1
Fundamentals of Optimization

HPLC Made to Measure: A Practical Handbook for Optimization. Edited by Stavros Kromidas

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1.1
Principles of the Optimization of HPLC Illustrated by RP-Chromatography

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First of all, some questions will be discussed, which should reasonably be answered before beginning method development. Subsequently, we will treat the principal possibilities for improving the resolution in HPLC. It follows a discussion about efficiency and the "right" sequence of such measures for the isocratic and the gradient mode. There is a particular focus on strategies and concepts for developing a method and checking peak homogeneity.

The last section will show ways to achieve other aims than "better separation": "make it faster", "raise sensitivity" or "save money". The chapter ends with a conclusion and an outlook.

1.1.1
Before the First Steps of Optimization

For economic reasons, one really ought to address the following questions prior to commencing the development of a method or the optimization of a given separation.

- What do I want? In other words, what is the true intention of the separation?
- What do I have? That is to say, what relevant information about the analytical purpose and the samples is available?
- How should I do it? Do I have all what I need, and is what I want to do really possible?

At first glance, these questions might appear too theoretical or even over-critical. Nevertheless, careful consideration of the actual aims and realistic possibilities for solving an analytical problem would seem to be important at the outset. An early discussion with my boss, a colleague or my client – if you are short, even with yourself – can later prevent a good deal of trouble, time expenditure, and last but not least costs. This time-saving can be considered a good investment.

As regards the first question: "What do I want?"

If it is at all possible, at the outset the following or similar questions should be answered:

- Do I need a method for the accurate quantification of *this* toxic metabolite, or is the aim that the authorities just accept my method?
- What is most important in *this* case: short analysis times, durable columns, robust conditions, or simply optimal specificity?
- Must the relative standard deviation S_{rel} be no higher than 2%? What loss of quality would be incurred if S_{rel} were to be 2.5%? Is there actually a correlation between the cost of the analysis and real improvement in the quality of the product?

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In other words, is the aim just to meet the requirements in this specific case or is the real "truth" at stake, i.e., are formal aspects or analytical questions in the foreground? This question should be consciously and truthfully answered because of the possible consequences.

How difficult it can be to stand by meaningful and well-considered decisions without being regarded as outlandish or as a troublemaker has been documented elsewhere [1]. Where possible, one should question all aspects. Unconventional questions frequently result in simple solutions.

As regards the second question: "What do I have?"

Information on the sample makes the design of a suitable method easier.

Some examples:

- What is written in the report of colleagues from the chemical development department on the light sensitivity and the sorption properties on glass surfaces of a new drug?
- Can I contact these colleagues quickly? That is to say, can I get relevant information with a minimum of effort?
- There may be information about similar separations in the past, which were not pursued further, in an internal database (which is perhaps rarely updated and even more rarely accessed).
- May I quickly calculate the pK_a value of the known main component in the sample with appropriate software (see Chapter 1.4)?
- Has a colleague in a neighboring department worked in the past with similar compounds and might therefore be able to provide valuable insights?

As far as possible, all means of communication with colleagues should be pursued to gather information. At times it may be helpful not to make this public.

As regards the third question: "How should I do it?"

One should assess the feasibility of the proposed work absolutely unconditionally.

Some examples:

- Can I convince my boss that it is useful from the overall company point of view to discuss in advance with the later routine users the design of the method and additional details? If fear of loss of know-how or questions of budget or other psychological and social barriers make impossible de facto a discussion with "the others", it is a bitter reality that one must accept.
- On the other hand, is it worth fighting for a change of the following well-known and accepted situation? A deadline is fixed and therefore a validation must be finished in two weeks. Later, the burden of subsequent, substantial costs for a repeat of the measurements, complaints, out-of-spec situations, etc., which inevitably result because an analytical method can hardly be validated within two weeks under real conditions, is not placed on "us" but on quality control, and as testing costs they have been accepted since decades in the absence of overall considerations. The reader may imagine the consequences, or viewed more positively, the possibilities for improvement.

- Is it really worthwhile in the case of the development of a routine method, which shall be applied all over the world, to opt for a polar RP-phase because of the frequently observed higher selectivity, even if one has to expect problems with the charge-to-charge reproducibility? Might a hydrophobic, more rugged column with a lower but still sufficient selectivity the better choice?
- Is it useful to demonstrate my analytical "knowledge" by further trimming the relative standard deviation of a method being used in diverse plant laboratories to a value of 0.7%?

Realities – and opinions are also realities – which determine the success or failure of an analytical activity should, wherever possible, influence the design of the method. It is useful if the number of meetings can be reduced to "a cup of coffee" or "lunch often together". The point is to improve the communication and this can turn out to be easier in a less formal situation.

In conclusion, two basic preconditions for successful method development may be noted:

1. Expert knowledge exists or can be loaned or sold.
2. The analytical possibilities correspond with the requirements, and it is possible to talk about them.

In the author's opinion, a clear definition of requirements, unequivocally formulated, understandable goals for all involved persons, shortcuts to information, and a critical estimation of possibilities/risks are more important, not only in analytics, than obtaining exemplary results such as low detection limit, correlation factors around 0.999, S_{rel} smaller than 1%, or 30% less expensive equipment.

1.1.2 What Exactly Do We Mean By "Optimization"?

Optimization of a separation is principally directed by the following goals:

- to separate better (higher resolution),
- to separate faster (shorter retention time),
- to see more (lower detection limit),
- to separate at lower cost (economic effort),
- to separate more (higher throughput).

The three first-named goals may be most important, and of these the improvement of the resolution is the prime concern. Therefore, we will treat this topic before we start to deal with the other aspects. Preparative HPLC is not the subject of this book.

Preliminary Remarks

The theory of chromatography is fundamentally valid for all chromatogaphic techniques. Therefore, basically the same principles are pursued. However, it is

evident that the priorities and the weighting of the specific steps are very different, for example in GPC and in μ-LC-MS(MS). In the following, the possibilities for optimization are presented and proposals for the most popular liquid-chromatographic technique, RP-HPLC, are given in short form.

The characteristics of the different modes are treated in Chapters 2.1 to 2.7. It is assumed that the reader is familiar with the chromatographic rules and the theory of HPLC, so these are not treated in detail, but where necessary some technical terms are briefly explained.

The immediately following remarks relate to isocratic separations.

1.1.3 Improvement of Resolution ("Separate Better")

Resolution (R), in simplest terms, is the distance between two neighboring peaks at the base of the peaks. An increase in this distance is what every chromatographer routinely strives for.

The corresponding equation is:

$$R = \frac{1}{4}\sqrt{N} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{k_2 + 1}$$

where N is the plate number, a measure of the performance or the efficiency of the column.

The number of plates is in effect a measure of the widening of the substance band because of diffusion effects. The basic question is whether the molecules of the analyte that reach the detector are contained in a small or a large (peak) volume, i.e., will one get sharp or broad peaks?

Strictly speaking, one should distinguish between the theoretical and the effective plate number. The theoretical plate number is the number of plates of an inert component (see below) and therefore a characteristic and constant value for a column under defined conditions. The effective plate number is the number of plates of a specified retained component, and the retention factor (see below) enters into the calculation. Today, however, this distinction is not made everytime; one speaks only about plate number. In the most cases, the theoretical plate number is calculated, but of retained substances. In this context, it should be made clear that the plate number depends on a lot of factors, e.g. the injection volume, the temperature, the composition of the eluent, the flow rate, the retention time, the analyte, and last but not least the equation used for the calculation, i.e. peak width at the peak base, at 10% or at 50% peak height. Therefore, the comparison of literature values of plate numbers is inherently difficult.

α: *Separation factor*, formerly *selectivity factor*.
α is a measure of the capability of a chromatographic system (chromatographic system: the actual combination of the stationary phase, the mobile phase, and the temperature) to distinguish two given compounds.

The α value is the quotient of two net retention times, i.e. the quotient of the dwell times of the two components within the stationary phase.

The point is that if *this* particular chromatographic system is selective for these two compounds, then in principle they are separable. Selectivity, in simplest terms, is the distance between two peaks, from the top of one peak to the top of the other. This is different from the resolution in that for the determination of the selectivity the form of the peak (plate number) is not considered, because α is only the quotient of two (retention) times. The separation factor depends only on the chemistry; see below on the subject of retention factor.

k: *Retention factor*, formerly *capacity factor k'*.
k is a measure of the strength of the interaction of a given compound in a given chromatographic system. It expresses for how much longer a given compound remains on the stationary phase compared to the mobile phase.

The *k* value is an index like the α value, and is thus independent of instrumental conditions such as the dimensions of the column or the flow rate. The *k* value changes only if parameters that have something to do with the interaction are changed, i.e., the chemistry: stationary phase, mobile phase, temperature. As long as these parameters are kept constant, the *k* value also stays constant, irrespective, e.g., of the flow rate or whether a 10 or 15 cm column is used.

Although the dead time does not appear explicitly in the equation for the resolution, it is useful for the following explanation to briefly deal with this term.

t_m: *dead time*, *breakthrough time*, *front*, "*air peak*": This is the dwell duration of an inert component in the HPLC equipment. A component is designated as inert if it is able to penetrate everywhere without steric hindrance, including, of course, in the pores of the stationary phase, but is not retained there. In other words, the dead time is the time of the presence of any not excluded component in the mobile phase – also in the standing mobile phase (i.e., within the pores), but again there is "no" interaction with the stationary phase. Therefore, the dead time only changes if something "physically" or "mechanically" is altered, e.g. a change in the length or the inner diameter of the column, the packing density, or the flow rate. The dead time is a time, which is independent from the particular compound just analysed. As all components move equally quickly in the eluent, the time that the compounds spend in the eluent makes no contribution to the separation. A separation is only possible if the substances stay in the stationary phase for different lengths of time.

The resolution *R* – the distance from peak base to peak base – depends only on the following three factors:

- the strength of the interaction between the compound and the stationary phase (if the peak comes soon or late), i.e. on the *k* value,
- the ability of the chromatographic system to distinguish between the two components of interest, i.e. the α value,
- if the relevant peaks are sharp or wide, i.e., the plate number.

Consequently, to improve resolution, there are in principle only three possibilities,

- namely a general increase in the interaction (k value increases),
- an analyte-specific change in the interaction (α value increases), or
- an increase in the efficiency of the separation (N value increases).

1.1.3.1 Principal Possibilities for Improving Resolution

The aforementioned topic is illustrated by way of a hypothetical example; see Fig. 1. Starting with a poor resolution (see Fig. 1, upper chromatogram), what are the principle possibilities for improving the resolution?

Remark: The dead time only changes in case 1 ("$\uparrow t_m$").

Possibility 1: One ensures that all components – including one possible inert component (increase of dead time, see above) elute later. Because now the dead time increases too a physical process must be responsible. This could be a longer column, a larger inner diameter of the column, decrease of the flow rate. (A larger inner diameter leads to peak broadening which rules it out for all practical purposes).

Possibility 2: The retention time stays largely constant, one seeks only a better peak form. Here, there are somewhat more possibilities: reduction of the dead volume (e.g., thinner capillaries, smaller detector cell), reduction of the injection volume (remark: local overload of the column happens more frequently than one

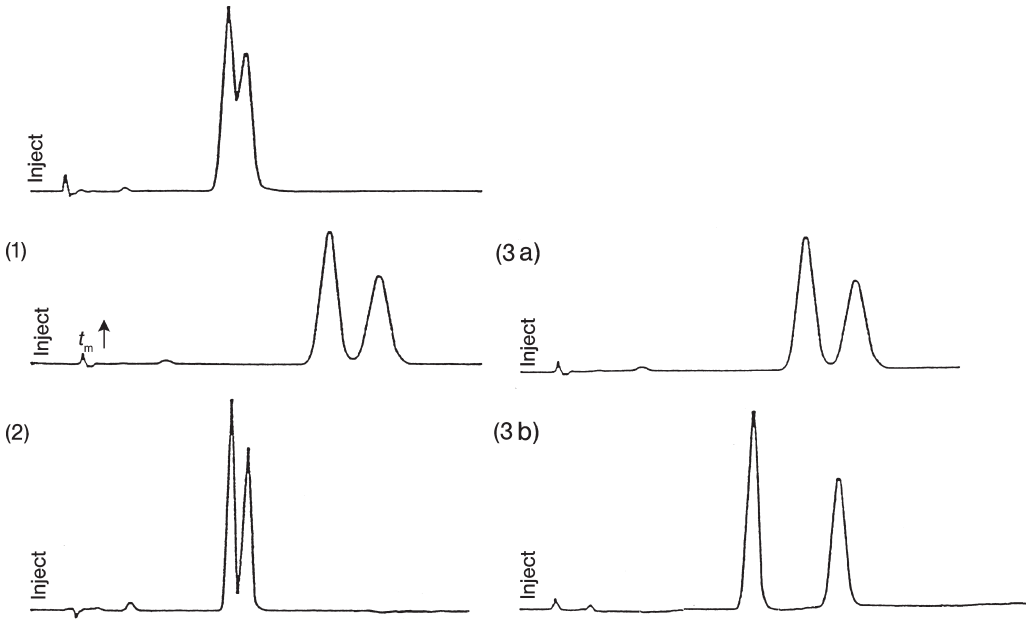

Fig. 1. Principle possibilities for improving resolution in HPLC; for comments, see text.

might imagine! Peak broadening caused by the injection is inversely proportional to the injection volume.) at an elution composition with equal solvent strength parameter, replacement of methanol with acetonitrile owing to the lower viscosity of the latter (approximately constant retention time can be expected), using smaller particles, or use of a newer, better packed column. In this context, one should also consider an optimization of the injection step as this also improves the peak form and consequently increases the plate number. The solvent of the sample should be weaker than the eluent; to this end, one uses a little bit more water in comparison to the eluent composition when preparing the sample solution.

In this way, it is possible to increase the concentration of the substance band at the head of the column, and the result is a better peak form. Finally, one should also consider various settings, such as sample rate (sampling time, sampling period), bunching factor, peak width, slit width in the case of a diode-array detector, etc. In this way, the peak form can also be improved measurably, without changing the "real" method parameters such as column or eluent.

Possibility 3a: This involves the increase of the interaction between the sample and the stationary phase, and for this there are only three "chemical" possibilities, as mentioned above: change of the eluent (e.g., increase of the water content), decrease of the temperature, and change of the stationary phase (e.g., use of a more hydrophobic phase).

The interactions increase for both/all components to be separated to the same extent, and so the retention times will also increase equally.

Possibility 3b: The same procedure as in Possibility 3a, but here it is possible to change the interactions of the two components to different extents, i.e. one component responds more strongly to a change, e.g., a change in the pH, than the other one.

Other possibilities do not exist in principle, because $R = f(N,a,k)$. This means that when trying to improve the resolution, one can only change consciously or intuitively these three factors.

1. One may successfully increase the interaction of the components of interest with the stationary phase per se, i.e. "the whole" comes later (case 3a, increase of k, e.g., by increasing the water content in the eluent) or one successfully increases the interaction of the components with the stationary phase individually, i.e. one component responds more strongly to the change than the others (case 3b, increase of α, e.g., change of the pH in the case of polar/ionic components). Both cases relate to the "chemistry": change of the temperature or change of the eluent (this includes the pH and other additives or modifiers, of course) or change of the stationary phase.
2. One may increase the plate number, either at (theoretically) constant retention time, case 2, or can concomitantly increase the retention time, case 1.

Other possibilities do not exist in principle.

Remark: In the case of a change of α and/or k, N varies simultaneously too, of course.

Having established how the resolution can be improved in principle, two questions now arise:

1. Which of the three possibilities has the greatest effect?
2. In which sequence should one try to optimize these parameters, i.e., which procedure is most economical?

1.1.3.2 What has the Greatest Effect on Resolution?

Consider once more the equation for the resolution:

$$R = \frac{1}{4}\sqrt{N} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{k_2 + 1}$$

As one can see from this equation, R responds most sensitively to a change of α. Therefore, a variation of the selectivity is the most effective, but frequently also the most difficult procedure for improving the resolution. For better illustration, consider two numerical examples:

1. If the α value is 1.01 for two neighboring peaks, some 160,000 plates would be needed to separate them perfectly (baseline separation). If the α value could be increased to 1.05, only some 2000 plates would be needed.
   In other words, if the selectivity can be slightly improved, far fewer plates will be needed for a given resolution, i.e., the flow rate can be increased or a shorter column can be used, and both will result in a shorter separation time. The "few" plates that are lost due to the increased flow rate or the shorter column, respectively, do not matter as the selectivity is improved.
2. Let us assume that when using a column of 9000 plates the second of two components to be separated elutes with a k value of 2 and an α value of 1.05. The resolution resulting from these values is 0.75.

   $$R = \frac{1}{4}\sqrt{9000} \cdot \frac{1{,}05 - 1}{1{,}05} \cdot \frac{2}{2 + 1} = 0{,}75$$

   The demand is: "a resolution of at least $R = 1$".

What are the possibilities now?

If it is possible to increase the α value to 1.10 at approximately constant interaction strength, then the resolution improves by a factor of almost 2.

$$R = \frac{1}{4}\sqrt{9000} \cdot \frac{1{,}10 - 1}{1{,}10} \cdot \frac{2}{2 + 1} = 1{,}44$$

If one considers the sequence of the most efficient steps for improving the resolution, then the efficiency comes directly after the selectivity. An increase in

the plate number can be achieved either by the classical approach of increasing the length of the column – in this case, a doubling of the column length and consequently a doubling of the retention time and the plate number increases the resolution by $\sqrt{2}$, that is, by a factor of just 1.4 – or by reduction of the particle size at constant dimensions of the column and therefore at constant retention time, which is the more frequently applied method today. Other possibilities for enhancing the plate number, such as better packing technique or reduction of the dead volume of the equipment (see below), should be common knowledge and are only mentioned here in passing.

The third, most simple method, although quite inefficient for k values > ca. 4, is the enhancement of the k value. This should also be shown by our numerical example. First, one might increase the interaction, in the first step to a k value of 4 and in a second step to 6. In this case, the resolution would improve to 0.90 and 0.97, respectively.

$$R = \frac{1}{4}\sqrt{9000} \cdot \frac{1{,}05 - 1}{1{,}05} \cdot \frac{4}{4+1} = 0{,}90$$

$$R = \frac{1}{4}\sqrt{9000} \cdot \frac{1{,}05 - 1}{1{,}05} \cdot \frac{6}{6+1} = 0{,}97$$

One would obtain a quite small improvement in the resolution as a result of a reasonable extension of the separation time (k value of 6). Therefore, the enhancement of the interaction (e.g., the increase of the water content of the eluent) is a common, easily implemented, but mostly quite inefficient procedure for routinely improving resolution. If, however, the plate number could be improved to 15,000, for example (smaller particles, injection tricks, etc.), in the case of a k value of 4, then the resolution would improve to 1.17.

$$R = \frac{1}{4}\sqrt{15\,000} \cdot \frac{1{,}05 - 1}{1{,}05} \cdot \frac{4}{4+1} = 1{,}17$$

1.1.3.3 Which Sequence of Steps is Most Logical When Attempting an Optimization?

In Fig. 2, an economical strategy is presented.

Remark: If the possibility of LC/MS coupling is available, it should be applied at the outset or at least at an early stage of the separation attempts; see below.

Explanations Relating to Figure 2

Question 1: "Reasonable" interactions, acceptable analytical run time?

After a first run with an unknown sample, or if one has to optimize an existing method, the first question ahead of any optimization steps should be: "Are there reasonable interactions?". In an isocratic separation, the components of interest

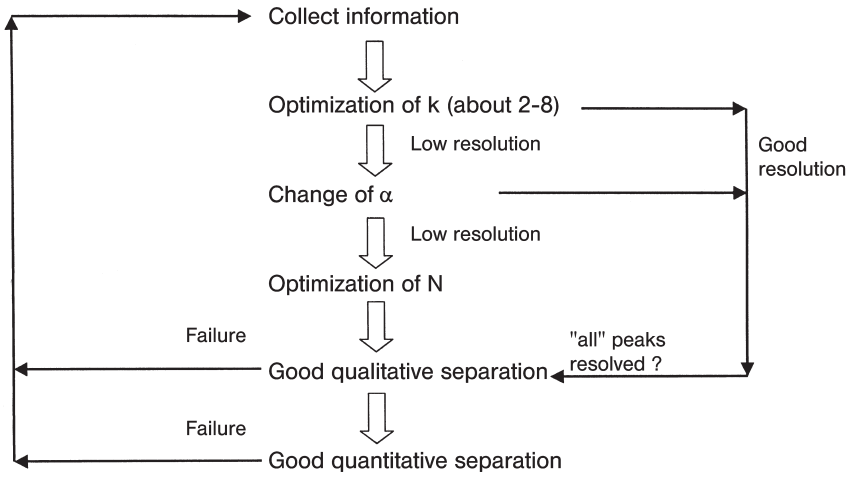

Fig. 2. Strategy for method development in HPLC.

should elute in the range of ca. $k = 2–8$. In the case of a gradient separation the $\bar{k}$ value should be ca. 5, in the middle of the chromatogram (see Section 1.1.3.4.2). These ranges are a good compromise between run time, robustness, and resolution. If the interactions are adequate (check k values) but the run time is not acceptable, then the flow should be increased or, alternatively, a shorter column should be used.

Question 2: Is it possible with the help of appropriate settings to get information on the peak homogeneity?

If one is satisfied with the run time but not with the resolution, then one has to consider whether or not the settings such as sampling time, sampling period, peak width or wavelength are optimal for this particular separation (early/late, narrow/broad peaks). One should then deal with the most effective of the three separation parameters, i.e. the selectivity.

Question 3: Is the selectivity sufficient?

If one obtains a short run time and a satisfactory selectivity (separation factor of the critical pair ca. 1.05–1.1) but the resolution is not yet satisfactory, then one should enhance the efficiency – to put it simply "make the peak sharper!".

Question 4: Is it now possible to enhance efficiency or must the selectivity be further improved in order to get a better resolution?

As a rule, this is a more economical way to improve peak shape than to continue to improve the selectivity (change of chemistry, which means a change of columns and eluents).

Two real examples, taken from Ref. [1], illustrate these statements:

Example 1

In Fig. 3a, the chromatogram of a validated method from a pharmaceutical company is shown.

The parameters of the method are as follows: linear gradient from 10% to 90% methanol, common commercial C_{18} phase, 5 µm, column 125 × 4 mm, flow rate 1 mL min^{-1}, ambient temperature, injection of 30 µL sample dissolved in THF/MeCN. The large peak at the dead time results from matrix components and is not a problem. The method, which is not really optimal, should be optimized in a quick and easy way.

Question 1: Is the run time OK?

No. The first troubling thing one notes is the really long run time: 16 min for two peaks is not acceptable for a routine method today. First, the flow was increased to 2.6 mL min^{-1}. As expected, a shorter run time was obtained without a loss of resolution: the gradient was changed in such a manner that the gradient volume was the same as at the start (see Chapter 1.2), Fig. 3b.

However, a retention time of 10 min for two peaks is still too long. While the pressure was ca. 345 bar at the flow rate of 2.6 mL min^{-1}, the starting conditions of the gradient were changed in the next step. It was not started at 10% but at 40% methanol; see Fig. 3c. The retention time of 3–4 min for two peaks was then satisfactory.

Questions 2 and 3: Are the settings and selectivity OK?

Yes, see the distance between the tops of the peaks in Fig. 3c (that is simply to say "selectivity"). Although the selectivity is obviously not bad, the resolution cannot be described as satisfactory because of the fronting.

Question 4: Can I improve the peak shape and hence the plate number?

A simple test was then performed: the sample solution was diluted twofold with the eluent ($MeOH/H_2O$, 40 : 60) and the resulting 120 µL was injected. Finally, the result thus obtained was satisfactory; see Fig. 3d.

Note: It is better to inject 100 or 150 µL of sample solution with an eluent-like solvent than to inject 20 or 30 µL of sample solution with a stronger solvent than the eluent (e.g., containing more MeCN than the eluent).

To sum up, in this example first the run time was reduced and then, due to the apparently adequate selectivity, resolution was improved merely through increasing efficiency (better peak shape).

