Chromatographic profiling: From samples to information

Peters, S.

Citation for published version (APA):
Peters, S. (2013). Chromatographic profiling: From samples to information
Chapter 8

Development of a resolution metric in comprehensive two-dimensional chromatography

This chapter has been published as:

Summary

A new resolution metric for two-dimensional chromatography is proposed and tested. This resolution measurement is based on the concept of the (one-dimensional) valley-to-peak ratio, which has been adapted and modified for two-dimensional chromatography. Two questions are considered related to the computation of the resolution of a given (two-dimensional) peak. First, the concept of peak neighbourhood is revised, since it changes drastically from one- to two-dimensional chromatography. In a chromatogram resulting from a two-dimensional analysis, one peak may be surrounded by more than two neighbouring peaks. However, the neighbouring peaks can be remote from the peak or some interfering peaks may be in between. In these cases, it is not meaningful to compute the resolution between them. A method is proposed to determine whether a resolution measurement between two two-dimensional peaks is reasonable. Second, a measurement of the valley-to-peak ratio in two-dimensional chromatography is proposed. The measurement is based on the concept of the saddle point (which is defined for two-dimensional surface plots). A study of the correlation of the valley-to-peak ratio with the error obtained for quantification is presented. The new metric can be used as an estimator of the quantification errors. Also, valley-to-peak ratios can be calculated for one or more target peak(s) to estimate the separation quality of the entire chromatogram. This makes the proposed measurement suitable for optimisation purposes.

Although the concept was developed for two-dimensional gas chromatography, its application to other two-dimensional separation methods should only require minor modifications – if any.


8.1 Introduction

The use of comprehensive two-dimensional chromatography for the analysis of complex mixtures has greatly increased during the last decade. Improvements in instrumentation and applications have made this set of techniques a powerful alternative to one-dimensional chromatography, especially when high peak capacities are needed [1]. High peak capacities are desirable for the analysis of complex samples, especially for chemical fingerprinting [2]. In comprehensive two-dimensional gas chromatography (GC×GC), two columns are connected in series, which separate the sample according to different properties of the analytes. The interface between the two columns is most commonly a cryotrapping modulator that traps the effluent of the first column during a given modulation period, focuses it, and then injects it as a narrow band into the second column. During the elution of a compound (peak) from the first dimension, several fast analyses are performed in the second dimension. The resulting chromatogram is usually presented as a three-dimensional contour or colour plot (first-dimension retention time, $t_{1R}$, second-dimension retention time, $t_{2R}$, and intensity). If the two columns are orthogonal, i.e. if the retention mechanisms of both columns are completely independent, the overall peak capacity of the system is obtained by the multiplication of the peak capacities of both columns: $n_c \times n_c$ [1,3,4,5]. Nevertheless, the probability of co-elution of two or more peaks is still quite high, especially if very complex samples are analysed, and if the neighbouring peaks have similar physical-chemical properties that cannot be readily differentiated by the chosen dimensions. Therefore, a resolution measurement in two-dimensional chromatography is still of significant interest.

Comprehensive two-dimensional separations require a redefinition of conventional one-dimensional interpretation criteria. We will take the resolution concept existing for one-dimensional chromatography as a starting point and adapt it for
two-dimensional (2D) chromatography. This is logical, since 2D chromatography can also be viewed as an evolution of one-dimensional (1D) chromatography.

Prior to developing the concept of resolution in two-dimensional chromatography, the concept of "peak vicinity" has to be revised. For one-dimensional chromatography, the resolution measurement is defined for any target peak and its neighbouring peak. Obviously, only two peaks (before and after the target peak) can be defined as "neighbours" of the target peak. In two-dimensional chromatography, and especially in the presentation of data in a 2D plane, it is obvious that peaks may be located anywhere around a given peak and that any number of neighbouring peaks may lie in any direction. In a complex GC×GC chromatogram comprising tens, hundreds, or even thousands of peaks, a significant proportion of them may be potential "neighbours" of the target peak. Criteria have to be developed to decide whether a resolution measurement between a given peak pair is of interest from the chromatographic viewpoint, for example when a third peak is (partially) located between the peak pair. An objective measure is required to decide whether the interference of the third peak renders the computation of the valley-to-peak ratio of the first two peaks obsolete.

There is scant information available in the literature on resolution measurements in two-dimensional chromatography. This is not surprising, since practical instrumental methods for column-based comprehensive two-dimensional chromatography were not available in the past. Existing resolution measurements can be divided into two groups, namely i) measurements evolved from the classical one-dimensional resolution concepts and ii) metrics derived from multivariate studies.

The first group of definitions tries to follow the chromatographer's intuition by projecting peaks to one dimension and applying conventional (1D) resolution measurements. Giddings et al. [6] developed a resolution function for two-
dimensional separations that was extended in 1996 by Schure [7]. Other groups that worked on similar approaches include Davis [8], and Shi and Davis [9].

