection**

Theoretically is possible to analyze some foods by direct injection into a gas chromatograph. One can inject a 2 to 3 μl sample into a GC and the GC has a detection limit of 0.1 ng (0.1 ng/2 μl), one could detect volatiles in the sample at concentrations that are greater than 50 ng/g.

Problems with direct injection that arises due to thermal degradation of any nonvolatile food constituents, damages the GC column, decreases the separation efficiency due to water in the food sample, contaminates the column and injection port by nonvolatile materials, and reduces column efficiency due to slow vaporization of volatiles.

**5.7.2.1.2. Sample derivatization**

The compounds must be thermally stable under the GC conditions. For some compounds (aroma compounds, volatile contaminants), the analyst can simply isolate the components of interest from a food as discussed above and directly inject them into the GC. For compounds that are thermally unstable, too low in volatility, or poor yield chromatographic separation due to polarity, a derivatization step must be included prior to GC analysis.

Some reagents are used for making volatile derivatives of food components for GC analysis: sylil reagents for hydroxy and amino carboxylic acid at sugar, sterol, and amino acid determination; esterifying reagents for carboxylic acids at fatty acid, amines, amino acids, triglyceride, wax esters, phospholipids, cholesterol esters analysis.

**5.7.2.2. Gas chromatographic hardware and columns**

The major parts of a GC are the gas supply system, injection port, oven, column, detector, electronics, and recorder/data handling system. Gas chromatographic hardware and operating conditions should be recorded for all GC separations.

**5.7.2.2.1. Gas supply system**

The gas chromatograph will require a supply of carrier gas, gases for the detector (hydrogen and air for a flame ionization detector, FID). The gases must be of high purity and all regulators, gas lines, and fittings must be of good quality. High-quality pressure regulators must be used to provide a stable and continuous gas supply. The regulators should be stainless steel.
All gas lines must be clean and contain no residual drawing oil. Nitrogen, helium, and hydrogen gases are typically used as the carrier gas to transport the analytes in the GC column. The carrier gas line should have traps (moisture trap, oxygen trap, and hydrocarbon trap) in line to remove any moisture and contaminants from the incoming gas.

### 5.7.2.2.2. Injection port

The injection port serves the purpose of providing a place for sample introduction, its vaporization, and possibly some dilutions and splitting. Liquid samples are always done by syringe injection. The injection port contains a soft septum that provides a gas-tight seal but can be penetrated by a syringe needle for sample introduction. Samples may be introduced into the injection port using a manual syringe technique or an automated sampling system.

#### Sample injection techniques

The sample must be vaporized in the injection port in order to pass through the column for separation. This vaporization can occur quickly by flash evaporation (standard injection ports) or slowly in a gentler manner (temperature-programmed injection port or on-column injection).

### 5.7.2.2.3. Oven

The oven controls the temperature of the column. In GC, one takes advantage of both an interaction of the analyte with the stationary phase and the boiling point for separation of compounds. The injection is often made at a lower oven temperature and is then temperature programmed to some elevated temperature. While analyses may be done isothermally, compound elution time and resolution are extremely dependent upon temperature, so temperature-programmed runs are most common. Higher temperatures will cause the sample to elute faster and oven temperature program rates can range from as little as 0.1 °C/min to the maximum temperature heating rate that the GC can provide. A rate of 2–10 °C/min is most common. The capillary column also can be directly heated with an insulated heating wire based on low thermal mass technology.

### 5.7.2.2.4. Column and stationary phases

The GC column may be is either packed or capillary. Early chromatography was done on packed columns.
Packed columns

The packed column made of stainless steel or glass and may range from 1.6 to 12.7 mm in outer diameter and be 0.5–5.0 m long. It is packed with a granular material consisting of a “liquid” coated on an allegedly inert solid support. The solid support is most often diatomaceous earth, skeletons of algae, that has been purified, possibly chemically modified. The liquid loading is usually applied to the solid support at 1–10% by weight of the solid support. The most common are silicone-based phases, methyl, phenyl, or cyano substituted, and ester based Carbowax. The liquid is of similar polarity to the analytes to be separated. The liquid coatings are somewhat volatile and will be lost from the column at high temperatures. As many as 200 different liquid phases have been developed for GC. The most durable and efficient phases are those based on polysiloxane (−Si–O–Si−).

Capillary columns

The capillary column is a hollow fused silica glass (<100 mg/g impurities) tube ranging in length from 5 to 100 m. The walls of the capillary columns are flexible. The column outer walls are coated with a polyamide material to enhance strength and reduce breakage. Column inner diameters are typically 0.1 mm (microbore), 0.20–0.32 mm (normal capillary), or 0.53 mm (megabore).

The most commonly used capillary columns are now 0.32 mm and 0.25 mm i.d. columns. Smaller diameter columns (0.10 mm and 0.18 mm i.d.) are used for fast GC analysis. The most common lengths of the GC column are 15, 30, and 60 m, although special columns can be over 100 m, but it's good to know that longer columns require longer analysis time, although a longer column gives improved resolution.

Liquid coating is chemically bonded to the glass walls of capillary columns and internally crosslinked to gives phase thicknesses ranging from 0.1 to 5 μm. Thicker films retain compounds longer in the stationary phase, thus the analytes will have longer interaction with the stationary phase to achieve separation.

A thin film (0.25 μm) column is usually used to separate high molecular weight compounds; the analytes will stay in the stationary phase at less time. Thin film columns are frequently used for GC-MS.
Most compounds can be separated by using nonpolar 5% phenyl 95% dimethylpolysiloxane-based columns. This column has a very wide temperature range (−60 °C to 325 °C) and is very stable. To separate very polar compounds, such as alcohols and free fatty acids, a polar column is needed.

A wax-type column has superior separation power; it has a narrow usable temperature range. Cyanopropyl-based columns (CP-Sil 88) are good for trans fatty acid esters. A cyclodex-based column is useful to separate stereoisomers of many flavour compounds.

**Gas-solid (PLOT) chromatography**

It has been applied both to packed and capillary columns. For the capillary column, the porous material is chemically or physically (by deposition) coated on the inner wall of the capillary and the column is called porous-layer open-tubular (PLOT) column. The most popular porous materials are alumina oxide, carbon, molecular sieve, and synthetic polymers. Separations usually involve water or other very volatile compounds such as headspace gas composition (N₂, O₂, CO₂, CO) in packaged food and ethylene during fruit ripening and storage.

### 5.7.2.2.3. Detectors

The most common detectors are the thermal conductivity (TCD), flame ionization (FID), electron capture (ECD), flame photometric (FPD), pulsed flame photometric (PFPD) and photoionization (PID) detectors.

**Thermal conductivity detector**

As the carrier gas passes over a hot filament (tungsten), it cools the filament at a certain rate depending on carrier gas velocity and composition. The temperature of the filament determines its resistance to electrical current. As a compound elutes with the carrier gas, the cooling effects on the filament that is typically less, resulting temperature increase in the filament and an increase in resistance that is monitored by the GC electronics.

**Flame ionization detector**

As compounds elute from the analytical column, they are burned in a hydrogen flame. A potential (often 300 V) is applied across the flame. The flame will carry a current across the potential which is proportional to the organic ions that present in the flame from the burning of
an organic compound. The current flowing across the flame is amplified and recorded. The FID responds to organics on a weight basis. The best response is compounds that contain C–C or C–H bonds. This detector is used for virtually all food analyses which a specific detector is not desired or sample destruction is acceptable. This includes flavour studies, fatty acid analysis, carbohydrate analysis, sterols, contaminants in foods, and antioxidants.

**Electron capture detector**

The ECD contains a radioactive foil coating that emits electrons as it undergoes decay. The electrons are collected on an anode, and the standing current is monitored by instrument electronics. As an analyte elutes from the GC column, it passes between the radioactive foil and the anode. Compounds that capture electrons reduce the standing current and thereby give a measurable response. The ECD has found its greatest use in determining PCBs and pesticide residues.

**Flame photometric detector**

The FPD detector works by burning all analytes eluting from the analytical column and then measuring specific wavelengths of light that are emitted from the flame by using a filter and photometer. The wavelengths of light are characteristic of sulfur (S) and phosphorus (P). This detector gives a greatly enhanced signal for these two elements (several thousand fold for S- or P-containing organic molecules vs. non-S or P-containing organic molecules). The FDP have found major food applications in determination of organophosphorus pesticides and volatile sulphur compounds in general.

**Photoionization detector**

The photoionization detector (PID) uses ultraviolet (UV) irradiation (usually 10.2 eV) to ionize analytes eluting from the analytical column. The ions are accelerated by a polarizing electrode to a collecting electrode. The small current formed is magnified by the electrometer of the GC to provide a measurable signal. The other detectors which are used in GC are electrolytic conductivity detector, and thermionic detector.
Hyphenated gas chromatographic techniques

Hyphenated gas chromatographic techniques combine GC with another major technique. Examples are GC-AED (atomic emission detector), GC-FTIR (Fourier transform infrared), and GC-MS (mass spectrometry). GC-MS has long been known to be a most valuable tool for the identification of volatile compounds. The MS serving as a specific detector for the GC by selectively focusing on ion fragments unique to the analytes of interest. The analyst can detect and quantify components without their gas chromatographic resolution in this manner.

Multidimensional gas chromatography

By simply coupling two GC columns, each of opposite polarity, an overall improvement in separation can be accomplished. This tandem operation of GC columns does not actually represent multidimensionality, but rather resembles the use of a mixed-stationary phase column. True MDGC involves a process known as orthogonal separation in which a sample is first dispersed by one column, and the simplified subsamples are then applied onto another column for further separation.

5.7.2.3. Chromatographic theory

GC may depend on several types (or principles) of chromatography for separation. GC depends adsorption, partition, and/or size exclusion for separation and solute boiling point.

5.7.2.3.1. Separation efficiency

A good separation has narrow-based peaks and ideally, but not essential to quality of data, baseline separation of compounds. Peaks broaden as they pass through the column – they broaden more, poorer is the separation and efficiency.

To achieve the necessary separation in the minimum amount of time, the following factors should be considered: Small diameter columns (packed or capillary) should be used since separation efficiency is strongly dependent on column diameter. Small diameter columns will limit column capacity, limited capacity often can be compensated by increasing phase thickness.

Lower column operating temperatures should be used – if elevated column temperatures are required for the compounds of interest to elute, use a shorter column if resolution is adequate.
One should keep columns as short as possible (analysis time is directly proportional to column length – resolution is proportional to the square root of length).

Use hydrogen as the carrier gas if the detector permits.

Operate the GC at the maximum carrier gas velocity that provides resolution.

**Relationships among column capacity, efficiency, resolution, and analysis speed:** The resolution is the best at small diameter, thin film, nonpolar phase and long column; the analysis is fastest at short column, thin film and small diameter, and the capacity is the best at large diameter, thick film, proper phase related to solutes.

One cannot optimize any given operating conditions and column choices to get one of these properties without compromising another property. Optimizing chromatographic resolution (small bore capillary diameter, thin phase coating, long column lengths, and slow or optimum carrier gas flow rate) will be at the cost of capacity (large bore columns and thick phase coating) and speed (thin film coating, high carrier gas flow velocities, and short columns).

### 5.7.2.4. Applications of GC

#### 5.7.2.4.1. Residual volatiles in packaging materials

Residual volatiles in packaging materials can be a problem both from health and quality standpoints. As the industry has turned from glass to polymeric materials, there have been more problems. GC is most commonly used to determine the residual volatiles in these materials.

#### 5.7.2.4.2. Separation of stereoisomers

GC has found extensive application in the separation of chiral volatile compounds in foods. Chiral separations are accomplished using cyclodextrin-based gas chromatographic columns. Stereoisomers of a given compound will be included in the cyclodextrin cavity of the gas chromatographic column to a lesser or greater extent as they flow through a cyclodextrin capillary column and become separated.
5.7.2.4.3. Headspace analysis of ethylene oxide in spices

Ethylene oxide is a highly volatile compound that has been found in the food industry as a fumigant for spices, suspect as a human carcinogen and thus its residual concentration in spices is of concern.

5.7.2.4.4. Aroma analysis of heated butter

GC has been widely applied to define a volatile chemical fingerprint to characterize the flavour quality of food products. Seven select aroma compounds reported to contribute to the flavour of heated butter, which was measured by GC. Changes in the concentrations of the volatile flavour compounds can be related to changes in the flavour properties of foods. An off-flavour defect in butter developed during storage that is related to an increase in lactone concentration, such as $\delta$-decalactone. The following components can be identified: 2-heptanon, dimethyltrisulfide, butyric acid, 3-methylbutiric acid, $\delta$ hexalactone, $\delta$ octalactone, $\delta$ decalactone.

5.7.2.4.5. Total fat by GC for nutrition labelling

**Principle**

Fat and fatty acids are extracted from food by hydrolytic methods (acidic hydrolysis for most products, alkaline hydrolysis for dairy products, and combination for cheese). Pyrogallic acid is added to minimize oxidative degradation of fatty acids during analysis. Fat is extracted into ether, then methylated to fatty acid methyl esters (FAMEs) by using BF$_3$ (boron trifluoride) in methanol. FAMEs are quantitatively measured by capillary gas chromatography. 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.

**Sample preparation procedures**

Samples are methylated by either a standard or alternative method to form the FAMEs prior to GC analysis. In both methods, the sample is combined with a specified amount of the internal standard. It is subjected to the appropriate fat extraction procedure. In the case of a sample that is extracted by the Soxhlet method, or if the sample is 100% fat, internal standard is added.
directly to the flask. Extracted samples can be stored and refrigerated (5 ± 3 °C), with added hexane, for up to 2 weeks if methylation will not be done immediately. The alternative sample preparation method is efficient for short chain fatty acids (butyric acid) and long chain fatty acids (eicosapentaenoic or docosahexaenoic acids) for products containing microencapsulated fatty acids.

**Chromatographic conditions**

Conditions may be modify as needed to optimize separation. Electronic pressure control of gases that may be are used.

**Calculation**

Calculations can be made by peak height or by integrated area. Fatty acids may be calculated as triglycerides, methyl esters, ethyl esters, or acids.

\[
\% \text{Fatty acid on lipid basis} = \frac{\text{mg/mg of fatty acid}}{x} \times 100 \quad (20)
\]

\[
\% \text{Fatty acid on a sample basis} = \frac{\text{mg/mg of fatty acid}}{x \times \% \text{ lipid}} \quad (21)
\]

**5.7.2.5. Summary of the GC**

GC is exceptionally well suited to the analysis of volatile that are thermally stable compounds. This is due to the outstanding resolving properties of the method and the wide variety of detectors that can provide either sensitivity or selectivity in analysis. Sample preparation generally involves the isolation of solutes from foods, which may be accomplished by headspace analysis, distillation, preparative chromatography (including solid-phase extraction), or extraction (liquid–liquid). Some solutes can then be directly analyzed, while others must be derivatized prior to analysis.

The gas chromatograph consists of a gas supply and regulators (pressure and flow control), injection port, column and column oven, detector, electronics, and a data recording and processing system. Traditional GC has reached the theoretical limits in terms of both resolution and sensitivity. GC as a separation technique has been combined with MS as detection techniques to make GC even more powerful tool.
5.7.3. Specific analysis of mono- and oligosaccharides

5.7.3.1. High-performance liquid chromatography

HPLC is the method of choice for analysis of mono- and oligosaccharides and can be used for analysis of polysaccharides after hydrolysis. HPLC gives both qualitative analysis 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. HPLC requires no prior derivatization of carbohydrates, but does require micronfilter filtration prior to injection. Complex mixtures of mono- and oligosaccharides can be analyzed.

5.7.3.1.1. Stationary phases

Anion-exchange chromatography (AE-HPLC)

Carbohydrates have pKₐ 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, monosaccharides, disaccharides, and higher oligosaccharides. AE-HPLC is most often used in conjunction with electrochemical detection. AE-HPLC has been used to examine the complex oligosaccharide patterns of many food components and products.

HPLC chromatogram of some common monosaccharides, disaccharides, alditols, and the trisaccharide raffinose at equal wt/vol concentrations are separated by anion-exchange chromatography and detected by pulsed amperometric detection. The elution order of the different components are: glycerol, erytritol, L-rhamnose, D-glucitol or sorbitol, mannitol, L-arabinose, D-glucose, D-galactose, lactose, sucrose, raffinose, maltose.