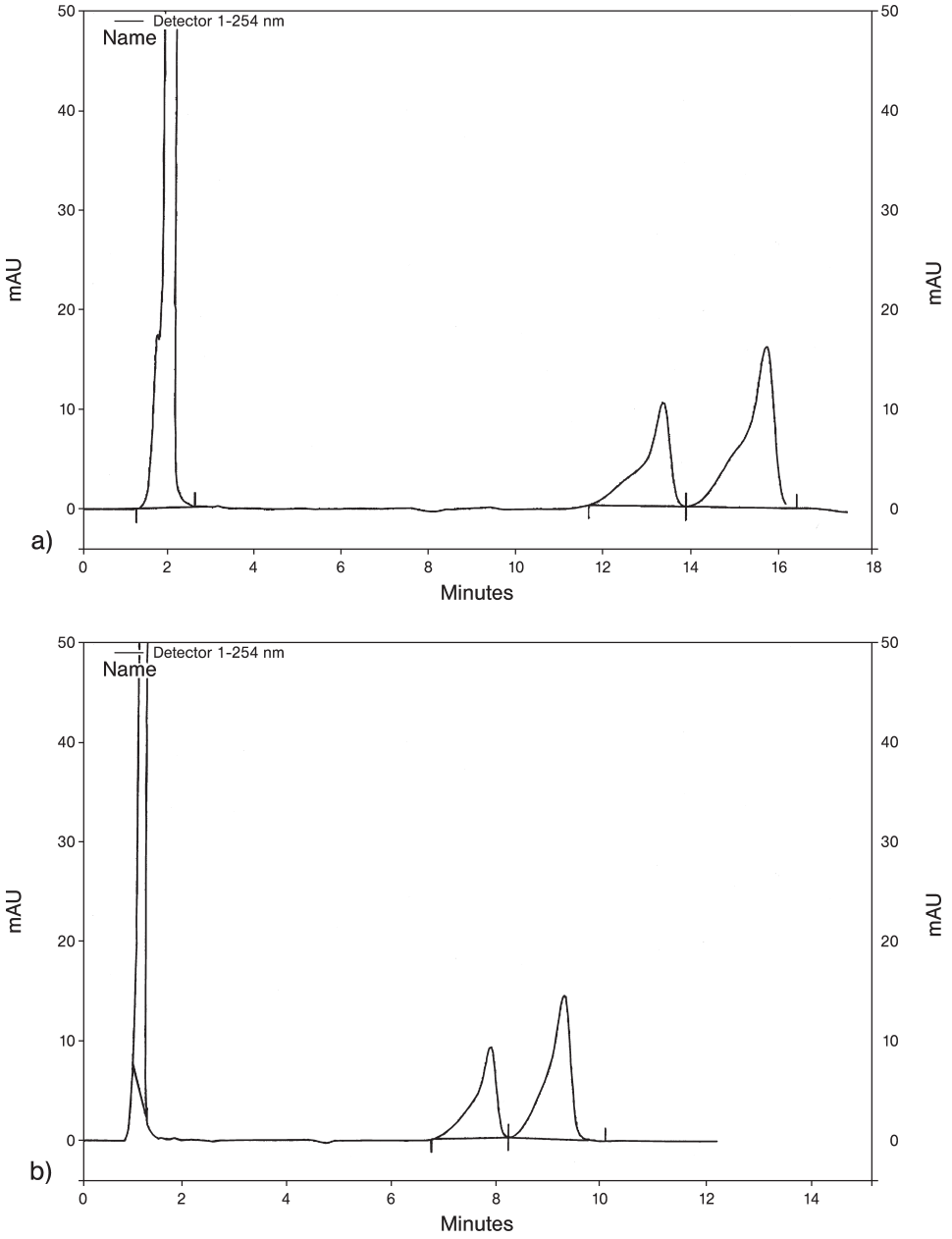

Fig. 3. Simple ways to improve the resolution of an existing RP method. For details, see text.

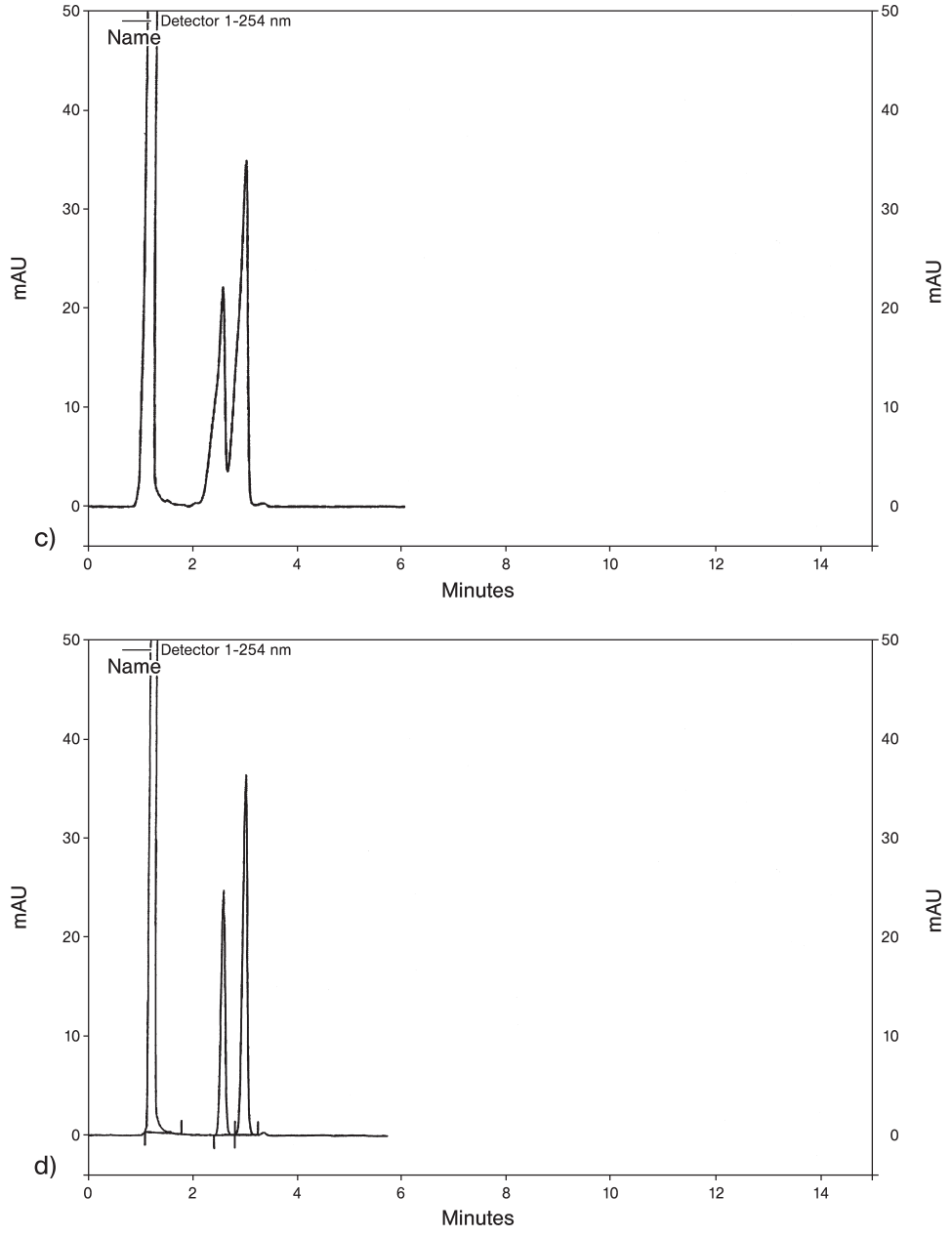

Fig. 3. (continued)

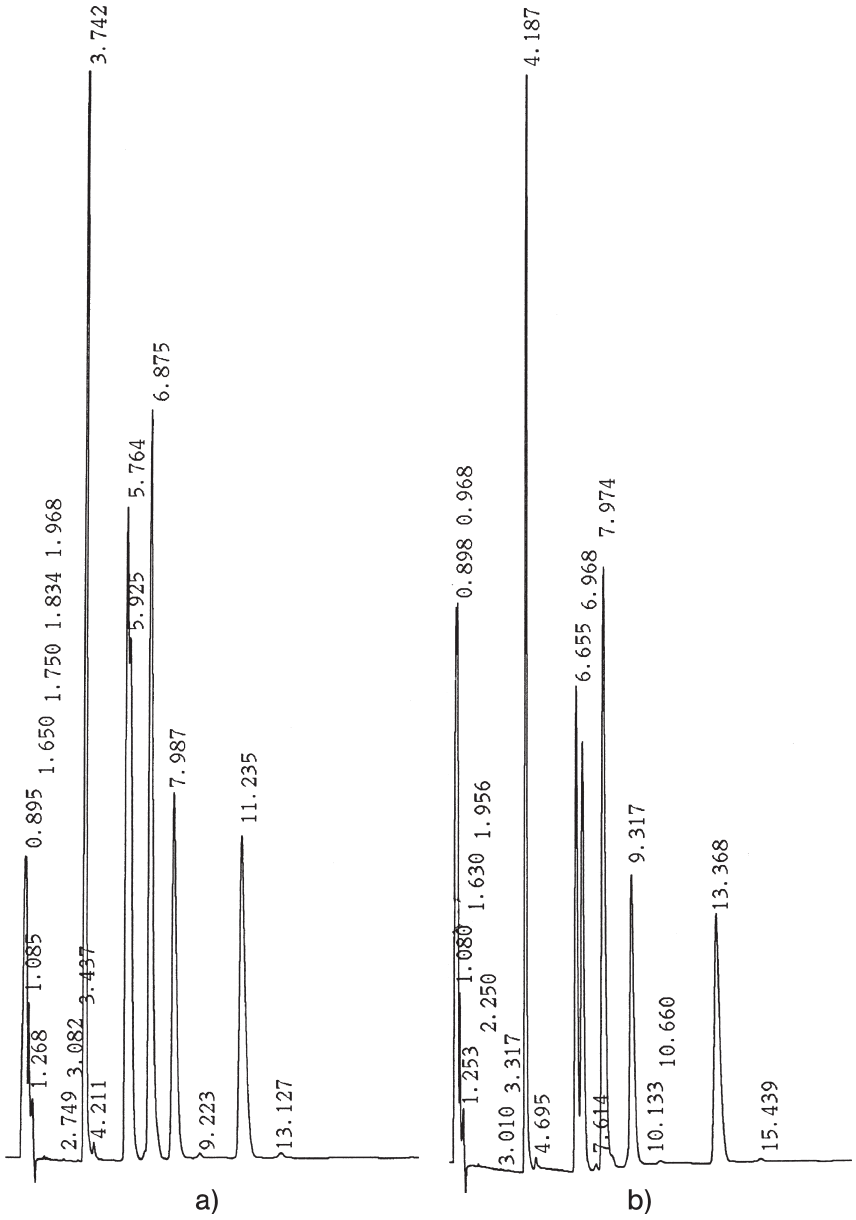

Fig. 4. Improvement of the resolution by increasing efficiency.

Example 2

Figure 4 shows the isocratic separation of metabolites of some tricyclic antidepressants. In (a), separation on a 5 µm Luna 2 C_{18} column is shown. The critical pair of peaks 2 and 3 at ca. 5.8 min (α value 1.05) cannot be separated.

Here, the resolution is not sufficient and hence there is a need for action. In this case, the answer to the first question, if the run time is okay, is "yes". As regards the selectivity, the decision is less clear-cut. If one were to try to improve the separation in this case by changing the column or eluent (selectivity, therefore "chemistry"), this would be unlikely to be a quick solution.

In (b), the separation obtained on a 3 µm column is depicted, under otherwise completely identical conditions. One nearly obtains baseline separation at similar selectivity (α value = 1.04).

The use of a column with smaller particle size is a quick and usually also an economical way to proceed. Even if the obtained selectivity is "only" 1.05, one should first consider an improvement of the resolution by efficiency (N), instead of continuing to try the more large-scale "chemical" possibilities: column, eluent, temperature.

As simplified conclusion and taking into consideration Examples 1 and 2, one can state the following ($k\alpha N$ principle):

1. First look for "reasonable" interactions (k) and acceptable retention times (e.g., by variation of the eluent composition and if necessary the flow).
2. Try to make the selectivity as good as possible through an acceptable degree of effort. This means an α value of around 1.05–1.1 (e.g., replace acetonitrile with methanol, add a modifier, change the pH).
3. If the resolution is still not satisfactory, improve the peak form (N). On the one hand, this can be accomplished by "scientific" procedures, e.g. using smaller particles, most effectively in the following combination: decrease column length and particle size, increase flow rate and possibly temperature. On the other hand, one should also consider simple but no less effective tricks that also result in a better peak form, i.e. smaller injection volume, weaker solvent to dilute the sample, thin/short connection capillaries between autosampler and detector.

1.1.3.4 How to Change *k*, α, and *N*

1.1.3.4.1 Isocratic Mode

Before we start to consider strategies for developing methods for unknown samples and for checking the peak homogeneity, let us consider the established procedures that are available to change or increase k, α, and N (see Fig. 5).

Remark: In the given sequence of the different possibilities in Fig. 5, the efficiencies (time factor and importance) generally decrease. That is not to say that the stationary phase plays a subordinate role with regard to selectivity. Not at all! However, one should test the other faster things before trying out a completely new column; see below.

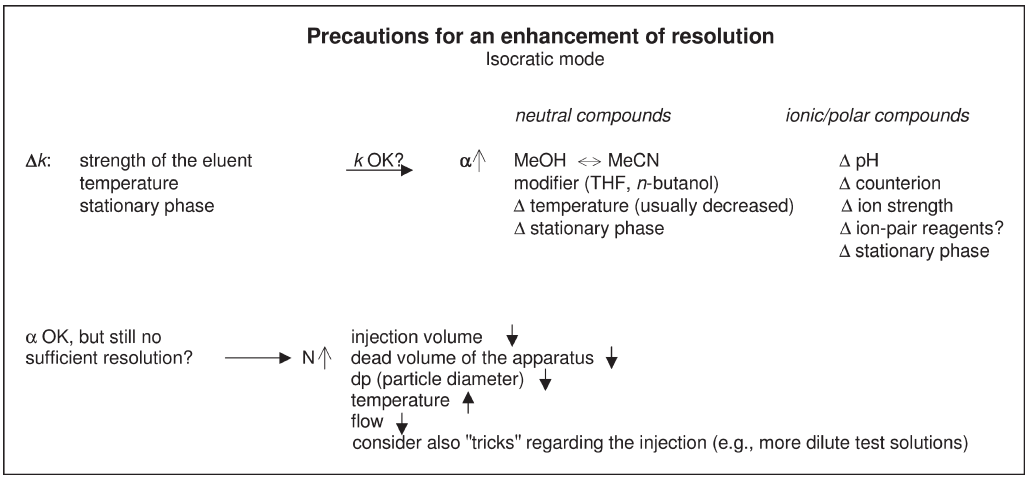

Fig. 5. Possibilities for improving resolution in the RP-HPLC (isocratic mode).

1.1.3.4.2 **Gradient Mode**

Preliminary Remarks

If in the case of a gradient separation one expects no more than 8–10 peaks and if the matrix under consideration is not a difficult one such as fermentation broth, urine, a cream, a plant extract, etc., then 100 or even 125 mm long columns are, as a rule, definitely too long; see Ref. [1] and Chapters 1.2 and 2.7.3. In many cases, we have been able to separate 4–6 peaks with a 10 mm/2 mm/2 µm C_{18} column and "everyday" equipment.

For gradient runs, the following equation applies:

$$\bar{k} = \left(\frac{t_G}{\Delta\%B}\right) \cdot \left(\frac{F}{V_m}\right) \cdot \left(\frac{100}{S}\right)$$

where

$\bar{k}$ = mean k value; the analyte is in the middle of the column (lengthwise)
t_G = duration of the gradient (min)
F = flow (mL min^{-1})
V_m = column dead volume
$\Delta\%B$ = changes in B from the beginning to the end of the gradient
S = slope of the $\%B/t_G$ curve; for smaller molecules, S is set at about 5

Bearing in mind that an optimal $\bar{k}$ value would be about 5, $\bar{k}$ and α can be changed in the following way:

- gradient volume (by the gradient time or more smartly by the flow rate),
- steepness of the gradient,

- % B, i.e. start and end conditions,
- profile of the gradient (linear, convex, concave); if a transfer of the method is intended, then only linear gradients should be used, because of their simple transferability. The inclusion of isocratic steps, on the contrary, should cause no great problems,
- temperature,
- stationary phase.

From a practical point of view, one should think in terms of high flow rates in the course of gradient runs. Increasing the flow while keeping the gradient time constant yields a better resolution because the gradient volume (flow × time) increases. Peak capacity (number of peaks per time unit) and thus resolution itself also increases with increasing gradient volume. Even if resolution is satisfactory, the flow should be increased. For example, if the flow is increased by factor 2 while simultaneously decreasing the gradient time also by a factor of 2 and adapting the gradient accordingly, the resolution will remain the same because the gradient volume remains constant – in only half the time! The disadvantages of using a higher flow rate are the higher pressure and the decrease in the peak area. In the case of gradient separations, the enhancement of the plate number is usually a minor goal because the peaks tend to be inherently sharp.

1.1.3.4.3 **Acetonitrile or Methanol?**

This question is treated in detail elsewhere [2, 3]; see also Chapter 2.1.4. Here, we consider only the results obtained through numerous experiments with various classes of substances under different conditions. It would seem that in the case of mixtures with similar solvent strengths, methanol yields the better selectivity in comparison with acetonitrile, possibly because of the preference for polar interactions (protic *vs.* aprotic solvent). This is most apparent in the case of small molecules such as primary amines.

At the same time, one generally observes a worse peak form because of the enhanced viscosity compared with acetonitrile. This is illustrated by two examples, see Figs. 6 and 7.

Figure 6 shows the injection of uracil, pyridine, benzylamine, and phenol in an acidic methanol/phosphate buffer (a) and in an acidic acetonitrile/phosphate buffer (b). These rather unsuitable eluents (strong bases in an acidic medium) have been deliberately chosen to test the selectivity of methanol and acetonitrile for polar analytes in difficult situations. In methanol, the bases were at least partly separated, which was not achieved in acetonitrile. Figure 7 shows the same separation under neutral conditions in methanol (a) and acetonitrile (b). Again, the better selectivity in methanol is striking. Not only are polar contaminants almost completely separated from uracil, whereas they are barely discernible in acetonitrile (see arrow), but in methanol phenol can also be separated from benzylamine, which is not achieved in acetonitrile. We can thus conclude that methanol gives rise to better selectivity while acetonitrile gives rise to better peak symmetry. This can be observed for many categories of substances.

Possible Explanation: An idea currently discussed is the existence of active or high energy sites on the surface of RP material. Although they take up only 0.4% of the total surface, they play a dominant part in selectivity, as these are the sites that are available to polar/ionic components at a methanol content in the eluent between 0 and 60%. Assuming an experimentally measured solvent layer of 2.5 Å (methanol) or 13 Å (acetonitrile) on the surface, in an acetonitrile scenario, the hydrophobic residue of polar/ionic molecules can only interact with the ends of alkyl chains anchored to the surface. With methanol in the eluent, the molecules can diffuse through the significantly thinner solvent layer, resulting in stronger/additional interactions. Furthermore, the formation of labile methanolates could facilitate polar interactions leading to good polar selectivity in methanol. Finally, we would like to point out three further observations made in our experiments:

1. Differences in selectivity are insignificant where the methanol/acetonitrile concentration in the eluent is low.
2. Selectivity differences between eluents containing methanol or acetonitrile are particularly noticeable in strongly hydrophobic stationary phases and least pronounced in polar stationary phases such as CN.
3. After the addition of methanol, the pH value of a solution shifts to the alkaline, see Fig. 7c (source: Dr. Bernhard Dreyer). See also comments in Chapter 1.3 and Table 1 in Chapter 1.4.

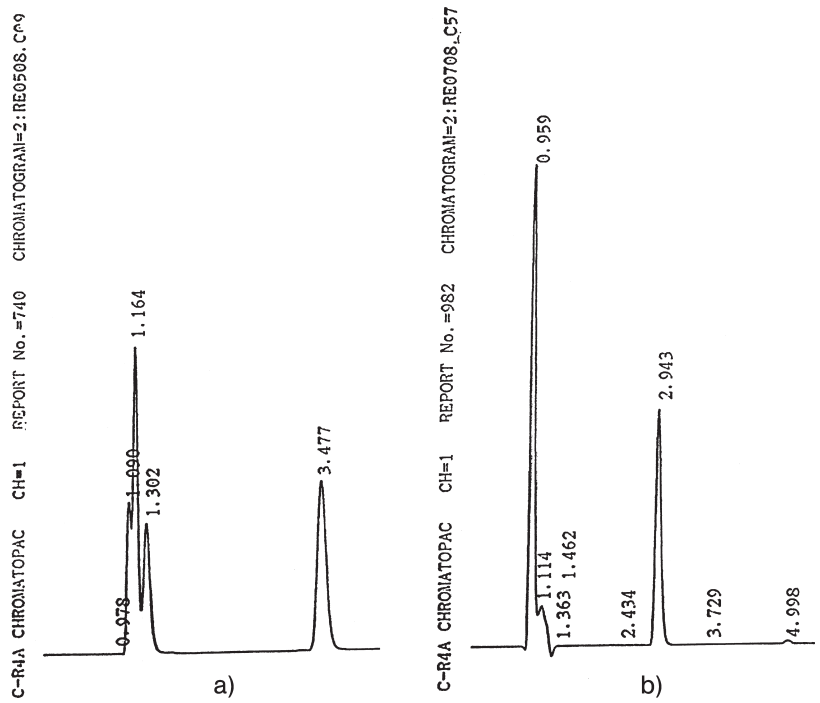

Fig. 6. Selectivity in phosphate buffer/methanol (a) and in phosphate buffer/acetonitrile (b); for comments, see text.

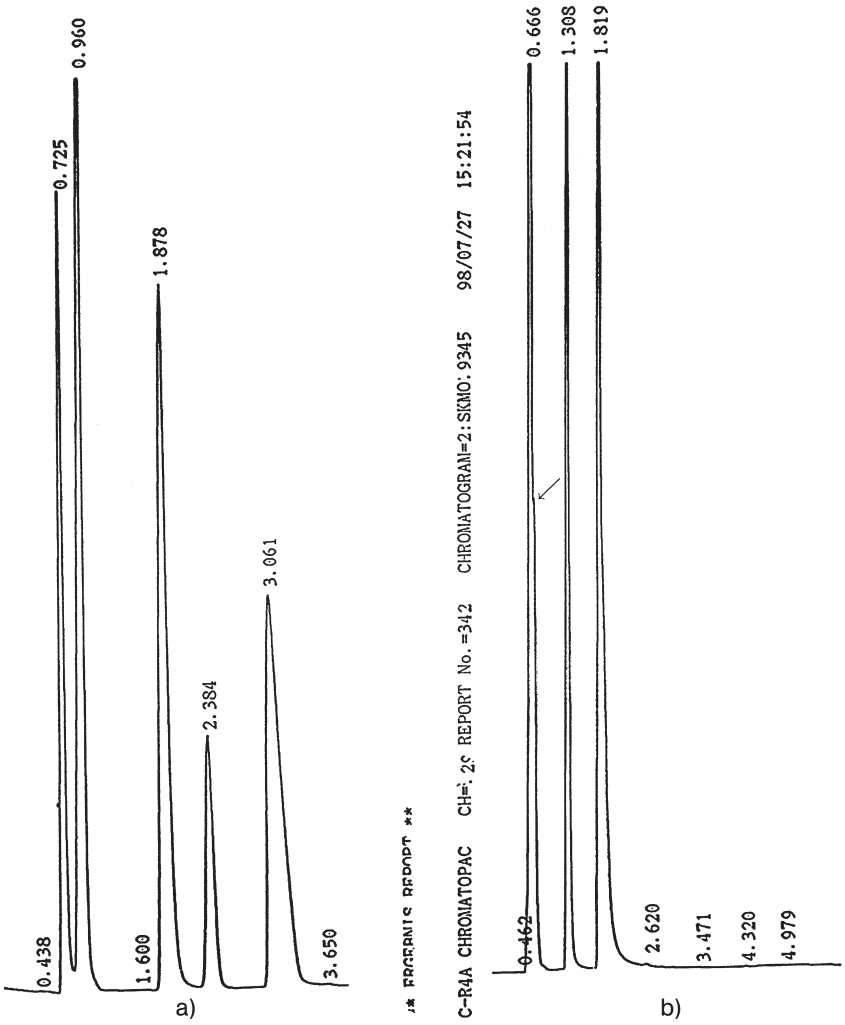

Fig. 7. Selectivity in water/methanol (a) and in water/acetonitrile (b), shift of pH value in a phosphate buffer solution after addition of methanol (c); for comments, see text.

Figure 7c shows the change in pH value of a 20 mMol Na buffer after the addition of methanol. This explains why a column that is run with an eluent at a nominal pH of 6 or 7 has a comparatively low lifespan. After the addition of methanol or acetonitrile, the pH value shifts towards the alkaline. Most silica gels detach and partially dissolve above a pH of 8.

1.1.4 Testing of the Peak Homogeneity

If a possible "rider" is seen on the flank of a peak, how can one improve the resolution? Testing the peak homogeneity and improvement of the resolution are related questions. For both, a three-step concept can be applied.

Step 1: A quick and cheap action for testing the peak homogeneity – the "1/2 hour method"

Supposing that time is limited and/or one has to work within given terms or is bound by requirements of a customer; in other words, the eluent, column, and temperature cannot be changed. What possibilities remain to better depict the chromatogram, or even to improve the separation? The use of contemporary standard equipment is assumed.

1. Make the most of the possibilities offered by the diode array/software, e.g., ratio plot, match factor, contour plot, 3D plot, first and second derivatives of the relevant peak.
2. One should ask whether all settings of the detector and of the software are optimal and whether the hardware can be further optimized.
   In the following, some possibilities are presented and some numerical values are recommended. For quite old equipment, reduction of the time constant to 0.1 s and use of the 10 or 100 mV output of the detector are recommended. With more modern equipment, a similar manipulation is possible by means of the settings "bunching factor" and "bunching rate"/"sampling period" of the software. Set the peak width during the integration to 0.01 min and the sample rate between 5–10 data points per second, i.e. a sampling period of ca. 200 ms. These settings are very important and necessary in the event of early, sharp peaks. If you use very short/narrow columns and obtain "really" fast peaks, 20–40 Hz sample rate are an absolute "must". Are wavelength, slit width, and reference wavelength optimally adjusted? Is the inside diameter of the capillary between the autosampler and detector 0.13 mm or at most 0.17 mm?
3. Remember the injection. To test the peak homogeneity, inject just 1 or 2 µL; there is frequently a risk of local overload of the stationary phase surface. Dilute the sample solution with water or with the eluent and inject again.