The second class of definitions tries to find a resolution measurement that predicts the error when multivariate techniques are applied to the two-dimensional data format. These definitions are based on the concept of selectivity of second-order instruments [10]. The definitions of Messick and Kalivas [11] and of Ho et al. [12] constitute examples of two-dimensional resolution measurements that can be applied to two-dimensional chromatography. Synovec et al. [13] demonstrated that Ho’s definition of resolution can be used as an estimator of the errors obtained when the generalized rank-annihilation method (GRAM) is used to deconvolve (partially) overlapping peaks. Some caution has to be observed when applying some of these measurements. With Ho’s resolution definition, for example, peaks that are completely separated in one dimension, but are completely overlapped in the other yield a resolution value of 0 (even if the two peaks appear on the two-dimensional map as two well-separated spots). This is in accordance with the performance of GRAM, which fails in this case. However, it is not reasonable from a chromatographic viewpoint.

Except Schure’s definitions, all resolution concepts require knowledge of the individual profiles of the (partially overlapping) peaks. This information is, however, not available for a two-dimensional separation, unless the individual peaks are injected separately or deconvolution procedures are applied. Alternatively, theoretical models may be used to predict the peak shape, but this decreases the practical applicability of the resolution measurements.

The scope of this work is to develop an algorithm for an easily-accessible resolution measurement that works with the raw experimental data acquired by the instrument. The first author proposing a valley-to-peak ratio measurement in two-dimensional chromatography was Schure [7]. However, his definition can only be applied to Gaussian-shaped peaks. In the present work, a general valley-to-peak-ratio measurement is proposed that is applicable to peaks of any shape.
The valley-to-peak ratio can be used for different purposes. In some cases, the valley-to-peak ratios for one target peak to all its surrounding peaks may be of interest. If the aim of the analysis is to get a single value representative of the separation of the whole chromatogram, the peak pair resulting in the worst resolution may be considered. This will especially be of interest when optimisation procedures are to be performed.

The calculated valley-to-peak ratios can be extended to an estimation of the error of quantification. Quantification of peaks in two-dimensional chromatography has been adequately described in the literature [14-16] and no further details will be given here. Here, peak quantification is based on the work presented in Chapter 4. The algorithm was developed and tested for real samples in two-dimensional gas chromatography. Advantages and drawbacks of the method are discussed. Although the algorithm was developed for GC×GC, its application to comprehensive two-dimensional liquid chromatography (LC×LC) data should only require minor modifications.

8.2 Theory

8.2.1 Peak detection in two-dimensional chromatography: peak regions and trajectory profiles

Prior to a resolution measurement in 2D chromatography, a chromatographic peak in the two-dimensional plane has to be defined. A peak eluting from the first-dimension (1D) column is sampled several times into the second-dimension (2D) column. Khummueng et al. [17] introduced the concept of the modulation ratio to describe the number of cuts required per first-dimensional peak. They come to the conclusion that three to four cuts are needed. This is in accordance with other authors [18,19].

A peak detection algorithm for 2D chromatography has been described in Chapter 4. Basically, this algorithm is a two-step process. First, the individual peaks are
detected, comparable to peak detection in 1D chromatography. Second, a decision tree is applied in order to decide whether these individual peaks have to be "merged" because they originate from the same compound (i.e. as sub-samples from the same peak). Some features of this approach are needed to illustrate the concept of resolution and these are described below.

Fig. 8.1A shows the result of the peak detection-algorithm when applied to a 2D gas chromatogram of an air-freshener sample. The one-dimensional (1D) peaks detected in the “raw” second-dimension chromatograms are depicted as pink dots. If two or more 1D peaks are found to be originating from the same compound, they are merged to form a 2D peak. This is represented by pink (“merging”) lines connecting the corresponding 1D peaks.

![Figure 8.1](image)

**Figure 8.1** (A) Region of interest of the GC×GC chromatogram of the air-freshener sample. The detected 1D peaks are depicted as pink dots. They are connected by pink lines if they form a "merged" 2D peak. The peak region of 2D peak 2 is depicted as white rectangles. Part (B) shows the (partially interpolated) intensity profile along the white, dotted line connecting peak 3 and 4.

In Fig. 8.1A, the "peak regions" of 2D peak 2 are depicted. The concept of a peak region is equivalent to the peak width in 1D chromatography. In this case, however, it is illustrated as a collection of boxes (see Fig. 4.2 for more details). Each box represents the peak width of a given 1D chromatogram. The edges of all boxes that belong to individual peaks that have been merged constitute the peak region of the 2D peak. This will be used to illustrate the concept of peak vicinity (next section).
In Chapter 4, the "peak-maxima profile" was introduced to describe the peak merging algorithm. This is obtained when plotting the trajectory along all maxima belonging to one 2D peak. If one were to monitor the signal of the chromatographic detector along the pink line, the "peak-maxima profile" of a given 2D peak is obtained. Because the number of data points available in the first dimension is limited (due to the low number of modulation cycles that sample the first-dimension peak), an interpolation method may be used. This takes advantage of the greater information (much higher number of data points) obtained in the second dimension to complement the information of the peak-maxima profile. The "peak-maxima profile" can be extended to represent the signal along any given trajectory between two points A and B located in the 2D chromatogram. Fig. 8.1B depicts the interpolated “trajectory profile” between peak 3 and 4 as shown in Fig. 8.1A (white dotted line).

