

CHROMATEC ANALYTIC SOFTWARE

BUILD 4.0

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1 INTRODUCTION

Chromatography is a physicochemical method to separate the components of analyzed sample. This method is based on the difference between the coefficients of their distribution between the two phases: stationary and mobile.

Carrier-gas is used in gas chromatography as a mobile phase. Stationary phase can be both solid (absorbent) and liquid (in the form of a film applied on the carrier surface).

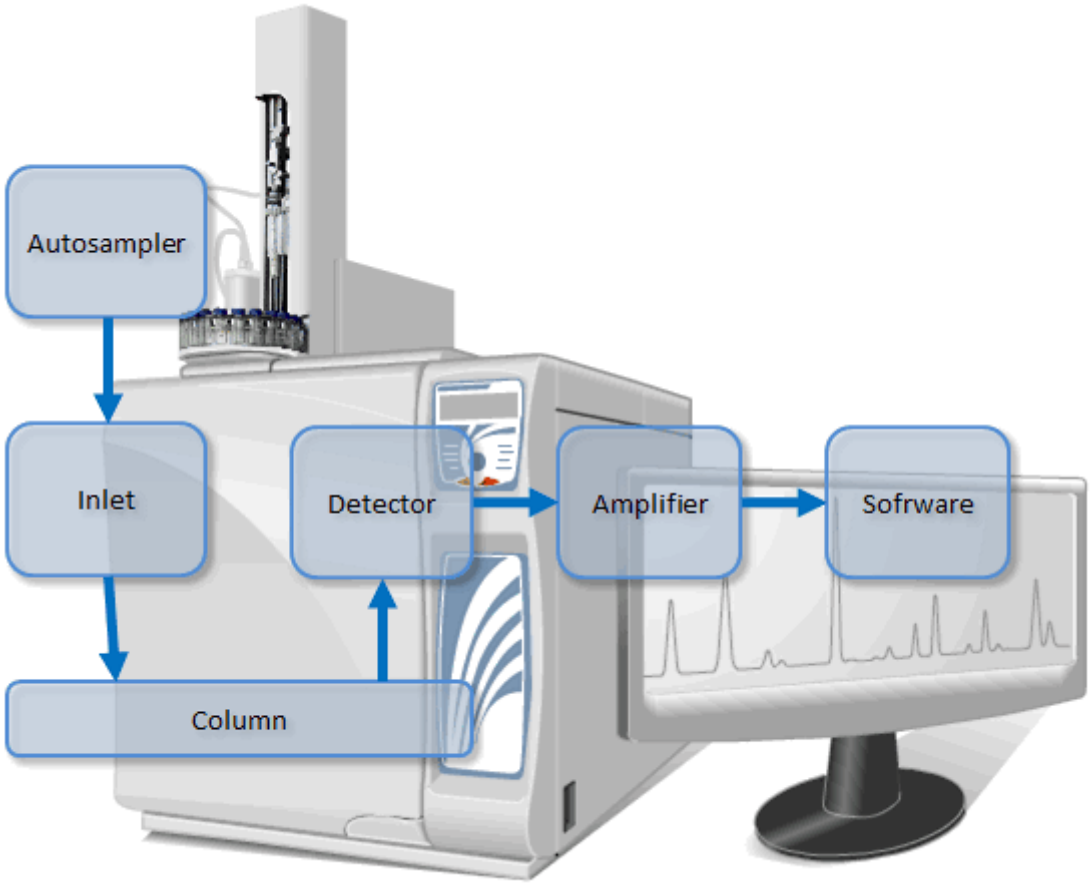


Figure 1 – Schematic diagram of a chromatograph

Sampling device supplies a certain quantity of analyzed sample in the gaseous state into the carrier-gas flow right before it enters the column.

The mixture is separated into components inside chromatographic column by sorption and desorption of stationary substances. Thus, poorly sorptive substances are carried around the column by mobile phase with higher speed or vice versa.

Sample components separated inside the column find their way to detector. The detector registers the substance that differs from carrier-gas by peculiar physical or physicochemical characteristics and converts the resulted changes into an electrical signal. Then, there is an amplification and analog-to-digital conversion of the received signal. The recording device (PC or self-recording device) builds the detector signal/time dependency diagram called chromatogram.