A change of settings or a repeated injection takes a matter of seconds or at most a few minutes. The following three examples show that it is profitable to remember such simple actions (see Figs. 8 to 10).

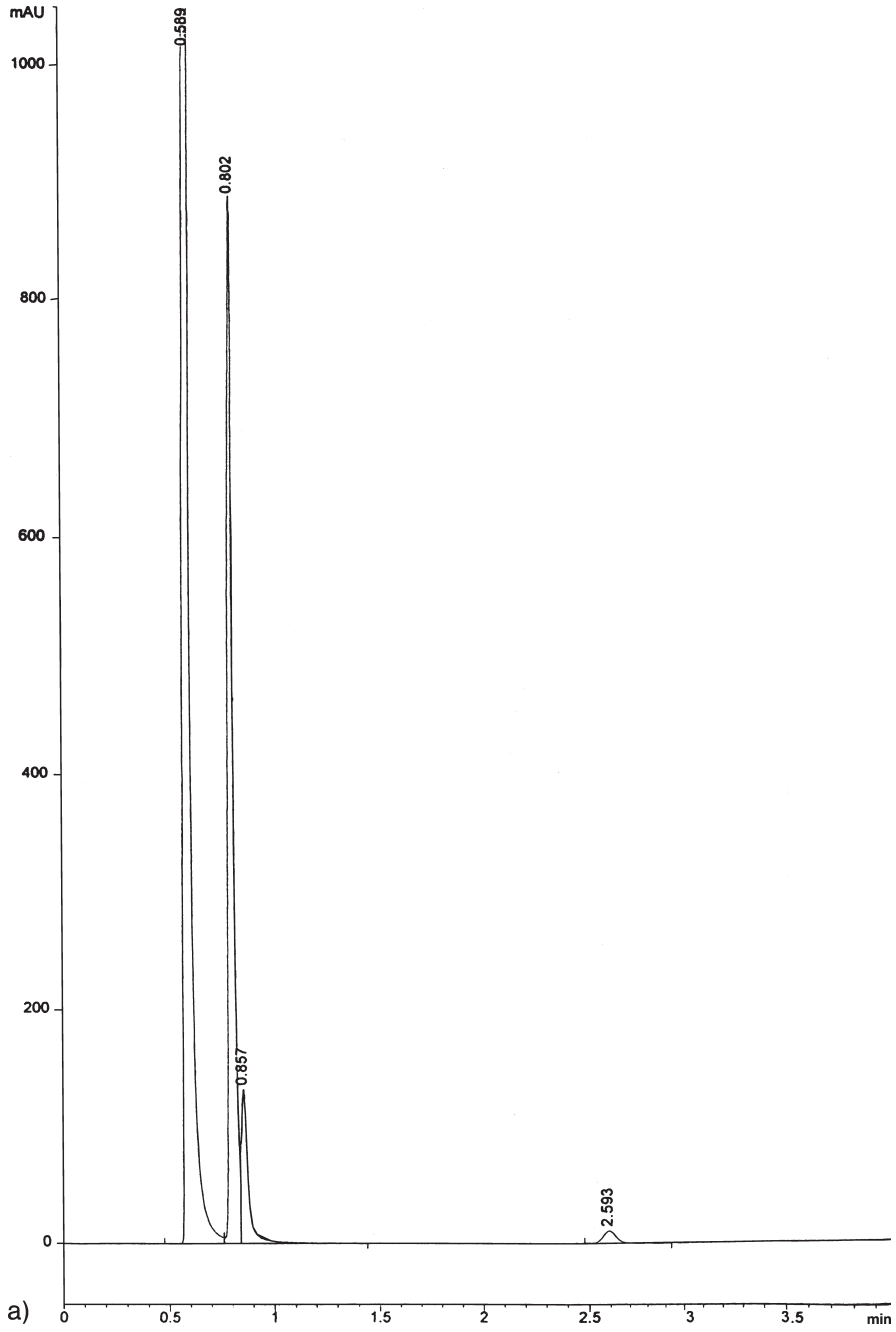

Fig. 8. Effect of sampling rate on the peak shape/resolution for early peaks; for comments, see text.
(a) Chromatogram taken at 0.01 min peak width.

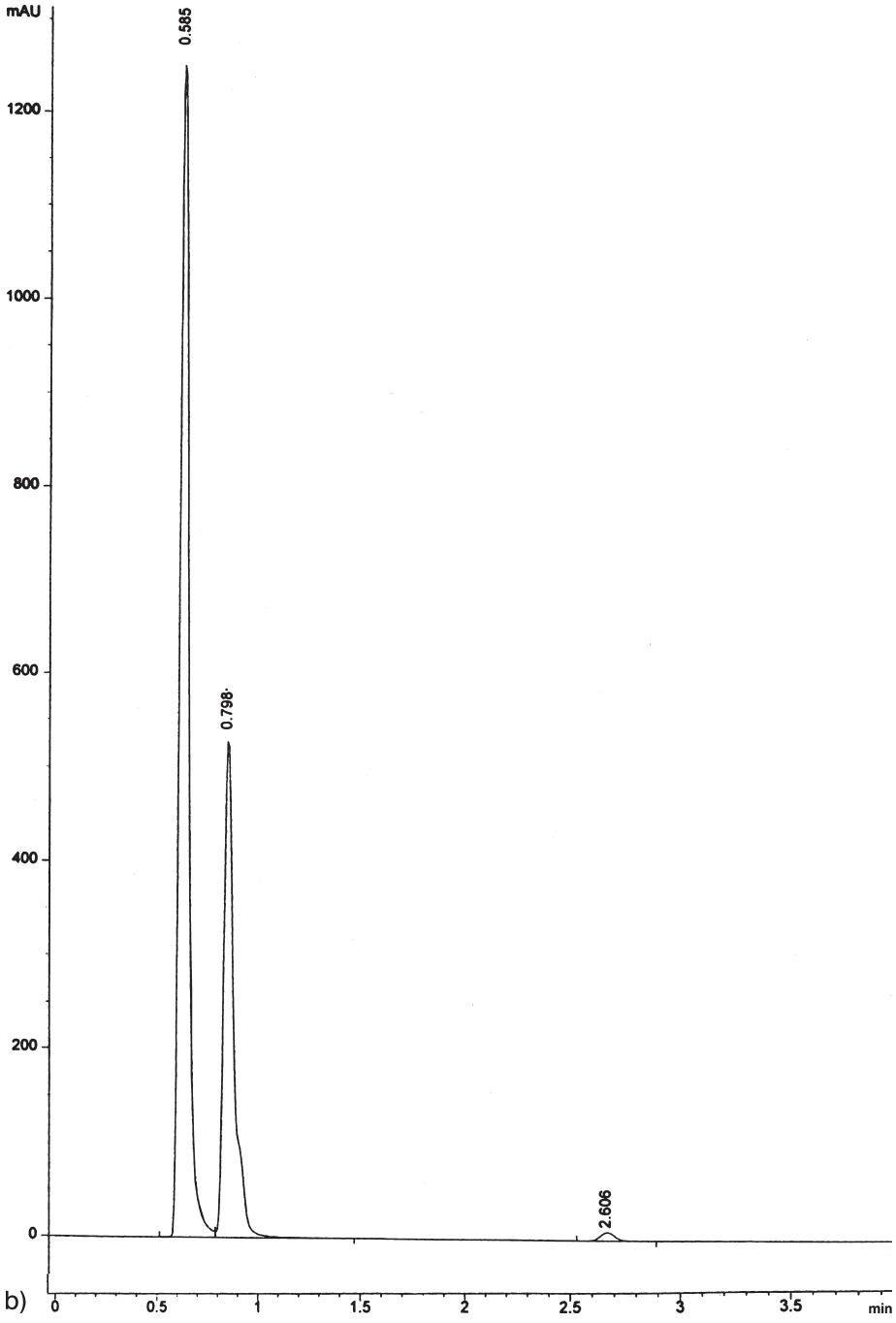

Fig. 8. (b) Chromatogram taken at 0.1 min peak width.

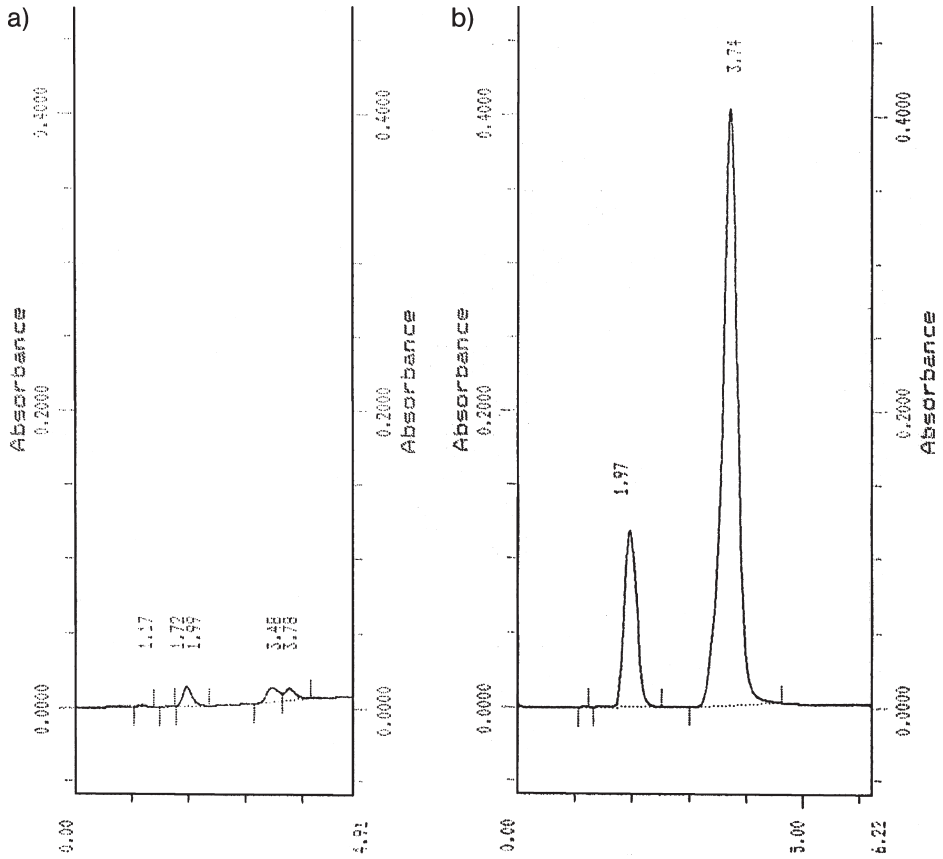

Fig. 9. Effect of injection volume on the resolution of neutral compounds.

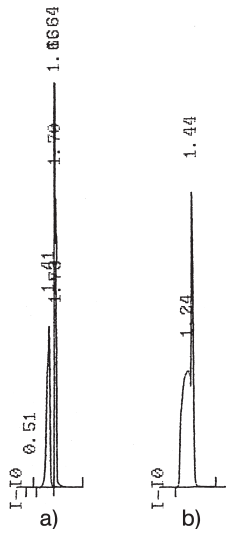

Fig. 10. Effect of injection volume on the resolution of polar compounds.

Figure 8 shows the same separation with 10 data points per second (a) and 1 data point per second (b). Using a low sample rate, there is an unnecessary loss of resolution for the early peaks.

Figure 9 shows the injection of 20 µL of acetophenone in a standard RP system (b). One may assume that the first peak relates to an impurity, and that the second one is acetophenone. Only the injection of 1 µL – see chromatogram (a) – "unmasks" the homogeneity of the impurity and reveals a possible impurity in the "acetophenone" peak.

Figure 10 shows the injection of 20 µL of benzoic acid (b). As can be easily identified, there is an unknown compound for which the peak is poorly resolved. In (a), one can see a much better resolution if 5 µL is injected. The risk of local overloading is increased in the case of polar compounds due to possible dual-interaction mechanisms.

Step 2: Change of chromatographic parameters

This step encompasses procedures that are usually performed within the scope of an optimization. In doing so, mostly the interactions between the sample and the stationary phase are changed. The intention is a change of the retention factor k (mostly enhancement), but ideally also of the separation factor α. Otherwise, at constant interaction strength ("chemistry" constant and therefore k and α as well), one attempts to enhance the plate number or in the case of a miniaturization to prevent dilution or to enhance the relative mass sensitivity. Using a trial-and-error procedure, one needs 1–2 weeks. Using a systematic procedure, aided perhaps by an optimization program, the time can be reduced measurably; see Part 4 for chapters on computer-aided optimization.

Some possibilities are listed below:

1. Eluent: Change of polarity, replacement of acetonitrile by methanol (or reverse), addition of ca. 5–10% of a modifier such as THF, isopropanol or *n*-butanol in the case of neutral components, or addition of amines or acid in the case of polar/ionic components; change of pH, buffer type, and/or molarity.

2. Stationary phase: The broad spectrum of commercially available columns is a great blessing and also a plague. For some rules and some theoretical background for the selection of RP columns, see Chapter 2.1. In this context, the double-column technique should be mentioned. An example of this is as follows: the separation of five quite polar components on a Nucleosil C_{18} column is not especially good; see Fig. 11. On a CN column, the separation is even worse (see Fig. 12). However, when the two columns are connected in series, a very nice separation under identical, constant conditions is obtained (see Fig. 13).

Better resolution is achieved, especially in the first part of the chromatogram. The last apolar peak is slightly delayed by the polar CN material, but this small increase in run time is a price worth paying. Moreover, we have been able to separate tricyclic antidepressants and their metabolites (a total of 12 peaks) in one isocratic run using Zorbax Bonus/Chromolith Performance and AQUA/Zorbax Extend coupled in series.

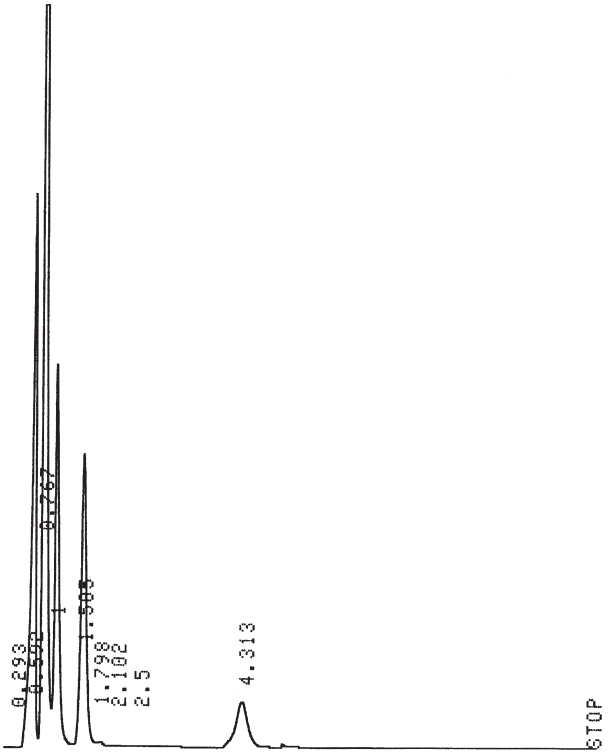

Fig. 11. Isocratic separation of five peaks on a Nucleosil C_{18} column.

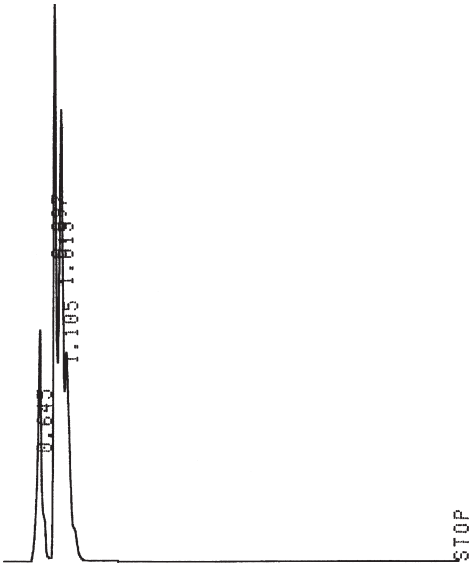

Fig. 12. Separation as in Fig. 11 on a CN column.

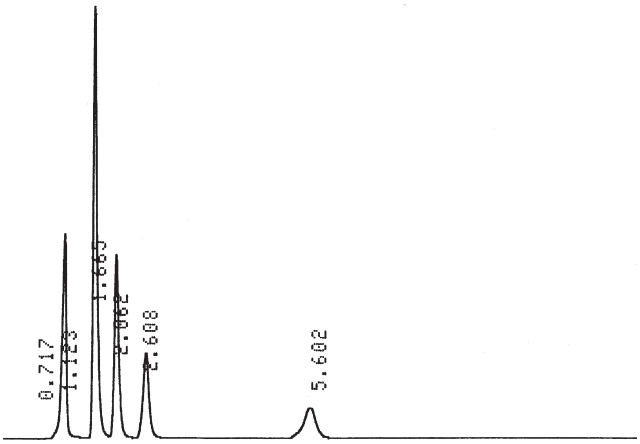

Fig. 13. Separation as in Figs. 11 and 12 on CN and C_{18} columns placed in series.

3. Temperature: At low temperatures, the influence of the mobile phase recedes into the background and the individual properties of the stationary phase come to the fore. The enthalpy differences in the interactions between the individual components and the stationary phase are larger than at higher temperatures and therefore a differentiation (= selectivity) is often easier.

If the temperature is reduced, then the kinetics decreases and therefore also the number of plates. The peaks become wider. On the other hand, the selectivity generally increases. The last noted advantage predominates mostly in the case of RP mode if isomers are to be separated, or with enantiomers (see Chapter 2.6), and therefore the best resolution for difficult separations is frequently obtained at low temperatures. In cases where very slow kinetics is seen as a result of the mechanism, one should work at higher temperatures. At temperatures well above 100 °C one enters the realm of high-speed kinetics, and this results in very good resolution.

The following can be established: Lowering the temperature seems to be an advantage where the steric aspect is crucial in otherwise similar components undergoing separation, e.g. enantiomers, spirally twisted structures, double-bonding isomers etc. In conventional RP separations, however, raising the temperature is usually the preferable option, as this decreases retention time as well as back pressure. The latter effect makes it possible to use 3 or even 1.7–2 µm particles without any trouble. Improved efficiency (higher number of theoretical plates) is achieved not only by using small particles, but additionally lowering the viscosity of the eluent, thus increasing kinetics. Accordingly, at a flow rate of 2 mL/min at 80 °C in four 10 cm columns in serial arrangement, the retention time is equal to that of a 25 cm column at 30 °C and a flow rate of 1 mL/min, but efficiency has been increased by a factor of 4. As the increase in temperature leads to a decrease of polarity in the eluent, fewer organic components are needed in the eluent ("green" HPLC). Finally, the use of acidic buffers suppresses the ionization of bases. This, in turn, increases hydrophobic interaction, and the resulting faster kinetics yield significantly improved peak shapes in ionic/ionizable species.

Step 3: Coupling orthogonal separation techniques

If the sample of a particular separation problem is very important and its identity is truly unknown, one should consider more reliable possibilities for testing the peak homogeneity. Because some of these possibilities are discussed in detail in subsequent chapters, they are mentioned here only in brief. Thus, one may couple HPLC with spectroscopy, e.g. LC-MS(MS) (Chapters 3.3, 5.1, and 5.4) or LC-NMR (Chapter 3.4).

Upon *coupling HPLC with a spectroscopic method,* it is the specificity, not the resolution, that is improved. Therefore, the reliability of a qualitative analysis strongly increases.

Orthogonal separation techniques (= combination of different separation principles/ different mechanisms), 2D or multi-D chromatography. By the coupling of two chromatographic techniques, e.g. LC-GC, gel filtration-ion exchange or LC-DC (2D separation, see Chapter 3.2), or the coupling of a chromatographic and another technique, e.g., gel electrophoresis-HPLC, HPLC-ELISA (see Chapter 3.1) or HPLC-CE, the (chromatographic) resolution increases. Possible subsequent coupling with spectroscopy, e.g. LC-GC-MS or LC-CE-MS, results in an additional increase in specificity.

The possibilities of the coupling technique are illustrated here through the example of LC-DC coupling.

Figure 14 shows the RP gradient separation of an emulsifier on a 2 mm C_{18} column (source: G. Burger, Bayer Dormagen, Germany). A lot of peaks are obtained, some of which are well-separated while others are poorly separated. Consider the last peak marked "D". This peak is quite broad, but there is no strong indication of peak inhomogeneity. If one applies this peak "D" on-line to a DC plate and performs a thin-layer separation with this fraction (two-step gradient; rotation by 90 degrees), one obtains the chromatogram illustrated in Fig. 15. The benefit should be obvious; on coupling two chromatographic techniques, the peak capacities are multiplied.

A "light" version of the orthogonal principle is as follows: one performs a second separation on a completely different column, using another eluent, a reduced injection volume, and/or at another wavelength. The possibility that two or more compounds will show the same behavior under different conditions, i.e. that the same chromatogram results again, is quite low. Guidelines for the selection of RP columns appropriate for orthogonal experiments can be found in Chapters 2.1.1, 2.1.3, and 2.1.6.

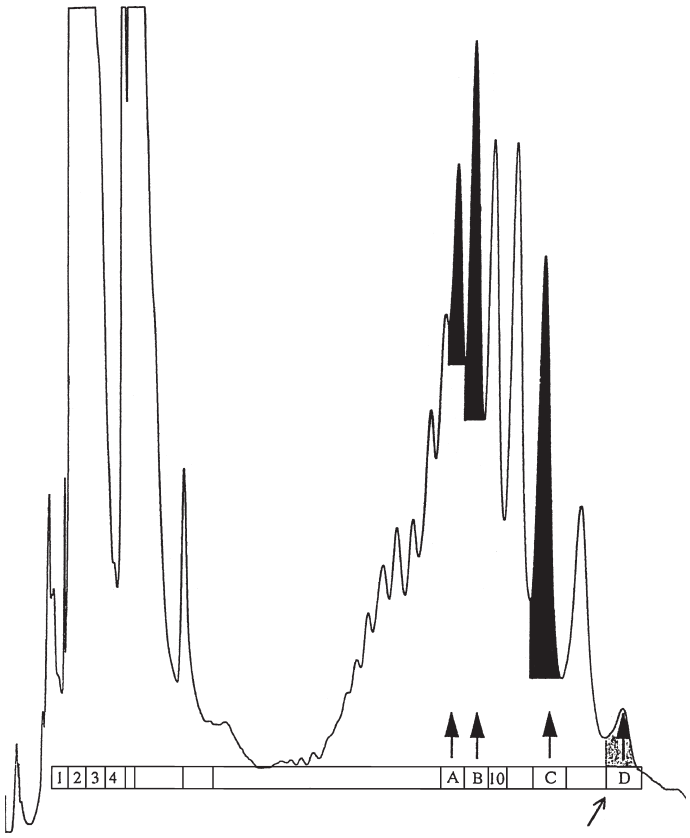

Fig. 14. Separation of an emulsifier in RP gradient mode; for comments, see text.

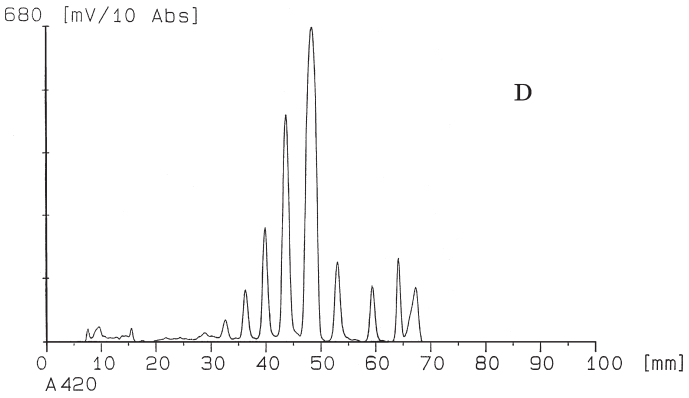

Fig. 15. Further separation of fraction "D" (see Fig. 14) on a thin-layer RP plate in two-step gradient mode; for comments, see text.

Example: An optimization has reached its final step. By injection of 20 µL of the sample on a hydrophobic phase, e.g. Symmetry, one obtains a nice chromatogram using an MeCN/water eluent at pH 3. For confirmation of the peak homogeneity/testing the selectivity, one now uses a polar column, e.g. LiChrospher, and an MeOH/water eluent, and injects 2 or 5 µL. If one obtains the same number of peaks, probably at different retention times and with different peak forms, one may assume that no information has been lost/obscured. In the simplest case, one can use a column with merely another surface, i.e. chemistry, under the same conditions as previously.