8.2.2 Peak vicinity

In 1D chromatography, only two peaks can be considered "neighbours" of a target peak (the peaks eluting immediately before and after). This does not hold for a 2D chromatogram. In that case, more than two peaks can be neighbours of a target peak. However, a resolution measurement between two peaks is only meaningful if no other peaks are located between the two peaks of interest. Implicitly, this provides a definition of what constitutes neighbouring peaks. Several criteria have been developed that define what constitutes peak neighbours. The examination of the peak vicinity is described by the peak-region test.

Consider two 2D peaks for which peak vicinities are to be tested. The goal is to establish whether a computation of the resolution between these two peaks is sensible. The peak-region test evaluates whether peak regions (defined in Section 8.2.1.) of third peaks are located between the two peaks. These third peaks are called interferents or interfering peaks. To this end, the non-interpolated trajectory profiles (see Section 8.2.1.) along all the connecting lines between the various 1D
peak maxima of the two target peaks are examined. Four situations can be distinguished, as illustrated in Figs. 8.2A to C and Fig. 8.3.

**Figure 8.2** Trajectory profiles (dashed lines) and peak regions (white boxes) of some peaks depicted in Fig. 8.1A. For more details, see text (Section 8.2.2).
Figure 8.3 Schematic representation of three 2D peaks (black solid dots) to highlight a feature of the peak-region test (Section 8.2.2). The peak region of peak 2 is depicted as black rectangles. The black empty dots represent non-interpolated points on the trajectory profile connecting peak 1 and 3.

Figs. 8.2A to C are taken from a chromatogram of an air-freshener sample (see Section 8.3.1 for sample description), whereas Fig. 8.3 depicts a simulated situation. In all these figures, the target peak is peak 3 and its vicinity is examined. In Fig. 8.2A, the vicinity of 2D peak 3 is tested against 2D peak 2. All connections between the peak maxima of both 2D peaks are depicted as pink dotted lines. In this case, no line crosses the peak region of another 2D peak and, therefore, 2D peaks 2 and 3 are considered "neighbours".

In Fig. 8.2B, the vicinity of 2D peaks 3 and 1 is tested. Some of the trajectory profiles (pink dotted lines) connecting the peak maxima of peaks 1 and 3 pass through the peak region of 2D peak 2. However, because not all lines pass through the peak region of the interfering peak 2D peaks 3 and 1 are considered "neighbours".

In Fig. 8.2C, the vicinity between 2D peaks 3 and 5 is tested. All trajectory profiles connecting the peak maxima of peaks 3 and 5 pass through the peak
region of peak 4. Therefore 2D peaks 3 and 5 are not considered "neighbours" and computing the resolution between peaks 3 and 5 is not considered meaningful.

There may be cases in which another 2D peak is not recognised as interfering when two peaks are connected. Consider the case depicted schematically in Fig. 8.3, in which the peak vicinity of 2D peaks 1 and 3 is tested. An interfering peak (peak 2) is located between peaks 1 and 3. The light grit represents the 1D chromatograms. The 1D peak regions of the interfering peak (peak 2) are depicted as rectangles. Due to the limited number of data points in the first dimension, the trajectory profile is only described by two points, depicted as empty dots, in addition to the start and the end of the trajectory (black dots, peak 1 and 3). None of the two empty dots lie within the peak region of peak 2. Therefore, peak 2 would not be recognised as an interfering peak. To accommodate this situation, an extra condition is introduced. The line crossing the two peaks of interest must not cross any line connecting the 1D peaks of another (interfering) 2D peak. Including this criterion will now exclude this combination and recognise 2D peak 2 as interfering. This situation rarely occurs in practice. However, it can happen and, therefore, it must be accounted for. Note that no interpolation is performed to obtain more information along the trajectory profile. One could suggest that the use of interpolated trajectory profiles would make this criterion redundant. However, this means that the peak vicinity would depend on the data acquisition rate of the instrument: low data acquisition rates may result in situations in which an interferent peak is not detected (as is the case in Fig. 8.3). Therefore, the use of this extra condition is always recommended to ensure the detection of all interfering peaks.

8.2.3 The valley-to-peak ratio in two-dimensional chromatography

Once the concept of the peak vicinity is established, computing the resolution between two peaks (that are defined as true "neighbours") is the next task. The definition of the valley-to-peak ratio between two 2D peaks is based on the
concept of the saddle point. A saddle point corresponds to the minimum when following a surface in one direction and the maximum when crossing it in a different direction (in case of a horse saddle, these directions are from the horses head to its tail and from right to left, respectively).