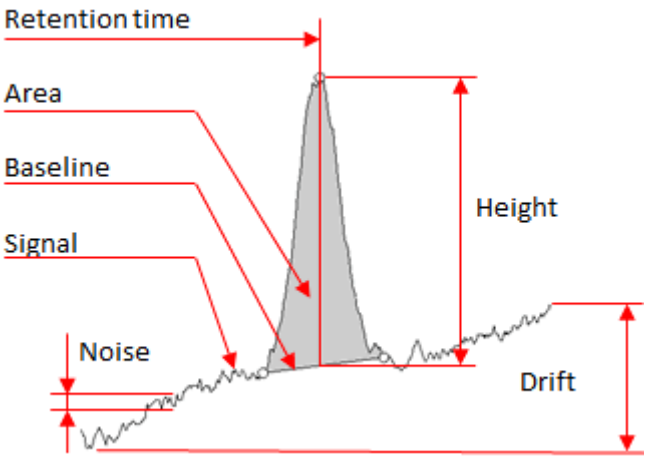


Figure 2 – Structure of a chromatogram

Carrier-gas passing through the detector with no sample injected is captured on chromatogram as background detector signal called baseline. The baseline is characterized with high-frequency vibrations – noise. Detector baseline signal variation in time is called drift.

When passing through the detector of analyzed sample detector signal level deviates from the baseline. This deviation forms a peak on the chromatogram. Chromatogram peak has the following characteristics:

Retention time. Time from analysis starts till the maximum peak. Retention time is a qualitative characteristic of analyzed sample, area and height – quantitative characteristics.

Area. The field bounded by peak profile and baseline.

Height. Distance from the peak top to the baseline.

Chromatogram processing involves several sequential steps.

Noise filtration. Chromatogram baseline smoothing with the purpose to increase the stability of automatic processing.

Noise filtration is normally applied in exceptional cases, for example, in the case of chromatograph outliers. In most of the cases, this operation is not necessary.

Peak integration. Definition of peak baseline and peak parameter measurement (retention time, area, height).

Peak identification. Assignment of the peaks on chromatograph to the particular component from the retention parameters table.

Calibration. Sample analyses with a known content of analyzed sample in order to calculate the response factors for these components.

In cases when calibration coefficients are known beforehand calibration is not performed. Thus, the known coefficients are listed in the component table.

Quantitative calculation. Final stage of quantitative analysis calculating the component concentration in analyzed sample.

Thus, the chromatogram (depending on the purpose) can be used for device calibration or concentration calculation of a sample with unknown components.

1.1 Peak integration

Peak integration is an operation designed to calculate parameters on the recorded chromatogram. Thus, the peaks are limited by the baseline (a straight-line connecting start and end points of the baseline peak).

Basing on these patterns you should estimate Retention time, Area and Height.

In some cases, you should set the peak width measured at the peak base and coincide with the baseline length. Peak width at half height can also be used (for example, to calculate column efficiency).

The correctness of peak integration has a sizable effect on the accuracy of the quantitative analysis result.

This program uses an automatic peak detection algorithm based on the first derivative (slope) of the chromatographic curve with integration events mechanism.

Keep in mind that neither of algorithms can (irregular baseline shape, poor peak separation, small "rider" peaks, high noise level) guarantee the correct automatic peak identification since the notion of "peak" itself is largely subjective and depends on the specific current issue. However, the accuracy of results often depends on operator experience. Therefore, automatic peak detection procedure must contain as much prior information as possible in order for chromatogram layout to be done according to operator's views.

Narrow peaks are typical for capillary columns, wide peaks – for packed columns. Therefore, various integration parameter values are used for peak identification of components split into capillary and packed columns.

To achieve the desired result, you need to vary integration parameters.

1.2 Peak identification

Identification is peak assignment to a particular component from the list. Thus, you should compare the calculated retention parameters of all the peaks detected on chromatogram with information stored in the component table.

Identification in "Chromatec Analytic 3.0" program is done according to the following algorithm:

1. Create a list of all the possible pairs (peak – component), for the peaks within the component search box. Thus, one peak in the list can refer to several components and vice versa.
2. This list should be sorted by meeting the criteria of peak selection from the component box. Sorting criteria: proximity on time and index scale.
3. Identify the reference peaks from the sorted pair list. The component is considered identified if there are confirming components with the similar name inside the pair list. If there are no respective peaks for confirming components identification is not performed. After identification of reference peaks is done the expected retention parameters (time, index) are recalculated for common and other types of peaks.
4. Identification process of common peaks is similar to step 3.