The efficiency of such simple testing is illustrated by three real examples:

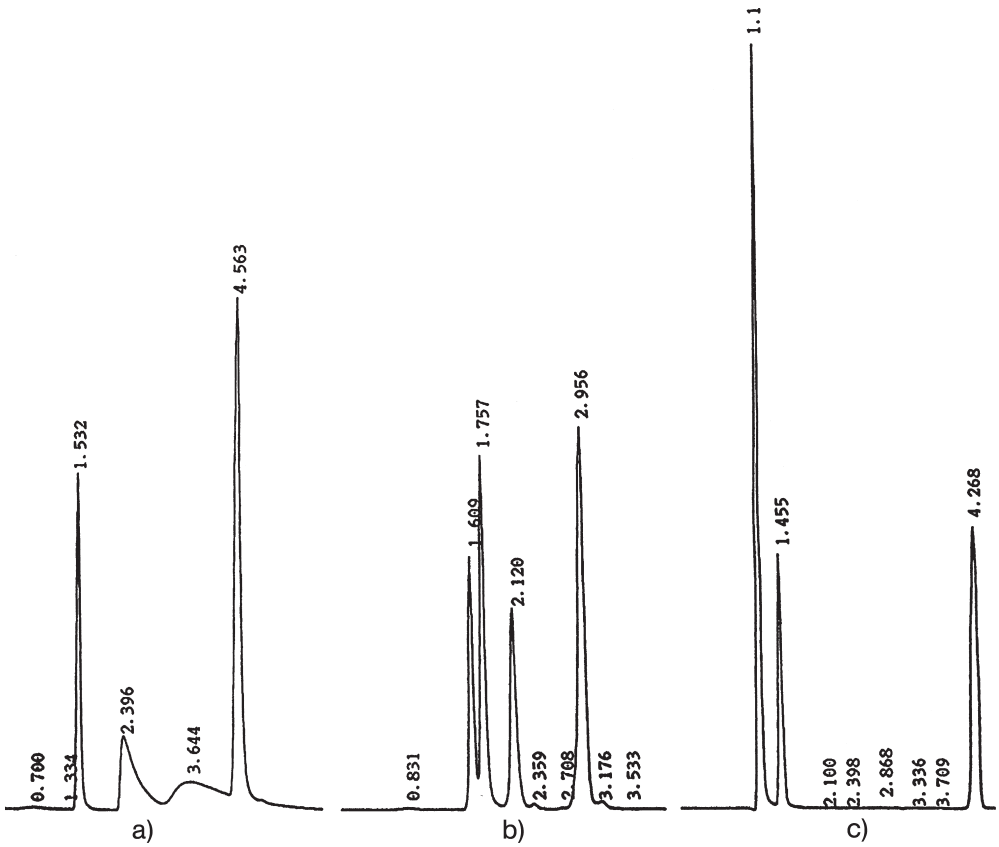

Fig. 16. Proof of peak homogeneity with the help of an "orthogonal" column. Pyridine/benzylamine/phenol test on three phase types in acidic methanol phosphate buffer. For comments, see text.

Example 1

In Fig. 16c , one can see the separation when a sample containing uracil, pyridine, benzylamine, and phenol is injected onto a modern, hydrophobic, endcapped column using an acetonitrile/phosphate buffer at pH 2.7.

If the fact that four peaks were to be expected was not known, no one would suspect the first, sharp, quite symmetrical peak at 1.1 min corresponds to more than one compound. In fact, this "single" peak corresponds to the two bases pyridine and benzylamine, which co-elute even before the dead time, i.e. they are excluded. Indeed, we can hardly expect such a hydrophobic stationary phase to show sufficient polar selectivity. Such a phase is unable to discriminate strong polar analytes in any case. In pharmaceutical laboratories one often sees the following situation: one uses modern, hydrophobic stationary phases because of their good peak symmetry when separating basic drugs. The chromatogram looks beautiful, the match factor of the PDA is around 990, and everything seems perfect, so peak homogeneity is claimed. However, the good peak symmetry also suggests good selectivity. Only by applying two polar stationary phases, Fluofix (Fig. 16 b), Hypersil ODS (Fig. 16 a), under constant conditions is the true situation revealed. Poor peak symmetry (Fig. 16 a) is not of concern here as the aim is just to ascertain the number of peaks.

Example 2

In Fig. 17, chromatograms obtained by the injection of metabolites of tricyclic antidepressants onto three columns are shown. The analytes are small, polar molecules, so two polar stationary phases were employed, an "old" packing (LiChrosorb (a)) and a new one (Reprosil AQ (b)). The apparent result is "five peaks", and so there is no reason for any suspicion. However, using a stationary phase with a pore diameter of 300 Å, one can see that the second, quite sharp peak is not homogeneous (six peaks in chromatogram (c)). Even for such small molecules, steric aspects may be relevant.

Another version of the orthogonal principle with only one column is as follows: one needs a stationary phase with a surface bearing two widely differing functional groups. This might be, e.g., an EPG phase (embedded polar group) with a hydrophobic alkyl chain along with a polar group in ionic form. Another possibility would bear usual C_{18}/C_8 alkyl chains together with very polar groups, e.g. "aggressive" (acid) silanol groups, terminal amino groups, etc. These different groups are responsible for different interactions, of course. Depending on the eluent composition, one or other of the mechanisms can dominate, i.e., through the choice of eluent one can determine whether hydrophobic or polar/ionic interactions prevail. As in the case of the version outlined above, it is very improbable that the analytes will show the same behavior in both cases, in other words that the resulting chromatogram will be almost the same. During these measurements, the wavelength can also be changed, of course. This version is also illustrated by an example (source: SIELC Technologies).

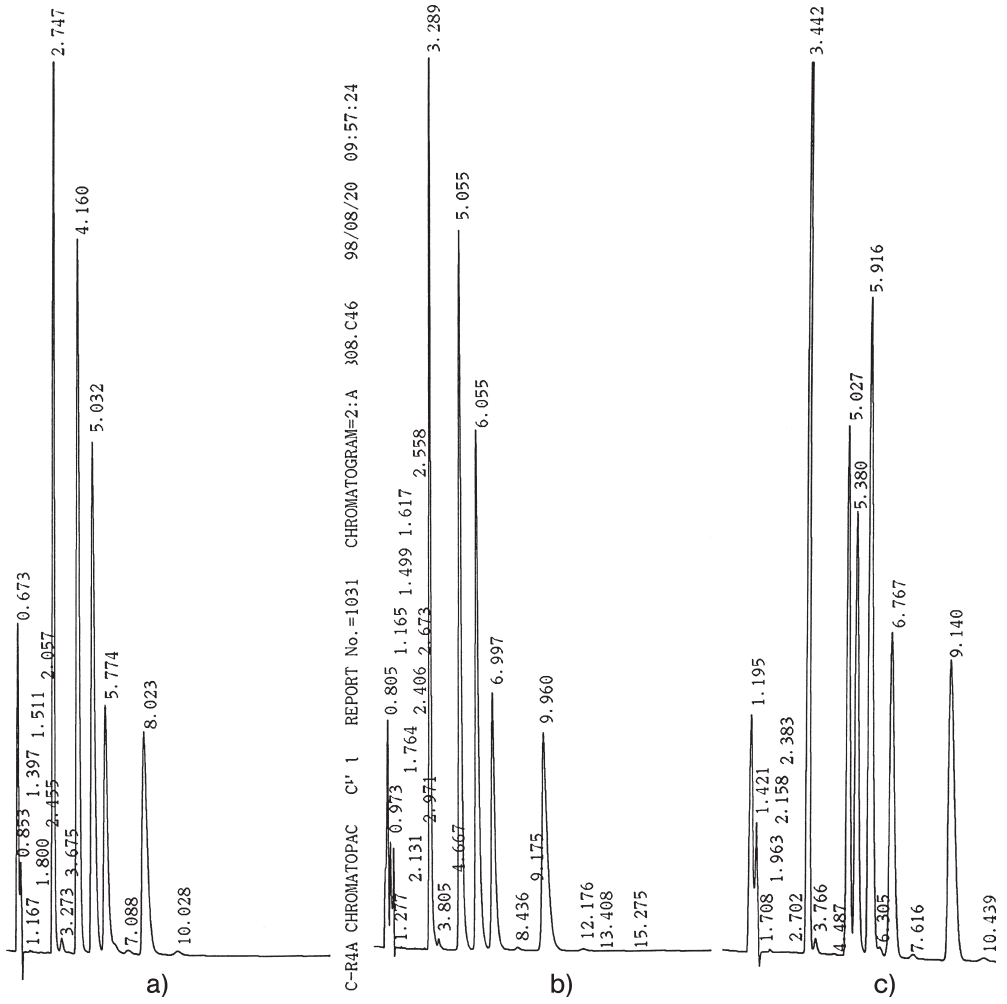

Fig. 17. On steric selectivity ("shape selectivity") even for small molecules; for comments, see text.

Example 3

In Figs. 18 and 19, the separation of six compounds on Primesep 100 with two eluents is illustrated. The surface of this "mixed-mode" stationary phase bears hydrophobic alkyl chains and embedded hydrophilic charged groups. Depending on the eluent, these two functionalities facilitate different interaction mechanisms. In other words, by choosing the appropriate composition of the eluent, one can dictate the mode of interaction.

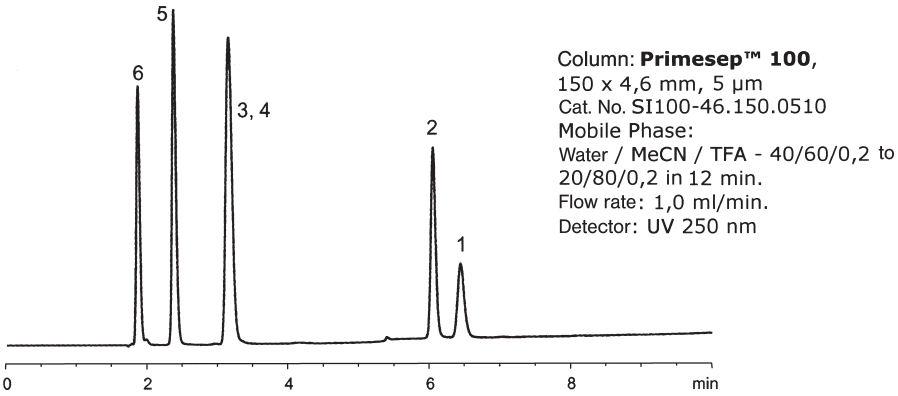

Fig. 18. Reversed-Phase Separation: Separation on a "mixed-mode" stationary phase with an RP eluent; for comments, see text.

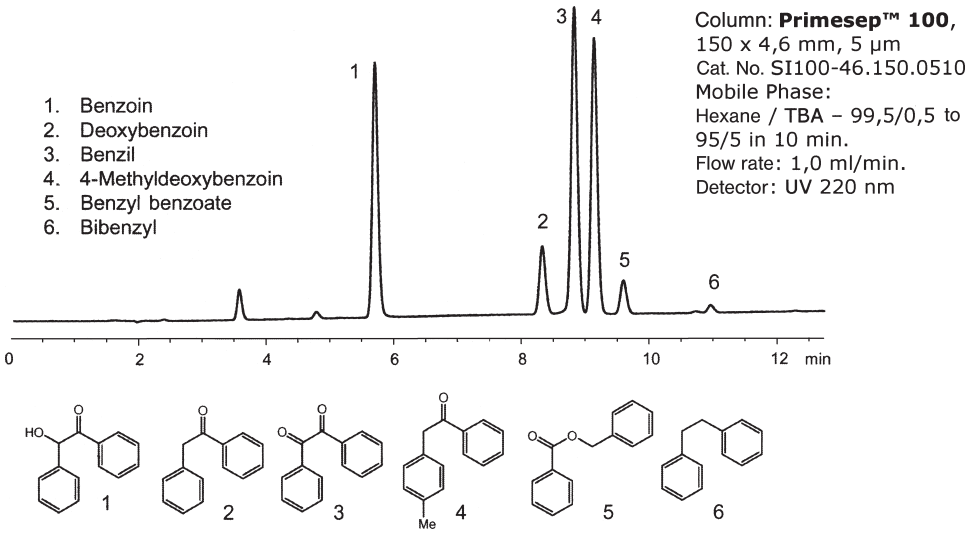

Fig. 19. Normal-Phase Separation: Separation on a "mixed-mode" stationary phase (same column and sample as in Fig. 18) with an NP eluent; for comments, see text.

Figure 18 shows the separation with a standard RP eluent; one observes five peaks. In Fig. 19, the separation is shown using the same column but with an NP eluent and at a different wavelength. Firstly, because of the different mechanism there is an inverse elution order, and secondly compounds 3 and 4 are now baseline-separated.

Summary of the "light" version of orthogonal separations

1. Employ your actual eluent on two as different as possible RP phases.
   Variation: Replace e.g. 50% MeCN by methanol.
2. Use a column with different functionalities on the surface of the stationary phase with two as different as possible eluents.

The probability of achieving a successful separation is further increased if one decreases the injection volume and/or changes the wavelength.

1.1.5
Unknown Samples: "How Can I Start?"; Strategies and Concepts

Obviously, there are several possible protocols for addressing this problem. If a procedure is established in your laboratory, which is adjusted to your samples and your environment, and which is effective according to your criteria, then there is no reason to change anything. However, if the development of the crude method for essentially similar samples needs more than about two weeks, then there is probably room for improvement.

In the following, some proposals for straightforward method development are presented, preceded by two preliminary remarks.

1. If it is possible and if it is relevant to the question, the pH of the sample solution should be measured. Sometimes, the conductivity can also be useful. A rough indication of the acidic or alkaline reaction of the sample solution is a welcome aid for the pre-selection of the mobile and stationary phases. Even more effective is the calculation of pK_a values in the case of known or expected substances on the basis of their structures. The most interesting pH range with regard to selectivity as well as robustness experiments is ca. ±0.5 pH units around the pK_a value. One should begin with experiments in this range (see also Chapters 1.3 and 1.4).
2. If the possibility exists, an LC-MS(MS) or even an LC-NMR run at the beginning of the development of a method can quickly yield extensive information. In general, the coupling LC-spectroscopy is suitable either for the last step in an analytical sequence to test the peak homogeneity or for the first step after a successful chromatographic separation (HPLC effectively serving as a better sample preparation technique) to get an initial impression of the sample. Considering that a given mass spectrum can rapidly be compared with ca. 250,000 spectra in spectral libraries (e.g., NIST) via the Internet or via one's

own intranet with spectra of similar company-specific compounds, one should always try to use this possibility. Incidentally, the costs of 1000–2000 Euro, typical for the measurement of an LC-MS(MS) or (LC)NMR spectrum by an external service, are small in relation to the overall analytical costs associated with an important sample.

1.1.5.1 The "Two Days Method"

Applying a systematic procedure and using adequate equipment, it is realistic to be able to develop a method in roughly two or three days.

Consider a hypothetical case, in which no information is known about the sample, only that it can be dissolved in MeOH/water, MeCN/water or THF/water mixtures and therefore an RP separation is possible. In the following, a possible strategy is outlined; see also Ref. [1].

Step 1: Development of a "useful" gradient run

One could start this procedure after lunch, let's say at 13.00–13.30. One applies a linear gradient, e.g. 10 to 90% MeCN with an acidic pH value (pH ≈ 2.5–3.5) at ca. 30 °C. The pH is usually adjusted with phosphoric acid, but perchloric acid, hydrochloric acid or trifluoroacetic acid are interesting alternatives. One will always like to start with one's favorite column, which is okay. However, it should be borne in mind that in this hypothetical case, one does not know how many peaks to expect, so the column should have ca. 8000–10,000 plates, which is obtained by filling a 125 mm column with 5 μm particles or filling a 100 mm column with 3 μm particles. If no more than five to ten peaks are expected, a shorter column can/should be used. The flow rate should be ca. 2 mL min^{-1} and the gradient time ca. 20 min. With these values, a gradient volume of ca. 40 mL results, which is sufficient in the first instance. Theoretically, at least 30–35 components would be separable under these conditions. In any case, one should inject a quite dilute solution and the volume of the injection should be no more than 20–30 μL. Assuming that one is equipped with a DAD, one should exploit its full potential, and this means more than just working at different wavelengths. If time permits, it is very desirable to also test methanol as organic solvent at a pH of ca. 7–8. It needs ca. 2.5–3 hours to get "any" chromatogram after these experiments. If nothing intervenes, the time is now ca. 16.30.

Step 2: Choice of a selective column I

A column selecting valve, which should be an indispensable tool in any development laboratory, can now be charged with 6 or 12 columns. In the case of a completely unknown sample – a situation that rarely arises in real life – one should use "a little bit of everything", e.g., two polar phases, two hydrophobic phases, as well as a 60 Å and a 300 Å material of both. For a detailed discussion of the choice of an RP column, see Chapter 2.1.1.

Under the conditions that result in the "best gradient" in the preliminary experiments (step 1), one should perform a run overnight using the six columns.

To get enough "buffer" in the run time, the analytical time should be set at 45 min. Six interesting chromatograms should be obtained in the morning.

Step 3: Choice of a selective column II

At the end of the working day, a further six columns should be inserted into the column selecting valve, preferably columns with phases that are similar to the best column of the previous night's experiment. This is the column that yielded the most peaks.

Step 4: Fine optimization

Using the most selective of the 12 columns from the two overnight experiments, one should perform additional experiments to achieve optimal resolution. This includes a change of the gradient, including pH, as well as a variation of the temperature. If method development software is available (see the chapters of Part 4), then finding the optimal conditions can be a smart and efficient procedure.

By treating the last step systematically, with or without method development software, there is a good chance of devising a more or less useful method at the end of the day. Clearly, the resulting separation will have to be examined and probably optimized as well.

Because the choice of the column is made using the column selecting valve overnight, the actual working time that has to be invested for this procedure is only a day and a half.

Recently, the major manufacturers have started to offer HPLC equipment with the possibility of automatic operation of the column selecting valve and optimization of the separation according to the requirements of the user by means of suitable software. For more details, see the chapters of Part 4.

As one might imagine, there are different versions of the aforementioned procedure. In the following, some of these are briefly outlined. The reader should decide which concept corresponds most closely with his or her requirements.

(A) The simplified principle of the concept is as follows:
"We want to use at most three different columns and to reach the optimization mostly using variations in the eluent. In this way, it is not necessary to buy a lot of columns." In such a case, one should use, for example, a hydrophobic phase, which one can designate as a "universal column" (Symmetry, Luna C_{18} 2, YMC Pro C_{18}, Nucleodur Gravity, Purospher, etc.), a polar "universal column" (LiChrospher, Zorbax SB C_8, Atlantis d C_{18}, SynergiPOLAR RP, Polaris, etc.), and a column that proved useful in similar cases in the past. If there is no experience of this type in the laboratory, for the third column one could use a completely different type, e.g., Nucleosil 50 or Jupiter (both of which give rise to a steric effect because of the small or large diameter of the pores), SMT OD C_{18} (polymer layer), Fluofix (fluorinated alkyl chain), Hypercarb, ZircChrom (different matrix and therefore

different chemistry). In particularly complicated situation where selectivity needs to be tested in extreme conditions, it would be advisable to use a column that retains its stability in the acidic as well as in the alkaline or at very high temperatures, such as Pathfinder MS/PS (Shimadzu) or $Blaze_{200}C_{18}$ (Selerity Technologies). In this case, one would also start with a linear MeCN gradient of the eluent at three different pH values, e.g. pH 2.5, 4.5, and 7.5. Alternatively, and this is recommendable, methanol can be used. If the pK_a values of the expected components are known or can be calculated, the pH can, of course, be carefully adjusted. If one expects neutral and/or weakly polar analytes, then one should use in any case a neutral gradient containing 3–10% modifier. THF, isopropanol, and *n*-butanol are suitable modifiers.

(B) Another concept is as follows:
"We vary the stationary phase and keep two quite different eluents constant in order to ascertain which phase type and therefore which separation mechanism yields the best selectivity. Thereafter, we make a fine adjustment, first by variation of the eluent and the temperature."

This procedure is the same as that described above. Versions of this are as follows:

Version 1

- Overnight, six polar columns should be inserted into the column selecting valve (e.g., from very polar, like Platinum EPS or Hypersil ADVANCE, to moderately polar, like LiChrospher or XTerra).
- Over the next night, one should test only hydrophobic phases (e.g., from quite hydrophobic like Purospher and Discovery C_{18} to very hydrophobic like Ascentis C_{18} or SynergiMAX RP).
- A further night is spent on "specialities" of selectivity. The following possibilities may be considered:
  - One should consider the steric effect (e.g., Novapak, Nucleosil 50, and Spherisorb ODS 1 up to Zorbax SB 300, ProntoSil 300, and Symmetry 300).
  - One uses six completely different columns, e.g., one of moderate hydrophobicity but elevated polarity (e.g., SynergiFUSION RP), a monolith (e.g., Chromolith Performance), one with a long alkyl chain and hydrophilic endcapping (e.g., Develosil), one with incorporated ionic groups (Primesep A), and two "high-speed" columns (20–30 mm, 2–3 mm, 1.5–2 µm).
  - One works with an alkaline eluent for this and therefore has to use an alkali-resistant phase, e.g., Gemini, Zorbax Extend, XBridge, Asahipak, ZircChrom, Hypercarb.

In this way, one can test overnight some quite different phases for selectivity for the given separation problem, without the necessity of working actively with the equipment.

Version 2

If a quaternary low-pressure gradient system and a six-column selector are available, a two-eluent-six-column combination can be tested overnight. For the four mixer chamber inlets use, e.g., the following: MeCN, "acidic" water, "basic" water, rinsing liquid, e.g.:

- First night:
  Eluent 1: acidic MeCN/water gradient
  Eluent 2: alkaline MeCN/water gradient ... on six columns
- Second night:
  Eluent 3: acidic MeOH/water gradient
  Eluent 4: alkaline MeOH/water gradient ... on six columns

Where necessary, additional eluent/column combinations and pH profiles should be tested.

Summing up the method development concept, the proof of peak homogeneity as well as the robustness of the method may be performed as follows:

1.1.5.2 "The 5-Step Model"

This approach draws on experiences from various method development and optimization projects. Variations in the description of the first steps reflect individual preferences of the laboratories involved as well as differences in the hardware used.

Preliminary Remarks

1. Supposing you have access to a powerful automatic gradient mixer with very little dead volume, featuring 2–4 solvent inlets with corresponding eluent switching valve, a coolable column oven, a column-switching valve and a PDA detector, LC-DAD-MS-coupling would be the method of choice. Although using a 12-column selector would provide a wider range of possible variation, we suggest using the more widely available 6-column selector.
2. Furthermore, for economical reasons, we urge you to use only short columns (20–30 mm, 1.8–2 µm) – at least in the initial stages when you are still trying to find your way. Thus, through standards and fast runs, even fairly conventional LC machines could detect trends and roughly identify important parameters such as pH value, solvent and gradient very quickly. Alternatively, if the opportunity arises, a UPLC (Waters), Ultra-Fast-LC (Agilent), X-LC (Jasco), UltiMate (Dionex) etc could be used. In a second step, Fast LC or UPLC results can be transferred to HPLC or those from a conventional LC machine adapted to the expected routine situation if necessary. Where complex samples can be expected in routine separations (mother liquor, stress solutions, contaminated samples, complex matrix etc) the acquired knowledge from testing stationary phases could be used to find appropriate longer and therefore more robust columns.

3. The model shown below is based on a worst case scenario, i.e. no additional information about the sample is available. If the components of a sample are known or within a certain range of expectation, it is worth making a reasonable effort to collect preliminary information about their chromatographic behavior from various sources (internal/external databases, the Internet, software tools such as acdlabs, ChromSword etc) or simply about compound data, e.g. pK_S value (see Chapter 1.4) and degree of dissociation. This could be helpful when deciding on what type of column and what eluent to use. Finally, no further persuasion should be needed in favor of using small columns. Otherwise the amount of time needed for the experiments will go up accordingly.

Step 1: Orientation (day experiment)

Start off running three quick linear gradients (e.g. 5 to 95% acetonitrile) at three different pH-settings (e.g. 3, 7, 9) on six different columns (e.g. C_{18} manufacturer 1, C_{18} manufacturer 2, phenyl, EPG 1 (EPG: phase with **E**mbedded **P**olar **G**roup), EPG 2, "AQ" (AQ: hydrophilic endcapped phase) at an optimum wavelength. Assuming that the gradient duration is 3–6 min, the timescale will be as follows: 3 pH values × 6 columns × approx. 14 min (10 min maximum separation time plus 4 min equilibration time) = approx. 250 min, which is roughly 4 hours. The optimum column-pH value combination (criteria: primarily number, secondarily shape of peaks) is used with the following changes:

1. The pH value is set using a second acid/base (e.g. formic or perchloric acid instead of phosphoric acid; ammonia or triethylamine instead of sodium hydroxide).
2. 50% of the acetonitrile component in the eluent is replaced by 50% methanol (e.g. instead of a 5 to 95% acetonitrile gradient 2.5% MeCN plus 2.5% MeOH to 47.5% MeCN plus 47.5% MeOH) or by 10% THF. The time required for these three runs is approx. 30 min.