This definition can be easily adapted to 2D peaks in two-dimensional chromatography. Each 2D peak is a three-dimensional “mountain” (1t_R, 2t_R and intensity). The saddle point between the top of the mountains (i.e. the maxima of the 2D peaks of interest) can be described as the lowest point that a hiker would reach when walking from the top of the first mountain (the maximum of the first 2D peak) to the top of the second mountain (the maximum of the other 2D peak), taking the path with the least possible descent.

Fig. 8.4A is an example of two peaks present in the chromatogram of the air-freshener sample. When applied to this example, the peak-vicinity test (Section 8.2.2) concludes that the two peaks are "neighbours", and therefore that the resolution between them is a meaningful parameter. In the calculation of the valley-to-peak ratio, the concept of trajectory profiles is again invoked. The trajectory profiles of all 1D maxima of peaks 1 and 2 are depicted as dashed pink lines in Fig. 8.4A. For the calculation of the valley-to-peak ratio, only the trajectory profiles that are free of interference (i.e. do not pass through the peak region of another peak) will be used. By definition, as the peaks are "neighbours", at least one of the trajectory profiles has to be free of interference of a third peak. In the example shown in the figure, all connections are considered, because there is no interfering peak located between the two 2D peaks. However, this may be different in other cases.
Figure 8.4 Chromatogram and trajectory profiles corresponding to two peaks of the air-freshener sample. (A) all 1D peaks are connected by trajectory profiles (pink dashed lines). The minima of these lines are marked as white dots (labelled a-j). (B) the overall maxima of the two 2D peaks (max 1 and 2) and the saddle point S are shown. For details, see text.

Figure 8.5 Chromatogram corresponding to three peaks of the fungicide sample. The trajectory profile linking 1D peak d of peak 2 with peak 1 is overlaid (pink dashed line). The crossing point of this trajectory profile with one merging line of 2D peak 2 is labelled C.

An additional criterion is applied that may result in trajectories being discarded prior to the next calculation steps. In certain cases, a trajectory profile can cross the "merging lines" (see Section 8.2.1) of either of the 2D peaks of which the resolution is being calculated (see Fig. 8.5). This can happen in two ways (i) the intensity of the trajectory profile at the “crossing point” C is below the intensity of the merging line at this point or (ii) the intensity of the trajectory profile is above
the intensity of the merging line. In case (i), this particular profile is excluded from
the calculation of the saddle point as it is not free of interference (peak 2 itself
constitutes an interference).

In Fig. 8.4A the minima of the (interference-free) trajectory profiles are depicted
as white crosses, named a-j. The saddle point is the most-intense (highest)
minimum (labelled e in Fig. 8.4A). The valley-to-peak ratio is now calculated
using the two overall maxima of the 2D peaks and the saddle point (labelled S in
Fig. 8.4B). Consider the lines that link max1, S and max2 in Fig. 8.4B. The
distance between max1 and S, d_{1,S}, and the distance between S and max2, d_{S,2}, can
be calculated using Eq. 1 and 2:

\[ d_{1,S} = \sqrt{\left(\Delta t_{R_{1,1}}\right)^2 + \left(\Delta t_{R_{1,2}}\right)^2} \]  
Eq. 1

\[ d_{S,2} = \sqrt{\left(\Delta t_{R_{2,1}}\right)^2 + \left(\Delta t_{R_{2,2}}\right)^2} \]  
Eq. 2

where \( \Delta t_{R_{1,1}} \) and \( \Delta t_{R_{1,2}} \) are the differences in time between max1 and S in the first
and second dimension respectively and \( \Delta t_{R_{2,1}} \) are the differences in time between
S and max2 in both dimensions.

Since the intensities of the two maxima, \( h_{\max 1} \) and \( h_{\max 2} \), and the saddle point, \( h_{S} \),
are known, a plot as shown in Fig. 8.6 can be created to compute the valley-to-
peak ratio. Note that the same features that can be identified in the concept of the
one-dimensional valley-to-peak ratio can be recognised in this figure. The
intensity \( g \) is defined as follows:

\[ g = \frac{d_{1,S} h_{\max 2} + d_{2,S} h_{\max 1}}{d_{1,S} + d_{2,S}}. \]  
Eq. 3

Once \( g \) is defined, \( f \) can be easily derived as it is the subtraction of \( h_{S} \) from \( g \).
Therefore, the valley-to-peak ratio is defined as

\[ V = f = \frac{g - h_{S}}{g}. \]  
Eq. 4
Figure 8.6 Schematic diagram of the calculation of the valley-to-peak ratio between two 2D peaks via their saddle point.