Identification by retention time

The simplest identification method is comparing the retention time of analyzed sample with the retention time of known compound with strictly defined analysis parameters. To identify the peak by its retention time the following fields are used in the component table:

- component name;
- retention time;
- time search box, %.

Search box – boundary of an area for peak detection with both positive and negative deviation from the retention parameter specified in the table.

The component with target retention time close to the real peak time is assigned to the peak.

Identification by reference peaks

Specify the reference peaks when identifying the retention time. For example, in the case of simultaneous retention time shift of all components, reference peaks can help to define the substances correctly regardless their retention time falling out of the time search box. Standalone or wide peaks are usually chosen as reference peaks. These peaks are identified as a reference in the component table and the advanced search box is assigned to them (2–5 %). In this case, identification is done in the following way:

- Search for reference peaks by retention time.
- Calculate the difference between the actual and target retention time of reference peaks and construct a linear function displaying the retention time offset.
- For common (not reference) peaks calculate the expected retention time referring to the retention time offset given in the component and function table.

Identification by retention indexes

Moreover, relative retention parameters less dependent (unlike retention time) on analysis conditions can be used for identification. One of such parameters is retention index – non-dimensional value characterizing the peak position on chromatogram with reference to peaks of selected standards. In case n-paraffin is used as standards retention index is called Kovats retention index. Index type (linear or logarithmic) depends on analysis conditions. Logarithmic dependence is typical for constant column temperature during the analysis, linear is typical for programming. However, these two extreme cases have no distinct difference between them.

To perform identification by retention indexes, add target component indexes and index search box into component table. In the component table assigns the “reference” type for reference peaks and set the advance time search box (2–5 %).

There should be two reference peaks, preferably appeared before and after analyzed sample. In case n-paraffin is used as reference peaks select the closest ones in time with regard to analyzed sample. If there are more than two reference peaks in the component table then during identification select those two (before and after the component) that are closer to the concerned substance as of time.

Identification by retention indexes is performed in the following way:

- Identify the reference peaks by retention time.
- Assign the respective indexes from component table to reference peaks.

- Using preset retention indexes of reference peaks calculate the retention indexes of common peaks and compare with table data.

Linear retention index:

$$l_i = l_n + \frac{(l_{n+1} - l_n)(t_i - t_n)}{(t_{n+1} - t_n)}$$

Logarithmic retention index:

$$l_i = l_n + \frac{(l_{n+1} - l_n)(\log(t'_i) - \log(t'_n))}{(\log(t'_{n+1}) - \log(t'_n))}$$

Where:

I_i – retention index of concerned peak,

I_n, I_{n+1} – indexes of the previous and following components with the known index value.

t_i – retention time of concerned peak.

t_n, t_{n+1} – peak retention time values corresponding to the previous and the following components with known indexes.

t' – normalized retention time.

Normalized retention time is equal to the difference of absolute retention time and hold-up time (unretained component residence time in chromatographic system). Hold-up time is defined in practice. In case of hold-up time is not preset the program takes first peak retention time instead of it.

If component has more than one retention parameter identification is done according to scale priority (time, linear index, logarithmic index) defined by an operator for the whole chromatogram. If reference peaks are not preset – identification of all peaks is done only by retention time.

Several peaks can fall into expected time screen or one component retention screen. In this case peak selection (depending on the settings) is done according to the following criteria:

- nearest to the expected retention time or index;
- with maximum height;
- with maximum area.

The same peak can fall into different component search boxes; in this case, it will be associated with a component of minor retention time.

In the general case, there can be the following peak identification scheme:

- reference components – with a maximum height,
- remaining components – nearest in time or index.

For more precise time adjustment and retention index calculation, it is recommended to select at least two reference components one in the beginning of chromatogram and one closer to the end.

The specified component recognition scheme is universal and flexible enough to avoid any problems with incorrect recognition in most of the cases. The only thing an operator has to do is to select the correct reference peaks at the stage of settings and then occasionally adjust their expected retention time on the current chromatogram.

1.3 Calibration

Calibration is performed in order to get the calibration dependence showing the connection between detector response value (peak area or height) and a number of components of a sample. Generally, component calibration dependence is as follows:

$$Q(R) = K_2R^2 + K_1R + K_0$$

Where:

Q(R) – component quantity in a sample.

R – detector response.