This first step which takes approx. 5–5.5 hours should answer the following question: Which stationary phase type, which modifier and which organic solvent in the eluent yields the highest number of peaks? If it emerges that the highest selectivity can be expected in the alkaline, six alkaline-stable columns should be tested at three higher pH levels (e.g. 10, 11, 12) by using two modifiers (e.g. ammonia, ammonium carbonate or borate buffer). Columns to be considered: Gemini, Pathfinder, Zorbax Extend, XBridge C_{18}/Shield, Kromasil, Hamilton PRP/Asahipak.

Note: In certain applications (ion exchange, RI detection, gel filtration), an eluent switching/low pressure valve is used to send naturally isocratic solvent mixtures along the six columns, modifying the solvent, pH value or salt content.

Variation

1. Three columns can be tested (e.g. C_{18}, phenyl, diol) in fast gradients using different solvents (usually acetonitrile and methanol or, rather than pure

methanol, 50% acetonitrile/50% methanol) and three different pH levels. Otherwise, proceed as described above. Number of runs: 3 columns × 2 solvents × 3 pH values = 18 runs. Required time: 18 runs × 14 min (10 min separation time plus 4 min equilibration time) = approx. 4 hours.

2. On the basis of the chromatograms obtained by these runs, it is then decided which parameter yields greater variance – the solvent or the pH value. Then a further test is run on three columns, with either two pH levels and the "better" solvent or with the "best" pH value and two solvent compositions. Number of runs: 3 columns × 2 pH levels or 2 solvent compositions = 6 runs. Required time: 6 runs × 14 min (10 min separation time plus 4 min equilibration time) approx. 1.5 hours.
3. Finally, the best column with the optimum pH level and the better solvent undergo a last small optimization process. Combine each of two gradients with two temperatures, resulting in 4 runs. Required time 4 runs × 14 min (see above), approx. 1 hour.

Total time required for the three steps: 4 + 1.5 + 1 = 6.5 hours. Allowing a little extra time for setting temperatures and other preparations, these tests would be feasible in a day. They also help recognize trends and yield similar information as the previously described runs – which stationary phase type, organic solvent, pH level, gradient and temperature are the most suitable to obtain the highest number of peaks.

Step 2: Choice of Column (Night Experiment)

The column-switching valve is equipped with the best of the six tested columns and five further columns (details about the choice of columns see Chapter 2.1). Two gradients are run in the six columns over night at a pH level ± 0.5 of the pH level previously considered as optimum. 2 pH values × 6 columns = 12 runs.

Step 3: Fine optimization, Method Robustness (Day Experiment)

The heretofore best combination of eluent, pH value and column now undergoes further fine optimization/adjustment – variation of gradient (initial and final conditions, slope, volume and profile of gradient), temperature and, where applicable, particle size and column length. This is also the time when for economic reasons, while chromatographic parameters undergo systematic variation, a first robustness check of the method used should take place (e.g. impact of small variations in pH level etc).Let me mention in this context that optimizing procedures and experiments to ensure method robustness are greatly helped by powerful optimizing programs that are commercially available (see Chapters 4 and 5.2). But even without such aids, it is realistic to set aside two days and one night to roughly work out the method. At this stage at the latest, critical input from an expert not involved in the project is needed.

Step 4: Checking Peak Homogeneity, Cross-Experiments (Night Experiment)

In principle, peak homogeneity can be checked using spectroscopy and orthogonal techniques, see above. Alongside PDA we recommend MS- (ESI-MS, TOF-MS, MALDI) and NMR offline/online coupling. 2D or Multi-D-chromatographic separation – often in connection with spectroscopic techniques – have become the tools of choice for a definitive check on peak homogeneity or for the separation of highly complex samples and/or matrices. For more details, see Chapter 5.3. How much work should go into these checks must be decided on a case-to-case basis. Evidence seems to suggest that orthogonal experiments (cross-experiments) are fairly reliable without the need for laborious coupling techniques. The peaks need not be identified, as long as their number is confirmed. The following schema gives an overview of simple and complex ways of checking peak homogeneity.

Note: It is understood that optimum hardware (capillaries, detector cell) and optimum settings have been chosen, e.g. wave length, reference wave length, data rate setting, bandwidth, time constant etc.

Checking Peak Homogeneity in RP HPLC

1. Make the most of what your machine has to offer without changing methodological parameters: PDA, perhaps LC-MS.
2. Here is what you can check in isocratic runs: Are the quotients peak width/retention time on a straight line for all peaks? Could an outlier be an indication of the inhomogeneity of the peak in question?
3. Easy and fast checks: decrease injection volume, dilute the sample with water/eluent and reinject, use the same stationary phase with smaller particles.
4. Orthogonal tests:
   - Same column, different eluent (e.g. methanol instead of acetonitrile, or pH level Y instead of X).
   - Same eluent, different column (e.g. nonpolar instead of polar stationary phase).
5. Fractionate main peak (front flank, tip, back flank), concentration of the fractions is hardly ever needed.
   a) Reinject the fractions one by one.
   b) After a second column has been switched in series, the fractions are again injected one by one.
   c) The fractions are examined using GC, DC or CE.
   d) The fractions are examined using IR, MS or NMR spectroscopy.

Tests 1 to 4 and/or 5a or 5b should always be carried out on important samples. Spending a day on checking points 1 to 4 or, respectively, about two days in addition for points 5a and b is worth the effort, while decision to invest in two additional days for point 5a and b depends on the particular circumstances.

Step 5: Robustness of Method (Day Experiment), Column Stability (Weekend Experiment)

The last step of a method development project should be a check of the ruggedness, if the method under consideration is not to be used for a single application. This means the capability of a method for routine work. The effort and the tests required depend of course on the actual question. Although robustness/ruggedness are very important issues, they are not topics of this book, and therefore the possible tests are only mentioned in brief.

Important Notice: It is vital that robustness is checked using real samples. If these are unavailable, samples should be prepared that resemble as closely as possible those that will be used in routine separations later: Matrix/placebos/solvents/excipients/stress solutions plus analyte. There is little point in testing the robustness of a method on standard solutions – or else there may be a few nasty surprises in store!

(A) Compatibility Sample-Stationary Phase

1. Catalytic Effect of Silica Gel?

Silica gel as a stationary phase or matrix in RP phase is a very good catalyst for solids. It is often found that many small peaks in the chromatogram stem from compounds that developed "in situ", i.e. through the catalytic impact of the column and could not be found in the original sample.

Double-check: Inject the sample as usual and switch off the pump while the sample is in the column. Leave the sample in the column for 20–30 min and then switch on the pump again. Do peak numbers and areas remain constant?

2. Irreversible Sorption in the Stationary Phase?

- Once the column has been equilibrated, inject the sample several times. Does the peak area remain constant?
- Inject a small sample volume, circumventing the column by connecting the sampler to the detector. Without the column, only one peak will appear. Make a note of its area. Now connect the column and inject the same sample volume. Depending on the column selectivity, 1, 2, 3 or more peaks will appear. Add all the peak areas and compare the sum to the peak area obtained from the run without column. If the difference is less than 5–8%, irreversible sorption in the stationary phase is negligible.

(B) Impact of the Sample Solvent

The sample solvent and/or the sample medium can have an impact on retention time, peak shape and peak area. Change one of the following parameters in the sample solution as appropriate: pH value, organic component, matrix, constitution of the sample, air content in sample solvent. When dealing with samples from a production plant, it is a good idea to be in close contact with the colleagues working

in the plant concerned who could provide current updates. It is the small changes in sampling or the actual medium of the sample, often regarded as negligible in day-to-day work, which can cause major problems in analysis.

(C) Compatibility of Eluent and Stationary Phase (Column Stability)

- An intentionally aggressive eluent is applied to a column over a whole weekend if a high durability of the column is important for routine work. In this way, ageing of the column can be simulated, and after just two days one will have a good idea of whether one is dealing with a selective and not a robust method suited for routine work, which is better than acquiring this knowledge later in the course of routine work. "Aggressive" conditions have to be defined for individual cases and result from the preceeding optimization experiments, e.g.:

  – high water content (e.g., 95–100%)
  – extreme pH values (e.g., pH 1.5/9.5)
  – high salt content (e.g., 50–100 mmol)
  – increased flow (e.g., 2.5–3.5 mL min^{-1})
  – high temperature (e.g., ca. 50 °C)

  or in the extreme case all of these conditions simultaneously.

With polar RP-phases, two or three charges should be tested in any case.

(The laboratory precision would probably be checked in the framework of an official validation procedure, along with the reproducibility, if necessary.)

To sum up the procedures described above – here are the key figures:

Time required: four days, two nights and a weekend
Labor input: approx. 50–55 runs
List of what could be tested:

- 5 pH levels including fine-tuning;
- 15 columns including columns for cross-experiments;
- 3 organic solvents, 2 modifiers, and, where strong bases are involved, three further pH levels as well as two modifiers and further special columns;
- optimization of gradient and temperature as well as column dimensions and particle size where applicable;
- finally, data concerning peak homogeneity, method robustness and column stability.

Of course, this list is open to variations on a theme. Let me just mention two of them:

Variation 1

Hardware required: Low pressure gradient with eluent-switching valve, i.e. six solvent inlets or, if a high pressure gradient is used, two pumps with three solvent inlets each.

Alternative to steps 1–2, as follows:

1. *Night experiment:* In order to get an overview, run a gradient at five different pH levels (plus a rinsing solution) on six columns (5 gradients × 6 columns = 30 runs)
2. *Day experiment:* The optimum column is run under optimum conditions (acid/base, modifier, see above) to serve as reference in a comparison to five further columns (6 runs).
3. *Night experiment:* Six further columns are tested at two pH levels (±0.5 pH units off the optimum pH level) 2 pH levels × 6 columns = 12 runs.

Step 3 to 5 (fine optimization and column stability) as above.

Time required: four days, three nights, one weekend
Labor input: approx. 54–58 runs
Comments: In this variation, two more pH levels would be tested on a higher number of columns – 17 rather than 11. This may be helpful in difficult samples containing a wide range of polar/ionic species.

Variation 2

Hardware required: Two quaternary pumps
Alternative to steps 1–2, as follows:

1. *Night experiment:* Three pH levels, three eluents (each of the two pumps has one solvent inlet reserved for the eluent), six columns, 6 eluents × 6 columns = 36 runs.
2. *Night experiment:* The best column so far is used as reference column for two pH levels (±0,5 pH unit of the optimum pH level and the optimum eluent to be compared with further five columns (6 runs) 2 pH levels × 6 columns = 12 runs. Step 3 to 5 (fine optimization and column stability) as above.

Time required: three days, three nights, one weekend
Labor input: approx. 50–53 runs
Comments: In this variation, variants of organic solvent could be tested on more columns. This could be helpful in laboratories who are short of staff (three nights and three days compared to two nights and four days as in variation 1). Also useful for samples containing many components tending to neutral.

Final Remarks

The scope of this five-step program for effective method development/optimization is based on a worst case scenario, and depending on the individual situation, the five steps could be modified and shortcuts taken. Usually, some information about a sample is available, perhaps also about its area of use. Such information must be used in order to shape the testing conditions, e.g.

- Known pK_S value – experiments regarding the pH level can be targeted and thus reduced in their number.

- Decreased pH stability at pH = X or in solvent Y? Avoid pH level X or solvent Y.
- Expecting many low-concentration impurities? Choose 3 µm material.
- Complex matrix? Take great care in preparing the sample and use 5 rather than 3 µm material.
- Expecting many components in a complex matrix? Go for 2D chromatography from the start.

The following alternatives to the approach described could be considered in certain situations or service laboratories:

- Fully automatic method development (see Chapter 4).
- Super fast separations (see Chapter 2.7).
- Developing a fast LC-MS method (see Chapter 5.1).
- Barely sufficient chromatographic selectivity (using very short columns) combined with spectroscopic specificity (NMR, MALDI-TOF, FTIR, X-ray fluorescence).

Successful combinations could be listed as in Table 1 for quick reference. Various optimizing parameters (pH level, solvent, column etc.) in the table columns and the relevant chromatographic key data or results after an optimization step in the rows could be summarized in such a matrix. Rows A to D are individual criteria that can be defined and amended as needed. For example, the higher the numeric value of row B, the more effective the separation (peaks per time unit, peak capacity).

It goes without saying that only those optimization parameters should be recorded that led to useful results, i.e. the matrix should be filled in after each experiment. If solvent THF does not yield a reasonable situation, column 6 in Table 1 should be left out altogether or a different optimization parameter should take its place. If desired, A to D can be arranged in order of relevance. But even

Table 1 Overview of results following an optimization step.

Criteria	*Parameter*					
	pH = 2	*pH = 2*	*pH = 5*	*pH = 5*	*pH = 8*	*pH = 8*
	MeCN	*MeOH*	*MeCN*	*MeOH*	*MeCN*	*THF*
A	4	5	3	5	etc.	etc.
B	0,4	0,5	0,3	0,6	etc.	etc.
C	1,3	1,5	1	1,1	etc.	etc.
D	Good peak shape	Drift, high pressure	Tailing at Peak X	broad Peaks	etc.	etc.

Examples for individual criteria regarding the quality of an RP separation
A: Absolute number of peaks
B: Number of peaks per time unit, e.g. 4 peaks in 10 min, 4/10 = 0.4
C: Critical pair resolution
D: Comments, e.g., narrow peaks, base-line drift, high pressure, tailing peaks etc.

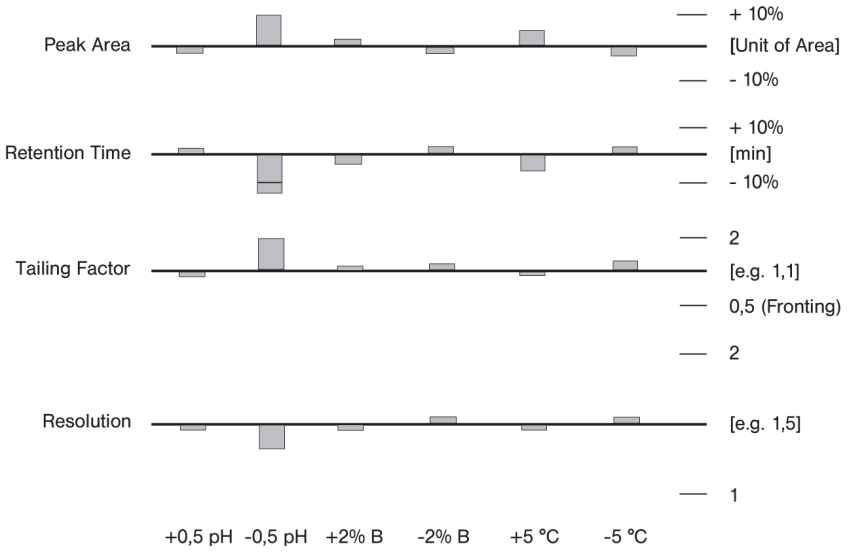

Fig. 20. Graphical representation of how specific parameters of a method influence chromatographic key data in a robustness experiment; for details, see text.

without such an order, one glance suffices to see which column-eluent combination is right for the specific criteria required.

A similar matrix gives a quick overview of the effect of various factors when testing rugedness (see Fig. 20). Chromatographic key data are entered on the left-hand side of the matrix. The changes they undergo due to variation in methodic parameters are to be analyzed. The changes carried out are entered into the line at the bottom of the matrix, which should also be specific – not only regarding the parameters to be analyzed (pH level, solvent ...) but also the scope of change (±05 pH units, X% B ...) When checking laboratory precision (intermediate precision), for example, "user 2" or "machine 2" etc. could be entered. The horizontal lines reflect the resulting numeric value (area, retention time ...) under the usual separation conditions – those that have been agreed upon when developing the method. The individual changes are visualized by hatched areas. Measurements can be given in % or as a numeric value. Based on the degree of change brought about by specific parameters, a decision can be taken which parameters should be considered as system suitability parameters for a later system suitability test and what criteria (accepted bandwidth of the numeric value in question) should be given. Figure 20 is an excellent example and should be interpreted as follows: The component is highly sensitive to a decrease in pH, apparently slipping near the pK_S region. This cuts the retention time by more than 10% of the time expected for similar separations. Similar findings apply to resolution – a neighboring peak is insufficiently separated. Furthermore, UV absorption seems to be pH level-dependent, as changes in the peak area suggest. It also seems that in this method, an increase in temperature can be critical (change in pH level – change through change in temperature?) As far as other parameters

are concerned, the method seems to be rather robust. This kind of documentation could be enclosed to a validation report, as it facilitates decision-making further down the line if the method needs to be changed and the question is whether such a change would be only an adjustment (does it meet criterion X?) or revalidation is called for.

1.1.6
Shortening of the Run Time ("Faster Separation")

A decrease of the resolution, except in the case of gradient runs with a constant gradient volume, is associated with a shortening of the retention time. This can be very small, e.g. enhancement of the flow using 3 μm material, or it can be considerable, e.g. a change in the pH. One has to decide in each specific case whether the advantages exceed the disadvantages. Experience shows that one frequently works too cautiously and accepts needlessly long retention times, e.g. at 1 mL min^{-1}. A lot of possibilities are discussed above and outlined in brief below.

- enhancement of the flow,
- reduction of the column volume (shortening of the column length and/or diameter),
- change of the eluent combination (e.g., water content, modifier, pH, ionic strength),
- increasing the temperature,
- change of the stationary phase (e.g., use of a more polar phase or choice of another matrix, e.g. non-porous material, monolith),
- use of a gradient technique (do not overlook flow gradients),
- change of the gradient (e.g., starting conditions, steepness, gradient volume).

The potential of modern columns should be emphasized once more; with a column of dimensions ca. 20–50 mm × 1.5–2.1 mm, 1.7–2.0 μm, 10–15 peaks can be separated within 2–4 min; see also Chapter 2.7.

1.1.7
Improvement of the Sensitivity ("To See More", i.e. Lowering of the Detection Limit)

Aspects of this possibility have already been discussed above. Some of them are collected in Table 2.

1.1.8
Economics in HPLC ("Cheaper Separation")

Remark: It should be obvious that "economical" means more than just "cheap". Thus, aspects at the periphery of HPLC analytics play a more important role than the actual analytical aspects. Some of these questions were addressed at the start of this chapter.

Table 2. Possibilities for enhancing sensitivity/lowering the detection limit in RP-HPLC.

(a) Aspects due to the HPLC equipment (e.g., use of the appropriate modules, optimal settings, avoid noise)
• Increase the sensitivity (range/attenuation) – the peak-to-noise ratio improves.
• Use a small time constant (< 0.5 s, or better 0.1 s).
• Are the settings for *this* separation optimal? That is, sampling rate, sampling period, rise time (bunching factor, response time), peak width, wavelength. Is the detector the right one? Is perhaps a derivatization necessary?
• Consider electronic damping; are protective covers on cables/interfaces O.K.?
• Miniaturization successfully realized? ($L\downarrow$, $ID\downarrow$, $dp\downarrow$) Are cell volume and design appropriate for *this* separation?
• Are the cell and the lens clean?
(b) Chromatographic conditions
• Increase the plate number.
• Apply steeper gradient.
• Increase the injection volume/mass (overloading?).
• Improve the peak shape (pH, modifier); remark: a tailing factor of 1.5 leads to a decrease of one-third in the peak height and thus to a decrease of one-third in the sensitivity.
• Avoid band spreading – aim for "optimal" injection techniques; on-column concentration possible/necessary?
• Are the membranes of the degasser still OK?
• Avoid baseline drift in gradient runs; is *this* eluent the right one for *this* wavelength? Might the use of a UV absorber in eluent A decrease the drift?

In the following, points are listed without any ranking, which one should remember when aiming for efficiency enhancement or cost saving. *Here* lies the big potential, not in the 20 or 30% discount when buying columns or equipment.

- In the event of method transfers or a co-operation with contract partners, one should aim for a standardization of the equipment.
- The validation should be performed under real and not under optimal conditions. From the information point of view, what can be gained if a skilled expert using optimal equipment and clean standard solutions measures several times during a week and thereby obtains a relative standard deviation of 1% and a correlation factor of 0.999?
- The person responsible for the project should attend to the whole project, e.g. from the development of the new substances up to the registration and should use his or her knowledge and up-to-date information in discussion with and between the various departments. Such a person can be a very important mediator, but it is important that this person has the ability and the permission.
- The sequence of operations should be adapted to the requirements and the realities. Single steps in the daily life of the laboratory should be intelligently combined.

- One should efficiently organize recurrent activities such as purchasing, sample management, equipment testing.
  One should endeavor to find reasonable guidelines for quality management.
- There should be a well-functioning interchange of information – think communication!

These points are not topics of this book and therefore are only mentioned here as a brief indication on effectiveness and efficiency in the HPLC environment. If the "company philosophy" permits, one should always reflect on the effectiveness and efficiency of one's own activities, almost instinctively and unconsciously.

In the case of effectiveness, the question concerns the correct choice of the means ("am I doing the right thing?"), while in the case of efficiency the issue is the correct use of this means ("am I doing it in the right way?").

Examples involving effectivity

It should be considered, for example, whether it is sensible to use HPLC for the entry control of raw materials, for identity checks, for the measurement of content, or for dissolution tests. In the case of the first two of these problems, NIRS might be a more sensible option, while for the latter two a titration or an on-line UV measurement would be more economical. It is not necessary to obtain the information through a "separation" in all cases.

Dissolution tests often result in a single peak, in some cases, can only be separated using the solvent. This begs the following questions:

- Do I really need a column, as this may be a case of identification rather than separation? Could I perhaps just use a capillary instead, as in a FIA (*F*low *I*njection *A*nalysis)?
- If it has to be a column, would perhaps a 10 mm × 2.1 mm precolumn do as a separation medium?
- Do I need to use the expensive and toxic solvents acetonitrile or methanol, or would ethanol be a possible alternative?

Examples involving efficiency

Is the use of the available equipment optimal, for example the DAD or the elusaver? Is it perhaps possible to work with methods in such a way that cleaning time and reflushing of the equipment can be reduced?

Are there "clever sentences" in the validation report that make it possible to denote small changes in the method as an adjustment by observation of the given requirements?

In this way, expensive, large-scale revalidation can be reduced to the absolute minimum. Experience shows that the margins given by organizations such as the FDA, EPA, Pharmeura, etc., are not always adhered to. On the contrary, fear and widespread alibi mentality has the result that one is frequently overcautious.

Consider now the purely chromatographic possibilities for saving time and reducing costs.

Is a "good" separation also an "optimal" separation?

If one is dealing with an isocratic method with an MeCN content in the eluent of 60–80%, then one should take a good look at the overall separation. Such a high MeCN content may be an indication of the following: the interaction of the compound(s) with the stationary phase is perhaps so strong that this high organic content of the eluent is necessary to obtain a short, acceptable run time. It is more economical in this case to use a shorter or thinner column. This could, for example, be a hardware change by a factor of two. In the first case, this would result in a saving of eluent used by a factor of two, in the second case by a factor of four. Even a change from a 4 mm to a 3 mm column would lead to a saving of solvent of ca. 45%. In this context, one should think not only of the purchase price of the solvent, but more of the costs of removal and disposal and of environmental concerns.

As already mentioned, several aspects fall under the umbrella of "economics", including time. Provided that the packing quality and the properties of the stationary phase are the same, one obtains the same resolution using a 150 mm, 5 µm column as using a 100 mm, 3 µm column, or a 50 mm, 1.7 µm column, respectively. In the second case, however, the time saving is ca. one-third, in the third case ca. two-thirds. For more time-saving procedures, see above ("faster separation").

Finally, the following remark: An optimization step, however it is achieved, only really yields results if one is willing and ready to draw conclusions.

To illustrate this, just think of an everyday example: one enhances the flow of a simple separation to reduce the retention time. Possibly this advantage yields absolutely nothing, because, if no other action happens, it is really unimportant, if a series of measurements ends at midnight or at 3 am. However, if the laboratory routine and other working processes (integrated laboratory production/raw material delivery) can be adapted to the obtained time saving, then, for example, the autosampler can be reloaded in the evening and the production unit can get required information directly in the morning. A time saving of 10 hours in the laboratory can be important; a time saving of 10 hours in production is a lot. However, these are decisions for management to make and are beyond the scope of this book.

1.1.9
Final Remarks and Outlook

The following topics would seem to be worthy of note:

- Even in a strongly regulated environment, there should be scope for searching questions. The sincere explanation of requirements in relation to the actual goals makes the relations with others easier and also makes general economic sense.
- Some checks of robustness should be made at an early stage of the project, even during the development of the method. The later a weakness of the method becomes apparent, the more expensive it is.