There is a clear link between the concept of valley-to-peak ratio defined here and the definition given by Schure [7]. In Schure’s work resolution is also calculated using the maxima of two 2D peaks. However (in contrast to what is proposed here), the 2D chromatogram of two peaks is generated by “slicing” through the three-dimensional chromatogram along a straight line connecting the maxima of the two 2D peaks (equivalent to the line connecting max1 and max2 in Fig. 8.7A). In Fig. 8.7B, the intensity of this slice is plotted against the distance in time.

Figure 8.7 Comparison of two resolution concepts (see text for details). (A) M is the minimum on the trajectory profile between the two peaks and S is the saddle point. The intensity profile from max1 to max2 following a line through the minimum M is depicted in (B). The intensity of the saddle point S is represented by the horizontal dashed line.
Schure proposed to calculate the valley-to-peak ratio using the minimum on this line, labelled M in Fig. 8.7. From the graph, the valley-to-peak ratio can be estimated as 0.922. If the new definition is applied, the minimum on this line is not used in the calculation, but rather the saddle point between the two 2D peaks, labelled S in Fig. 8.7. Using the saddle point, the valley-to-peak ratio is 0.868. The definition given by Schure is equivalent to the new one if the 2D peaks are Gaussian shaped. In that special case the saddle point is located on the straight line between the two peak maxima \((\text{i.e. M and S would coincide in a figure such as Fig. 8.7})\). The definition given in this article is more general, as it can also correctly accounts for non-Gaussian peaks. Because the peaks in Fig. 8.7 are not Gaussian, the valley found on the straight line between the two maxima (M) gives an erroneous impression of the separation \((\text{i.e. M is lower than S and, therefore, the valley-to-peak ratio is larger, suggesting a better resolution})\).

Assuming Gaussian-shaped peaks, the valley-to-peak ratio and the resolution may be combined [7]:

\[
Rs = \sqrt{-\frac{1}{2} \ln \left(\frac{1-V}{2}\right)}
\]  

Eq. 5

where \(Rs\) is the resolution in two-dimensional chromatography [6], and \(V\) is the valley-to-peak ratio defined by Eq. 4. We will use this function to calculate the resolution that corresponds to a specific valley-to-peak ratio, even in the case of non-Gaussian shaped 2D peaks. Since chromatographers are more familiar with resolution than with valley-to-peak ratios, the definition given in Eq. 5 will also be used in the “Results and Discussion”-Section.

As mentioned before, more than one peak can be considered as a “neighbour” of a target peak for which the resolution may be calculated. If the objective is to give an overall value of resolution for a target peak, all the values of valley-to-peak ratios calculated between the target 2D peak and its neighbouring 2D peaks have to be collected in a single value. Since the valley-to-peak ratio is normalised
(varies between 0 and 1), the overall valley-to-peak ratio of a 2D peak can be represented by the product of all valley-to-peak ratios calculated for that peak.

8.3 Experimental

8.3.1 Chemicals

Two groups of samples were used. A commercially available air-freshener bought in Melbourne, Australia was used in this study to illustrate the application of the concept of valley-to-peak ratios to a real sample (Section 8.2). The sample was diluted in n-hexane to a concentration resulting in suitable peak heights. The 2D peaks were detected using the program described in Chapter 4 using the following parameters: \( \text{Thr}_0 = 30 \text{ pA}, \text{Thr}_1 = 2 \text{ pA.s}^{-1}, \text{Thr}_\text{OV} = 0.2 \) and a unimodality option of 1. \( \text{Thr}_0 \) and \( \text{Thr}_1 \) are thresholds used for peak detection in the one-dimensional chromatograms, whereas \( \text{Thr}_\text{OV} \) and the unimodality option are used for the peak-merging algorithm.

For the discussion of the performance of the developed valley-to-peak ratio measurement (Section 8.4), a pesticide-mixture containing eight fungicides was used. It was provided by the Department of Primary Industry (Melbourne, Australia). The standard mixture was diluted with n-hexane yielding a concentration of about 2.5 mg/L for each fungicide. Nine different sets of conditions were created by varying the flow rate and the temperature-programming rate (see Table 8.1). The parameter for peak detection and peak merging here were set as follows: \( \text{Thr}_0 = 40 \text{ pA} \) and \( \text{Thr}_1 = 2 \text{ pA s}^{-1} \), \( \text{Thr}_\text{OV} = 0.2 \) and the unimodality option was 1.
Table 8.1 Experimental design carried out to create the different peak-separation situations used in Section 8.4.

<table>
<thead>
<tr>
<th>Condition number</th>
<th>Flow rate [mL/min]</th>
<th>Temperature rate [°C/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td>1.5</td>
<td>12</td>
</tr>
</tbody>
</table>

8.3.2 Instrumental details

The instrument used in this study was an Agilent Model 6890 gas chromatograph (Agilent Technologies, Burwood, Australia) with a flame-ionisation detector (FID) operated at a data-acquisition frequency of 100 Hz. The GC was retrofitted with a longitudinally modulated cryogenic system (Everest Model, Chromatography Concepts, Doncaster, Australia).