Normal-phase chromatography

The stationary phase is polar and elution is accomplished by employing a mobile phase of increasing polarity. Silica gel that has been derivatized with one or more of several reagents to incorporate amino groups is often used. Acetonitrile–water (50–85% acetonitrile) is effective in carbohydrate separations. The elution order is monosaccharides and sugar alcohols, disaccharides, and higher oligosaccharides.
Cation-exchange chromatography

Sulfonated resins are used for cation-exchange stationary phases. The mobile phase used with these columns is water plus varying amounts of an organic solvent such as acetonitrile and/or methanol. Carbohydrate elution from cation-exchange resins takes place in the order of decreasing molecular weight.

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 or a phenyl group. Reversed phase chromatography has been used for separation of mono-, di-, and trisaccharides by groups. A wide variety of stationary phases is available. Both normal- and reversed phase columns have long lives, good stability over a wide range of solvent compositions and pH values that make them suitable for the separation of carbohydrates range with relatively low cost.

5.7.3.1.2. Detectors

Refractive index detection

The refractive index (RI) detector is commonly employed for carbohydrate analysis. RI measurements are linear over a wide range of carbohydrate concentrations and can be universally applied to all carbohydrates. With RI detection gradient elution cannot be used.

Electrochemical detection

The triplepulsed electrochemical detector, called a pulsed amperometric detector (PAD) is universally used with AE-HPLC. It requires a high pH. Gradient and graded elutions can be used with the PAD. The detector is suitable for both reducing and nonreducing carbohydrates. Limits are approximately 1.5 ng for monosaccharides and 5 ng for di-, tri-, and tetrasaccharides.

Postcolumn derivatization

The purpose of pre- and postcolumn derivatization is to increase detection sensitivity by addition of a substituent whose concentration can be measured by using an ultraviolet (UV) or fluorescence detector.
Precolumn derivatization

Reactions must be stoichiometric. Oligosaccharides are derivatized with aromatic groups and often separated with higher resolution in normal-phase HPLC.

5.7.3.2. Gas chromatography

GC (gas-liquid chromatography, GLC), provides both qualitative and quantitative analysis of carbohydrates. Sugars must be converted into volatile derivatives. The most commonly used derivatives are the alditol peracetates. Conversion of sugars into peracetylated aldononitrile and peracetylated ketooxime derivatives for GC have also been done. A flame ionization detector is the detector of choice for peracetylated carbohydrate derivatives.

5.7.3.2.1. Neutral sugars 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. During acetylation of alditols acetic anhydride and 1-methylimidazole (as a catalyst) are added. At determination of alditol peracetates by GC alditol acetates may be chromatographed isothermally and identified by their retention times relative to that of inositol hexaacetate. Inositol is being added as an internal standard prior to acetylation.

5.7.3.2.2. Hydrolyzates of polysaccharides containing uronic acids

The hydrolyzate is evaporated to dryness. The residue is dissolved in sodium carbonate solution and treated with an excess of sodium borohydride. Excess borohydride is decomposed by addition of glacial acetic acid; borate may be removed by addition and evaporation of methanol. This procedure reduces uronic acids to aldonic acids and aldoses to alditols.

5.7.3.2.3. Preparation and chromatography of trimethylsilyl (TMS) derivatives

The aldonic acids are converted into per-TMS ethers rather than peracetate esters. Trimethylsilylation of free aldonic acids gives derivatives of lactones, while trimethylsilylation of the sodium salt produces the ester.
5.7.3.3. Thin-layer chromatography

Thin-layer chromatography has been used for identification and quantitation of the sugars that present in the molasses from sugar beet and cane processing.

5.7.4. Analysis of vitamins by chromatographic methods

5.7.4.1. Commonly used regulatory methods for vitamin analysis

Vitamin A and precursors can be determined by liquid chromatographic methods with UV-VIS detection at 313, 328, 340, 444 and 445 nm, or fluorometric detection at E<sub>x</sub> λ=325nm, E<sub>m</sub> λ=475 nm. Vitamin D also can be determined by liquid chromatographic methods by UV-VIS detection at 265 nm. The fluorescence detection seems to be the best for vitamin E and K, and there are many liquid chromatographic methods for determination of the water soluble vitamins with fluorencence or UV-VIS detection.

5.7.4.2. Determination of vitamin A by HPLC

Vitamin A is sensitive to ultraviolet (UV) light, air (and any prooxidants), high temperatures, and moisture. Therefore steps must be taken to avoid any adverse changes in this vitamin due to such effects. Steps include using low actinic glassware nitrogen, and/or vacuum, as well as avoiding excessively high temperatures. The addition of an antioxidant 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 first step of saponification determination of the sample with ethanolic KOH, after 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. Critical points of the determination: All works must be performed in subdued artificial light. Care must be taken to avoid oxidation of the retinol throughout the entire procedure. Solvent evaporation should be completed under nitrogen, and hexadecane is added to prevent destruction during and after solvent evaporation. Chromatography parameters: Column: 4.6 mm × 150 mm packed with 3 μm silica, mobile phase: isocratic elution; heptane containing 2-propanol (1–5%, v/v), injection volume: 100 μl, detection: UV, 340 nm, flow rate1–2 ml/min.
Steps of the determination: Inject 100 μl standard working solutions into the HPLC. Inject 100 μl test extract. Measure peak areas for all-trans-retinol and 13-cis-retinol. The exact mobile phase composition and flow rate are determined by system suitability test to give retention times of 4.5 and 5.5 min for 13-cis-retinol and all-trans-retinol, respectively.

Calculation of the results:

\[
\text{All-trans-retinol (ng/ml milk or diluted formula)} = \left( \frac{A_t}{A_{st}} \right) \times W_t \times C_t \times DF \tag{22}
\]

where

- \(A_t\) = peak area, all-trans-retinol in test sample,
- \(A_{st}\) = peak area, all-trans-retinol in standard,
- \(W_t\) = weight, mg, oil solution used to prepare working standard solution,
- \(C_t\) = concentration, ng/ml, all-trans-retinol in oil solution,
- \(DF\) = dilution factor.

\[
\text{13-cis-retinol (ng/ml milk or diluted formula)} = \left( \frac{A_c}{A_{st}} \right) \times W_c \times C_c \times DF \tag{23}
\]

where

- \(A_c\) = peak area, 13-cis-retinol in test sample,
- \(A_{st}\) = peak area, 13-cis-retinol in standard,
- \(W_c\) = weight, mg, oil solution used to prepare working standard solution,
- \(C_c\) = concentration, ng/ml, 13-cis-retinol in oil solution,
- \(DF\) = dilution factor.

5.7.4.3. Determination of vitamin E (tocopherols, tocotrienols) by HPLC

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. Principle of the determination for 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, \(E_x \lambda = 290\) nm, \(E_m \lambda = 330\) nm (\(E_x\), excitation; \(E_m\), emission).

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.

Oils: Oil is dissolved in hexane and injected directly onto the HPLC column.
Critical points of the determination: Vitamin E is subject to oxidation, therefore saponification is completed under reflux, in the presence of the antioxidant, pyrogallol, with the reaction vessel that is protected from light.

5.7.5. Determination of the fatty acid composition of the fats

The lipid can be characterized by measuring the amount of its various fractions, which include fatty acids, mono-, di-, and triacylglycerols, phospholipids, sterols, lipid soluble pigments and vitamins.

Another means of categorizing lipid fractions are the measurement of saturated fatty acids (SF), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and trans isomer fatty acids (TFA). GC is ideal for the analysis of many lipid components, because it can be used for determinations such as total fatty acid composition, distribution and position of fatty acids in lipid, sterols, studies of fat stability and oxidation, assaying heat or irradiation damage to lipids, and detection of adulterants and antioxidants.

GC combined with mass spectrometry (MS) is a powerful tool used in identification of compounds. HPLC also is useful in lipid analyses, especially for components that are not readily volatilized, such as hydroperoxides and triacylglycerols. Thin-layer chromatography (TLC) is still useful, although many assays may be more quantitative or have better resolution using GC or HPLC.

5.7.5.1. Determination of the fatty acid composition as fatty acid methyl esters

The fatty acid composition, or fatty acid profile; of a food product is determined by quantifying the kind and amount of fatty acids that are present, usually by extracting the lipids and analyzing them by using capillary GC. To increase volatility triacylglycerols are typically esterified to form fatty acid methyl esters (FAMEs). Acyl lipids are readily transesterified using base such as sodium hydroxide and methanol. Sodium methoxide that is produced by this combination will create FAMEs from acyl lipids rapidly, but will not react with FFAs. Acidic reagents such as methanolic HCl or boron trifluoride (BF₃) react rapidly with FFAs, but more slowly with acyl lipids.
Procedure: The FAMEs are prepared by combining the extracted lipid with sodium hydroxide-methanol in isooctane and then heating at 100 °C for 5 min. The sample is cooled and then excess BF$_3$-methanol is added with further heating (100 °C for 30 min). After addition of saturated aqueous sodium chloride, additional isooctane and mixing, the upper isooctane solution containing the FAMEs is removed and dried with anhydrous Na$_2$SO$_4$, then diluted to a concentration of 5–10% for injection onto the GC.

Applications: Determination of the fatty acid composition of a product permits calculation of percent saturated fatty acids, percent unsaturated fatty acids, percent monounsaturated fatty acids, percent polyunsaturated fatty acids, conjugated linoleic acids (CLAs), and percent trans isomer fatty acids. During the calculation or normalization, the areas of all of the FAMEs are summed and the percent area of each fatty acid is calculated relatively to the total area.

The separation of FAMEs on GC columns depends on the polarity of the liquid phase. On nonpolar liquid phases FAMEs are separated largely based on their boiling points. This results in the elution order 18:3n3 > 18:3n6 > 18:1n9 > 18:0 > 20:0. On phases of medium polarity the order of elution is changed because of the interaction of the pi electrons of the double bonds with the liquid phase. The order of elution on these columns would be 18:0 > 18:1n9 > 18:2n6 > 18:3n3 > 20:0. When the polarity of the liquid is increased further greater interaction of the double bonds with the very polar liquid phase results an elution pattern 18:0 > 18:1n9 > 18:2n6 > 20:0 > 18:3n3. As the liquid phase polarity increases, the effect of double bonds on retention time increases.

Trans fatty acids interact less effectively with the liquid phase than cis acids for steric reasons, so trans acids will elute before the corresponding cis acid. 18:1Δ9 trans (elaidate) elutes before 18:1Δ9 cis (oleic acid) and 18:2Δ9 trans, Δ12 trans (linolelaidate) elutes before linoleic acid (18:2n6, 18:2Δ9 cis, Δ12 cis). Analysis of FAMEs of a vegetable oil is quite simple and can easily be accomplished in less than 20 min by using a column with a medium polarity liquid phase.

The fatty acids present in most vegetable oils range from C14 to C24. Coconut and palm kernel oils also contain shorter chain fatty acids such as C8–C12. Dairy fats contain butyric acid (C4) and other short chain fatty acids whereas peanut oil contains C26 at around 0.4–0.5% of the total FAMEs.
Trans fatty acids in foods originate from three main sources: biohydrogenation in ruminants, incomplete hydrogenation in the conversion of liquid oils to plastic fats, high-temperature exposure during deodorization. Separation of trans FAMEs is facilitated by selection of the most polar column phases available.

Mono-, di-, and triacylglycerols may be determined by using HPLC and GC. Short nonpolar columns and very high temperatures are needed for analysis of intact triacylglycerols by GC.

**Determination of the fatty acid composition of sunflower oil and lard**

The method is suitable for the determination of fatty acid composition of sunflower oil, lard as well as other fats of plant and animal origin. It can be examined by means of gas chromatographic methods mainly those, which are volatile or can be volatilized until reaching 300 °C. Triacylglycerols do not meet this requirement, and free, long-chain fatty acids are also unsuitable. In the case of the aforementioned, there are synthesized fatty acid methyl esters from the fatty acids released after the hydrolysis of ester bonds of the triacylglycerols, and the resulting compounds are analyzed by gas chromatography. The process of hydrolysis and esterification are shown in the Figure 63.

![Figure 63](image-url)  
**Figure 63 The triglyceride hydrolysis and its esterification process**

During the determination of 0.2 g sunflower oil and lard that are dissolved in 2 cm$^3$ of n-heptane, and for the purpose of dewatering a little calcined sodium sulphate as to be added to it. From the dewatered heptane solution 0.5 cm$^3$ has to be pipetted into a vial, and we add 0.5 cm$^3$ of sodium methoxide reagent, and heat it for one hour at 60 °C, and shake it every 10 minutes. After this, 1 cm$^3$ of n-heptane and 1 cm$^3$ of distilled water are added and shaken for 1-2 minutes. After removing the excess reagent, has to be injected into the gas chromatograph from in the
upper organic phase. The areas under the obtained chromatographic peaks are proportional with
the amount of fatty acid methyl esters (Figure 64). The results are given as a percentage of
weight relative to the fatty acid methyl esters accordingly.

\[
\text{Rel}\% = \frac{T_{\text{fatty acid}}}{\sum T_{\text{fatty acid}}} \cdot 100
\]  

(24)

where

\text{Rel}\% = \text{relative amount of the fatty acid methyl esters}

\text{T}_{\text{fatty acid}} = \text{area under the chromatographic peak of the fatty acid methyl ester}

\Sigma T_{\text{fatty acid}} = \text{the sum of areas under the chromatographic peak of the fatty acid methyl ester}

Difference between the measures of two identical samples is 5% of the result.

Figure 64 The standard chromatogram of saturated and unsaturated fatty acids
Gas chromatographic analysis of conjugated linoleic acids

The physiological effects of conjugated linoleic acid isomers (CLA) are in the scope of interest and as a consequence, there is an increasing need for the quantification of CLA from food. At the beginning of this decade, it has been shown that silver ion liquid chromatography (Ag-HPLC) possesses the best resolving power for the separation of CLA-isomers. Now, when it is coming to the end, one question arises that why Ag-HPLC does not become the dominant analytical technique for CLA analysis. Authors in the previous years still often have used GC alone or Ag-HPLC together with GC. The possible cause in Ag-HPLC may be is potential source of errors that could not be only the variations in factors that are common in LC, but other parameters (e.g., sample size, solvent composition and even storage times) could also hamper obtaining reproducible results. Moreover, the batch-to-batch variation in the silver loadings of the columns is also a problem. On the contrary, the gas chromatographic determination of fatty acid composition is easier to implement and proper identifying tools that are available. The serious disadvantage of this technique is the improper resolution of CLA-isomers. The best gas chromatographic separation of fatty acid methyl esters (FAME) has been achieved with use of 100 m long, 100% cyanopropyl polysiloxane stationary phase columns, and recently fast gas chromatographic methods have been developed in order to achieve the same resolution in much less time.

The main steps of the method are lipid-extraction, transesterification, extraction of fatty acid derivatives and gas chromatographic analysis. Lipid extraction is achieved by using a mixture of hexane/isopropanol, because it has been shown to have more advantages over extraction with chloroform/methanol that are rapid phase separation, less proteolipid contamination and less toxic solvents. CLA and the other fatty acids present in the glycerides are transesterified by an alkali-catalysed reaction with sodium methoxide in order to avoid changes in the ratio of the CLA-isomers.

General scheme of sample preparation and analysis. Lipids are extracted with a mixture of n-hexane/i-propanol 3:2 (v/v)[10]. 80 mL n-hexane/i-propanol mixture (HIP) was added to approx. 10 g meat sample (max. 0.3 g fat); then a suspension is prepared with an Ultra-Turrax T25 basic type dispersion tool (IKA WERKE GMBH, Germany). Solid particles are removed with vacuum filtration. When filtration had been completed, the filter funnel is rinsed three times with 10 mL HIP. Liquid phase is clarified from the soluble non-lipid fraction: it is extracted with 60 mL 0.47 M sodium sulphate in water. The organic phase is separated, dried
under water-free Na_2SO_4 and the solvent is then removed under vacuum in a rotary evaporator. The crude lipid extract is dissolved in 10 mL of n-hexane.

Transesterification of glycerides is carried out with sodium methylate in methanol: 0.5 mL lipid extract in n-hexane and 0.5 ml sodium methoxide solution (0.5 M) is mixed and warmed at 50 °C for 30 min. When the reaction had been completed, 1 mL of distilled water is added and FAME is extracted four times with 1 mL hexane and diluted up to the final volume of 5 mL. All solvents and reagents are of analytical grade, the '37-component FAME mix' was obtained from Supelco, while 'conjugated linoleic acid mixture' was purchased from Sigma.