- A small improvement of the communication – communication in this context means more than just the exchange of words, arguments, and opinions – can yield an remarkable effect. A 5% improvement of real communication can lead to an improvement of 30% in earnings [3]. Admittedly, it is difficult to prove such an improvement or the opposite (frustration of the employees) in numerical terms, but it is possible.
- In the case of an unknown sample, do not trust any one peak, even if it is very sharp.
- After a successful separation, one should use a completely different column-eluent combination and inject 1–2 μL, exploiting the possibilities of the DAD. Orthogonal separations yield more security than match factors, etc., of the DAD after a one-dimensional separation.
- If one is dealing with a really important sample and the truth is at stake, then 1000 or 2000 Euro for an LC-MS(MS) or LC-NMR/2D-NMR measurement or the consultation of an expert frequently represents a profitable investment.
- Is one aware that by injecting standard solutions one just measures the precision of the instrument and this test has nothing to do with *the* "system suitability test", even though everyone in the laboratory speaks as if this were the case? Unless, that is, I could prove in the validation process that matrix, all excipients, degradation products etc have no impact on resolution and sensitivity. If, in addition, the standard deviation is not exceeded more than 1–2 times a year, I could conclude that my system is robust and consider increasing the time span between system checks. This is often accepted by national authorities as well as by e.g. the FDA, as long as it can be documented through historic data and control cards that the control process is ISC (in statistical control).
- Is "top value" the same as "good analytics"? A lot of us are probably of the opinion that the proportions are doubtful if someone drives a Ferrari for 500 m from home to work place everyday or if a private modern baseball stadium is only used twice a year only because of a lack of time. In the same way, a critical discussion of top values/top performance is advisable in analytics: is it meaningful to equip the instruments for quality control with DADs only for the reason of image or the like, if de facto these facilities will be used at only 20% capacity, if at all? Is the requirement "$R > 2$" meaningful? Is it necessary to strive for optimal resolution in every case, or can it sometimes be better just to aim for resolution that is adequate for the analytical problem at hand? How many "out-of-spec" situations do I produce for my colleagues in quality control, if as method developer I claim a relative standard deviation of 0.8% for a method with a biological matrix or a contaminated process sample?

Outlook

From today's point of view, the following trends in HPLC in the context of method development/optimization can be discerned:

Coupling techniques: For the separation of complex samples, more and more coupling techniques will be used, partially in miniaturized form, e.g. "RP-ion exchange" in µ-bore design and subsequent on-line coupling with MS. Through the further development of interfaces in mass spectrometry, applications of MS as the dominant partner of LC will become more diverse. The LC-NMR coupling will only prevail in pure research laboratories, because of the high prices of 600–900 MHz NMR equipment. For automation in relation to coupling techniques, see below.

2D/Multi-D Chromatography: 2D- and multi-D-chromatography will become increasingly important, and there are two reasons for this: (1) Such systems can easily be put together using commercial modules, and any easily available software can be used for control functions. The wide variety of available separation media (columns, capillaries) makes practically every imaginable combination possible. (2) The complexity of samples and thus the need for chromatographic resolution is ever-increasing. All sorts of combinations are possible, as long as the basic requisite is met – the second dimension must be much faster than the first. If, for example, a micro bore column in the first dimension has an injection volume of 1 µL, the negative effect of a possible incompatibility of eluents in an NP-RP combination will be barely noticeable. Assuming a peak capacity of $n = 9$, switching a second column in series would improve peak homogeneity "only" by a factor of 1.4 ($n \cdot \sqrt{2}$, thus $10 \cdot 1.4 = 14$ peaks), whereas orthogonal switching would yield a (theoretical) increase by a factor of 10 ($n = 10^2 = 100$ peaks). Here is a list of successfully used combinations:

NP-RP, HILIC-RP, ZrO_2-RP, SEC-LC, IC-LC, LC-GC, LC-GC-GC, LC-LC-APCI-MS.

Miniaturization: Columns of length 20–30 mm packed with < 2 µm particles can be expected to become increasingly popular, at least in method development, because of the short retention times and the low consumption of solvent. The long-expected breakthrough has been held back because of the well-known circumstances in analytics, and may start in the next 5–8 years. Only recently have the major HPLC providers seriously addressed the issue of miniaturization techniques, not only in the field of the columns on offer, but also with regard to the accessory equipment. UPLC (ultra-performance liquid chromatography), Ultra Fast LC, HSC (high-speed chromatography), RR- and RR-HT (rapid resolution high-throughput) columns are some examples. The handling of columns filled with particulate material, even in the case of 1.5–1.8 µm particles, is easier than that of other separation media.

Therefore, for the time being, CLC (with particulate material or monolith), chip-LC, and non-porous materials remain interesting but specialized niche products.

Conclusion: The future of HPLC lies without any doubt in fast separations. From today's perspective, the most promising routes towards this goal are UPLC, Xtreme LC (Specific hardware, high pressures, ca. 1.7 µm material), Ultra-Fast-LC (conventional HPLC machine, perhaps with minor hardware/software modifica-

tions and 1.8–2 µm material), chip HPLC and monoliths, preferably in capillaries. Some of these technologies are already quite sophisticated. When taking a decision about possible equipment changes, the user should think very carefully if the time is right and which of the methods on offer meet the actual needs. Here are some of the questions that need to be answered before coming to a decision:

- How important is a retention time of 30 or 60 sec if the overhead time (injection, needle-washing etc) takes up 2 min or the sample preparation time is even 5 min? How many samples do I have to deal with on a daily basis?
- Am I prepared to become long-term dependent on a specific – albeit excellent – technology provided by a single firm?
- What seems to be more acceptable to my colleagues/contract partners – a flow rate of 10–20 µl/min or 5 ml/min? Depending on the matrix used and the dimension of the column/capillary, the desired decrease in separation time can be achieved in any case.
- What about validation/method transfer? Will the real life samples in routine separations provide the same consistency? How experienced is my staff?
- Can I make use of the substantial time savings and the faster information or would the advantage just lie in the very short analysis time?

In a situation where faster data availability helps to optimize the production process – by enabling me to analyze more experiments (e.g. stress samples under a variety of conditions, intermediate products of a synthesis process etc.) and thus underpin my conclusions, if the time for method development can/must be cut by a factor of five, or if I do not have to deal with difficult matrix, then I would take the plunge sooner rather than later. The potential scientific and economic benefits are immense.

Automation: A high level of automation in method development and effective time-saving procedures look set to become increasingly indispensable.

Some examples for illustration:

1. Instruments for automatic overnight method development with computer-aided optimization and a column selecting valve are now associated with high robustness and will be applied more and more. On the basis of calculated substance data, a computer-aided informed choice of column and eluent is possible, at least the "assiduous" choice of column and eluent, until a given chromatographic resolution is reached. In the future, more will be calculated before the measurements start: e.g., pK_a and log P values, and other chromatographically relevant data. One can expect that more and more evolutionary and generic algorithms are implemented in optimization software. After this, the appropriate chromatographic conditions (column, eluent, temperature) can be selected manually or automatically by means of optimization software. The chromatographic software of the future will use its "memory" to optimize the separation according to given requirements through interaction with databases via the Internet or an intranet, using individual filters and with the

modules of the HPLC equipment including columns containing inserted microchips. Communication with the user will be possible at all stages and the software will be able to "ask" the user in difficult situations. The systems will be self-starting, self-testing, capable of independent calibration and measurements, and, through built-in diagnostic tools, capable of recognizing errors that arise and in simple cases of compensating for these. The sample may then proceed to a second, parallel connected, preconditioned column – in case the first no longer provides the required quality (selectivity, efficiency) for the separation problem at hand.

The technological challenges of the future lie in:
- handling micro/nano capillaries and nano connectors,
- reproducibility of microcolumns and capillaries,
- packing chiral micro bore columns and capillaries,
- chip technology.

2. After a rough separation on a 10–20 mm column, one obtains the necessary information by a subsequent step having a higher level of specificity. This requires a splitting system; the sample is transferred in parallel or optionally to DAD-MS(MS), NMR-MS, ICP/OES, ICP/MS, FT-IR, fluorescence/X-ray fluorescence and, if necessary, also to a second (chromatographic) separation system (e.g., IEC, GPC or CE). By using sophisticated LC-UV-NMR-MS coupling techniques, a dream of separation scientists seems at last to be becoming reality – at least in specific cases: "enter the sample and out comes the structure!". Such systems have been available for some years in different versions. In contrast to the systems listed under 1, such coupling techniques are very complex and usually only usable to their full potential by the user who designed them. In general, one can conclude the following: as far as is possible, "physics" should be preferred to "chemistry". From the user's point of view, the handling of "physics" is easier in daily work. The measurement/testing has fewer variables and therefore fewer sources of error and results are obtained immediately. Frequently, the relationship effort/specificity = information is better.

"Chemistry" in the column: As is explained in detail in Chapter 2.1.1, polar/ionic interactions – being highly specific – play a rather more important role in RP-HPLC than hydrophobic interactions. For this reason, in the last decade more polar than "classical" hydrophobic RP phases have been developed. This trend looks set to continue. Any type of alkyl chain that enables the necessary retention of the components may be attached to the phase surface. On the other hand, polar groups reside on the surface of the stationary phase which are important for the differentiation of ionic to moderately polar analytes. Polar character of a phase can be imparted in different ways: polar and ionic groups in the alkyl chains ("embedded", EPG, zwitterion chemistry at the alkyl chain), polar terminal groups, polar groups on the matrix surface, low coverage, hydrophilic endcapping, sterically protected surfaces, etc. Because of the dual character of such phases, one or other

mechanism can be selectively invoked by the selection of the eluent and the number of columns that need to be used decreases. Also, to decrease costs and for more environmentally friendly work, thermally stable columns will be developed (mostly based on zirconium oxide, and less frequently based on titanium oxide or polymer based) for use at 100–200 °C:

1. Supercritical water and/or ethanol can be used as cheap and ecologically benign "hydrophobic" eluents.
2. Instead of solvent gradients, temperature gradients can be used. Finally, types of stationary phases that undergo a change of a surface functional group from polar to hydrophobic depending on temperature are solvent-conserving. In this way, the selectivity can be obtained primarily by a temperature change. Such a column can be used with either a polar or an apolar eluent at either a lower or a higher temperature. The aim is to achieve a broad range of selectivities with only four runs and using a minimum number of different columns and different (cheap, non-toxic) eluents.

Final Comments

In analytics, there is a trend away from High Throughput Analysis towards High Content of Information. Producing simply large amounts of data of the same quality (e.g. chromatographic data following an RP separation) has not provided the desired results in difficult cases, as demonstrated by combinatorial chemistry. The density of information will enhance the quality of analysis if the data obtained from a sample are robust rather than numerous and can be correlated to each other (multiple statistical approaches, improving data analysis techniques). It is thus possible to achieve maximum resolution without unreasonable effort, and peak purity can be checked in 2D separations. Spectroscopic data (DAD, MS, ICP, MALDI-TOF) can be used to achieve very high specificity. Further information about the target component such as chirality, biorelevance is available at an early stage. Chemometrics, generic algorithms, optimizations tools etc. will be used to an ever larger extent. For economic reasons, there is a trend towards to preliminary calculations in order to predict results, discover interdependencies and approach method development more efficiently. Pro-active thinking and calculations have the edge over laborious and expensive measuring experiments. Rather than being achieved by testing at the end of the production chain, quality should be achieved through intelligent process design adapted to the problem at hand. Post factum quality control is going to be replaced by continuous optimization of the individual stages in the process, known as quality assurance. This allows early corrective steps to be taken where needed. It is a matter of establishing quality earlier in the process. The earlier critical factors can be discovered and checked, the more robust the method or process can become, which reduces quality control costs – in other words stop and think before you experiment. Not for the first time does such advice come from unexpected quarters, e.g. from authorities such as the FDA. Rather than insisting on compliance with formal requirements and strict reglementation (which was not very successful), continuous gentle pressure is

supposed to help implement quality assurance in the early stages of development (Modern Quality Management Techniques, Real Time Quality Assurance). Another buzz word on these lines is PAT (Process Analytical Technology) – analytic facilities should be available all along the process, but high-tech is not always needed, as long as pH value, temperature, humidity, particle size etc. are checked at the critical points to identify potential flaws. Minimizing risks by preventative checkups using intelligent simple analytical tools is the order of the day.

The realization of perfected solutions clearly depends on a lot of factors, in analytics as in other fields. Therefore, a lot can be realized but not applied. Nevertheless, many of the trends outlined above are reality today. However, it is difficult to predict which of them will find their way into the laboratories most widely. Economic preconditions may on the one hand enhance the formalism, while on the one hand reducing the amount of regulatory requirements to a normal level. Frequently, both trends coexist. It is to be hoped that common sense can be given precedence over formal regulations.

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1.2
Fast Gradient Separations

Uwe D. Neue, Yung-Fong Cheng, and Ziling Lu

1.2.1
Introduction

The time involved in the execution of analytical procedures is a subject of major interest in today's pharmaceutical industry. It is also of increasing importance in other industries as well. The general goal is to reduce analysis time as much as possible in order to reduce the cost of the analysis and to increase sample throughput. At the same time, the quality of the analysis must not be compromised. In chromatography, the key parameters are the resolving power of a separation as well as the sensitivity of an assay. In this chapter, we show how the peak capacity of a gradient can be maximized through appropriate choice of the gradient operating conditions. We cover both the theoretical basis of this technique as well as solutions to practical difficulties.

The principles outlined here are especially relevant for the rapid analysis of plasma or urine samples by means of liquid chromatography coupled with mass spectrometry [1]. In the last few years, understanding of this analytical technique has continued to grow, and investigators have realized that ion suppression plays an important role in signal suppression and signal enhancement in HPLC/ESI-MS analyses [2]. This problem can often be avoided completely if the chromatographic resolution is maximized [3]. At the very least, the solution to this problem can be simplified by maximizing the separation power of the chromatographic system. The thought processes involved in this solution include the manipulation of the separation through pH change as well as the optimization of instrumental parameters such as gradient delay volume or detector sampling rate.

1.2.2
Main Part

1.2.2.1 Theory

The principles of fast gradient separations have already been outlined in the past [4, 5]. Here, we only reiterate the most important results. As a measure of the quality of a separation under gradient conditions we use the peak capacity P, which is defined as follows:

$$P = 1 + \frac{t_g}{w} \tag{1}$$

Here, t_g is the gradient duration, and w is the (average) peak width. The peak width itself is a function of the column plate count N, the retention factor at the column outlet k_e, as well as the column dead time t_0:

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$$w = 4 \cdot \frac{t_0 \cdot (k_e + 1)}{\sqrt{N}} \tag{2}$$

The retention factor at the column exit depends on the gradient steepness G:

$$k_e = \frac{k_0}{G \cdot k_0 + 1} \tag{3}$$

where k_0 is the retention factor at the beginning of the gradient. The gradient steepness G is defined as follows:

$$G = B \cdot \Delta c \cdot \frac{t_0}{t_g} \tag{4}$$

Δc is the difference in the concentration of the organic modifier at the beginning and end of the gradient. B is the slope of the linear relationship between the logarithm of the retention factor and the solvent composition. The ratio of the gradient duration to the column dead time is called the gradient span.

In all cases of practical interest, the gradients extend over a large difference in solvent composition, and the analytes have a high retention at the start of the gradient. Under these circumstances, we can further simplify Eq. (3):

$$k_e = \frac{1}{G} \tag{5}$$

If we now combine all of these equations in order to express the peak capacity as a function of the gradient parameters, we obtain:

$$P = 1 + \frac{\sqrt{N}}{4} \cdot \frac{B \cdot \Delta c}{B \cdot \Delta \cdot \frac{t_0}{t_g} + 1} \tag{6}$$

With this equation, and the simplifications mentioned above, we can now estimate how the peak capacity changes as we vary the gradient parameters. Thus far, we have assumed that the plate count remains constant. However, what we would like to do is vary the flow rate at a constant run time. This means that the plate count will change as well. To introduce this variation, we use the van Deemter equation:

$$H = A \cdot d_p + \frac{B \cdot D_M}{u} + \frac{d_p^2}{C \cdot D_M} \cdot u = A \cdot dp + \frac{B \cdot D_M \cdot t_0}{L} + \frac{d_p^2}{C \cdot D_M} \cdot \frac{L}{t_0} \tag{7}$$

Here, d_p is the particle size, u the linear velocity, L the column length, and A, B, and C are the constants of the van Deemter equation. The diffusion coefficient of the analytes, D_M, depends on the sample and the composition of the mobile phase. In order to avoid excessive complications, we assume typical diffusion coefficients for the types of analytes that we are using. For the coefficients of the van Deemter equation, the following typical values can be found in the literature [6]: $A = 1.5$,

$B = 1$, $C = 6$. Using the known relationship between plate count and theoretical plate height, we obtain the peak capacity:

$$N = \frac{L}{H} \tag{8}$$

$$P = 1 + \frac{1}{4} \cdot \sqrt{\frac{L}{A \cdot dp + \frac{B \cdot D_M \cdot t_0}{L} + \frac{d_p^2}{C \cdot D_M} \cdot \frac{L}{t_0}}} \cdot \frac{B \cdot \Delta c}{B \cdot \Delta \cdot \frac{t_0}{t_g} + 1} \tag{9}$$

This form of the equation for the peak capacity can now be used to examine how the separation power under gradient conditions (= the peak capacity) changes as we vary the analysis time ($t_a = t_0 + t_g$) or the flow rate (which determines the column dead time t_0). We discuss these situations in the following paragraphs.

1.2.2.2 Results

1.2.2.2.1 General Relationships

Experience tells us that at constant flow rate the peak capacity improves when we increase the gradient duration. This is shown in Fig. 1. Here, we have plotted the measured peak capacity for a 3.5 µm column *versus* the gradient span. The strongly eluting solvent in the gradient was acetonitrile, and the sample was a mixture of typical pharmaceutical compounds with molecular weights of around 300. One can see that the peak capacity improves as the gradient span increases. It should be mentioned that a standard gradient as applied in a typical analytical laboratory typically extends over a gradient span of 20 or at most 40.

The relationship shown in Fig. 1 follows from Eq. (6). However, we need to realize that the gradient span can be expanded through two different approaches.

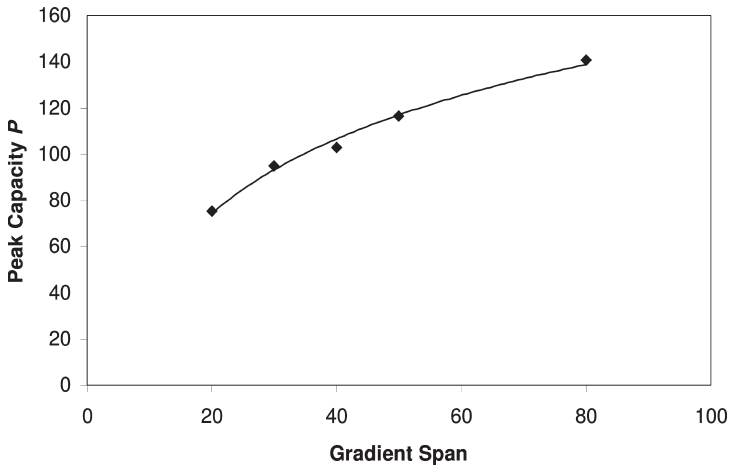

Fig. 1. Peak capacity as a function of the gradient span.

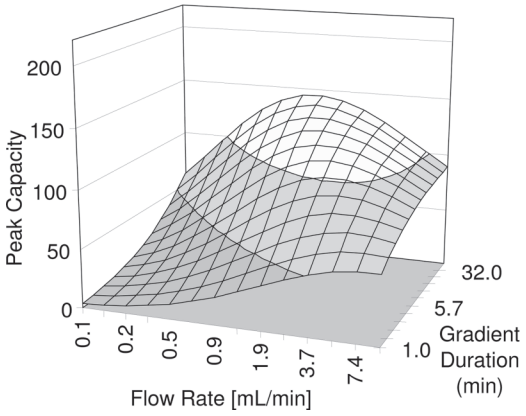

Fig. 2. Peak capacity as a function of flow rate and gradient duration.

On the one hand, we can increase the gradient duration while keeping the flow rate constant. On the other hand, we can decrease the column dead time by increasing the flow rate. However, a change in the flow rate changes the plate count. If we want to understand the influence of flow rate on the peak capacity at a constant analysis time, we need to examine Eq. (9) in its entire complexity. In order to do this, we can look at the dependence of peak capacity on flow rate and gradient duration in a three-dimensional graph. An example of such a graph is shown in Fig. 2 for a 4.6×50 mm 5 µm column. Both flow rate and gradient duration have been plotted using logarithmic scales. The flow rate covers the range from 0.1 to 10 mL min^{-1}, and the gradient duration extends from 1 min to 32 min. Let us examine the conditions for the best peak capacity for different gradient run times. For long gradients, the optimal flow rate is slightly lower than 1 mL min^{-1}. Under these conditions, we achieve a peak capacity of about 150. If we now run a fast, 1 min gradient at the same flow rate, the peak capacity is poor, only about 20. For such a rapid gradient, the optimum flow rate is around 5 mL min^{-1}. With this flow rate, we reach a peak capacity of around 65 – much better than at the slow flow rate. For somewhat less extreme gradients with a gradient duration between 2 and 4 min, one should use flow rates between 4 mL min^{-1} and 2.5 mL min^{-1}. This results in peak capacities ranging from 75 to more than 90. This means that the peak capacity for a 2 min gradient is only half of what can be achieved with a 30 min gradient, but with a 15-fold time saving this is surely not a disadvantage.

1.2.2.2.2 **Short Columns, Small Particles**

As we have seen, we need to use fast flow rates for fast gradients if we want to maximize the separation power. This also means that we should use short columns. In this section, we examine this proposition in more detail.

The plot in Fig. 2 stops at a flow rate of 10 mL min^{-1}. The reason for this limit is that at this flow rate we reach the pressure limit of the HPLC instrument. What will happen if we pack our 5 cm column with smaller particles? This is shown in

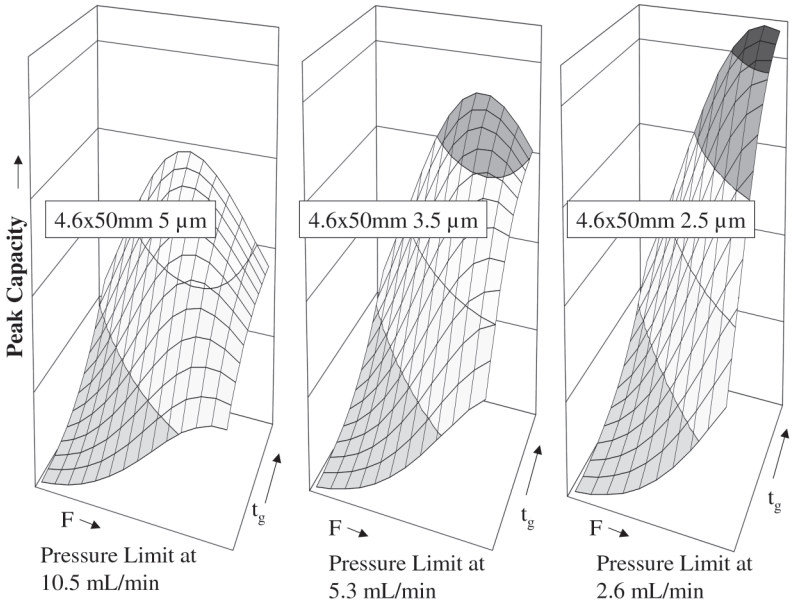

Fig. 3. Peak capacity as a function of flow rate and gradient duration for three columns of length 5 cm packed with 5 µm, 3.5 µm, and 2.5 µm particles.

Fig. 3. Here, we have created the same diagram as discussed above for a series of 5 cm columns packed with 5 µm, 3.5 µm, and 2.5 µm particles. As we can see, for slow analyses the separation power increases with decreasing particle size. With the 5 µm column, we reach a peak capacity of nearly 150 for a 30 min analysis, while with the 3.5 µm column a value of around 180 is reached. This performance is surpassed by the 2.5 µm column, with a peak capacity of roughly 220 for this half-hour analysis. However, for very rapid analyses, the column back-pressure limits the performance of the column. For a 1 min separation, the 3.5 µm column shows a somewhat better performance than the 5 µm column, but the peak capacity for the 2.5 µm column is lower than what was achievable with the 3.5 µm column. The reason for this is that the 2.5 µm column reaches the pressure limit imposed by the instrument. The main explanation for this is that, at a fixed column length, smaller particles reduce the flow rate that can be used. This can also be seen in Fig. 3. On the other hand, the 5 cm 3.5 µm column is ideal for analysis times in the 2–4 min range. Under these conditions, the 3.5 µm column exhibits a better separation power than the 5 µm column and still exceeds the performance of the 2.5 µm column.