The column set included a low-polarity BPX5 first-dimension column (30 m x 0.25 mm; 0.25 µm film thickness) and a BPX50 second-dimension column (1 m x 0.1 mm; 0.1 µm film thickness). Both columns were from SGE International (Ringwood, Australia).

A modulation period of 4 s was applied for all analyses and the cryogenic trap was set to -10 °C.

Unless stated otherwise, the GC was operated under temperature-programmed conditions from 90 °C to 250 °C at 3 °C/min, held at 250 °C for 15 min. Hydrogen was used as the carrier gas at a flow rate of 1 mL/min. The injection volume for all samples was 1 µL. A split injection with a split ratio of 20:1 was applied for the air-freshener and a pulsed splitless injection was applied for the fungicides.
8.3.3 Software

The instrument was controlled by Chemstation software (Agilent, Waldbronn, Germany) and data treatment was performed by home-built routines, written in MATLAB 6.5 (The Mathworks, Natick, MA, USA). The raw data set can be exported from the instrument software in comma-separated-values (*.csv) format and then processed with the developed program. A personal computer with a 1.4 GHz Intel® Celeron® M Processor and 512Mb RAM was used for all data treatment.

8.4 Results and discussion

8.4.1 Performance of the valley-to-peak ratio measurement

In order to test the performance of the valley-to-peak ratio in practice, the chromatogram obtained for the pesticide mixture was selected. Fig. 8.8 depicts the region of interest of the resulting chromatogram. Peak 2 is the target peak for which the valley-to-peak ratios are calculated (see Table 8.2).

Figure 8.8 Chromatogram corresponding to the region of interest of the fungicide sample (eluted under condition 2). Pink dotted lines indicate peaks that are considered neighbours of peak 2.
The inspection of Fig. 8.8 is instructive to illustrate the operation of the peak-vicinity algorithm. Only five peaks are considered "neighbours" of peak 2. Consequently, the valley-to-peak ratio is calculated between peak pairs 2-1, 2-3, 2-4, 2-6 and 2-8. For peak pair 2-1 and 2-3, no other 2D peak is located between the pair and therefore, the resolution can be calculated between them. This is in accordance with the chromatographer's intuition. The algorithm also considers the peaks 6 and peak 8 to be neighbours of peak 2, even though these peaks are far apart. This is because at least one trajectory profile from peak 2 to peak 6 (or 8) was found to be unobstructed (i.e. no peak regions of other peaks were crossed). In our experience, the developed criteria are suitable to determine meaningful peak-pair combinations, for which the valley-to-peak ratio is then calculated. Note that the concept of peak vicinity does not consider how far the peaks are separated, but only whether a peak is located in between. Therefore, it can happen that peaks that are located far apart (such as peak pair 2 and 6 in Fig. 8.8) are still considered neighbours. Another remark is that often a peak pair is excluded (meaning the resolution is not calculated) even though inspection of the colour plot may suggest that the peaks involved could be neighbours. This can easily happen when the integration thresholds are below the threshold of plotting the peaks. Interfering peaks can often only be identified using the concept of peak regions, rather than by inspecting the colour plot. Peak pair 2-7 is an example. Intuitively, one may think that peaks 2 and 7 are neighbours (especially because peaks 2 and 6 also are). However, when the algorithm examines all the trajectory profiles between peaks 2 and 7, it is found that the peak region of peak 6 is always in between. This would certainly bias the value of the valley-to-peak ratio (resolution) if it was to be calculated. The saddle point would be higher than expected, not because the peaks are less resolved, but because of the interfering peak.

Even though the values for the valley-to-peak ratio theoretically range between zero (no resolution) and one (perfect resolution), the values for this calculation for two-dimensional peaks vary only slightly. This is not surprising, since all peaks
are well resolved (see Table 8.2). This again is in accordance to the chromatographer's intuition. A valley-to-peak ratio of 0.98 equates to a \( R_s \)-value of about 1.5, which is considered as well-resolved. For peaks 6 and 7, which are clearly not completely resolved, the valley-to-peak ratio is 0.87, which corresponds to a resolution \( R_s \) of 1.17. One should be careful when using of Eq. 5, since the peak distance is not considered when the valley-to-peak ratio is computed. Therefore, once two peaks are baseline-separated, the valley-to-peak ratio approaches unity and the conversion to \( R_s \) loses accuracy. This starts to happen with values of valley-to-peak ratio above 0.97 (which is equivalent to \( R_s > 1.5 \)).