The separation of FAME was accomplished with a Chrompack CP 9000 gas chromatograph. The injection was manual, the split ratio was 16:1 and the injected amount was 6 μL at 270°C. The column was a CP-Sil 88 (FAME) with a dimension of 100 m x 0.25 mm and the film thickness of the stationary phase was 0.2 μm. The final temperature programme: the temperature of the column was immediately increased from 130°C to 225 °C at a rate of 2 °C min^{-1}. At 225 °C, isotherm conditions were applied for 20 min; the carrier gas was He (230 kPa, 16.1 cm/s). The temperature of FID was 270 °C.

The initial temperature programme that was developed for other FAMEs was changed in order to optimize the resolution of CLA-isomers. The shifts in retention times were observed with use of the '37-component FAME mix' and also with 'conjugated linoleic acid mixture'.

**Checking the reliability of the sample preparation steps.** Meat samples were extracted with HIP and the fat contents were determined. These values were compared with the crude fat contents obtained with the Soxhlet method, which is applied for the determination of the fat content of meat. In case of fat determination with HIP-method, sample preparation was carried out as described above, until the evaporation of the solvent from the clarified HIP solution. The residuum was kept at 98 °C for 2 hours in a drying chamber, let to cool down in a desiccator, then the extract was weighed. The drying was repeated until the weight was constant.

The precision of transesterification and FAME extraction with hexane was determined with the parallel methylation of the same HIP-extract following extraction with hexane. The accuracy was determined with the transesterification of known amount of glycerides. The ideal model solution would be an ‘artificial beef fat’ with similar fatty acid pattern as in beef, or at least CLA glycerol esters with a similar ratio as in beefs fat. Due to purchasing difficulties, the efficiency of the above processes were determined with use of myristic acid glycerol ester
(MGE). The methyl ester of myristic acid (MME) appears at the beginning of the chromatogram and its peak area ratio within the sum of the FAMEs in beef is approx. 2%. During the determination of efficiency of transesterification and extraction, 0.150 g MGE was dissolved in 5 mL n-hexane and 0.5 mL from this solution was used for transesterification with 0.5 mL 0.5 M sodium methylate in methanol following the same method as in the case of the meat samples. The amount of the resulting MME derivatives was determined with external standard calibration.

Besides the examination of particular parts of the sample preparation, the reliability of the whole procedure was also evaluated. The MGE stock solution contained 199.2 mg MGE in 100 mL HIP and 2.5 mL of this solution was added to the initial HIP solution, then extracted, clarified, transesterified, and the resulting MME was extracted and measured. The recovery of MGE was determined in form of MME.

During the examination of specificity of the analytical method, it was observed – as in the case of the other studies – by using of the above means the separation of the CLA-isomers cannot be achieved perfectly, though the CLA-isomers proved to be separable from the other fatty acids that are present in the beef in significant quantities related to CLA. The most abundant isomer, c9,t11-CLA-ME, coelutes with t8,c10-CLA-ME, while the separation of c11,t13-CLA-ME and t10,c12-CLA-ME is adequate; then, the minor c,c- and t,t-isomers come after.

The resolution could be improved but the cycle time would be too long, more hours. In the chromatogram of the mixture of the two test solutions, it can be seen that heneicosanoic acid methyl ester (C21:0-ME) eluates between the first and the second CLA-ME peaks. In the beef samples, only the signal of the first CLA peak (c9,t11-CLA-ME/t8,c10-CLA-ME) was big enough for quantification, although more minor isomers were present, but their signal was about or less than the limitation of detection.

5.7.6. Determination of volatile acids (volatile fatty acids) by gas chromatography

Volatile acids are called the mono carboxylic acids containing 2-6 carbon atoms (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, caproic acid and iso-caproic acid). These “volatile acids” can be easily volatilized by heating their aqueous solution, thus they distill off. Due to their volatility their smell is intense too; since acetic acid is even
pleasant at low concentrations but rest of it is unpleasant. From the practical aspect, the determination of volatile acidity of fermented foods have high significance. During the preservation by means of fermentation, the sugar content is converted into lactic acid due to anaerobic degradation. During the fermentation process, acetic acid, propionic acid, and butyric acid because of protein degradation, volatile fatty acids with higher molecular weight may occur in food. It is desirable that the concentration of lactic acid in the diets prepared by fermentation should reach 1-1.5%; the presence of propionic acid is not harmful, and even contributes to food preservation. The presence of the butyric acid in itself is not harmful, but reduces the organoleptic properties, and refers to poorly executed fermentation. Iso-butyric acid, and volatile fatty acids with higher molecular weight appear as the result of amino acid degradation processes.

The amount of volatile fatty acids was determined by fractional distillation in the second part of the last century. However, recently gas chromatography is used more and more for this purpose. In the first method the various acids were distilled off from their aqueous solution, retained separately according to their boiling point, and quantified by alkaline titration. During the distillation, various acids did not separate perfectly, so this method was only used for the determination of acetic acid, propionic acid and butyric acid, the rest of the volatile acids was ignored.

Nowadays, the determination of volatile fatty acids is carried out by gas chromatography. As a first step 100 g of food preserved by fermentation that is measured into a 1000 cm$^3$ volumetric flask, poured into it 900 cm$^3$ of distilled water and allowed to stand for a day, while shaking it periodically. 4 cm$^3$ of the filtrate is measured into a centrifuge tube, and 0.2 cm$^3$ of phosphoric acid is added. The mixture is centrifuged at 8000 g for 10 minutes. The supernatant is decanted, and 1 µl of it is injected into the gas chromatograph. The separation of the volatile fatty acids occurs at 120 °C, in a 2 m long, filled column, where the stationary phase is silicone oil that is applied on diatomaceous earth.

Before each analysis, a standard solution is injected daily, which contains all of the components to be determined at a known concentration. The retention times and the factors require calculation of the concentration that is determined. After completion of the verifier chromatogram, a small part of the aqueous extract is injected, by using the integrator or the computer the acids that are present can be determined, and their concentrations are calculated.
by comparing their calibration chromatogram. The Figure 65 on the left side shows a standard chromatogram on the chromatogram of the volatile fatty acids of a fermented food.

![Figure 65](image)

**Figure 65 The determination of volatile fatty acids by gas chromatography (1 = acetic acid, 2 = propionic acid, 3 = isobutyric acid, 4 = butyric acid, 5 = isovaleric acid, 6 = valeric acid, 7 = kapronic acid, 8 = isocapronic acid, 9 = heptanoic acid)**

**5.7.7. Determination of antioxidants**

Butyl hydroxy toluene (BHT) can be transformed easily into its gas state because of its low boiling point, and therefore it can be determined by gas chromatography. During the procedure there is prepared solution from the antioxidant-containing material, in particular fat, margarine, oil, which is directly injected into the gas chromatograph. The separation by gas chromatography takes place at a relatively high temperature (200-300 °C), where both the solution and the solute convert into the gas state, and can be separated on an appropriately chosen gas chromatography column.

During the procedure, 100 mg of the examined lard, margarine, or cooking oil is dissolved in 10 cm$^3$ of hexane, and 1 µl from the resulting solution is injected into the gas chromatograph. The temperature of the gas chromatograph injector is 250 °C, the carrier gas is helium, the pressure in the injector is 180 kPa, the length of the column is 10 m length, and its 0.25 mm diameter quartz capillary that is filled with silicone oil packing, the temperature of the column
area is 220 °C, ITD-800 and mass spectrometer is used as a detector, set to a temperature mode of 220 °C. After identification of the peak of the BHT, the result is calculated by considering the fact, that the chromatographic peak obtained during the measurement which is proportional for amount of BHT present. BHT content is given in mg/kg according to the following formula:

\[ BHT = \frac{T_{\text{sample}}}{T_{\text{standard}}} \cdot 100 \]  

(25)

where

- \( T_{\text{sample}} \) = the area of the BHT peak from the sample
- \( T_{\text{standard}} \) = the area of the BHT peak from the standard

The maximum deviation between the two parallel measurements can be only 5% of the result.

Various antioxidants can be separated and determined simultaneously when the appropriate chromatographic conditions are adhered. The separations that are shown on the Figure 66 are determinations of various antioxidants. The first peak in the chromatogram is butyl hydroxy anisole, and the second peak represents the amount of butyl hydroxy toluene, and the last one is propyl gallate. The peaks 3-6 belong to various antioxidants.

The conditions of the gas chromatographic separations are the followings (from the 200 µg/cm\(^3\) concentration of each antioxidant 2 µl was injected into the gas chromatograph):

- 30 m x 0.25 mm SAC-5 column,
- temperature 200 °C,
- 30 cm/s flow rate of helium gas,
- FID on 200 °C.
Modern methods of food analysis

5.7.8. Determination of the amino acid content by gas chromatography

The "traditional" method of amino acid analysis is the separation that is carried out on the ion-exchange column chromatography (IEC), followed by post-column ninhydrin derivatization and spectrophotometric detection. Although this technique is widely spread and reliable, it also has disadvantageous properties. The analysis costs are relatively high, the sensitivity is limited, the analysis duration is long, and the IEC device is not very flexible: only a few types of analysis can be performed with it. To eliminate these drawbacks, there has been experimented with the amino acid analysis carried out by gas chromatography (GC) already at the end of the sixties century. Due to the possibilities of gas chromatographic technology, using capillary GC with excellent resolutions can be achieved, and by selecting the appropriate detectors high sensitivity can also be provided. The ninhydrin derivatives can be detect in quantities above 10 ng, and o-phtaldialdehyde-derivatives can be detected by fluorescence detector in quantities above 1 ng after the separation by HPLC. **The gas chromatography derivatives can be detected in quantities of 10-100 pg** by electron capturing detector, and this sensitivity can be further improved by attaching mass spectrometer (MS).

The gas chromatographic analysis of amino acids is only feasible, if prior to the analysis there are such formed derivatives from the amino acids, which are volatile and less polar than the
original amino acids. Therefore, in addition to the optimized GC system parameters, appropriate derivatization procedures are also necessary for the gas-chromatographic analysis of the amino acids. In which case can the ion exchange chromatography be used as an alternative to the gas chromatographic technique? Generally speaking, those derivatization techniques should be used, by the advantages of the GC analysis that can be exploited, while the results are in good agreement with the data obtained from the analysis carried out by IEC.

In addition to examining the total amino acid content, the volatile derivatives of amino acid enantiomers can also be determined by gas chromatography. In the latter case, there are two possibilities for the realization of the chiral selection: the use of chiral stationary phase column, or by the creation of diastereoisomers from amino acid enantiomers by using chiral reagents, which is separated from one another on a non-chiral column. As the mobile phase cannot be manipulated in this case, the third option generally is used in liquid chromatography - using a chiral mobile phase - is not an option for this time.

There should be used such a derivatization method, through by using of a given reagent at the same reaction conditions, the complete conversion of each amino acid can be achieved simultaneously, and there will be generated only one product from each amino acid. This task could not be fully achieved in all cases yet. **During the generation of achiral derivatives** the polar groups of amino acids should be converted in less polar groups, so that they become more volatile, and get less prone to chemical reactions. One possibility is the *carboxyl group is esterified with a short carbon chain alcohol, and the amino group is acylated with halogenated acid anhydrides*. By means of entering the halogen atoms, there will be possibility to use the electron capture detector (ECD), which means high increase in sensitivity. Another option is blocking a single step, of the active groups by one reagent, forming trimethylsilyl derivatives.

Overall, has been gathered much experience in the biological analysis of samples using TFA (N-(O, S)–trifluoroacetyl (N TFA) -n-butyl ester) and HFB (N heptafluorobutiril (N-HFB) isobutyl ester) derivatives, but the implementation of these derivatization procedures is often difficult, because of the need of a dry medium and high temperature that involves the transformation of amides (asparagine, glutamine), and the slight solubility of some amino acids in alcohol that can cause also a problem. The trimethylsilylated amino acids form the other major classes of achiral derivatives. The generation of simple silylated derivatives, such as *trimethylsilyl and N(O)-t-butyldimethylsilyl* (terc-BDMS) takes place in one step, but it
needs a water-free medium, high temperature and a long time. In the case of some amino acids, more derivatives can also be formed, therefore the chromatogram can also contain more peaks, and some molecules with high-boiling point which can partially decompose during the high temperatures of the CG analysis. In contrast, the iso-BOC ((O,S)-izobutiloxikarbonil methyl ester) derivatives are considered to be promising, as their synthesis requires no water-free medium or high temperature or a long generation time.

After the generation of chiral derivatives, there is a possibility to separate the amino acid enantiomers in the form of diastereoisomers. During this process either carboxyl group is reacted with a chiral amine or alcohol, or the amino group is acylated with a chiral acid. The formation of \( \text{N(O,S)}\text{-TFA(+)-2-butyl esters and the (+)-3-methyl-2-butyl esters} \) belongs to the first group, and the derivative formation with \( \text{N-TFA-L-prolyl chloride, N-α-chloro isovaleryl and N-α-chloro propionyl} \) reagents belongs to the second group. The volatility of diastereoisomers that are generated by reaction of carboxyl group of amino acids is better than those of the molecules formed by acylating the amino group, as the size of the entered chiral groups is smaller. The existing most volatile derivatives can be formed by the coupling of the 2-butyl group.

The separation of amino acid enantiomers is also possible without chiral derivatization, if there is available corresponding column which contains the appropriate, optically active compound. During the direct separation of enantiomers there are no chiral reagents required, thus not sufficient optical purity and the racemisation which may occur during the derivatization reaction, does not cause a problem. The main purpose of developing chiral stationary phases are creation of such phases, which provides appropriate enantiomer selectivity for all the amino acids, and can also be used at the high temperatures of the gas chromatographic process as well. The high enantiomeric selective \( \text{L-valine-t-butylamide group} \) was linked to \text{carboxy-silicone}, which forms a stationary phase (Chirasil-Val) usable in the temperature range of 30 to 250 °C, allowing the elution of all the amino acid isomers in half an hour.

5.7.9. Determination of cholesterol and phytosterols

During the first step of determination the lipid is extracted from the food and saponified. Next, acyl lipids being converted to water-soluble FFA salts. Other components (the unsaponifiable or nonsaponifiable matter) do not change in solubility after hydrolysis, they remain soluble in organic solvents. Cholesterol (in the unsaponifiable fraction) is extracted and derivatized to
form trimethylsilyl (TMS) ethers or acetate esters. This increases their volatility and reduces problems of peak tailing during chromatography. Quantitation is achieved by using capillary GC. GC quantitation of cholesterol is recommended since many spectrophotometric methods are not specific for cholesterol. Samples such as eggs have had their cholesterol contents that are overestimated by relying on less specific colorimetric procedures.

5.7.10. Separation of lipid fractions by TLC

TLC is performed by using silica gel G as the adsorbent and hexane – diethyl ether – formic acid (80:20:2 vol/vol/vol) as the eluting solvent system. Plates are sprayed with 2’,7’-dichlorofluorescein in methanol and placed under ultraviolet light to view yellow bands against a dark background. This procedure permits rapid analysis of the presence of the various lipid fractions in a food lipid extract.

5.7.11. Protein separation and determination by chromatographic methods

Before starting a separation is necessary to learn about the biochemical properties of a protein, molecular mass, isoelectric point (pI), solubility properties, and denaturation temperature. The first separation step is often a technique that utilizes the differential solubility properties of a protein. The most common methods include precipitation, ion-exchange chromatography, affinity chromatography, and size-exclusion chromatography.

5.7.11.1. Separation by adsorption

Adsorption chromatography: the separation of compounds by adsorption to, or desorption from, the surface of a solid support by an eluting solvent.

5.7.11.2. Ion-exchange chromatography

It is defined as the reversible adsorption between charged molecules and ions in solution and a charged solid support matrix. A positively charged matrix is called an anion exchanger, it binds negatively charged ions or molecules in solution. A negatively charged matrix is called a cation exchanger, it binds positively charged ions or molecules. The protein of interest is first adsorbed to the ion exchanger and buffer conditions maximize the affinity of the protein for the matrix. Contaminating proteins pass through the exchanger unabsorbed. Proteins that are bound to the exchangers are selectively eluted from the column by gradually changing the ionic strength or
pH of the eluting solution. As the composition of the eluting buffer changes, the charges of the proteins change and their affinity for the ion-exchange matrix decreased.

**5.7.11.3. Affinity chromatography**

Affinity chromatography is a type of adsorption chromatography, in the course of a protein that is separated in a chromatographic matrix containing a ligand covalently bound to a solid support. Ligands include enzyme inhibitors, enzyme substrates, coenzymes, antibodies, and certain dyes. The protein is passed through a column, in doing so the contaminating proteins and molecules that do not bind the ligand are eluted. The bound protein is then desorbed or eluted from the column under conditions that decrease the affinity of the protein for the bound ligand, by changing the pH, temperature, or concentration of salt or ligand in the eluting buffer.