There is still a way to increase the separation power for very rapid analyses, even within the pressure limits of older instrumentation. This is best accomplished by simultaneously varying the column length and the particle size [6, 7]. If the particle size is varied while the ratio of column length to particle size remains constant, the maximum achievable plate count remains the same. Thus, for example, a 10 cm 10 µm, a 5 cm 5 µm, and a 3 cm 3 µm column all have the

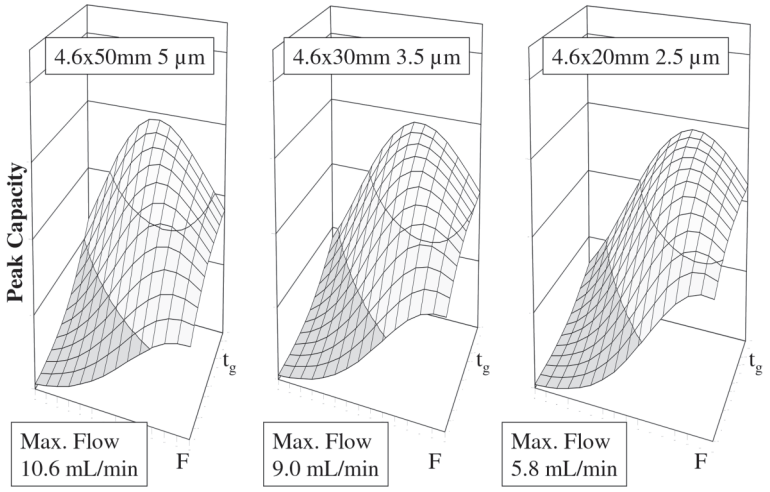

Fig. 4. Peak capacity as a function of flow rate for three columns of approximately equal ratio of column length to particle size; gradient duration from 1 min to 30 min.

same maximum plate count, and the same pressure drop for a particular column dead time. The difference between the three columns lies in the fact that columns packed with the smaller particles reach this plate count maximum at a higher analysis speed [6, 7]. The same thought process can be applied to gradient separations. In Fig. 4, we compare the performances of three columns of nearly equal column length to particle size ratio: a 5 cm 5 µm column, a 3 cm 3.5 µm column, and a 2 cm 2.5 µm column. For a 30 min separation, the peak capacity is practically identical for all three columns. However, for rapid, 1 min separations, better results are achieved with the smaller particles packed into shorter columns. The 2 cm 2.5 µm column reaches a peak capacity of nearly 90 under these conditions, while the 5 cm 5 µm column barely reaches a value of 65. The shorter column reaches the maximum at a flow rate that is twice as high as that for the 5 µm column, but this value is still far from the pressure limit of the HPLC instrument (see Chapter 2.7.3). For rapid separations, one should therefore use the shortest column with the smallest particles, unless extra-column effects, e.g. bandspreading and detector sampling rates, put a limit to such an endeavor.

1.2.2.2.3 **An Actual Example**

For the practitioner of HPLC, it is often best to view a problem in the form of an actual application example. For this purpose, we have selected the separation of five pharmaceuticals on a 3 cm 3.5 µm XTerra MS C_{18} column (Fig. 5). We used a high flow rate of 1.5 mL min^{-1} for the column with an internal diameter of 2.1 mm. We changed the gradient duration at this constant flow rate, which means that we moved parallel to the *y*-axis in Figs. 2 to 4. For a 4 min gradient the peak capacity was 140, for a 2 min gradient it was just above 100, and for a 1 min gradient we still achieved a peak capacity of 75. These results are in complete

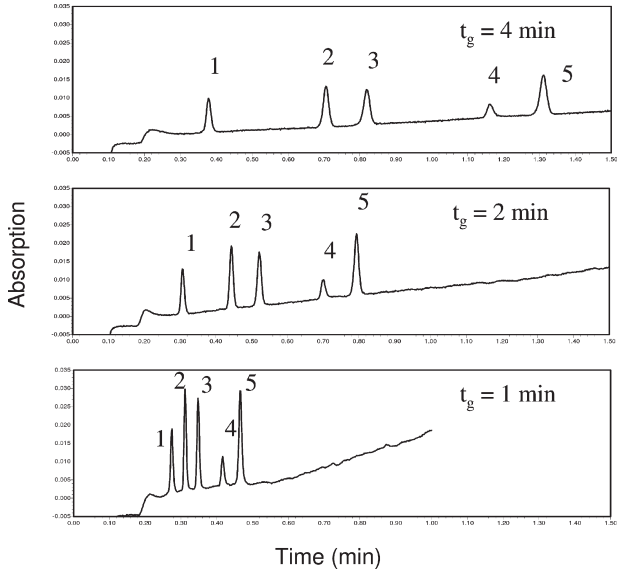

Fig. 5. Gradient separation of lidocaine, prednisolone, naproxen, amitriptyline, and ibuprofen. Column: 2.1 mm × 30 mm XTerra MS C_{18}, 3.5 µm. Temperature: 60 °C. Flow rate: 1.5 mL min^{-1}. Gradient from 8 to 95% acetonitrile. Top: 4 min; center: 2 min; bottom: 1 min.

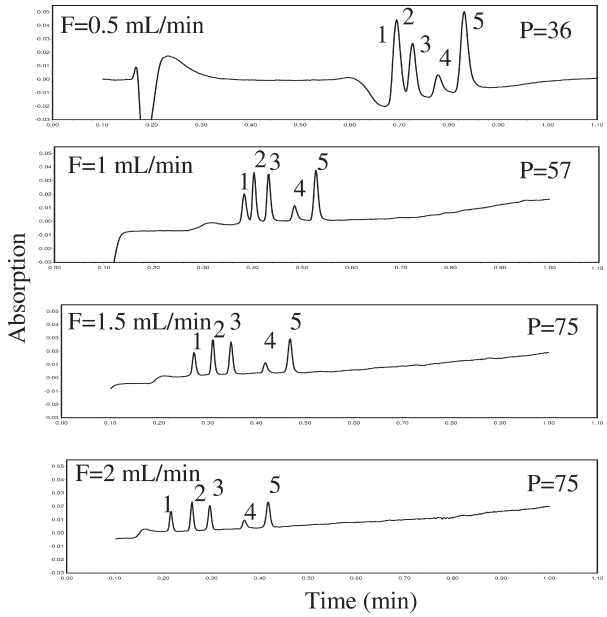

Fig. 6. Gradient separation as in Fig. 5. Gradient duration: 1 min. Flow rates (from top to bottom): 0.5 mL min^{-1}, 1.0 mL min^{-1}, 1.5 mL min^{-1}, 2.0 mL min^{-1}.

agreement with our expectations. As one can see, we were still able to achieve the separation even with the fastest gradient, despite the fact that it deteriorated on going from the slower to the faster gradients.

Figure 6 shows how the same separation changes as we change the flow rate at a constant gradient duration of 1 min. The flow rate was varied in steps of 0.5 mL min^{-1} from 0.5 mL min^{-1} to 2 mL min^{-1}. The peak capacity improved from 36 at 0.5 mL min^{-1} to 57 at 1 mL min^{-1} to 75 at both 1.5 mL min^{-1} and 2 mL min^{-1}. These values also agree with the theoretical expectations.

This real example shows that the thought processes outlined above are fundamentally correct. In our laboratory, we have demonstrated the same effects many times over, although not in the same detail as shown here. If one wants to achieve optimal results with rapid gradients, one needs to work at high flow rates. Often, these flow rates are at the limit of existing HPLC instrumentation. We discuss the limitations in the next paragraph.

1.2.2.3 Optimal Operating Conditions and Limits of Currently Available Technology

In Table 1, we have assembled a few guideline values as a rule of thumb for the selection of optimal gradient conditions, if the gradient is carried out over a large difference in solvent composition. We have selected several short columns with commonly used internal diameters of 4.6 mm and 2.1 mm and lengths of 50 mm, 30 mm, and 20 mm. The commercially available XTerra packing was used as the model for available particle size distributions: 5 µm, 3.5 µm, and 2.5 µm. The table shows the approximate values of the optimal flow rates for 1 min, 2 min, and 4 min gradients from 0% to 100% organic.

The table also shows that the optimal flow rates for these rapid gradients lie outside the pressure limit of current HPLC technology, if one wants to use columns of length 5 cm or more. It should be pointed out that the estimates assume that we want to separate typical pharmaceutical compounds with molecular weights between 250 and 600 with a water/acetonitrile gradient. The exact values are somewhat dependent on the solvent composition at which the compounds are

Table 1. Optimal flow rate as a function of the gradient duration and the column dimensions.

d_p	*5 µm*			*3.5 µm*			*2.5 µm*		
t_g	1 min	2 min	4 min	1 min	2 min	4 min	1 min	2 min	4 min
4.6 × 50	7.5	5.0	2.5	10.0 [a)]	6.0 [a)]	3.5	10.0 [a)]	6.0 [a)]	3.5 [a)]
4.6 × 30	5.0	3.0	2.0	6.5	3.5	2.5	7.0 [a)]	4.0	3.0
4.6 × 20	4.5	2.0	1.5	5.0	2.5	2.0	6.0	3.0	2.0
2.1 × 50	1.5	1.0	0.5	2.0 [a)]	1.2 [a)]	0.7	2.0 [a)]	1.2 [a)]	0.7
2.1 × 30	1.0	0.6	0.4	1.3	0.7	0.5	1.4 [a)]	0.8	0.6
2.1 × 20	0.9	0.4	0.3	1.0	0.5	0.4	1.2	0.6	0.4

[a)] Outside the pressure limits of the technology available at the time of writing.

eluted. Higher flow rates should be used if the elution starts at high acetonitrile content. This means that for practical use the values in Table 1 should be taken only as guidelines, and that the actual optimal flow rates should be determined experimentally. Nevertheless, these values clearly demonstrate that optimized rapid separations require high flow rates and higher pressures than are available today. In addition, we should also think about separations that are faster than 1 min (see Chapter 2.7.3).

1.2.2.4 **Problems and Solutions**

An optimal execution of rapid gradients also requires optimal settings of instrumental parameters. In the following, we discuss some important details.

1.2.2.4.1 **Gradient Delay Volume**

In order to execute rapid gradients, the gradients have to arrive at the column inlet without delay. However, most modern HPLC instruments operate with a single pump, and the gradients are generated in a gradient mixing chamber upstream of the pistons. This means that, before it starts to influence the separation in the column, the gradient needs to migrate through the volume of the mixing chamber, the connection tubing to the pump heads, the pump heads themselves, the tubing of the injector, and finally the tubing to the column. The sum of all these volumes is called the gradient delay volume, and the time that it takes to purge this volume at a particular flow rate is called the gradient delay time. Older instruments may have a gradient delay volume of several mL. In modern HPLC instruments, it is of the order of 1 mL. If a 4.6 mm i.d. column is used, a gradient delay volume of 1 mL has only a small effect on the separation. On the other hand, a short, 2 cm column with an internal diameter of 2 mm has a column dead volume of only 40 µL. This means that the gradient delay volume of the instrument has a value of over 20 times the column dead volume. This leads to a significant delay before the gradient reaches the column. The consequence is that the analysis takes much longer than it should. How can we avoid this time delay?

The solution to this problem is a delayed injection. The sample is injected shortly before the gradient reaches the column inlet. Now, the instrument executes the gradient as desired, and a prolonged analysis time is avoided. In addition, one can use the time saved under normal operating conditions and continuous gradient runs for column re-equilibration with the initial starting conditions. The way to do this is to program the end of the gradient as desired, perhaps with the inclusion of a column wash step, and then convert directly to the starting conditions of the gradient. The re-equilibration is then executed in exactly the same way as if the gradient delay volume did not exist. This is best shown by way of a diagram (Fig. 7). The upper trace shows the execution of the analysis without the delayed injection. The gradient only reaches the column after the gradient delay volume has been purged. The gradient is then executed, and the column is washed and re-equilibrated with the starting mobile phase. The next gradient is started only after completion of the entire procedure. The lower trace shows the same gradient scheme, but now we take advantage of the delayed injection feature. The first

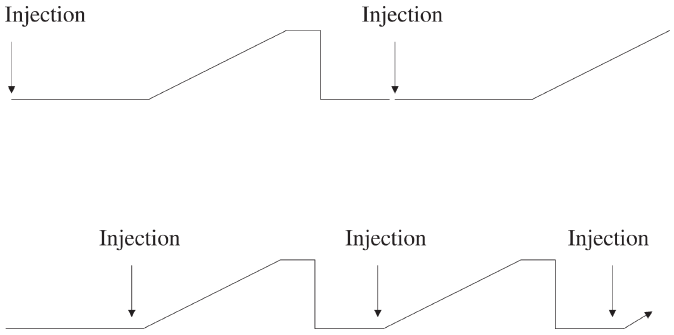

Fig. 7. Delayed sample injection to eliminate the gradient delay volume.

injection is executed just before the gradient reaches the column. In every subsequent gradient, the gradient run time, the purge cycle, and the column re-equilibration are all programmed as if the gradient delay volume does not exist. The point at which the gradient reaches the column only needs to be calculated once, and then one programs the injector to inject again at this time thereafter. With this trick, the influence of the gradient delay volume on the gradient run time can be largely eliminated.

In order to take full advantage of this feature, one needs to know the gradient delay volume. This is best measured by programming a linear gradient from water to water containing a small amount of a UV-absorbing compound, such as 1% acetone, without a column. The gradient delay time is then the difference between the time at which the start of the gradient was programmed and the time found by linear extrapolation of the UV trace recorded by the detector. The gradient delay volume is then calculated by multiplying this time by the flow rate.

1.2.2.4.2 **Detector Sampling Rate and Time Constant**

When running very rapid gradients, the peak width may be as little as 1 s. In the rapid gradient example shown above, the peak width was only 0.8 s. It is generally assumed that a peak can be represented well and is not unduly broadened if about 40 data points or more are measured over its width. This means that the sampling rate needs to be faster than 20 ms per data point. A similar argument can be put forward for the time constant, which is the measure of the speed of signal processing with older detectors. The time constant should be smaller than 50 ms. Today, the detector noise is often suppressed through an accumulation of data points obtained by the use of digital filters. Key characteristics of this process are the width of the filter, i.e. the number of data points used, as well as the filter algorithm, i.e. the way in which the data points are accumulated.

The experienced chromatographer will recognize two of the phenomena resulting from signal processing without difficulty. Edged peak shapes are a sign that the sampling rate is too low. A problem with the time constant results in tailing peaks. This tailing increases when the speed of the analysis is further increased. Difficulties arising from digital filtering are more difficult to recognize

since the peaks are not visibly deformed. In order to check for this problem, it is best to run two identical chromatograms with different filter settings. If this results in a difference in the peak widths, less filtering should be used to maximize resolution. If these difficulties arise, it is highly advisable to carefully read the care and use manuals of the detector and the data system.

1.2.2.4.3 Ion Suppression in Mass Spectrometry

A completely different detector problem arises if one wants to perform a quantitative analysis of a complex sample, such as a plasma sample, by means of an LC/MS method. LC/MS coupling has been a major breakthrough for this type of analysis. Analysis times of as little as 1 min can be achieved nowadays. However, the simplicity and the speed of the analysis have resulted in a complete disregard for the value of the chromatographic separation. In due course, it was found that "invisible" sample constituents cause a falsification of the signal, for the most part a suppression of the MS signal [1, 2]. This problem can be minimized or even eliminated through the use of more efficient separation techniques or better sample clean-up. In any case, it is worthwhile to measure the magnitude of this effect. If one knows the problem, one can search for ways to eliminate it through improved sample preparation techniques or an improved chromatographic separation.

The method used for measuring signal suppression is rather straightforward (Fig. 8), and the relatively little effort is always justifiable. A solution of the analyte(s) of interest is mixed post-column with the column effluent via a T-connection. The concentration of the analytes should be in the middle of the detection range of interest. Then, a blank plasma sample (i.e., without analytes) is injected onto the column, and the analysis is executed as usual. The mass spectrometer monitors the signal for the analyte(s) during this process. If the substances that elute from the column cause signal suppression, the signal declines. If there is no suppression, the signal remains constant. From the chromatographic analysis, we know at which point in time the analyte(s) elute. If there is no ion suppression during this time, the method can be used for the analysis of the compound(s) of interest in plasma samples. If one encounters a significant amount of ion suppression, one needs to re-work some part of the analytical method. Either the separation of the analyte from the matrix interferences needs to be improved, or more effectively, the sample preparation procedure can be refined. Possible ways of improving the HPLC method are often an adjustment of the pH value or an expansion of the gradient. Using an effective solid-phase extraction procedure instead of a simple protein precipitation will reduce the matrix interferences carried over into the HPLC method. If an improvement needs to be made, it is often best to examine several alternative methods and to compare them with respect to ion suppression. The method described here for the determination of ion suppression is universally applicable. The only disadvantage is the fact that the sample is often in the form of a specific salt, whereas during elution in the HPLC method it is associated with the counterions of the HPLC buffer. However, we are not aware of any negative consequences of this fact.

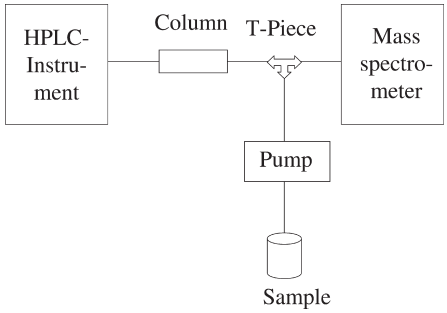

Fig. 8. Method for the determination of ion suppression.

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1.3
pH and Selectivity in RP-Chromatography

Uwe D. Neue, Alberto Méndez, KimVan Tran, and Diane M. Diehl

1.3.1
Introduction

The majority of the sample compounds analyzed by reversed-phase (RP)-chromatography have ionizable functional groups, such as carboxylic acid, sulfonic acid or amino groups. The retention of a sample strongly depends on the ionization of the functional group [1]. Often, there is roughly a 30-fold difference in the retention time between the neutral and the ionized form of the same analyte. The degree of ionization is determined by the pH of the mobile phase. It is important to understand all of the various influences on the degree of ionization if one wants to obtain good chromatographic selectivity and reproducible retention.

In this chapter, we discuss these influences in great detail. In our treatment, we rely on the latest information available; for example, the state-of-the-art knowledge on the influence of an organic solvent on the pH of the mobile phase and the pK_a values of analytes. This information has become available in recent years, largely through the investigations of the Barcelona group of researchers [2]. In addition, we discuss buffers used in classical HPLC as well as buffers useful for LC/MS applications. A few rules on the dependence of retention on the functional groups of the analytes are included as well.

1.3.2
Main Section

1.3.2.1 Ionization and pH

The retention of an ionizable analyte depends on its degree of ionization. For simple substances, one can state that the non-ionic form of the analyte always has a much higher retention than the ionic form. If the analyte has multiple stages of ionization, the form with the higher degree of ionization usually exhibits lower retention. The degree of ionization of the analyte depends on the pH of the solution and on the pK values of the ionization stages of the analyte.

The dependence of the retention on the degree of ionization is shown in Fig. 1 for both an acidic and a basic analyte. For both compounds, the retention changes by more than an order of magnitude. This is typical: for most compounds, the change in retention between the ionized and the non-ionized form is of the order of 10- to 30-fold. The ionized form always has lower retention in RP-chromatography. Therefore, the retention is lowest under acidic conditions for a basic analyte and under basic conditions for an acidic analyte. On the other hand, high retention is observed when the analyte is in its neutral form, i.e. under acidic conditions for acidic analytes and under basic conditions for basic analytes. There is a transition

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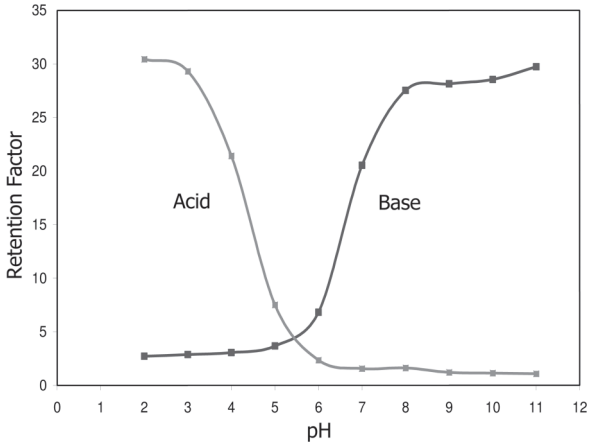


Fig. 1. Dependence of the retention factor of an acidic and a basic analyte on the pH value of the mobile phase. Column: XTerra® RP_{18}, 3.9 mm × 20 mm.

range between the two stages, in which the retention depends on the degree of ionization of the analyte. The following equation describes this behavior [1, 3]:

$$k = \frac{k_0 + k_1 \cdot d}{1 + d} \tag{1}$$

Here, k is the retention factor of the analyte, k_0 is the retention factor of the protonated form of the analyte, and k_1 is the retention factor of the deprotonated form; d is the degree of deprotonation and is defined as follows:

$$d = 10^{pH - pK_a} \tag{2}$$

In this equation, pH is the pH value of the solution and pK_a is the pK value of the relevant dissociation step of the compound. For multiply-charged compounds, the equation can be expanded in a simple way. This is shown in the following equation for a doubly-charged compound, such as an acid or base with two dissociation steps or a zwitterion:

$$k = \frac{k_0 + k_1 \cdot d_1 + k_2 \cdot d_1 \cdot d_2}{1 + d_1 + d_1 \cdot d_2} \tag{3}$$

The retention factors usually decrease with the degree of ionization. For example, a zwitterion is less retained than the singly-charged forms of the same analyte. Such a case is illustrated in Fig. 2. The sample is fexofenadine, a compound with a carboxylic acid group and a tertiary amino group. In the strongly acidic pH range, the carboxylic acid is protonated and therefore not charged. The compound therefore bears just one positive charge. In the intermediate pH range, both the carboxylic acid group and the amino group are ionized. Due to the fact that there is now a dual charge on the molecule, the retention is lower. In the alkaline pH range, the amino group is deprotonated, the molecule bears only a single, negative charge, and the retention increases again.

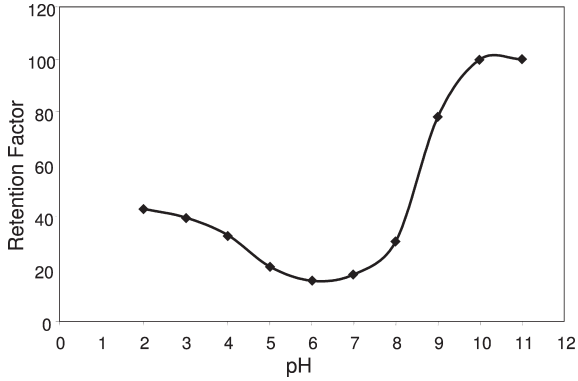


Fig. 2. Dependence of the retention of a zwitterionic sample on the pH of the mobile phase [1]. Column: XTerra® RP_{18}, 3.9 mm × 20 mm. Mobile Phase: 20% acetonitrile, 80% 30 mM buffer (from [1] with permission of Elsevier Science B.V.).

The pK values of the analyte determine the pH range in which the charge, and therefore the retention, changes. If the pH value is outside ± 2 pH units around the pK_a of the compound, the analyte is either 99% dissociated or 99% undissociated. This means that outside this pH range the retention does not change with a change in pH. However, within this range, especially within ± 1.5 pH units around the pK_a, the retention changes markedly. For the practice of chromatography, this means that good pH control is absolutely essential in order to achieve reproducible retention times. We will discuss this in more detail a little later in this chapter.

Aliphatic carboxylic acids have pK_a values around 5. For example, ibuprofen has a pK_a of 5.2 [4, 5]. Typical pK_a values for amines are around 9. The pK_a value of amitriptyline, for instance, is 9.4. Phenols, on the other hand, are very weak acids (the pK_a of phenol is 10.0). Similarly, anilines are very weak bases (the pK_a of aniline itself is 4.7). These data are good reference points for an estimation of the pK values of unknown compounds in the absence of measured data. It should be mentioned that the pK values of aromatic compounds need to be estimated by taking into account the conjugation with other groups through the aromatic ring(s).

1.3.2.2 **Mobile Phase and pH**

In Fig. 1, we have shown that the retention of an analyte may depend strongly on the pH of the mobile phase. In order to maintain reproducible pH values, one needs to use buffers in all pH ranges except at very acidic or strongly alkaline pH values. Buffers are solutions of ionogenic compounds that contain a conjugated pair of a proton donor and a proton acceptor. Thus, they stabilize the pH against the addition of small amounts of acid or base [6]. Let us discuss an acetate buffer as an example. This buffer contains an equimolar amount of acetic acid, the proton donor, and acetate, the proton acceptor. The pH of such a solution in water is 4.75. If one adds small amounts of acid or base (below the total buffer concentra-

Table 1. HPLC buffers.

(a) Buffers based on acids (neutral or anionic proton donors)	
Name	pK_a
Trifluoroacetate	0.5
Phosphate I	2.15
Phosphate II	7.20
Phosphate III	12.38
Acetate	4.75
Formate	3.75
Hydrogencarbonate	10.25
Borate I	9.24
(b) Buffers based on bases (cationic proton donors)	
Name	pK_a
Ammonium	9.24
Trimethylammonium	9.80
Triethylammonium	10.72
Pyrrolidinium	11.30

tion), the pH changes very little. If one prepares a solution of dihydrogenphosphate in water, one will also obtain a solution with a pH of around 4.5. However, this solution is not a buffer, because it does not stabilize the pH. If one adds a small amount of acid or base to the dihydrogenphosphate solution, the pH will change instantaneously to a more acidic or more neutral value. This does not happen with the acetate buffer. As we can see, a buffer is a solution that stabilizes the pH. If it does not do this, it is not a buffer, merely a salt solution.

The pK_a values of some commonly used HPLC buffers are shown in Table 1. The table is divided into two parts: buffers based on acids, and buffers based on a base. Of course, this list can be expanded at will. The buffers selected here cover the entire pH range of interest to the chromatographer. All organic buffers as well as hydrogencarbonate and ammonium buffers are volatile and are therefore compatible with MS detection, provided that a suitable counterion is used.