**Table 8.2** Valley-to-peak ratios and resolution values for 2D peak 2 of the fungicide sample.

<table>
<thead>
<tr>
<th>2D peak combination</th>
<th>Valley-to-peak ratio</th>
<th>Resolution ( R_s )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 – 1</td>
<td>0.995</td>
<td>1.73</td>
</tr>
<tr>
<td>2 – 3</td>
<td>0.993</td>
<td>1.68</td>
</tr>
<tr>
<td>2 – 4</td>
<td>0.992</td>
<td>1.66</td>
</tr>
<tr>
<td>2 – 6</td>
<td>0.999</td>
<td>1.95</td>
</tr>
<tr>
<td>2 – 8</td>
<td>0.999</td>
<td>1.95</td>
</tr>
<tr>
<td>Overall resolution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(minimum)</td>
<td>0.992</td>
<td>1.66</td>
</tr>
<tr>
<td>Overall resolution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(product)</td>
<td>0.973</td>
<td></td>
</tr>
</tbody>
</table>

In Table 8.2, the values of the overall valley-to-peak ratio (the minimum and the product of all values) are also given for the target peak. These values can be used as an estimate of how well the target peak is resolved from all other (neighbouring) peaks. This may be useful in certain applications, for example when the objective of the analysis is to resolve only a few target peaks. Note that the product as a single value for one target peak is dependent on the number of peaks that are considered as neighbours. This calls for caution for well-resolved peaks. In this case, the overall valley-to-peak ratio is a result of multiplying several quantities close to 1. The reason why the valley-to-peak ratio is not exactly equal to unity for well-resolved peaks is random noise. The more neighbours
present, the lower the overall valley-to-peak ratio may become, which does not follow the chromatographer’s intuition. This can be remedied by setting all individual valley-to-peak ratios that exceed a certain threshold to 1.

There may be applications in which a chromatographer needs to establish an overall quality criterion for the entire chromatogram. In such cases, since all peaks are target peaks, the overall valley-to-peak ratio of each peak is calculated. Subsequently, the minimum, the product, or the mean of all values may be computed, to obtain a single value expressing the resolution of the whole chromatogram. In our case, the computing power was inadequate to compute all valley-to-peak ratios for all 2D peaks in a two-dimensional chromatogram of average complexity.

8.4.2 Relation between the error of quantification and the valley-to-peak ratio

Valley-to-peak ratios provide an objective estimate of the separation between peaks. However, this may not be adequate. The chromatographer may want to relate this value to the error obtained in the quantification of the target peaks. This would add additional meaning to the computation of valley-to-peak ratios. In order to investigate the relation of the error of quantification and the valley-to-peak ratio, a target mixture of eight peaks was considered. Nine different experimental situations were created, for which the separation (and therefore the resolution) of the peaks was changing. This allowed a measurement of the error of quantification in different situations of partial overlap. In order to generate the different experimental situations, an experimental design was carried out. For simplicity, only two factors were considered, namely the flow rate and the temperature-programming rate (see Section 8.3). These factors are expected to greatly influence the separation. Three levels were considered for each factor, which resulted in a design with $3^2 = 9$ experiments (see Table 8.1). Some attention has to be paid to the "intensity threshold" $\text{Thr}_0$ when changing experimental conditions (see Chapter 4 for details on this threshold). If $\text{Thr}_0$ is chosen too low, noise might
be detected as peaks. The noise level is different under different conditions and, therefore, care must be taken to either set the threshold correctly for each set of conditions, or to discard peaks that are known to be due to noise.

Fig. 8.8 represents the separation obtained at condition 2. It should be noted that only peaks 6 and 7 showed significant variation in resolution at the nine different sets of conditions described in Table 8.1. Therefore, only these two peaks plus peak 8 were selected as target peaks for this study. Peak 8 was included to also examine one well-resolved peak.

Under certain conditions (e.g. condition 6), the resolution between peaks 6 and 7 was so low that the peak-detection algorithm merged the two peaks to form one 2D peak. The peak-detection algorithm separates peaks when a valley is found in between them (the first derivative is monitored). Strongly overlapping peaks (showing just a shoulder, not a valley) will be merged to a single peak and therefore, no resolution measurement can take place.

To evaluate the error in determining the area, a quantification method described elsewhere in Chapter 4 was applied to compute the volume of the target 2D peaks (equivalent to the area in 1D chromatography). Quantification of 2D peaks is performed by integrating the 1D peaks individually and then summing the areas of the 1D peaks that were merged to form the 2D peak of interest. In order to minimise quantification errors, the sample was prepared only once and the same sample was injected under the nine different conditions. Then, one of the experiments (condition 1, in which all peaks were perfectly separated) was selected as a reference. The quantification error was calculated as % of relative error (ε), as follows:

$$
\varepsilon = \left| \frac{a_{\text{expected}} - a_{\text{experimental}}}{a_{\text{expected}}} \right| \times 100
$$