**5.7.11.4. High-performance liquid chromatography**

The use of high performance liquid chromatography to separate proteins was made possible by development of macroporous, microparticulate packing materials that withstand high pressures.

**5.7.11.4.1. Separation by size**

Protein molecular masses range from about 10,000 to over 1,000,000; size is a parameter to exploit for separations. Actual separation occurs based on the Stokes radius of the protein, not on the molecular mass. Stokes radius is the average radius of the protein in solution and is determined by protein conformation.

**5.7.11.4.2. Size-exclusion chromatography**

Also known as gel filtration or gel permeation chromatography. It can be used to separate proteins on the basis of size. A protein solution is allowed to flow down a column packed with a solid support of porous beads that are made of a cross-linked polymeric material. Beads of different average pore sizes that allow for efficient fractionation of proteins of different molecular masses are commercially available. Size exclusion chromatography is used to remove salts, change buffers, fractionate proteins, and estimate protein molecular mass.
5.7.12. Separation and determination of the amino acids by ion exchange column chromatography by applying postcolumn derivatization

5.7.12.1. Introduction

Amino acids have several biological functions and they are building up peptides and proteins. The amino acid content of different materials can be determined by paper-, thin layer-, gas-, liquid- and ion exchange chromatography. The methods of amino acid determination were continuously developed from the classical methods of chemical analysis to ion exchange column chromatography (IEC) or high performance liquid chromatography (HPLC) or gas chromatography (GC). In opinion of author of this part of the book, all of the three methods are very good and suitable for precise and fast determination of the amino acids, but the most widespread and most accurate is the IEC.

Moore and Stein devoted plenty of time to separation and very precise determination of amino acids in the middle of the 20th century. In 1958, together with Spackman they published the description of the automatic amino acid analyser for quantitative and qualitative determination of amino acid content of the protein based on IEC after postcolumn derivatization with ninhydrine. For this work in 1972 they have been awarded the Nobel Prize. After they published their method, many researchers tried to improve it, so a lot of improvements were elaborated, but the principles of the method were unchanged. Most of the amino acid analysers operates by the traditional principle of Moore and Stein and use ninhydrine or some different postcolumn derivatization methods.

During recent time HPLC has become very popular in the field of amino acid analyses, but the determination of the amino acids by means of HPLC brought a number of problems in comparison with the classical Moore and Stein method. These problems explain the small number of HPLC methods in the practice. For HPLC analysis of amino acids perfectly clean samples are required, otherwise the impurities of the sample destroy the prewash or analytical columns, or the derivatization of the amino acids is not successful. The IEC method is not so sensitive for the impurities of the sample, and there is no need for precolumn derivatization of the amino acids. By using of IEC method all of the process of amino acid determination is fully automatic, so IEC surpass HPLC method both in reliability and accuracy of determination.
Since the original improvements in the technique were published by several researchers, but these meant no fundamental changes. They try to improve the sample preparation method, the hydrolysis of the protein, the determination of the sensitive amino acids (methionine, cystine, tryptophan) by different protein hydrolysis methods, and mark the trend to faster analysis and higher sensitivity. The original two column systems were described by Moore and Stein that had been used for a long time, but after that the single column system has been spread. An accelerated single column lithium buffer system was elaborated for determination of the ninhydrine positive compounds of biological fluids, and some authors investigated the different postcolumn derivatization method with different agents for improving the sensitivity of the determination. This chapter deals with the determination of the amino acids by IEC.

5.7.12.2. Sample preparation

The most correct separation of the samples is the base of the accurate and repeatable analysis of amino acids by automatic IEC. Before the preparation of the samples the protein content or the approximate content of amino acids should be known for the selection of the optimum weighing of the original sample. The sample has to be as pure as possible, because some of the constituents of the sample can assist to destroy the sensitive amino acids. The volume of the sample which can be applied to the ion exchange column vary for the different instruments. With refinements in instrumentation the tendency has been pointing towards a decrease of the sample volume to 50 µl or less. The preparation of the sample can be divided into two parts dependent on the purpose of investigation: releasing the amino acids from protein and peptides by means of hydrolysis, and preparation of samples containing free amino acids when the protein and other disturbing substances are removed.

5.7.12.2.1. Hydrolysis of the protein

Hydrolysis of the protein means cleavage of the peptide bonds of a polypeptide or a protein. The total acid hydrolysis has extreme conditions during this reaction, therefore some amino acids are partially or totally destroyed. Most of the destruction can be avoided, if oxygen is completely removed before the hydrolysis tubes that are sealed. The break down of amino acids is influenced by the type of proteins, the presence of carbohydrate, minerals or fats and the purity of the hydrochloric acid. Furthermore the resistance of peptide links between the amino acids against the hydrolysis differs depending of the type of amino acids and the structure of the protein. Some linkage between the longer chain aliphatic amino acids may not be cleaved.
completely. For very accurate determination of amino acid content different hydrolysis times have to be applied and hydrolysis curves have to be made for each sample. After that the recovery that is determined by amino acid analyser (AAA) is plotted against different times of hydrolysis and the extrapolated maximum recovery is estimated from the diagram. There are easily hydrolysed amino acids which are not destroyed (glycine, proline), some of them are partially decomposed during the hydrolysis (threonine, serine, tyrosine, phenylalanine), and the third group contains amino acids that are the most difficult to release from a peptide bond (isoleucine, leucine, valine).

The tryptophan is very sensitive to acid hydrolysis and almost totally decomposes during the condition of hydrolysis. Alkaline hydrolysis does not affect the tryptophan in the same extent, but some part of the other amino acids can be destroyed during the alkaline hydrolysis. The loss of threonine and serine during the acidic hydrolysis is reproducible, but varies from protein to protein. Some parts of two aromatic amino acids, tyrosine and phenylalanine, decomposes during the hydrolysis particularly if oxygen is at present. An entirely different problem is the cleavage of the peptide bonds between isoleucine–leucine, isoleucine–valine and valine–valine, and even extended times of hydrolysis may not split these bonds completely.

**Acidic hydrolysis.** The most popular method of hydrolysis is by 6 M hydrochloric acid (HCl) which can be obtained by diluting the 37% hydrochloric acid in the ratio 1:1 with distilled water, or by distillation of azeotropic mixture of HCl with water, 1:1, in a glass rotation evaporator with the addition of a small amount of SnCl₂. To avoid the losses of methionine small amount of mercaptoethanol or to prevent destruction of aromatic amino acids 0.1% of phenol can be added to 6 M HCl. Before the hydrolysis the test tubes or special hydrolysis vessels have to be rinsed with a chromium sulphur mixture, after distilled water and 1 M HCl. HCl residue has to be removed by drying at a temperature of 100 °C. The test tubes have to be kept in closed polyethylene bags to avoid the deposition of ammonium-chloride from the air of the laboratory.

One mg of protein contains 0.3–1 μmol amino acids, therefore different sample sizes have to measured into the test tubes to receive the optimum amino acid concentration for determination by IEC. After measuring the sample, pipette 6 M HCl is two-hundred times higher surplus into the tube. It is advisable to bubble analytical grade nitrogen into the bottle containing the hydrolysing agent to removal the trace of oxygen. (For making the sample liquid, add 12 M HCl in the volume of the sample, and supply with 6 M HCl to the final volume.) Narrow the top of the test tubes about 2 mm, then freeze the solution in a freezing bath, and evacuate the
sample through a valve. Allow the sample to thaw, the vacuum valve should be closed, and allow nitrogen to the test tubes to saturate the sample solution with oxygen free gas. Repeat these steps for three times to remove all of the oxygen from the sample. After the final evacuation close and fuse the test tubes at its neck, and insert the test tubes into the hydrolysing block or into a thermostat and leave it for 24 hours at the temperature of 110±1 °C. It is advisable to prepare two other samples in the same way which will be hydrolysed for 48 and 72 hours. After the hydrolysis cool the test tubes down, and evaporate the HCl in a vacuum rotary evaporator. The test tubes should be opened just before the evaporation, therefore the samples have to be stored in a deep freezer. It is necessary to dissolve the residue of the sample in a small amount of distilled water, the pH has to be set to the value 6.5 by means of a phosphate buffer, and has to be leave for four hours at room temperature if the cysteine wants to be oxidised to cystine. After the oxidation pH of the samples has to be set by 1 M HCl and after that by citrate buffer of the pH value 2.2. If the cysteine oxidation is not necessary, dissolve the evaporation residue directly in this buffer. After filtration and dilution of the amino acid solution the sample is ready for analysis of amino acids by IEC.

Some errors can occur during the hydrolysis. The presence of heavy metals, the losses of threonine, serine and sulphur that containing amino acids increase. In the case of threonine and serine the losses are 3–15% during 24 hours of hydrolysis and the serine is less stable than the threonine in most cases. The imperfect evacuation of the sample or the presence of oxygen will cause losses of the sulphur containing amino acids by oxidation and tyrosine by chlorination. The tyrosine losses is presented in the literature between 1 and 14%. More accurate results can be obtained if the sample is oxidised by performic acid before hydrolysis, and the oxidised forms of the two sulphur containing amino acids are determined. The presence of sulphate ion supports the losses of threonine and serine as well as glutamic acid and aspartic acid by means of esterification. The other considered amino acids are stable during 24 hours hydrolysis, or their losses are negligible.

For obtaining some data concerning the real content of non-stable amino acids from the results of hydrolysis experiments some methods were elaborated. One of the methods presupposes a reaction kinetics of the first order, carrying out of the two hydrolysis at the time \( t_1 = 24 \) hours, and \( t_2 = 72 \) hours, and the computation of the original area \( A_0 \) is:

\[
\log A_0 = \frac{t_2}{t_2 - t_1} \log A_1 - \frac{t_1}{t_2 - t_1} \log A_2
\]  

(26)
Where $A_1$ and $A_2$ are the computed areas for amino acid A at the times $t_1$ and $t_2$. The correction on the basis of graphic interpolation or by means of the method of nonlinear least squares require 4–5 periods of hydrolysis.

### 5.7.12.2. Performic acid oxidation before hydrolysis

Preparation of the performic acid: one part of 30% hydrogen peroxide is mixed with 9 parts of 88% formic acid, the mixture stands for the time of 30 minutes at room temperature, and cooled down to 0 °C. Then 1 cm$^3$ of performic acid is added to 0.04–0.08 mg of cystine, which is equal about of 2 mg protein, and is left standing for 4 hours at 0 °C. The samples, which will not be dissolved during this time, should be left at 0 °C for overnight. Then the oxidation mixture is evaporated by rotary vacuum evaporator at 40 °C up to a syrup consistence. Complete evaporation of the mixture can cause damage in the cystine content. After this, two hundred times higher surplus 6 M HCl solution is added to the residue, and the hydrolysis of the protein is subjected as described earlier. The oxidised hydrolysate will be used only for determination of the sulphur containing amino acids, even though the other amino acids, except for tyrosine and tryptophan, are stable against oxidation. The yield of cysteic acid content can be slightly increased by means of reduction of the plus performic acid with help of HBr and avoiding over oxidation of the sample. It is not necessary to evaporate the whole sample, but sufficient to dry an aliquot part of the sample, which accelerate the sample preparation.

### 5.7.12.2.3. Hydrolysis methods for the determination of tryptophan

In the case of pure proteins it is possible to prevent the Trp from its decomposition during the acid hydrolysis by adding mercaptide compounds, or using aril- or alkyl sulphonic acid as hydrolysis agents. In sugar that is containing samples, basic hydrolysis is used. In the practice 4 M sodium hydroxide or 4 M barium hydroxide is used for protein hydrolysis to determine the Trp. Barium hydroxide hydrolysis yields are the highest Trp recovery, but removing of barium hydroxide from the mixture is quite difficult, and some parts of the Trp can absorb at the surface of the precipitated barium sulphate or carbonate, and it can be a source of further losses.

During the acidic hydrolysis 4% of thioglycollic acid is added to 6 M HCl, and proceed at the same way as in the case of a standard acid hydrolysis. If the sample does not contain sugar, the Trp yield is over 90%. Cysteine that is arising during the hydrolysis interferes in the determination of proline. Thioglicollic acid provides two peaks on the chromatogram, the first
is at the area between cysteic acid and aspartic acid, the second one is at the place of carboximethylcystein. 3 M p-toluenesulphonic acid instead of 6 M HCl was applied for protein hydrolysis, but this method cannot be used if the carbohydrate content exceeds 50%. The recovery of the other amino acids are as good or better than the generally used acidic hydrolysis. 3 M mercapto ethane sulphonic acid was used for the mild acid hydrolysis of peptides and proteins in order to increase the precision of determination of tryptophan.

During the basic hydrolysis the same hydrolysis tubes are used at acidic hydrolysis. Into the sample containing 1–5 mg protein 0.5 cm$^3$ of 4 M sodium hydroxide solution is added freshly prepared from 50% NaOH. Narrow the top of the test tubes about 2 mm, then freeze the solution in a freezing bath, and evacuate the sample through a valve. Allow the sample to thaw, the vacuum valve should be closed, and allow nitrogen to the test tubes to saturate the sample solution with oxygen free gas. Repeat these steps for three times to remove all of the oxygen from the sample. After the final evacuation close and fuse in the test tubes at its neck, and insert the test tubes into the hydrolysing block or into a thermostat and leave it for 48 hours at the temperature of 110±1 °C. After cooling add 0.5 cm$^3$ of sodium citrate buffer (pH=4.25) to the sample and mix thoroughly. Then the solution is transferred with the buffer to the 5 cm$^3$ measuring vessel containing 420 μl 6 M HCl placed in the dry ice, then fill the content of the flask up. Any possible turbidity will be removed by centrifuging for the time of 30 min at 40 000 g. The clear solution is ready for Trp determination by IEC.

The yields of Trp reach 98–100% on the condition of a sufficient time of hydrolysis. At most of the proteins 24–48 hours is sufficient to cleavage of peptide bonds between Trp and the other amino acids, but the link between Val and Trp or Ile Trp requires 98 hours hydrolysis at 110 °C, or 48 hours hydrolysis at 135 °C. The alkaline hydrolysis method is not used for other amino acids, because some of them significantly changes during the hydrolysis. Arginine is partially transformed to ornithine, cystine is transformed to lanthionine, cystein and serine decomposes to dehydroalanine and react with lysine arise lysinoalanine.

Hydrolysis of foodstuffs and fodders is the most difficult because these materials are heterogeneous, therefore usually necessary to work with large weighing amount and the evacuation of the sample is often omitted and replaced by a nitrogen atmosphere. These samples can be hydrolysed under reflux in stream of nitrogen for 24 hours, or special ampoules can be employed with great volume. The hydrolysing agent in both cases is 6 M HCl, and the volume
is 150–250 cm³. Usually not the whole sample is evaporated but an aliquot, which contents approximately 1–10 mg of nitrogen is optimal for determination of the amino acids by IEC.

5.7.12.2.4. Recent developments in the hydrolysis of the proteins

Hydrolysis of the protein is the most limiting factor in determination of amino acids, because some of them are destroyed during the hydrolysis, and some converted to other amino acids or to ninhydrine positive compounds. The standard hydrolysis method use 6 M HCl at 110 °C, for 24 hours to hydrolyse the protein. This method is widely used for protein hydrolysis, but there is no method to give optimum values for all amino acids. Most of the methods give satisfactory results in the case of pure proteins, but to determine the amino acid content of feeds, digesta and faecal samples is the most difficult because of the fats, saccharides and minerals that are present in great concentration.

Methionine and cysteine undergo partial oxidation during the protein hydrolysis, therefore controlled oxidation with performic acid before the acidic hydrolysis has to be carried out. Some of the authors used phenol as a scavenger, and others 3,3’-dithiopropionic acid during the hydrolysis which give satisfactory result for cystine.

Tryptophan is normally measured after barium, lithium or sodium hydroxide hydrolysis. To improve the acidic hydrolysis for tryptophan determination, addition of pyridine boran to 6 M HCl was suggested, which reduced the decomposition of tryptophan. It is stated that in the case of pure protein the tryptophan and the other amino acids can be analysed from the same solution. No differences in the sodium or lithium hydroxide hydrolysis were found, and 20 hours of hydrolysis time at 110 °C was suggested as an optimum. An alternative acid ninhydrin method has been described. This method uses acidic hydrolysis in the presence of ninhydrin which tryptophan reacts before it that can be decomposed. A rapid fluorometric detection system is called optical multichannel analyser for determination of tryptophan from plasma was also elaborated.