K_2, K_1, K_0 – calibration component coefficients (coefficients of detector response factor to component).

To plot the calibration dependence, perform the analysis of one or several samples with a known content of analyzed samples.

Component concentration in calibration mixtures theoretically should cover the whole range of measurable concentrations. To achieve the consistent results the analysis of each calibration mixture is done at least twice.

Basing on the achieved results plot the calibration dependence curve of peak response from concentration.

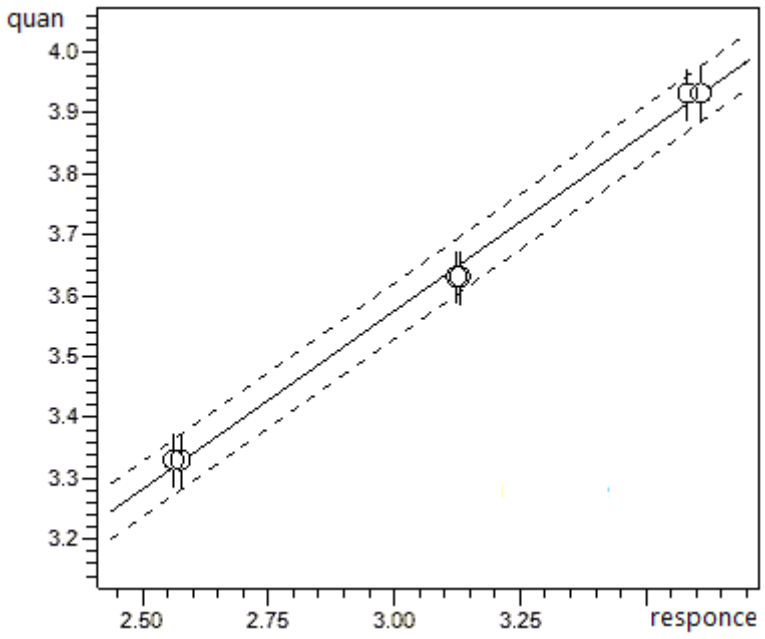


Figure 3 – The calibration graph

In the case of multipoint calibration, the dependence can be approximated to any type of curve, not necessarily linear one. Calibration coefficients are calculated using the least square method for a curve most accurate for experimental data description. The analyst selects the type of a curve for calculation.

If calibration dependence is represented by a straight line passing through the origin of coordinates $Q(R) = K_1R$, this dependence can be built at one point (using one calibration mixture with known component concentrations).

The choice of response (area or height) to build the calibration dependence curve is not always clear. In most cases, the area is a more objective factor, however, height reproducibility also shows positive results. Common patterns are as follows:

- If the peaks are narrow and high you can measure their height more precise than area.
- In the case of unsymmetrical peaks, height-based calculation is invalid.
- Peak areas are more resistant to analysis condition fluctuation.

Two dependence curves (for height and area) are parallelly built in "Chromatec Analytic" program. Thus, having performed the calibration you can evaluate the reproducibility of results for each curve and select the response best suited to analysis conditions.

When applying absolute calibration method, the component substance amount is calculated using the following formula:

$$Q = C \times V$$

Where:

- C – component concentration.
- V – injected sample volume.

When applying the internal standard calibration method curves are built on the same axis, but component substance amount is defined using the following formula:

$$Q = \frac{CW_{is}(R_{is})}{C_{is}}$$

Where:

$W_{is}(R_{is})$ – estimated amount of internal standard.

C_{is} – known concentration of internal standard within the mixture.

Herewith, the *K1* value is 1 for internal standard, while the calculated K1 for the component is represented with a relative coefficient of detector response factor to an internal standard.

In some calculation methods, for example, internal normalization method, detector response relative coefficients known in advance are used quite often. In this case, you can select the view function $Q(R) = K_1R$ without calibration measurements and manually set the K_1 coefficient value.

1.4 Quantitative calculation

The quantitative calculation is the final stage of quantitative analysis, which calculates component concentrations in a sample with known analyzed sample content. When performing the quantitative calculation change the analyzed sample response and calculate its concentration basing on the current calibration dependence.

The following quantitative calculation methods are the most frequently used:

1.4.1 Percent normalization method

According to this method, the sum of all peak areas and heights on chromatogram are taken for 100 %.