Eq. 6

where $a_{\text{experimental}}$ is the sum of the (1D) areas of the target peak and $a_{\text{expected}}$ is the sum of the areas of the target peak at the reference condition (*i.e.* condition 1). An
internal standard was used to minimise errors due to non-repeatable injection volumes or instrumental errors, and to compensate for the variations in signal response at different experimental conditions. Peak 5 was chosen as an internal standard, as it was well separated from all other peaks under all conditions. Prior to applying Eq. 6, the areas were normalised using the internal standard procedure. In Fig. 8.9 the relative error in quantification is plotted vs. the valley-to-peak ratio of the target peaks (6, 7 and 8). For each target peak, the product of all valley-to-peak ratios in which the target peak was involved was used (Section 8.2.3). We expect that the error of quantification is related to the valley-to-peak ratio; the lower the value of the valley-to-peak ratio, the greater the error in quantification is expected to be. In Fig. 8.9, this trend is clearly observable.

![Figure 8.9](image.png)

**Figure 8.9** Relative error of quantification – using Eq. 6 – versus the valley-to-peak ratio for peaks 6 (open diamonds), 7 (open squares) and 8 (open triangles) under the nine different experimental conditions described in Table 8.1. To compute an overall value of the valley-to-peak ratio of each target peak, the values are multiplied.

For valley-to-peak ratios greater than 0.6, the plot becomes more scattered and a trend is harder to define. This is probably due to the fact that the error expressed by Eq. 6 includes all instrumental errors, which are more relevant in situations of low overlap. However, it can be concluded that for almost all peaks with a valley-to-peak ratio above 0.85, a relative error of less than 10% is observed.
**Table 8.3** Valley-to-peak ratios (v2p) for 2D peaks 6, 7 and 8 analysed under nine experimental conditions.

<table>
<thead>
<tr>
<th>2D peak comb.</th>
<th>v2p cond.1</th>
<th>v2p cond.2</th>
<th>v2p cond.3</th>
<th>v2p cond.4</th>
<th>v2p cond.5</th>
<th>v2p cond.6</th>
<th>v2p cond.7</th>
<th>v2p cond.8</th>
<th>v2p cond.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-5</td>
<td>0.9999</td>
<td>0.9997</td>
<td>0.9998</td>
<td>0.9996</td>
<td>0.9997</td>
<td>-</td>
<td>0.9992</td>
<td>0.9995</td>
<td>0.9997</td>
</tr>
<tr>
<td>6-7</td>
<td>0.9377</td>
<td>0.8712</td>
<td>0.8171</td>
<td>0.7728</td>
<td>0.6773</td>
<td>-</td>
<td>0.7918</td>
<td>0.5520</td>
<td>0.2954</td>
</tr>
<tr>
<td>7-8</td>
<td>0.9996</td>
<td>0.9996</td>
<td>0.9992</td>
<td>0.9992</td>
<td>0.9992</td>
<td>0.998</td>
<td>0.9992</td>
<td>0.9992</td>
<td>0.9983</td>
</tr>
</tbody>
</table>

Table 8.3 shows the valley-to-peak ratios for the target peaks under all experimental conditions. As can be seen, the valley-to-peak ratios of peak 7-8 are always greater than 0.998. Accordingly, the relative errors associated with the quantification of peak 8 are always lower than 7%. In contrast, for 2D peaks 6 and 7, the valley-to-peak ratios decrease to 0.29 for situation 9 (flow rate of 1.5 mL/min and a temperature-programming rate of 12 °C/min) with a relative error of quantification up to 61% for peak 6 and 47% for peak 7.

### 8.5 Conclusions

The concept of the valley-to-peak ratio was successfully adapted to two-dimensional separations. Two problems were tackled in this context, namely *i)* determining which are neighbouring peaks and *ii)* the calculation of valley-to-peak ratio for non-Gaussian peaks.

In a two-dimensional separation space, one peak is surrounded by more than two peaks. Therefore, prior to the calculation of the valley-to-peak ratio, criteria have to be applied to determine which peak pairs have meaningful resolution values (*i.e.* which peaks can be considered neighbours). In this paper, a method has been proposed to investigate the peak vicinity. The method assures a calculation of valley-to-peak ratio free of artefacts due to interferents. In addition, the peak vicinity concept proved to be in accordance with chromatographic intuition.

A measurement of valley-to-peak ratios in two-dimensional separations was proposed, based on the saddle-point concept. The use of the saddle point allows the calculation of valley-to-peak ratios for real (experimental) peaks, which are not
of Gaussian shape. For the samples analysed, reasonable values for the valley-to-peak ratio were obtained. In addition, an approximate conversion of the valley-to-peak ratio to the resolution as given by Schure was applied, yielding indicative values for the resolution of peaks in comprehensive two-dimensional chromatography.

The error associated with the quantification of (modulated) 2D peaks could be related to the valley-to-peak ratios. The lower the valley-to-peak ratio the greater the expected error. Although this relation is not exact, it does provide a useful estimate. For the sample analysed, the error of quantification was below 10% when the valley-to-peak ratio was above 0.85.

References