Some years ago attempts have been made to reduce the analysis time by elevated temperature. The significant reduction in analysis time can be achieved by carrying out the hydrolysis at 145 °C for 4 hours with 6 M HCl. The result achieved after the standard and elevated hydrolysis that was very similar therefore some systems have been elaborated for hydrolysis of protein at an elevated temperature. A Reacti-Therm III manual hydrolysis system was introduced by Pierce (Rockford, Illinois, USA) which 24 samples can be hydrolysed at 110 to 200 °C with 6
M HCl from 15 minutes to 72 hours. 48 and 72 hours hydrolysis time is recommended for
isoleucine and valine since the peptide bonds of these amino acids are resistant to hydrolysis.
During this very long time hydrolysis some parts of sensitive amino acids are destroyed. The
Waters Associates (Milford, Massachusetts, USA) have produced a new hydrolysis system for
their Pico-Tag RPC analysers, which can hydrolyse the samples at 180 °C for 5 minutes with 6
M HCl.

30% higher value was found for the tryptophan content after hydrolysis with 3 M
mercaptoethanesulphonic acid (MES-OH) than after hydrolysis with para-toluenesulphonic
acid (containing 0.2% tryptamine). Different temperatures (160, 170, 180 °C) and time periods
(15–90 min.) were compared under standard conditions to minimise the side reaction during 3
M mercaptoethanesulphonic acid hydrolysis for the best recovery of the amino acids (especially
tryptophan and methionine). It was stated that the highest temperature and the shorter time give
results which are very similar to the original method (125 °C, 24 h). In some cases, such as
tryptophan and methionine determination at 160–170 °C for 15–30 minutes, the results were
better than those which were obtained by the original method. A disadvantage of MES-OH
hydrolysis method is reducing cystine to cystein, which coelute with proline from the ion
exchange column and it may interfere with determination of proline in high-cystine proteins.
MES-OH can be applied not only for hydrolysis of the proteins but also for derivatization of
amino acids with help of OPA. Hydrolysis of proteins are performed with 6 M hydrochloric
acid at high temperature and for short times in order to reducing the racemization of the amino
acids during the analysis of amino acid enantiomers.

New development was the vapour phase protein hydrolysis. At rising temperature the
hydrochloric acid is vaporised and only the vapour of the HCl comes into contact with the
sample, which avoids contamination from present amino acids. The Applied Biosystems
(Warrington, England) also developed a vapour phase system which the samples are hydrolysed
at 155 °C for 75 minutes, after derivatized automatically. It was concluded that for pure proteins
the automated hydrolysis system gave better accuracy than manual hydrolysis. The vapour
phase hydrolysis seems to be very good, if performic acid pretreatment of the sample is not
necessary, and if volatile additives, which prevent the distraction or oxidation some amino
acids, cannot be used.

The use of the microwave irradiation have been introduced into the protein hydrolysis 25
years ago both in liquid or vapour phase with 6 M HCl at approximately 180 °C for 5–10
minutes by using commercial microwave ovens. 4 M methanesulphonic acid and 6 M HCl were compared in liquid phase and a good correspondence between the results and obtained by the standard method that was reported. The use of methanesulphonic acid hydrolysis gave very good correspondence to the theoretical values in case of methionine, cysteine and tryptophan. Five minutes at 180 °C was the optimum time for hydrolysis because shorter time provided unknown peaks, which was probably the result of an insufficient hydrolysis. In the another study the amino acid composition of the methionyl human growth hormone was determined by hydrolysing the protein in microwave oven by using 6 M HCl in the vapour phase. The optimum conditions of the hydrolysis were 180 °C for 8–10 minutes but this time was not sufficient for the bounds of valine and isoleucine.

5.7.12.3. Ion exchange chromatography of amino acids

After sample preparation, which means in most cases hydrolysis of the protein or preparation of the sample for free amino acid analysis, is depending on the amino acids that are present in the sample, sodium or lithium buffers are prepared for separation of the amino acids by IEC. The eluate from the ion exchange column is passed through a teflon coil that is placed in a boiling water bath, or other heating apparatus. Before entering the column, the column effluent is mixed with reduced ninhydrine reagent which is dissolved in acetate buffer. The ninhydrin reacts with amino acids that are forming a dye complex. The absorption is determined in a flow photometer, and registered on the chart of a recorder or a computer. The area under the peaks corresponds the amounts of amino acids that are present in the sample. The evaluation can be done manually or automatically with an integrator or a computer. The circumstances of the analysis make it possible to quantitate as little as one nanomol amino acid with a high degree of accuracy.

The original two column systems for separation all of the proteins that are building amino acids, were described first by Spackman et al., and this method was used for manual and automated systems for many years. Nowadays this method is not used, because of its problems that are related to reliability, accuracy, sensitivity and sample loading system. Nowadays the simple single column system is generally used. By the method of Moore and Stein amino acids are separated on a cation exchange resin with buffers of carefully defined salt concentration and pH. The ion exchange takes place on resin, which consists of small spherical beads of polystyrene that are reacted with divinylbenzene to achieve the required degrees of cross
linkage between two polymerised chains of styrene, and sulphonated to provide an electrical charge.

The chromatographic column is filled with resins of negative charge, and the amino acids are put on the column at a low pH value (pH=2.2), hence all of them bear a positive charge. In these conditions all of the amino acids will link to the resin, no chromatographic division will occur, and the amino acids are waiting at the beginning of the column for a change in conditions. If the pH and the ionic strength of the elution buffers increase, the isoelectric point of the amino acids will be reached, and the attraction of the ions towards the resin diminishes and the amino acids will be eluted from the column. The isoelectric point of an amino acid molecule is defined as the pH value, at the molecule in the solution that does not have any charge. The isoelectric point of amino acids is a function of the pH values of the ionisable groups in the molecule. The conditions of the separation of the amino acids can be modified in such a way that the isoelectric points, for all amino acids, will be reached at various times. For example for aspartic acid the different charges at different pH is following:

\[
\begin{align*}
\text{H}_3\text{N}^+\text{CH}—\text{COOH} & \quad \text{pH 1} \\
\text{CH}_2\text{COOH} & \quad \text{pH 2.8} \\
\text{H}_3\text{N}^+\text{CH}—\text{COO}^- & \quad \text{pH 6.6} \\
\text{CH}_2\text{COO}^- & \quad \text{pH 11}
\end{align*}
\]

At pH = 1 the molecule has one positive charge, but if the pH value is increasing, larger number of molecules are situated in the α-carboxil group that will have a negative charge up to the limit of pH = 2.8, when all of them will have it. This is the isoelectric point of the aspartic acid. The carboxylic group in the side chains are less acid than α-carboxilic acid, and the concentration of the hydrogen ions is sufficient to prevent its ionization. If the pH value rises to 6.6, the carboxylic group of the side chain will be ionised, and the molecule will have two negative and one positive charge, and if the pH rises to 11.0, the molecule has only two negative charges.

The lysine has an amino group on its side chain, its isoelectric point is at pH = 9.7. At pH = 1 the lysine has two positive, at pH = 5.6 two positive and one negative, at pH = 9.7 one positive and one negative and at pH = 11 one negative charge.
The theoretical treatment of the separation of amino acids supposes the concentration of the individual amino acids is small, therefore the ratio between the amino acids bound to the resin and free in the solvent has to be regarded as an independent concentration. The process of ion exchange is following:

\[
\text{Matrix-SO}_3^-\text{Na}^+ + \text{H}_3\text{N}^+\text{CH(R)COOH} = \text{Matrix-SO}_3^-\text{H}_2\text{N}^+\text{CH(R)COOH} + \text{Na}^+ \tag{27}
\]

The distribution coefficient \( a_{\text{amino acid}^+} \) for the amino acid is defined as the ratio between free and bound ion in a given section of the column.

\[
a_{\text{amino acid}^+} = \frac{[\text{Matrix-SO}_3^- - \text{H}_2\text{N}^+\text{CH(R)COOH}]}{[\text{H}_3\text{N}^+ - \text{CH(R)COOH}]} \tag{28}
\]

Where: \([\text{Matrix-SO}_3^- - \text{H}_2\text{N}^+\text{CH(R)COOH}]\) is the concentration of amino acid bound to the resin, \([\text{H}_3\text{N}^+ - \text{CH(R)COOH}]\) is the concentration of the free amino acids in the buffer.

For the ion exchange process the law of mass action can be applied, and from the equilibrium constant \((K)\), the amino acid concentration is bound to the resin, the free amino acid concentration, the counter ion concentration is bound to the resin, and the free counter ion concentration that we can get information about the elution of the amino acids and the retention time.

### 5.7.12.3.1. Ion exchange resins

Nowadays spheroidal ball shape ion exchange resins are used. The synthesis is carried out by means of co-polymerization of styrene and divinylbenzene. The share of divinylbenzene that is used in the synthesis is approximately 8%. The concentration of divinylbenzene is very important that it forms cross links in the styrene chains and leading to formation of the ball shape, and that is depending on the quantity of cross links of the resin that has more or less favourable properties: terms of rigidity, swelling capacity and porosity. The structure of the resin and the procedure of the ion exchange is the following:
The cross-linked resin structure is referred to resin matrix, and if it is sulfonated, then the strongly acid cation exchange resin is obtained. The sections which are situated inside of the skeleton are called pore and for the charged ions $-\text{SO}_3^-$ the term linked ions are used. The ions that are bearing the opposite charge are referred to exchangeable ions which are assigned to the matrix by means of heteropolar links. These are positively charged groups in buffers or amino acids. During the ion exchange the buffer ions that are bearing opposite charges penetrate to the matrix pores, and exchange places with the ions with opposite charges which are linked there.

The dimension of the particles, the level of sulphonation and cross linking varies in the case of resins are used for the amino acid analysis. As the divinylbenzene concentration increase, the cross-linking occurs at shorter intervals and the effective particle size or permeability reduce, contrary the anchor group is brought closer to each other so that the separating power will increase. The low cross-linking resins with 1–4% divinylbenzene have a higher permeability, their equilibrium will reach more rapidly, and they are capable to handling larger molecules. The capacity of the resins, because of the swollen volume is smaller, the separation power for certain ions will reduce, and the physical stability of the resin is also smaller. The low cross-linking resins with 8–16% divinylbenzene have small pore size, smaller permeability, but it is sufficient for smaller ions, and the swelling is slight.

Examining the particle size of the resin is advisable, that the smallest possible particle size is the best. The exchange rate increases with decreasing particle size, since the diffusion path between the active groups become shorter. Short diffusion values improve the sharpness of the
separation, and permit to use shorter columns which reduce the separation time. Smaller particles have a higher mechanical stability and this is very important, because the resin expands and contrasts in the column through the continuous changes in pH and concentration during the analysis.

The dimension of the separating column is very important as regard to the high separation resolution between the amino acids. The diameter of the columns nowadays is 1–2 mm, but earlier columns with 5–9 mm diameter were widely used. The larger diameter columns are preparative columns. The separating performance depends in addition to the diameter of the ion exchange particles, on a length factor and the column diameter. It is preferable to keep the column as narrow as possible in order to have the largest possible number of the theoretical plate number in the column.

The flow rate of the eluting buffer on the column is very important, because it determines the time of the analysis. If the flow rate through the column is more than the optimal, the fractions leave the column to become unsymmetrical, leading to tailing, in addition the amino acid peaks can overlap. Increasing flow rate leads to a higher back pressure which is undesirable for safety.

The regeneration of the ion exchange column is indispensable after the sufficient number of amino acid analysis. During the regeneration sodium hydroxide or lithium hydroxide that is used to wash the impurities from the column and replace used Na\(^+\) or Li\(^+\) ions during the analysis. Some authors suggest 0.2–1.0 M, but the optimum concentration seems to be 0.4 M for sodium hydroxide and 0.3 for lithium hydroxide. If cation resins contaminated with heavy metals, proteins or other bigger molecules, the resin have to be removed from the column, treated with 1% EDTA in 2 M hydrogen chloride solution for some hours at room temperature, regenerated by boiling the resin in 6 M HCl for half an hour, cooled at room temperature, diluted to 3 M HCl, filtered and washed with 500 cm\(^3\) two times by distilled water. Remove the resin from the filter and suspend in 2 M NaOH or LiOH depending on Na or Li system. Boil the resin for some minutes, and dilute to 0.5 M base. This resin is ready to fill in the analytical column.

The chromatography activity of the amino acid analysers is still influenced by the column dimensions, eluent flow rate, temperature and present of organic solvent in the buffers.
5.7.12.3.2. Buffer systems for separation of the amino acids

Choice of buffer system

Commonly protein hydrolysates contain most of all 18 amino acids that normally are found in proteins, they easily separate with three sodium buffer systems. Physiological fluids contain some of all 40–50 ninhydrin positive compounds that are present in different physiological mixtures. For this purpose four or five sodium buffer systems are suitable to achieve the satisfactory separation between the ninhydrin positive compounds. The lithium buffer system is suitable for these purposes, but the use of this system is justified rather in the case that simultaneous separation of aspartic acid, asparagine, glutamic acid and glutamine is required. The lithium system is more sensitive to variations than the sodium system.

The salts that is used for making buffers should be in the highest purity. The salts should be dissolved in deionized or carefully distilled water. Not only the ninhydrine positive impurities, but also others may cause irregularities in the baseline, for this reason freshly drawn deionized water is preferred. The acidic buffers have a tendency to take up ammonia and other ninhydrin positive compounds, therefore it is advisable to add HCl as late as possible to the buffers. The source of ammonia is tobacco smoke, cleaning fluids, urine of the laboratory animals and toilets, and vapour of different chemicals.

Sometimes thiodiglycol is added to the buffers to prevent oxidation of methionine, which can be under certain circumstances that influence the baseline shifts. Organic solvents (ethanol, propanol, 2-methoxyethanol) that are in the case of some resins are also added to the first buffer to improve the separation between threonine and serine. These peaks become slightly broader as the column ages and further additional organic solvent may be is necessary later. It appears that different solvents are better for different resins. Preservatives are added to the buffers to inhibit the growth of micro organisms. Several different chemicals (0.1% phenol, 0.01% pentachlorophenol, 0.01% caprylic acid) can be used for this purpose.

Effect on separation by pH, temperature, organic solvents and column flow rate

pH of the buffer is very critical for separation of various amino acids. All of the peaks of amino acids emerge earlier and sharper if the pH is too high, and the peaks chromatograph later if the pH is too low. The cystine is the most sensitive for pH, temperature and the concentration of
the ions with an opposite charge of the buffer. Cystine should be eluted and completely separated directly after alanine. With increasing pH and temperature the column accelerates the cystine, thereby shortens its elution time and if the temperature and pH are lower its elution times become longer, and cystine falls behind. The pH value and temperature must be selected in a way, that cystine can just be positioned between alanine and valine. The pH change has a greater influence on the cystine movement than a change in temperature.

The temperature affects the separation in two different ways: by changing the pH and by altering the affinity of the amino acids to the ion exchange resin. The separation between threonine and serine can be improved by lowering the temperature, but at the same time the backpressure is increased substantially, and this influences the separation of the glutamic acid. Therefore it is important to have a temperature gradient after the separation of two hydroxy amino acids. Cystine is also sensitive to temperature, but any changes in the retention time caused by the temperature can easily be compensated by pH. In the system for hydrolysates increase of the temperature from 50 °C to 70 °C or higher is recommended to decrease the time of analysis, but the rise should not take place before the separation of isoleucine and leucine. The optimum temperature for separation of aspartic acid, hydroxy proline, threonine, serine, asparagine, glutamic acid and glutamine is 37–38 °C with both sodium or lithium buffer system, because glutamic acid is particularly sensitive even to minor changes of temperature.

The organic solvent that is added to the first buffer, changes the solubility of the different amino acids. It is particularly the extra –CH₃ group of threonine in compared to serine that results an improvement in separation. The most frequently used compounds are methanol, ethanol, propanol, isopropanol and methyl cellosolve. The drawback of these techniques are slight loss of separation between glycine and alanine and an increased back pressure. It is possible to use as much as 25% of organic solvent, but the normally used concentration is between 2% and 5%. The analysis should be started at a rather low percentage of organic solvent, which gives an acceptable separation between threonine and serine, and increases the amounts when the column becomes older, and the peaks slightly broader. The limiting factor should be the separation between glycine and alanine.