This method does not require any predetermined calibration and suggests a number of conditions:

- all the components of analyzed sample are eluted from a column;
- all the components have similar detector response factor coefficient. Concentrations are calculated by the following formula:

$$C_i^{\%} = \frac{R_i}{\sum R}$$

Where:

R_i – peak response (area or height)

$\sum R$ – the sum of all peaks in chromatogram channel.

1.4.2 Internal normalization method

Internal normalization method registers relative coefficients of detector response factor to different components of analyzed sample known in advance. The response of each component is recalculated taking account of these coefficients. The sum of responses received is taken for a value equal to normalization coefficient. In case all substances of analyzed sample are detected normalization coefficient is equal to 100 %.

Otherwise, define the content of undetected substances using the other methods and reduce the normalization coefficient by the value of undetected components. Internal normalization method is invalid if off-scale peaks appear on the chromatogram.

Substance concentration is calculated by the following formula:

$$C_i^{\%} = \frac{NM_iW(R_i)}{\sum\left(MW(R)\right)}$$

Where:

N – normalization coefficient.

W(R) – component calibration dependence built in advance.

M – multiplier.

In the simplest case, calibration dependence is as follows:

$$W(R) = K_1 R$$

Where:

K_1 – a relative coefficient of detector response factor to the component.

The formula has the following form:

$$C_i\% = \frac{NK_{1i}R_iM_i}{\sum(K_iRM)}$$

1.4.3 Method of internal normalization for multi-channel chromatograms

When applying the internal normalization method, it is sometimes necessary to summarize the components detected in different channels. To do so you need to bring the channels to common response factor scale.

This is done by calculating the correction factors for each of summarized channels. These components are automatically calculated by a program using correction components detected simultaneously on 2 or more channels. The formula to calculate the component concentration by internal normalization method used for multi-channel chromatograms is as follows:

$$C_i\% = \frac{NW_i(R_i)K_nM_i}{\sum(W(R)K_nM)}$$

Where:

K_n – correction factor for a channel involved in the calculation of component concentration.

1.4.4 Absolute calibration method

In absolute calibration method, the component quantity in a sample is defined by calibration dependence received in advance. The most important requirements when performing quantitative analysis by absolute calibration method are sampling accuracy along with strict observance of chromatographic conditions during calibration and analyzed sample determination.

You can define the substance quantity by calibration dependence and adjust it with an account for multiplier and dilution:

$$Q_i = W_i(R_i)M_iD$$

Component concentration equals to the relation of component quantity to injected sample volume:

$$C_i = \frac{W_i(R_i)M_iD}{V}$$

Where:

V – injected sample volume.

W(R) – component calibration dependence built in advance.

M – multiplier.

D – dilution.

1.4.5 External standard method

When applying the external standard method first define calibration dependence (by absolute calibration method) for component taken for external standard. For other components use relative coefficients (known in advance and defined in practice) of detector response factor to external standard. Use the following formula to calculate the substance concentration by external standard method:

$$C_i = \frac{W_s(K_{1s}R_i)M_iD}{V}$$

Where:

K_{1i} – sensitivity factor i-th component given detector relative to an external standard.

$W_s(R)$ – calibration curve for external standard.

M – multiplier.

D – dilution.

1.4.6 Internal standard method

Internal standard method is based on adding a known quantity of certain substance (called internal standard) to analyzed samples. If relative coefficients of detector response factor to internal standard components are unknown perform the calibration using a mixture with known analyzed sample content and internal standard.

Apply either absolute calibration method or internal standard method to define the calibration dependence. The following factors determine the choice of calculation method for calibration coefficients:

- Absolute coefficients are used for quantitative calculation both by absolute calibration method and internal standard method. However, precise sample injection is required in this case.
- When calculating coefficients by internal standard method precise sample injection is not required while carrier-gas speed and column temperature variations are not influential. However, only linear dependence can be used in this case.

When performing the quantitative analysis of unknown sample add certain amount of internal standard to this sample and calculate the analyzed sample content according to the dependence built in advance. Take account of multiplier and delution factor in this calculation:

$$Q_i = \frac{Q_{is} W_i(R_i) M_i D}{W_{is}(R_{is})}$$

or

$$Q_i = \frac{Q_{is} W_i(R_i) M_i D}{W_{is}(R_{is})}$$

Where:

$W_s(R)$ – calibration curve for external standard.

M – multiplier.

D – dilution.

Note that in this formula Cis – internal standard concentration in analysis, it should not be recalculated with account of multiplier.