A steady buffer flow rate is required for successful and reproducible separations of amino acids by IEC. This can be achieved with a constant pressure or a constant displacement pump. At most of the analysers the pumps are pulse-free and feature an even power output and their use guarantees conformity of the retention times of individual peaks. The pressure limitation of the
pumps is 1 to 8 MPa, and this is controlled by the software. The choice of flow rate is dependent upon the type of resin, the dimensions of the column and overall design of the instrument, and it varies between models.

**Preparation of the sodium citrate buffers**

Sodium citrate buffers are mainly used for the determination of amino acids in protein hydrolysate. List of necessary chemicals: citric acid, sodium citrate, sodium chloride, sodium hydroxide, boric acid, thiodiglycol, sodium azide. The Table 10 for computation of the quantity of the individuals for the preparation of the sodium citrate buffers is below.

<table>
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<th>4</th>
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<td>M citrate</td>
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<td>0.066</td>
<td>0.066</td>
<td>0.066</td>
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<td>pH</td>
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</tr>
<tr>
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<td>30</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>Sodium citrate (g/dm$^3$)</td>
<td>19.6</td>
<td>19.6</td>
<td>19.6</td>
<td>19.6</td>
</tr>
<tr>
<td>Sodium chloride (g/dm$^3$)</td>
<td>11.7</td>
<td>11.7</td>
<td>23.4</td>
<td>52.6</td>
</tr>
</tbody>
</table>

*Diluting buffer of 0.2 M sodium with pH=2.2* will be used for the dilution of both the samples and standards to a required concentration. The *regeneration solution is 0.2 M sodium hydroxide*. The *first sodium buffer* (0.20 M Na, pH=2.95) elutes the following amino acids: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine and cystine. This buffer is designed for the determination of amino acid content of hydrolysate, and suitable for the determination of cysteic acid and methionine sulphone as well. It is also used when it is necessary to determine proline exactly, or when you want to determine the amino acids with the best separation, and the time of the analysis is not a limiting factor. In this buffer smaller ionic strength is used, therefore cystine is eluted after glycine and alanine. With an increased value of pH and increased temperature, cystine elutes earlier. The separation of threonine and serine as well as glycine and alanine are very good in the case of this buffer. These two groups of peaks behave in the same way as a balancing mechanism, if the separation is improved at one pair, the separation of the other ones become worse. It means if cysteine is separated well, both of the pairs will be separated very well.
The second sodium buffer (0.30 M Na, pH=3.50) elutes the following amino acids: aspartic acid, threonine, serine, glutamic acid, proline, cystine, glycine, alanine and valine. This is a classical buffer that is designed for the single column system of the determination of the hydrolysate. The cysteine is very sensitive for pH, temperature and concentration of ions with an opposite charge. An increasing pH and temperature accelerates its movement on the column and cystine thereby shortens its elution time. The pH value of the buffer and temperature must be selected in a way that cystine can just be positioned between proline and glycine.

The third sodium buffer (0.40 M Na, pH=4.25) elutes the following amino acids: methionine, isoleucine, leucine. This buffer is not problematic, all of the amino acids are separated very well. The fourth sodium buffer (1.12 M Na, pH=7.9) elutes the rest of the amino acids: tyrosine, phenylalanine, histidine, lysine and arginine, and among the amino acids elute ammonia (Figure 67).

Figure 67 Determination of the amino acids from hydrolysate after performic acid oxidation of the sample by sodium buffer system. The standard contains 25 nmol of each component except for ammonia. The amino acids in order of appearance on the chromatogram are: 1. cysteic acid, 2. methionine sulphone, 3. aspartic acid, 4. threonine, 5. serine, 6. glutamic acid, 7. proline, 8. glycine, 9. alanine, 10. cystine, 11. valine, 12. methionine, 13. isoleucine, 14. leucine, 15. tyrosine, 16. phenylalanine, 17. histidine, 18. lysine, 19. ammonia, 20. arginine
Operating parameters are: Instrument: INGOS AAA400, packing of column: OSTION Lg ANB, column height: 35 x 0.37 cm, buffers: 1: pH 2.7, 0.2 M Na⁺; 2: pH 4.25, 0.5 M Na⁺; 3: pH 6.9, 1.12 M Na⁺; 4: 0.2 M NaOH.

Program:

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<th>Time (min)</th>
<th>Temperature (°C)</th>
<th>Buffers</th>
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</thead>
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</tr>
<tr>
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</tr>
<tr>
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</table>

Preparation of the lithium citrate buffers

Lithium citrate buffers are used especially for the determination of the free amino acids from physiological samples. List of necessary chemicals are: citric acid, lithium citrate, lithium chloride, lithium hydroxide, boric acid, thiodiglycol, lithium azide. The Table 11 for computation of the quantity of the individuals for the preparation of the lithium citrate buffers is below.

Table 11 The composition of the lithium buffers

<table>
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<th>Buffers</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
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<td>M Li</td>
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<td>0.20</td>
<td>0.35</td>
<td>0.33</td>
<td>1.20</td>
</tr>
<tr>
<td>M citrate</td>
<td>0.053</td>
<td>0.060</td>
<td>0.070</td>
<td>0.100</td>
<td>0.220</td>
</tr>
<tr>
<td>pH</td>
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<td>3.10</td>
<td>3.35</td>
<td>4.05</td>
<td>4.65</td>
</tr>
<tr>
<td>Citric acid (g/dm³)</td>
<td>27.26</td>
<td>30.07</td>
<td>35.17</td>
<td>38.48</td>
<td>41.65</td>
</tr>
<tr>
<td>Lithium citrate (g/dm³)</td>
<td>14.92</td>
<td>16.92</td>
<td>19.74</td>
<td>28.20</td>
<td>62.04</td>
</tr>
<tr>
<td>Lithium chloride (g/dm³)</td>
<td>7.62</td>
<td>8.47</td>
<td>14.83</td>
<td>13.98</td>
<td>50.87</td>
</tr>
</tbody>
</table>
Diluting buffer of 0.1 M lithium with pH=2.2 will be used for the dilution of both the samples and standards to a required concentration. The regeneration solution is 0.3 M lithium hydroxide. The first lithium buffer (0.18 M Li, pH=2.80) elutes the following amino acids: cysteic acid, taurine, phosphoethanolamine, urine, aspartic acid, hydroxyproline, threonine, serine, asparagine, glutamic acid, glutamine. Elution is carried out at the basic temperature of 37 to 40 °C. The most sensitive in terms of pH and temperature are asparagine, glutamic acid and glutamine. Glutamic acid is the most sensitive and most moveable by changing pH and temperature, therefore pH and temperature must be prepared in a way that glutamic acid can just be positioned in the middle between asparagine and glutamine.

The second lithium buffer (0.20 M Li, pH=3.05) elutes the following amino acids: α-amino adipic acid, proline, glycine, alanine, citrulline, α-amino butiric acid and valine. Citrullin is very sensitive to temperature and pH and its position can be set by pH of the buffer. The third lithium buffer (0.36 M Li, pH=3.35) elutes the following amino acids: cystine, methionine, cystathionine, isoleucine, leucine. At this buffer only the cystathionine is problematic, which is sensitive for both pH and temperature. It is recommended to switching to the higher temperature (60 °C) so that the cystathionine will be positioned in the middle between methionine and isoleucine. In the case of later switching of temperature cystathionine is eluted later and it is not sufficiently separated from isoleucine, in opposite case that is eluted with methionine.

The fourth lithium buffer (0.33 M Li, pH=4.05) elutes the following amino acids: tyrosine, phenylalanine, β-alanine and β-amino butyric acid. This buffer is not accompanied by any problem if the buffer change has been performed in a right place. The fifth lithium buffer (1.20 M Li, pH=4.65) elutes the following amino acids: γ-amino butyric acid, ornithine, lysine, histidine, 1-methyl histidine, 3-methyl histidine and arginine. This buffer is trouble free. The buffer change must be performed after β-amino butyric acid (Figure 68).

Lithium buffers are much more aggressive than Na buffers, that shows why is suitable to rinse approximately once a month with distilled water at the maximum throughput of the pump. Because Li buffers are more aggressive towards all metals, it is not recommended to leave them for longer times in contact with surfaces of varnishes and metals.
Figure 68 Determination of the free amino acids by lithium buffer system. The standard contains 25 nmol of each component except for ammonia. The amino acids and the ninhydrin positive compounds in order of appearance on the chromatogram are:


Operating parameters are: Instrument: INGOS AAA400, packing of column: OSTION Lg FA, column height: 20-22 x 0.37cm, buffers: 1: pH 2.8, 0.18 M Li⁺; 2: pH 3.1, 0.20 M Li⁺; 3: pH 3.35, 0.35 M Li⁺; 4: pH 4.05, 0.33 M Li⁺; 5: pH 4.65, 1.20 M Li⁺; 6: 0.3 M LiOH.
5.7.12.3.3. Recent developments in the chromatographic separation

For separation of the amino acids after deproteinization or hydrolysis of the sample, column chromatography is the best method. This means high performance liquid chromatography (HPLC) which consists of ion exchange column chromatography (IEC) and reversed phase chromatography (RPC) with post- or precolumn derivatization of the amino acids, and gas liquid chromatography (GLC). During IEC the amino acids are separated by sulphonated polystyrene cation exchange resin, mixed with derivatization agent (mainly ninhydrin), passed through a coil and a detector and depending on derivatization agent spectrophotometer or fluorometer.

During two last decades the analysis time of IEC reduced by improvement of the ion exchange resins. The shorter analysis time achieved by the use of complex buffer and column temperature systems. During the short time analysis the resolution of the peaks sometimes was not sufficient, and very expensive instruments, ready to use buffers and ninhydrin produced by the manufacturers.

The detection of the amino acids was mainly based on ninhydrin system but instead of methylcellosolve, sulfolane was used as solvent agent of the reduced ninhydrin. This solution buffered with lithium acetate that is not so toxic, and the stability, the signal to noise ratio, the resolution of the peaks and the baseline are also better than normal ninhydrin. This reagent does not form precipitates and blockages in the flow lines and in the reaction column, but it is three times more expensive than the normal ninhydrin solution.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°C)</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>38.00</td>
<td>2</td>
</tr>
<tr>
<td>33.00</td>
<td>38.00</td>
<td>3</td>
</tr>
<tr>
<td>45.00</td>
<td>70.00</td>
<td>3</td>
</tr>
<tr>
<td>50.00</td>
<td>70.00</td>
<td>4</td>
</tr>
<tr>
<td>63.00</td>
<td>70.00</td>
<td>5</td>
</tr>
<tr>
<td>95.00</td>
<td>74.00</td>
<td>5</td>
</tr>
<tr>
<td>120.00</td>
<td>74.00</td>
<td>6</td>
</tr>
<tr>
<td>136.00</td>
<td>53.00</td>
<td>5</td>
</tr>
<tr>
<td>139.00</td>
<td>74.00</td>
<td>1</td>
</tr>
<tr>
<td>144.00</td>
<td>38.00</td>
<td>1</td>
</tr>
<tr>
<td>160.00</td>
<td>38.00</td>
<td>1</td>
</tr>
</tbody>
</table>
Other derivatization reagents (fluorescamine, dabsylchloride, 4-fluoro-7-nitro-2,1,3-benzoxadiazole and o-phthalaldehyde) were introduced to improve the sensitivity and the accuracy of the method, but many problems, particularly with derivatization of proline and hydroxiproline had to be solved. From these reagents only the OPA/mercaptoethanol and the OPA/3-mercaptopropionic acid could be used widely for postcolumn derivatization of the amino acids.

### 5.7.12.4. Detection systems

The colour or fluorescence that is produced of amino acids varies for different amino acids and this has to be determined for quantification. It can be made by loading a mixture of amino acids containing the same concentration of each amino acid (including the chosen internal standard) and from the areas of the peaks on the recorder trace that is calculating each response factor in the used way. Sometimes an internal standard, which is absent from the sample, is used for every analysis that is carried out. For instance the non-physiological amino acids norleucine or α-amino-β-guanidinobutyric acid may be used. This should be added in a known amount to the sample prior to any sample pre-treatment. If the amount of the internal standard is known, the concentration of the unknown amino acids can be determined by using peak area relationship.

### 5.7.12.4.1. The reaction of the amino acids with ninhydrine

The reaction of ninhydrin with amino acids is very important for the detection and quantitative estimation of the amino acids. Ninhydrin is an oxidising agent, which elicits the oxidative determination of α-amino group liberating ammonia, carbon dioxide and an aldehyde and the reduced form from the ninhydrin.

\[
\text{RCHCOOH} + \text{ninhydrin} \rightarrow \text{RCHCOOH} + \text{CO}_2 + \text{H}_2\text{O} + \text{R}CH=\text{C(OH)}\text{O}_2\text{N} \quad (\text{Ruheman's purple})
\]

\[
\text{RCH}=\text{N} \quad (\text{reduced form of the ninhydrin with } \alpha\text{-amino group})
\]

\[
\text{RCH}=\text{N} \quad (\text{original ninhydrin})
\]

\[
\text{RCH}=\text{N} \quad (\text{Ruheman's purple})
\]
The ammonia reacts with an additional molecule of ninhydrin to yield a purple substance (Ruheman’s purple) which has a maximum absorption at 570 nm. This absorption is a linear function of the amount of $\alpha$-amino groups that originally presents, and this reaction provides a quantitative colorimetric assay for compounds with NH$_2$ group on an $\alpha$-carbon atom. At imino acids a bright yellow product is formed which has a maximum absorption at 440 nm. The colour recovery for the individual amino acids differs slightly which is influenced by pH, ionic strength and reaction time.

Before the reduction of the ninhydrin with stannous (II) chloride was mostly used, after that titanous (III) chloride was suggested instead. TiCl$_3$ has two practical advantages compared to SnCl$_2$: The 15% solution can easily be pipetted to ninhydrin, and it is possible to use ninhydrin reagent almost immediately compared after the few hours for SnCl$_2$ reduced ninhydrin. Ninhydrin is protected with nitrogen and bubbling through the solution. It is advisable to keep gas flow as low as possible and the lowest possible volume of 50 cm$^3$ of N$_2$ per hour is suggested. The teflon tubing is used for ninhydrin lines that is slightly permeable to oxygen molecules, but during the continuous analysis it does not cause any detection interference. If the analyser is left for a weekend, it is advisable to pump out of the ninhydrin standing in the short line between the valve and the pump before starting the first analysis.

**Preparation of the ninhydrin reagent**

Necessary chemicals for the preparation of the ninhydrin reagent are: ninhydrin, 2-metoxi ethanol (ethylene glycol monomethyl ether, methylcellosolv), 4 M acetate buffer and hydridantin dihydrate. The chemicals should be dissolved in the specific order during the continuous bubbling through by nitrogen directly in the reagent bottle. Hydridantin is very difficult to be dissolved in the mixture of acetate buffer and methoxy ethanol, therefore it is necessary to pour out 200 cm$^3$ methylcellosolv into a small baker, and it is necessary to add dissolved hydridantin in methylcellosolv under preparation. After the dissolving
hydrindantine in methylcellosolv, immediately pour it into a bottle with the prepared ninhydrin, and bubble for a short time with nitrogen.

During the preparation of the ninhydrin reagent all of the chemicals must be very pure for amino acid analysis. Methoxyethanol can be replaced with dimethylsulphoxide but in this case it is necessary to use 4 M lithium acetate buffer at pH=5.2. Instead of hydrindantin other reduction agents (SnCl₂, TiCl₃) can be used. Nitrogen must be fully free of oxygen, and instead of nitrogen, argon is also possible to use, but helium is not recommended.

5.7.12.4.2. The reaction of the amino acids with other reagents

The fluorescamin reacts with amino acids and primary amines at room temperature under alkaline conditions to form fluorescent pyrrolidones derivatives ($\lambda_{ex} = 390$ nm, $\lambda_{em} = 474$ nm). The detection limit is at 50–100 pmol. The excess of the reagent is very quickly hydrolysed into water soluble non fluorescent compounds. The reagent is of great interest because of its fast reaction rate with amino acids at room temperature; it does not offer any greater sensitivity than the ninhydrin reaction.

![Chemical reaction]

The reaction of an amino acids with o-phthaldialdehyde (OPA) and mercaptoethanol leads to fluorescent isoindol derivatives ($\lambda_{ex} = 330$ nm, $\lambda_{em} = 455$ nm). The OPA derivatives the amino acids that can be used for postcolumn and precolumn derivatization of the amino acids with a limit detection of 1 pmol. Mercaptoethanol can be replaced with other thiols, such as N,N-dimethyl-2-mercaptoethylamine. If the chiral thiol is used (for example tetra-O-acetyl-β-D-glucopyranoside) the separation of the amino acid enantiomers is possible.
The OPA reaction is favourable compared to ninhydrin reaction in several respects. The reagent is stable and is in an aqueous form which eliminates the use of toxic organic solvents such as methylcellosolv and storage under nitrogen. There is no need for heating apparatus because the reaction proceeds quickly at room temperature. The big disadvantage of the method is proline and hydroxyproline are not converted to fluorescent isoindole derivatives, therefore these two amino acids as well as cystine and cysteine must first be treated with a suitable oxidizing agent such as chloramine T or sodium hipochlorite to convert them to compounds which will react.

5.7.12.5. Controlling of the apparatus and evaluation of the chromatograms

The most modern amino acid analysers is a software serving that helps for controlling the apparatus and subsequent assessment of the results. At INGOS Amino Acid Analyser 4000 (AAA 4000) the system develops in the DELPHI environment for WINDOWS NT or WINDOWS 2000 and an extension for LINUX is expected. The ChromuLan program for the AAA 4000 uses standard assessment and a special program module for control of AAA 4000. The program can operate in a fully automatic mode. Set a sequence in a program, within the framework of each sample that is assigned as an analytical program and assessment method. The apparatus will process samples in an automatic manner, ensure the equilibration of the column during the passage be tween analytical program and will automatically assess the results according to the pre-set method.

The evaluation of the results can be done manually or automatically. On a good chromatogram, amino acids with the exception of tryptophan give almost symmetrical peaks. For quantitative evaluation the curve with the highest absorption values is used, in most cases the 570 nm curve. Proline and hydroxiproline give their highest absorption at 440 nm, for this reason the suggested evaluation of these two peaks is at 440 nm if it is possible. When two amino acids are not completely separated, an error is introduced. If the separation is better than 65% of the peak height, it is possible to assume that the two peaks are symmetrical and to calculate the width of the peak at a height where the influence of the neighbouring peak is negligible.

During the manual peak evaluation the baseline, total height, net height, half height and the width of the peak at the half height have to be determined, and from these data the basic area
of the peak can be calculated by multiplying the net height with width. This value represents
the area under the peak, which is linear in function to the concentration of the amino acids. If
the area is known for a given amount of an amino acid, the amount corresponding to any peak
size can be determined. If computer program is used for determination of the quantity of amino
acids the peak parameters can be edited directly in the graph or in the peak table. In the graph
you can also edit the baseline and the integration marks of the peaks.

In the case of AAA 4000 it is necessary to use always a computation with the standard. This
computation is carried out according to the following formula:

\[
\text{Amount} = \frac{\text{Area}}{\text{Response}} \cdot \text{UsrPeakCoeff} \cdot \text{Factor} \cdot \frac{\text{Multiply Factor}}{\text{Divide Factor}}
\]  

(29)

where

Area = the peak area

UsrPeakCoeff = the coefficient from the peak table

Factor = set in the method that is heading and is same for all peaks and for all samples
that are assessed by using the method in question

Multiply Factor and Divide Factor = set for each sample separately in a sequence or in
the heading of the sample are all for all peaks

Response = computed from the standard according to the formula (30):

\[
\text{Response} = \frac{\text{Area}_{\text{std}}}{\text{Amount}_{\text{std}}} \cdot \text{UsrPeakCoeff}_{\text{std}} \cdot \text{Factor}_{\text{std}} \cdot \frac{\text{Multiply Factor}_{\text{std}}}{\text{Divide Factor}_{\text{std}}}
\]  

(30)

The meaning of individual members is the same; they are only taken from the standard.

5.7.12.6. Summary of amino acid analysis

There is no perfect method for the determination of the amino acid composition of pure
protein, feeds or biological fluids. The major problems are hydrolysis of the protein, the
deproteinization of the biological fluids and the partial decomposition of methionine, cysteine
and tryptophan during the sample preparation for analysis. The moment of the traditional IEC
with postcolumn ninhydrin derivatization seems to be the best for both pure proteins and
feeds and complex mixtures. It is suggested that it would be the best if the existing methods of
amino acid analysis were applied with the most possible care.
5.7.13. Determination of the amino acids by precolumn derivatization with HPLC

Precolumn derivatization of the amino acids are followed by reversed-phase HPLC that is a very useful method, if amino acid analyser with ion exchange column chromatography is not available. Amino acids are derivatized prior to chromatography with many derivatizing reagents, separated by reversed-phase HPLC, and quantified by UV spectroscopy or fluorometry. Methods which are using precolumn derivatizations can detect picomole quantities of amino acids, and the chromatographic runs usually take 30 min or less.

The proliferation of high-performance liquid chromatography began at the end of the 1960s and early ‘70s. Through the implementation of a corresponding pump, the high pressure flow of the liquid could also be achieved, while the detection of the effluent components was realized by developing detectors with high sensitivity. Using the method it was possible to separate and quantitatively determine the protein constituent amino acids. Most of the methods developed operating with reverse phase chromatography, where the polarity of the mobile phase is greater than stationary phase. In the case of reversed phase chromatography the stationary phase is usually a hydrophobic packing covered with octadecyl (C18) or octyl- (C8) groups, while the mobile phase is a polar group.

Derivatization

During the elaboration of methods which are suitable for the detection of amino acids by HPLC, different methods have developed, which are in this followings: the determination is preceded by a derivatization step, which not only just improves the separation of amino acids, but also makes the amino acid derivatives suitable for the detection with UV of fluorescence detectors. (It is well known among of amino acids only tryptophan, tyrosine and phenylalanine that show ultraviolet absorption.) Several possible derivatizing reagents have been described in the scientific literature, from which the most commonly used are the followings: OPA (o-phthalaldehyde), FMOC (9-flourenil methyl chloroformic acid ester), DABS-Cl (4-dimethylamino-azobenzene-4’-sulfonyl chloride; Dabzil chloride), DNA-Cl (1-dimethylamino-naphthalene-5-sulfonyl chloride, dansyl chloride), DNFB (2,4-dinitro-fluorobenzene), PITC (phenyl isothiocyanate).
Amino acid determinations are generally carried out by use of gradient elution, however, to resolve small problems, the simple isocratic elution can also be used. For the determination of amino acids by HPLC the derivatization methods listed below are widespread in practice. The ideal **pre-column derivatizing reagents** must meet the following requirements:

- Quantitative reactions between simple reaction conditions.
- Quantitative reaction with all of the protein-building amino acids, including the imino acids.
- Stable reaction products.
- High fluorescence activity or UV absorption of the derivatives.
- The excess reagent and by-products should separate well from the amino acid derivatives.
- Reagent should allow easy automation of the method.

The derivatizing reagents are not always able to meet these optimal expectations, but despite of small shortcomings, most of them can still be used well in their application filled.

**5.7.13.1. Determination of protein building amino acids by pre-column derivatization**

**Derivatization with o-phthaldialdehyde and mercaptoethanol**

A great advantage of the OPA performed derivatization is the fact that OPA in itself does not have a fluorescence activity, however, its amino acid derivatives **have a high fluorescence activity**, consequently, the method is not sensitive to the reagent in excess, and even by the addition of large amounts of OPA, the amino acid derivatives can be perfectly analyzed. Some OPA derivatives are unstable, and another disadvantage of the reaction is imino acids that does not form derivatives with OPA. The instability of the derivatives is only a problem if the device itself does not have an automatic derivatization system. But in the possession of such a system, the standard and the sample derivatization is performed with the same method, therefore the results are well reproducible. Using this method, 15 from the 17 amino acids of the protein hydrolisates are well detectable and measurable, however, the error of the method is proline (an imino acid) that does not form a derivative with OPA, and the fluorescence signal of cystine OPA derivative is also very low (Figure 69). In the case of proline the method is unsuitable for the determination, while in the case of cystine the problem can be solved by blocking sulphydryl
group of cystine with iodoacetic acid during the derivatization process, and resulting highly fluorescent isoindole derivative.

Figure 69 The separation of amino acids by HPLC after pre-column derivatization with OPA/mercaptoethanol.

To enhance the stability of the OPA reagent ethyl mercaptan, 2-mercaptoethanol or 3-mercaptopropionic acid is added to the reagent. Among the derivatives obtaining OPA/mercaptoethanol has the highest fluorescence signal. OPA/mercaptopropionic acid is also considered as a good derivatization agent, that resulting amino acid derivatives which can be measured well both with UV and fluorescence detection. By comparing the obtained results and using OPA/mercaptoethanol method and those of ion exchange column chromatography, has been found matching between the results of two methods is very good. Of course, proline and cystine were not measured by the OPA/mercaptoethanol method, however, the other amino acid residues could be analyzed at a concentration of two orders of magnitude lower. Since the method is more sensitive to the ion exchange column determination, therefore, the OPA/mercaptoethanol derivatization was used widely to measure primarily free amino acids. It was used to determine the amino acid content of tissues and plasma, blood and urine, as well as various body fluids. It was also used to determine the soluble amino acid content of different waters, the amino acid compositions of coffees and teas, and it was a widespread method for the analysis of amino acid content in food and feed. The method has the following advantages:
- The reaction between the amino acids and OPA is very fast even at room temperature.
- OPA itself has no fluorescence activity, therefore there is no need to remove the excess reagent, and the derivative can be created in one step.
- The derivatives can be separated easily by gradient elution during the determination with HPLC.
- Tryptophan is also detectable with the OPA method.
- Extreme high sensitivity: 0.1 pmol of amino acid is detectable by use of this method.

The disadvantages are:

- Proline and hydroxyproline cannot be measured with the OPA method.
- OPA reagent is less stable, therefore the OPA derivatives are also unstable, they decompose relatively quickly, thus, a good reproducibility is only conceivable by use of automated systems.
- The fluorescence signal of cysteine and cystine OPA derivatives is very low, fluorescence detection is not sensitive enough for these amino acids, but they can be measured with UV detection at 330 nm.

**Derivatization with o-phthaldialdehyde and mercaptoethanesulphonic acid.**

**Mercaptoethanesulfonic acid as the reductive thiol-containing reagent employed for the derivatization of amino acids with o-phthaldialdehyde**

Hydrolysis of peptides and proteins with mercaptoethanesulfonic acid (MES-OH) has often applied prior to determination of amino acid content of food and feed samples (with the exception of cysteine), and tryptophan content of samples with low amount of carbohydrates. Side-chains of amino acids which are susceptible to oxidation during hydrolysis can be protected with use of MES-OH due to its thiol group with reducing properties. With the use of MES-OH oxidized forms of methionine were converted to methionine and the indole group of tryptophan was protected against oxidation.

Prior to HPLC analyses samples that were precolumn derivatized with OPA/ME or OPA/MES-OH with the programmable autosampler. First 200 µL (0.4M; pH=9.5) borate buffer was added to 450 µL sample solution, then 100 µL derivatization reagent (100 mg OPA and 100 µL ME/or 100 µL 3M MES-OH/ in 9 cm³ methanol and 1 cm³ borat buffer) was added and mixed. After three min 20 µL of the mixture was injected. The HPLC analyses were accomplished with
a MERCK-Hitachi HPLC comprising L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, and AIA data conversion utility for the D-7000 HPLC system manager. The compounds were separated on a 250mm x 4 mm column packed with Superspher 60 RP-Select B, the temperature of thermostat was 40 °C. The mobile phase was composed from methanol, sodium acetate 50mM (pH=7.0) and acetonitrile, the flow rate was 1 cm$^3$ min$^{-1}$. The derivatives were detected with a fluorescence detector ($\lambda_{\text{ex}}$ 330 nm, $\lambda_{\text{em}}$ 450 nm).

When the OPA/MES-OH derivatization procedure was applied, the sensitivity was about fivefold higher than in case of OPA/ME-method. In fact, there were no significant differences among the results of IEC and HPLC analyses of irrespective—choice of hydrolysis or derivatization methods. Although in case of samples that are analyzed by HPLC and derivatized with the OPA/ME or with the OPA/MES-OH methods the concentration of some amino acids (threonine, serine, methionine, tyrosine) were slightly higher than in case of IEC analysis with ninhydrin derivatization. When samples were hydrolyzed with MES-OH, in most cases, the concentration of MES-OH after dilution was enough also for derivatization and therefore the addition of OPA was solely required. If the solution after hydrolysis contains at least 1 μL of 3M MES-OH, the protein content of the sample was below 50%, and an extra addition of MES-OH was not necessary for derivatization. In case of corn samples with crude protein content of 9-10 % the amount of MES-OH in diluted sample (approx. 4 μL 3M) was sufficient for derivatization, and also for meat meal and soybean samples with 46,6-45,2% crude protein content. If the protein content of the sample is less than 5% the peak of MES-OH can disturb the evaluation of acidic amino acids in IEC.

The intensity of OPA/MES-OH derivatives on chromatograms (Figure 70) begin to decrease after 10-12 hours of the reaction, therefore analysis should be achieved within this time period. After that time a significant decrease was detected mostly in case of aspartic acid and glutamic acid derivatives.
Einarsson et al. published the derivatization of amino acids with 9-fluorenymethyl chloroformate (FMOC) reagent and the determination of the derivatives by reverse phase HPLC analysis in 1983. The reaction between amino acids and FMOC is very fast, and *imino acids also form derivatives with the reagent*, and even FMOC derivative of cysteine gives an easily measurable fluorescence signal. The disadvantage of FMOC derivatization is excess reagent that has to be removed with extraction with pentane before the detection by chromatography. The implementation of extraction makes this method more difficult, and it is also possible, that a part of the amino acid derivatives will also be extracted with pentane. Instead of pentane of other extraction reagents that have also been proposed, but they did not significantly improve the reproducibility of this method.

The combined use of the OPA and FMOC overcomes the problems of the methods. If we are not interested in the proline, hydroxyproline and cysteine content of the sample, it is better to perform the derivatization with OPA. When there is interest in the determination of these amino
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acids too, it is better to use the FMOC reagent. If the fluorescence signal is weak, it is appropriate to perform the detection with in line bound fluorescence and UV detection.

**Derivatization with dabsyl chloride (DABS-Cl)**

The derivatization with dabsyl chloride (DABS-Cl) is very popular in the field of amino acid analysis, because the amino acid derivatives are stable at room temperature more than one month, and they can be detected in the visible range. The advantage of this method is a very simple derivatization procedure, very good stability, reproducibility, and complete separation of each amino acid by liquid chromatography and its possibility to perform the detection in the visible range. The dabsyl chloride forms a stable derivative both with amino acids and imino acids. The lower limit of sensitivity is less than 1 pmol; sometimes the values are given in fmol range too. Due to the stability of the derivatives it does not require automatic derivatization and dispensing system, the manual derivatization procedure gives also well reproducible results.

**Derivatization with dansyl chloride (DNS-Cl)**

Dansyl chloride (DNS-Cl) was one of the first reagents that applied for pre-column derivatization of amino acids during the separation and determination of these. It reacts both with amino acids and imino acids, and forms dansyl-amino acids with high fluorescence activity, which can be detected easily and with high sensitivity. The disadvantage is that the dansyl-amino acid derivatives are sensitive to light, and therefore during the reaction and until the start of the analysis, they must be protected from light. During the reaction and after the reaction, the dansyl chloride hydrolyzes continuously to dansyl-sulfonic acid, which may interfere in the separation. It is also a problem that the reaction of dansyl-chloride with lysine, histidine and tyrosine can result in many forms of reaction products, which may call question for reliability of the method for these amino acids.

**Derivatization with phenyl-isothiocyanate (PITC)**

During the reaction of amino acids with phenyl-isopropyl isothiocyanate (PITC), phenyl-thiocarbamyl (PTC) derivatives are formed, which will reorder to phenyl thiohydantoyl- (PHT) derivatives by their exposure to acid. Both derivatives are suitable for the separation and determination of amino acids by liquid chromatography, but in most cases the phenyl thiocarbamyl derivatization is performed, where the detection will be in the UV range. The elution order of the amino acids is determined by high-performance liquid chromatographic
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analysis of phenylthiocarbamyl-derived amino acids from infant formula that are separated on a reversed-phase column is the following: Asp, Glu, internal standard, Ser, Gly, His, Tau, Arg, Thr, Ala, NH₃, Pro, internal standard, Tyr, Val, Met, Ile, Leu, Phe, reagent, Lys. The quantity of each amino acid in a peak is usually determined by spiking the sample with a known quantity of internal standard. The internal standard is usually an amino acid, such as norleucine, and not commonly found in a food product. This type of amino acid analysis is used to determine the amino acid composition of a protein, determine quantities of essential amino acids to evaluate protein quality, identify proteins based on the amino acid profile, detect odd amino acids, corroborate synthetic or recombinant protein structures.

Comparison of the derivatization methods used for the determination of amino acids by HPLC

A comparison of five aforementioned methods can be summarized based on the sensitivity, stability of the derivatives, the reaction time and the measurability of imino acids as follows:

- OPA, FMOC and DNS-CL derivatives can be detected by fluorescence methods, PITC derivatives can be detected in the ultraviolet range, while DABS-Cl derivatives in the visible range.
- OPA, FMOC and the DNS-Cl methods allow the detection of amino acids in the fmol range, DABS-Cl and PITC methods in the pmol range.
- The stability of FMOC and DABS-Cl derivatives are outstanding, that PITC is good, while OPA and DNS Cl is poor.
- The reaction with OPA and FMOC is rapid, the reaction with PITC has a medium speed, while the reaction with DABS-Cl and DNS-Cl is slow.
- With OPA method exception, each one is suitable for the detection and measurement of proline, hydroxyproline and cysteine.

5.7.13.2. Determination of D- and L-amino acids by using high performance liquid chromatography

According to earlier concepts D-amino acids may not occur in food and feed proteins, or only in traces. However, recent studies have shown up to microbiological degradation, or even due to technological interventions, the amount of D-amino acid of proteins in certain food and feed products can be significant, therefore, there is an increasing demand toward the
determination of these. D- and L-stereoisomer amino acids could not be separated by conventional methods (ion exchange column chromatography, high performance liquid chromatography), whereas the stereoisomer amino acids are same in all physical and chemical properties - except of rotating the plane of polarized light. The separation of amino acid enantiomers was initially performed by using the method of polarimetry, which was mainly used to study the racemisation of pure amino acids. Enzymatic techniques were also used to determine D- and L-amino acid. The essence of this method is in the enzymatic oxidation of D- or L-amino acids that is followed by determination of the remaining amino acids. These methods have the fault, as usually in the case of protease enzymatic methods, that they cannot be used for the determination of trace amounts of D-amino acids, and the L-amino acids which are derived from enzyme pollution that can also be a major source of error.

**Methods developed for the separation and determination of D- and L-amino acids**

The reaction of the optically active (chiral) amino acids with a chiral reagent results in **diastereoisomeric compounds**, which can be separated even on non-chiral columns. If the chiral reactant is another amino acid, it may be formed diastereoisomeric dipeptides, which can be separated by ion exchange column chromatography. The essence of this method is in the reaction between an L-amino acid N-carboxy anhydride or an active ester of one of the amino acids and the examined D- and L-amino acids. During this reaction **diastereoisomeric dipeptides** can be formed, which are suitable for the ion exchange separation. This is how L- and D-aspartic acid that was also routinely analyzed in the diastereoisomeric dipeptide form of L-Leu-D-Asp and L-Leu-L-Asp.

In addition to the method of separating D- and L-amino acids by high performance liquid chromatography, one of the best method is doing this with gas chromatography. Enantiomers may be are separated in the form of diastereoisomer couples that are created with a suitable asymmetric reagent, or the volatilized derivatives which have to be separated on an optically active stationary phase. Charles et al. used n-trifluoroacetyl (±)-2-n-alcohols to create diastereoisomers, and this method improved by Pollack et al. who used it for the derivatization (+)-2-n-butanol. The first optically active stationary phase that was used in gas chromatography, also considered to be very effective in the separation of optical isomers, was n-trifluoroacetyl-L-isoleucyl-lauryl ester, followed the use of N-lauryl-L-valyl-t-butylamide.
The techniques of gas chromatography developed to such a high perfection, that the error of enantiomer determination is less than 5%, and reproducibility is extremely good.

Today, high performance liquid chromatography is increasingly used for the separation and detection of enantiomers, at the expense of the aforementioned methods. Weinstein and Weiner created from amino acids the **fluorescent derivative 5-dimethyl-aminonaphthalene-1-sulfonyl**, and could separate all the D- and L- enantiomers by using reversed phase liquid chromatography, chiral packing of N, N'-di-n-propyl-L-alanine (L-DPA) and copper acetate. The method is sensitive and rapid, and it is well suitable for determination of amino acid enantiomers. Knabe and Gübitz developed one direct method to detect the optical purity of biologically active materials, by using high performance liquid chromatography, that was equipped with a chemically bounded L-hydroxyproline-Cu$^{2+}$ chiral column and Cu$^{2+}$ containing mobile phase. Using this stationary and mobile phase is possible to control the optical purity of all compounds, which form chelate complexes with Cu$^{2+}$-ions, such as amino acids. The fault of the method is that it can be used for the detection of D- and L- variant of only one amino acid at the same time.

Marfey also developed a method by using high-performance liquid chromatographic for the separation of amino acid enantiomers. Using **1-fluoro-2,4-dinitrophenyl-5-L-alanine amide**, which contains highly reactive fluorine atom, that were created diastereoisomeric derivatives from D- and L-amino acid mixtures. The formed derivatives could be separated and quantitatively determined with very good results by HPLC, and using the adequate gradient of acetonitrile and triethylamine aminphosphate eluents. He reports about the separation of D- and L-aspartic acid, glutamic acid, methionine, phenylalanine and alanine mixture, but, by modifying the conditions appropriately, is possible to separate other amino acid enantiomers too.

To quantitatively determine of amino acid enantiomers is not enough to separate the enantiomers, it must be taken care to the fact, that the enantiomers should be well separated from other amino acids or from the derivatives of these. Furthermore, to achieve an adequate sensitivity, it is necessary to create amino acid derivatives which are detectable even in small quantities. Recently, the **pre-column derivatization with fluorescent reagents** and the reverse phase chromatography of the derivatives are widely used for this purpose. The limit detection of this method is very small, and the flexibility of the analytical system is fraught with extraordinary benefits. After the derivatization with chiral reagent, is possible to separate the
protein building amino acids and to determine them by RP-HPLC. Since the chromatographic separation usually takes 50-70 minutes, it is very important that the developed analytical method is fully automatic. The simple derivatization reaction which takes place in a short time at room temperature is also a prerequisite.

Aswad made the first suggestion to use chiral thiols for the separation of amino acid enantiomers. He used OPA and N-acetyl-L-cysteine (NAC) to separate D- and L-aspartic acid. The derivatives can be separated in a copper-proline system too, during forming complexes with copper. In addition to NAC tert-butyloxy-carbonyl-L-cysteine has also been used for derivatization. Nimue et al. used the chiral 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-isocyanate (GITC) to separate D- and L-amino acids. They obtained a very good separation by the use of RP-HPLC, which allowed the separation and identification of the protein-building amino acid enantiomers in one step.

Einarsson et al. used the pre-column derivatization of amino acids in the OPA reaction that is commercially available thiol analogue of GITC, the 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (TATG). The aim of their experiment was to synthesize diastereoisomers, which can be easily separated and detected both with fluorescence detector and electrochemically.

Einarsson used the chiral 1-(9-fluorenyl) ethyl chloroformate (FLEC) to separate and to determine the amino acid enantiomers. This method has the advantage that not only forms a stable derivative with α-amino acids, but also with imino acids. Therefore, using this method both imino and amino acids can be determined, in fact is possible to selectively derivatize and detect in very small amounts of imino acids, even in the presence of high quantities of amino acids. During the separation and determination of enantiomers hydroxyproline, threonine and isoleucine, which contain two asymmetric centers, have to be specially mentioned. Among these, the isoleucine transformation has practical significance, where D-allo-isoleucine is formed. This behaves as the diastereoisomer of isoleucine, and appears between isoleucine and methionine in the chromatogram during the routinely applied ion exchange column chromatography, and giving a well separated and evaluable peak.

From the aforementioned methods, the fluorenyl ethyl chloroformate (FLEC) and ortho-phthalaldehyde and 2,3,4,6-tetra-O-acetyl-1-thio-D-β glucopiranozide (OPA/TATG)
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method, published by Einarsson et al., was applied practically (Csapó et al), and used for determination of D- and L- amino acid content of thousands of food and feed samples.

The separation and determination of amino acid enantiomers by RP-HPLC after derivatization with 1- (9-fluorenyl) with ethyl chloroformate (FLEC)

Two different methods were used during the process of derivatization. The first method was used both for the derivatization of both α-amino acids and imino acids, however, the second method was only used for the derivatization of imino acids. The selectivity is based on the reaction between the α-amino acids and OPA, which is followed by the derivatization of imino acids by using FLEC. OPA blocks the α-amino acids, and resulting in fact that FLEC will react only with imino acids. Due to the different fluorescence properties of OPA derivatives, the imino acids can be determined without interference from α-amino acids.

The selective detection of imino acid enantiomers includes three quick reactions. The blocking of amino acids takes place in reaction with OPA/mercaptoethanol, which paves the way for imino acid FLEC reaction. The role of iodoacetate are only removing the excess mercaptoethanol, and protecting the FLEC reagent from thiols. The first two reactions require less than a minute, and it takes less than five minutes until the reaction with FLEC proceeds fully.

To separate the proline and hydroxy-proline enantiomers, low pH mobile phase is required. The separation can be performed perfectly by a simple isocratic elution, and the hydroxyproline cis and trans isomer can also be separated under the same conditions, after the derivatization with optically inactive FMOC-Cl.

To measure the purity of the amino acid enantiomers and the ratio of racemization after the protein hydrolysis, is necessary that the enantiomers can be determined in a wide range of concentration. The detection of trace amounts of D-amino acids can be problematic due to possible overlaps, which may be a cause of the reagents or the impurities arising from other amino acids. It is highly preferable if both of the enantiomers of the derivatizing reagent can be used, because it increases the reliability of the identification of the peaks, whereas performing the derivatization with the other enantiomer of the reactant, and the elution order of the diastereoisomer derivatives changes. Another advantage of the use of both enantiomers of the reagent is the possibility to determine the retention time of the amino acid enantiomer which is not present in the sample, by performing the chromatography after the reaction which occurs
with the opposite folded reagent. The identification of the peaks is particularly of high importance in the food and feed analysis, since in this case many peaks appear on the chromatogram, then is very difficult to decide whether it is a D amino acid that present in low concentrations or something else. If there is a change in the elution order, we can be sure of the presence of D-amino acid, if there is no change in this order, then most likely it is not D-amino acid.

The separation and determination of amino acid enantiomers by RP-HPLC after derivatization with o-phthalaldehyde and 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (OPA/TATG)

During the derivatization the reaction between amino acids and OPA/TATG occurs in a similar manner as that has been seen in the case of reactions between OPA/mercaptoethanol and amino acids. Studying the reaction time of L-alanine, L-lysine, L-methionine, L-aspartic acid, L-histidine, L-serine and D-/L-threonine can be concluded the reaction that occurs in less than three minutes except in the case of threonine. However, in the case of threonine at least six minutes is required for the occurrence of the perfect reaction. The quick reaction between the OPA and the high-molecular weight TATG as well as the amino acids, supports the observation, that increase in weight does not necessarily decrease the reaction rate. During the use of OPA reagent for amino acid analysis, will arises the serious problem that the derivatives are unstable, whereas the OPA/TATG derivatives have been proved are quite stable. Alanine, glycine and serine derivatives showed no decrease in their fluorescence, even one and half hours after the reaction that took place. After 10 hours of storage at room temperature, the fluorescence of 68% in the case of lysine and 37% for ornithine were measured (Figure 70).

In most of the applications, we are interested in only a few amino acids and their D- and L-enantiomers, and there is no need to separate and determine all of the protein building amino acid enantiomers. Therefore, by modifying the pH and the ionic strength of the mobile phase, or by the use of other organic solvents, or by varying the analytical column size and charge, it is possible to separate and to determine each of the protein building amino acid enantiomers. Of course, this should always be done after the system that is optimized on the examined amino acid. In the followings there is shown such an optimization on the separation of aspartic and glutamic acid, as well as alanine enantiomers (Figure 71).
Figure 71. Separation of amino acid enantiomers after derivatization with OPA/TATG

Figure 72 Detection of trace amounts of D-amino acids in the presence of high amounts of L-amino acids
In food proteins the amount of **glutamic acid** can reach 20-30% of the proteins, and **aspartic acid** can also reach a rate of 8-10%. From the D-enantiomer of these two amino acids, it is possible to take conclusions on the amount of all other amino acid D-enantiomers, as if cannot be detected D-glutamic acid and D-aspartic acid in the sample, we can almost be sure that in case of a natural proteins the quantity of all other D-amino acids are negligibly small. On the other hand the amount of D-alanine is of interest, because **D-alanine (together with D-aspartic acid and D-glutamic acid) is the marker of bacterial protein synthesis**, since the bacterial cell wall constituting peptidoglycans contain as a significant amounts of these three D-amino acids. The chromatogram shows that these examined six amino acids separate from each other and the other enantiomers are quite highly satisfactory, and it can also be seen, that the separation and determination of D-amino acids is performable by using the conventional fluorescence detection methods even at 20-fold amount of L-amino acids. The limit detection of D-amino acids in this defined system is between 0.2-0.5 µmol, although in the case of D-aspartic acid and D-glutamic acid this concentration is lower than 0.1 µmol, in the case of lysine it is around 2 µmol.

Comparing the FLEC and the OPA/TATG method and regarding the separation and determination of amino acid enantiomers following results can be concluded:

- Both methods are very suitable for the separation and determination of amino acid enantiomers, because none of them presents significant racemization during the derivatization process.

- The FLEC method might be more suitable in the case of a greater sample quantity, and when there is no particular interest in the enantiomers of aspartic acid. The FLEC method is extremely useful if there is interest to separate the enantiomers of basic amino acids, in particular lysine enantiomers.

- The OPA/TATG method is preferable where there is the need to determine the aspartic acid enantiomers, or if there are very small quantities of material available, or the sample contains many minerals. The FLEC method is very sensitive to minerals, and the resulting precipitate thwarts the derivatization reaction and the analysis itself.

- A great advantage of the FLEC method is suitable for the selective derivatization of imino acids. By using this method L-proline, D-proline and trans-L-hydroxyproline, cis-L-hydroxyproline, trans-D-hydroxy-proline and cis-D-hydroxyproline are perfectly separable.
During the determination of amino acids there is also an advantage that by the use of (+)FLEC and (-)FLEC the security of identifying the peaks increases due to the changed elution order, and the retention time of the enantiomer are not present in the sample which can also be determined.

As a final summary, it can be concluded that there is hardly any amino acid analysis task in the field of amino acid enantiomer separation, which after proper optimization of the system could not be resolved by derivatization either with FLEC or OPA/TATG.

5.7.14. Mycotoxin analysis

Moulds can develop on food commodities and produce various types of chemical toxins, collectively known as mycotoxins. The main producers of mycotoxins are the genera Aspergillus, Fusarium, and Penicillin. Crops can be directly infected with fungal growth and subsequent mycotoxin contamination as a result of environmental factors temperature, humidity, weather fluctuations, mechanical damage of kernels, and pest attack. More than 300 mycotoxins, belonging to various chemical classes, are known. The major classes of mycotoxin with a toxicological impact on human health include aflatoxins (B1, B2, M1, M2, G1, and G2), ochratoxins (ochratoxin A, OTA), trichothecenes [deoxynivalenol (DON), T2, and HT-2], fumonisins (FBs, FB1, FB2, FB3), patulin (a mycotoxin that occurs mainly in apples and apple products), zearalenone (ZEA).

5.7.14.1. Detection and determination of mycotoxins

Sample preparation commonly includes extraction, clean up, and concentration. Conventional TLC techniques are commonly used for screening purposes, with detection limits reaching 2 ng/g. When results are positive, confirmatory and more sensitive quantitative analysis follow.

5.7.14.2. Quantitative and confirmative chemical methods

For quantitative determination, HPLC is the methodology of choice for most mycotoxins, specifically, aflatoxins, DON, OTA, ZEA, FBs, and patulin. Pre- or postcolumn derivatization is required for the fluorescence detection of aflatoxins OTA, ZEA, and FBs, and direct UV detection is used for DON and patulin. Reversed-phase chromatographic separation is normally employed for multimycotoxin analysis. Coupling of HPLC with MS analysis, especially LC-
MS/MS, provides greater sensitivity and selectivity and allows for simultaneous analysis of multiclass mycotoxins.

Gas chromatography is not widely used for the detection of mycotoxins, except in the case of trichothecenes. Capillary column GC is commonly employed for the simultaneous detection of different trichothecenes, DON, T2, and HT-2, by using trifluoroacetyl, heptafluorobutyryl, or trimethylsilyl derivatization that are coupled with electron capture detection. GC is often linked to MS for peak confirmation.

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