1.3.2.2.1 Buffer Capacity

Buffer capacity is a measure of the strength or quality of a buffer. It is defined as the reciprocal value of the slope of the pH curve (or titration curve) of the buffer [6]. It depends on two factors: (1) the concentration of the buffer; (2) the distance between the pH and the pK_a of the buffer. In Fig. 3, we show the buffer capacities of acetate buffers at concentrations of 5 mM, 10 mM, 20 mM, and 40 mM. The maximum of the buffer capacity is always at the pK_a, i.e. 4.75 for the acetate buffer. The maximum buffer capacity increases in direct proportion to the buffer concentration. At the same time, the pH range over which a particular buffer capacity is achieved increases with increasing concentration. For example, a 10 mM acetate buffer has a buffer capacity in excess of 0.005 in the pH range from

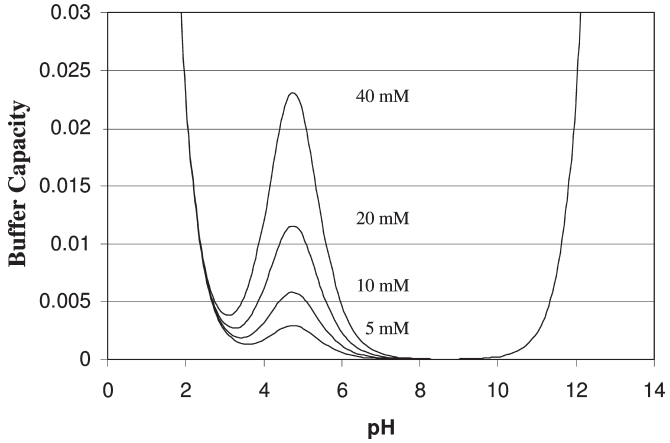


Fig. 3. Buffer capacities of acetate buffers of various concentrations. From top to bottom: 40 mM, 20 mM, 10 mM, 5 mM.

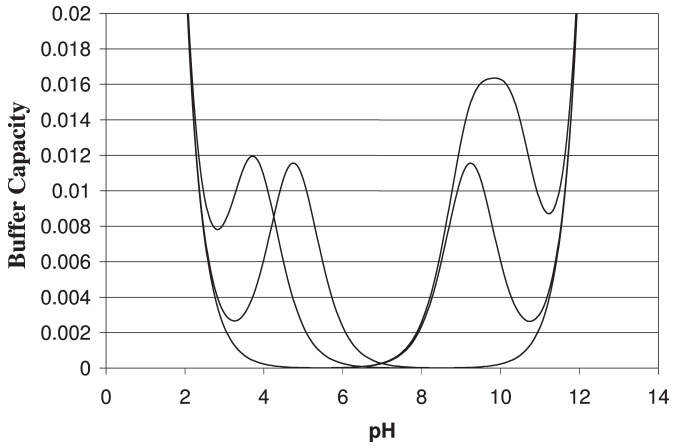


Fig. 4. MS-compatible buffers, 20 mM; from the left: formate, acetate, ammonium, ammonium hydrogencarbonate.

4.5 to 5.0, while a 40 mM buffer has the same buffer capacity between pH 3.5 and pH 6.0. This is important for understanding the quality of a buffer. Figure 3 also shows the buffer capacities of the H^+ and OH^- ions at the extremes of the pH scale.

Figure 4 shows the buffer capacities of a few MS-compatible buffers: formate, acetate, ammonium and ammonium hydrogencarbonate, all at a concentration of 20 mM. Formic acid or ammonium formate are preferentially used at acidic pH. Acetic acid and acetate are useful for a slightly higher pH range, but they are less frequently employed. In the alkaline pH range, one can use ammonia or ammonia together with a volatile ammonium salt such as formate, acetate or hydrogencarbonate. Ammonium hydrogencarbonate is the preferred buffer for

LC/MS applications in the alkaline pH range. The reason for this is the overlapping and additive buffer capacities of the ammonium cation and the hydrogencarbonate anion. Above 60 °C, ammonium hydrogencarbonate decomposes into water, ammonia, and carbon dioxide.

1.3.2.2.2 Changes of pK and pH Value in the Presence of an Organic Solvent

The pH and pK_a values of buffers as well as the pK_a values of analytes found in the literature have commonly been measured using water as the solvent. It is important to understand that both the pH values and the p*K* values change when an organic solvent is added [2]. Generally, the pK_a values of acids increase, while the pK_a values of bases decrease with the addition of the organic modifier. This phenomenon has been described in detail in the literature [2]. For the practitioner, it is important to understand that the buffer capacity does not change in the presence of the organic solvent. A buffer that is a good buffer in water is also a good buffer in the presence of an organic solvent. Contrary to recommendations in the literature [2], we do *not* recommend the measurement of the pH of the buffer *after* the addition of the organic solvent. The most important value for the practitioner of HPLC is the buffer capacity, and this does not change with the addition of the organic solvent, while the pK_a value of the buffer and the optimal pH do change. The true pH value after the addition of the organic solvent is certainly of interest to the theoretician, but for the practitioner the only relevant value is the buffer capacity, and this value can be estimated without difficulty with reference to the aqueous p*K* and pH values. The aqueous p*K* values of buffers can be found in textbooks.

It is nevertheless of value to review the phenomena described in Ref. [6]. If one is interested in an understanding of the changes in retention time occurring at different pH values in the presence of an organic solvent, it is absolutely necessary to measure the pH after the organic solvent has been added. To do this, two pH scales can be used. For the first pH scale, the pH meter is calibrated with standard buffers in the presence of the organic solvent. This pH scale is called the ${}^{s}_{s}\mathrm{pH}$ scale. The upper index designates the method used for the measurement, and the lower index the method of calibration. If one calibrates the pH meter with standard buffers in water and measures the pH after the addition of the organic solvent, one obtains pH values on the ${}^{s}_{w}\mathrm{pH}$ scale. The difference between the two scales is the activity coefficient of the hydronium ion γ^0_H:

$$ {}^{s}_{w}\mathrm{pH} = {}^{s}_{s}\mathrm{pH} - \log({}^{s}_{w}\gamma^0_H) \tag{4} $$

The difference between the pH value in water and in a mixture of an organic solvent with water is caused by a change in the autoprotolysis constant of water due to the addition of the organic solvent.

Figure 5 shows the change in the p*K* values of several buffers for methanol/water mixtures. The data for this figure have been taken from the literature [7, 8]. As mentioned above, one can see that the pK_a values of acids, e.g. formic acid, increase upon the addition of the organic solvent. For buffers generated from bases, the p*K* values change to more acidic values over a large part of the solvent

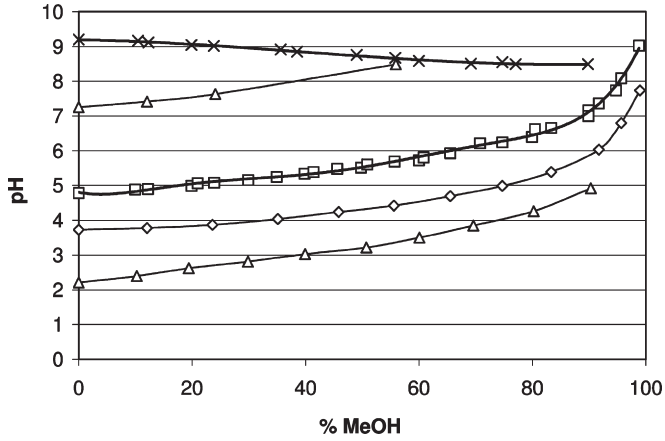


Fig. 5. ${}^{s}_{s}pK_a$ values of several buffers in methanol/water mixtures: △ phosphate (aqueous pH 2 and 7), ◇ formate, □ acetate, × ammonium. (Adopted from [8] with permission from the authors).

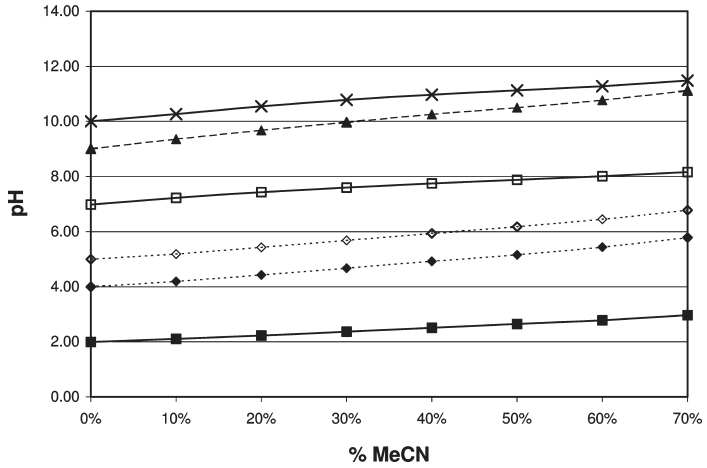


Fig. 6. ${}^{s}_{w}pH$ values of several buffers in acetonitrile/water mixtures: □■ phosphate (aqueous pH 2 and 7), ◇◆ acetate (aqueous pH 4 and 5), △▲ borate, × hydrogencarbonate.

composition. This means that the addition of the organic solvent results in a weakening of both acids and bases. Figure 6 shows the changes in the pH of buffered mobile phases based on acetonitrile measured in our laboratory. All the buffers shown in this chart are based on acids. Therefore, the pH values shift towards alkaline pH as the concentration of acetonitrile is increased. An interesting situation is obtained when the pK_a values of the acidic and basic components of a buffer overlap, as is the case for an ammonium hydrogencarbonate buffer (Fig. 7). The ${}^{s}_{w}pH$ value of this buffer does not change much as the concentration of the organic solvent is increased. This is due to the fact that the pK_a value of the

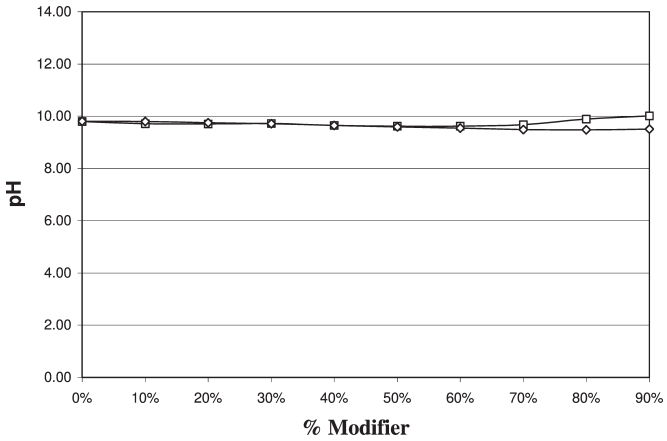


Fig. 7. $^{s}_{w}$pH values of an ammonium hydrogencarbonate buffer with an aqueous pH of 9.8: ◊ methanol, □ acetonitrile.

hydrogencarbonate shifts towards more basic pH, while the pK_a value of the ammonium ion shifts towards more acidic pH.

The pK_a values of analytes shift upon the addition of an organic solvent in exactly the same way as described for the buffers. We will discuss this subject in more detail below in the discussion of the influence of pH on retention.

1.3.2.3 Buffers

1.3.2.3.1 Classical HPLC Buffers

Which buffers are preferred for an application depends primarily on the choice of detector. For this reason, we distinguish between classical HPLC buffers, which are used with UV detection, and MS-compatible buffers, which are highly volatile. We discuss first the classical buffers.

Phosphate buffers are the preferred buffers for UV detection. Their pK_a values are 2.15 and 7.20. They can be used without difficulty at low UV wavelength, for example 210 nm. At weakly alkaline pH, buffers based on the ammonium ion (pK_a = 9.24) with a suitable counterion with low UV absorption can be used. Borate buffers can be used in the same pH range and also have a low UV absorption. In the more alkaline pH range, some simple amines can be used: pyrrolidine has a pK_a of 11.3, and triethylamine a pK_a of 10.7, and both can be used at low UV wavelengths. With all amines, their purity is an important factor for their use at such UV wavelengths.

Other buffers often exhibit a substantial UV absorption below 215 nm. However, for the normal UV detection range around 254 nm a large number of buffers is available. Acetate with its pK_a of 4.75 is probably the most popular buffer, since its pK is exactly intermediate between the first and second dissociation constants of phosphate. Ammonium hydrogencarbonate is a preferred buffer for the pH range around 9 to 10, since the buffering ranges of the ammonium ion (pK_a = 9.24) and the hydrogencarbonate ion (pK_a = 10.25) overlap.

1.3.2.3.2 MS-Compatible pH Control

We have already mentioned some of the commonly used mobile phase additives for MS-compatible pH control. The most important attribute is the volatility of all buffer components. The most frequently used mobile phase additives are formic acid and acetic acid, together with the true buffers formic acid/ammonium formate and acetic acid/ammonium acetate. In the alkaline pH range, the preferred additive is ammonia, and the buffer of choice is ammonium hydrogencarbonate. One can also use the ammonium ion with volatile counterions such as formate or acetate to establish a true buffer at the pK_a of the ammonium ion (pK_a = 9.24). One finds sometimes in the literature the reported use of ammonium formate or acetate at neutral pH. It needs to be pointed out that this has little to do with pH control, since these salt solutions have no buffer capacity at pH 7!

Trifluoroacetic acid is used in the very acidic pH range. It should be mentioned that this additive often contributes substantially to ion suppression, and it should therefore only be used when there are no viable alternatives. In our laboratory, we have also used semi-volatile buffers such ammonium phosphate at pH 7. However, a very low concentration should be used, and one should plan for a more frequent clean-up of the MS inlet and source.

1.3.2.4 Influence of the Samples

The retention of an analyte strongly depends on its ionization. As mentioned above, the non-ionized form of the sample has a much higher retention, up to a factor of 30 higher, than the ionized form. However, the difference is not the same for all analytes. For some analytes, the retention changes more, for others less. This is shown in Fig. 8, where we have plotted the retention times under gradient conditions for more than 70 samples under basic *versus* acidic conditions.

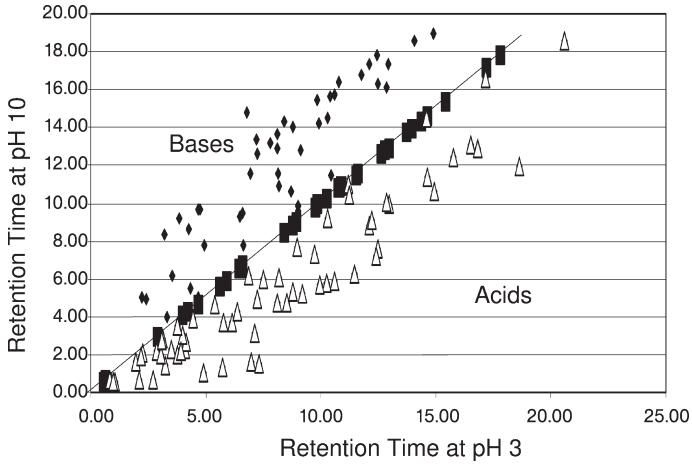


Fig. 8. Differences in the retention values of a large number of samples under gradient conditions as a function of pH.
■ neutrals, ♦ basic compounds, △ acidic compounds.

The *x*-axis corresponds to the retention times at pH 3, and the *y*-axis to those at pH 10. In the center of the figure we have drawn a line through the data points for non-ionizable compounds for which retention does not change with pH. Analytes with an acidic functional group are neutral under acidic conditions and thus are retained more. Conversely, basic analytes are neutral at basic pH, and therefore exhibit a higher retention in the alkaline pH range.

1.3.2.4.1 The Sample Type: Acids, Bases, Zwitterions

How much the retention of an analyte changes with pH depends both on the degree of ionization as well as on structural parameters. Sulfonic acids are very acidic. Benzenesulfonic acid has a pK_a of 0.7. The pK values of aliphatic sulfonic acids are higher. Aliphatic carboxylic acids commonly have pK_a values around 4.5 to 5. Aromatic carboxylic acids, on the other hand, are more acidic. Sulfonamides are weakly acidic. The pK_a values of phenols can be found in the mildly alkaline pH range (phenol: pK_a = 9.99) and depend strongly on the substituents on the aromatic ring.

Basic analytes are protonated at acidic pH, and lose their charge under alkaline conditions. Anilines are very weak bases: the pK_a of aniline itself is 4.6. Pyridine too is a very weak base with a pK_a of 5.2. On the other hand, aliphatic amines are rather strong bases: methylamine pK_a = 10.6, dimethylamine pK_a = 10.8, trimethylamine pK_a = 9.8. An analysis of the retention time shifts of the strong bases in Fig. 8 has shown that the retention of tertiary amines changes more than the retention of secondary amines, when the data at acidic pH are compared with those at alkaline pH. This may be due to the fact that the charge on a protonated amine creates a strongly bound hydration shell around the amino group, which "hides" the adjacent methyl or methylene groups. On the other hand, the methyl groups do contribute to retention when the amino group is not protonated. Another possibility could be that under the measurement conditions only the tertiary bases were completely deprotonated.

The retention of zwitterions also changes predictably with pH. We have already highlighted the example of fexofenadine earlier in this chapter. The retention of this analyte decreased by a factor of three on going from the acidic pH range to neutral pH, and increased again by a factor of seven on going from neutral pH to alkaline pH. For analytes that contain an aniline and a phenol function, one obtains the opposite result. Under acidic conditions, the sample is positively charged. In the intermediate pH range, the analyte loses its positive charge, the molecule becomes neutral, and the retention increases. In the alkaline pH range, the phenol group becomes negatively charged, and the retention decreases again. As one can see, one can obtain significant changes in the retention and thus in the selectivity of a separation by manipulating the pH of the mobile phase.

1.3.2.4.2 Influence of the Organic Solvent on the Ionization of the Analytes

In Section 1.3.2.2.2, we established that the pH of a buffer changes upon the addition of an organic solvent. So does the pK_a value of an analyte. It is important to keep these things in mind when developing HPLC methods. The combined effect of the pH shift of the buffers and the pK_a shift of the analytes can yield surprising results. For example, let us look at the retention of amitriptyline in a phosphate buffer in 65% methanol [9]. The pH value of the phosphate buffer as measured in water was 7.0. The pK_a value of amitriptyline in water is 9.4. Since there is a difference of 2.4 pH units between the pK_a of amitriptyline and the pH of the mobile phase, one would expect amitriptyline to be completely protonated in this buffer. However, measurements of the retention behavior have shown that under the conditions of the measurement in 65% methanol only one-third of the amitriptyline molecules are ionized. Under these conditions, the second pK_a of phosphoric acid is around 9 [8]. This means that the pK_a of amitriptyline shifted from 9.4 in water to around 8.5 in 65% methanol. The combination of both effects has the result that amitriptyline is only partially protonated under the measurement conditions.

Consequently, it is important to keep these pH and pK shifts in mind when developing a new method. A good control over the degree of ionization of the analytes is an important requisite for method reproducibility and robustness.

1.3.3 Application Example

As we have seen, the pH value plays a major role with regard to the separation of ionizable compounds. This means that the pH is the most important factor in method development, and one should explore its effect at the beginning of the method development effort. We have devised a procedure that does exactly that: it demonstrates relatively early on which combination of pH and mobile phase composition is most promising for a further fine-tuning of the method [10]. The foundations of this principle had already been developed earlier [11].

In this method, a separation is explored using two pH values, two organic modifiers, and several columns with significant selectivity differences. If one uses a pH-stable hybrid packing such as XTerra®, one can combine an alkaline pH (pH 10 with ammonium hydrogencarbonate) with an acidic pH (e.g., pH 3.5 with an ammonium formate buffer). Such a method can be used with MS detection [10]. With classical silica-based columns and a UV detector, it is proposed that phosphate buffers at pH 2.5 and 7.0 are used [11]. In both cases, the large change in pH results in a change in the ionization of the analytes and in a striking change in the selectivity of the separation.

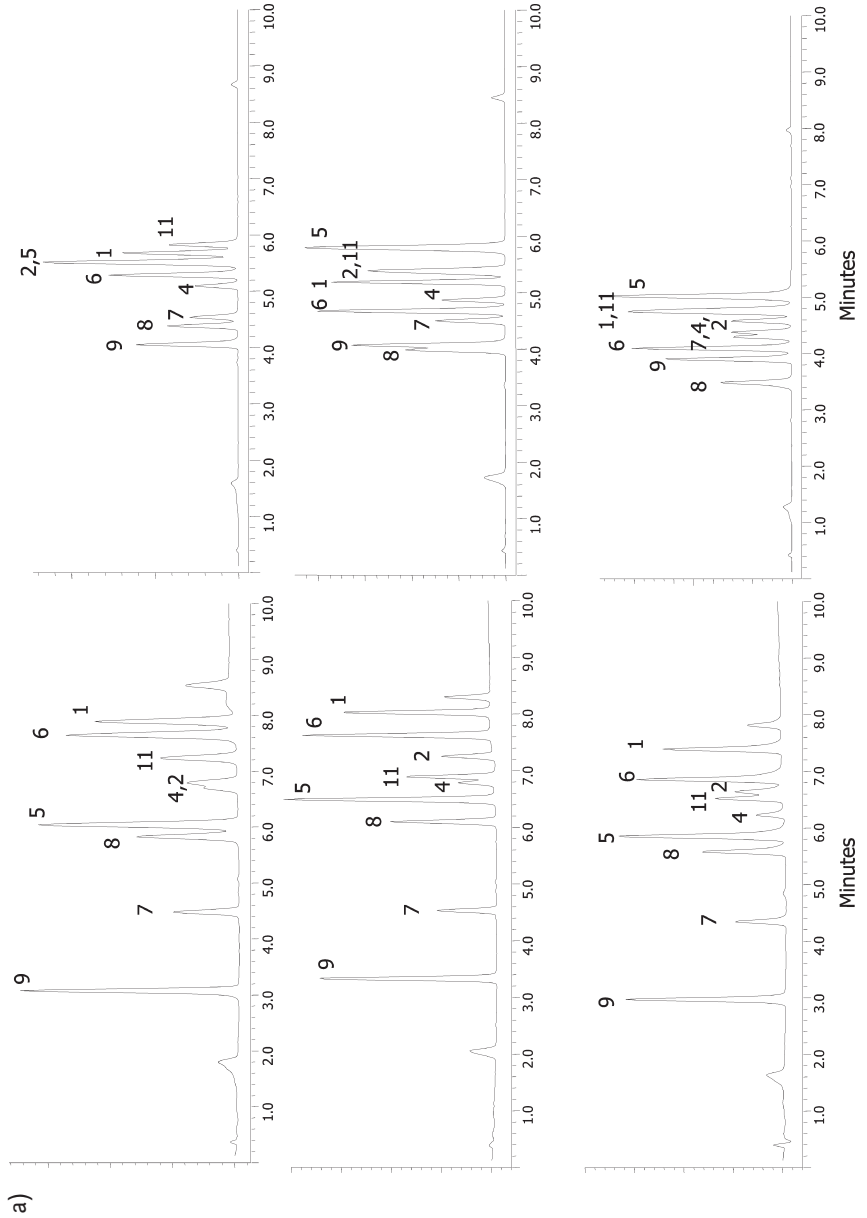
Acetonitrile and methanol are used as the organic solvents. Methanol is a proton donor, and acetonitrile is a proton acceptor. Significant selectivity differences can therefore be achieved with these two solvents. In addition, the solvent selectivity can be varied continuously, and a fine tuning of the selectivity can thus be obtained using mixtures of the two solvents. In classical method development, THF was

used as well, but in most cases this is not necessary. Moreover, one can often use a fine-tuning of the pH, i.e. around the p*K* of the buffer, to improve the separation. However, this is only possible if the p*K* values of the analytes are not too far away from the pH value.

In addition, this method permits a rapid exploration of the selectivity of the stationary phases. We have generally used three different XTerra® columns: XTerra® MS C_{18}, with a trifunctionally bonded C_{18} chain; XTerra® RP_{18}, with an incorporated carbamate group; and XTerra® Phenyl, with an aromatic group. This column selection maximizes the selectivity differences within the given family of packings. Alternatively, one can use the silica-based packings Symmetry® C_{18} and SymmetryShield™ RP_{18} with acidic and neutral buffers. The relevant selectivity differences between these two packings are discussed in Chapter 2.1.2 of this book.

In the following, we discuss the separation of nine diuretics as an example of our method development strategy. In order to rapidly evaluate the influence of pH, organic solvent, and column chemistry, the same gradient (from 0 to 80%B in 15 min) was run under acidic (pH 3.64) and basic (pH 9.0) conditions on three short 4.6 mm × 50 mm XTerra® MS C_{18}, XTerra® RP_{18}, and XTerra® Phenyl columns, using both methanol and acetonitrile as organic modifiers. The separations are shown in Fig. 9. One can clearly see the influence of the pH on the selectivity of the separation. Generally, the separation extends over a larger range of the solvent composition at acidic pH than at alkaline pH. If the final separation can be a gradient separation, then one should select the acidic pH range. Indeed, it was possible to optimize the separation without much additional effort using the XTerra® MS C_{18} column under acidic conditions. If one wishes to achieve an isocratic separation, the alkaline pH range is more promising. However, in this case it is necessary to use solvent selectivity, i.e. mixtures of acetonitrile and methanol, to achieve the final optimized isocratic method.

Such a separation is shown in Fig. 10. We used a mixture of 13% acetonitrile and 4% methanol and an ammonium hydrogencarbonate buffer at pH 9.0. A short 5 cm XTerra® MS C_{18} column was sufficient for this separation. All peaks are cleanly separated. This example demonstrates the value of pH adjustment for the separation of ionogenic compounds. This method development strategy can be employed generically for all separation problems involving ionogenic analytes.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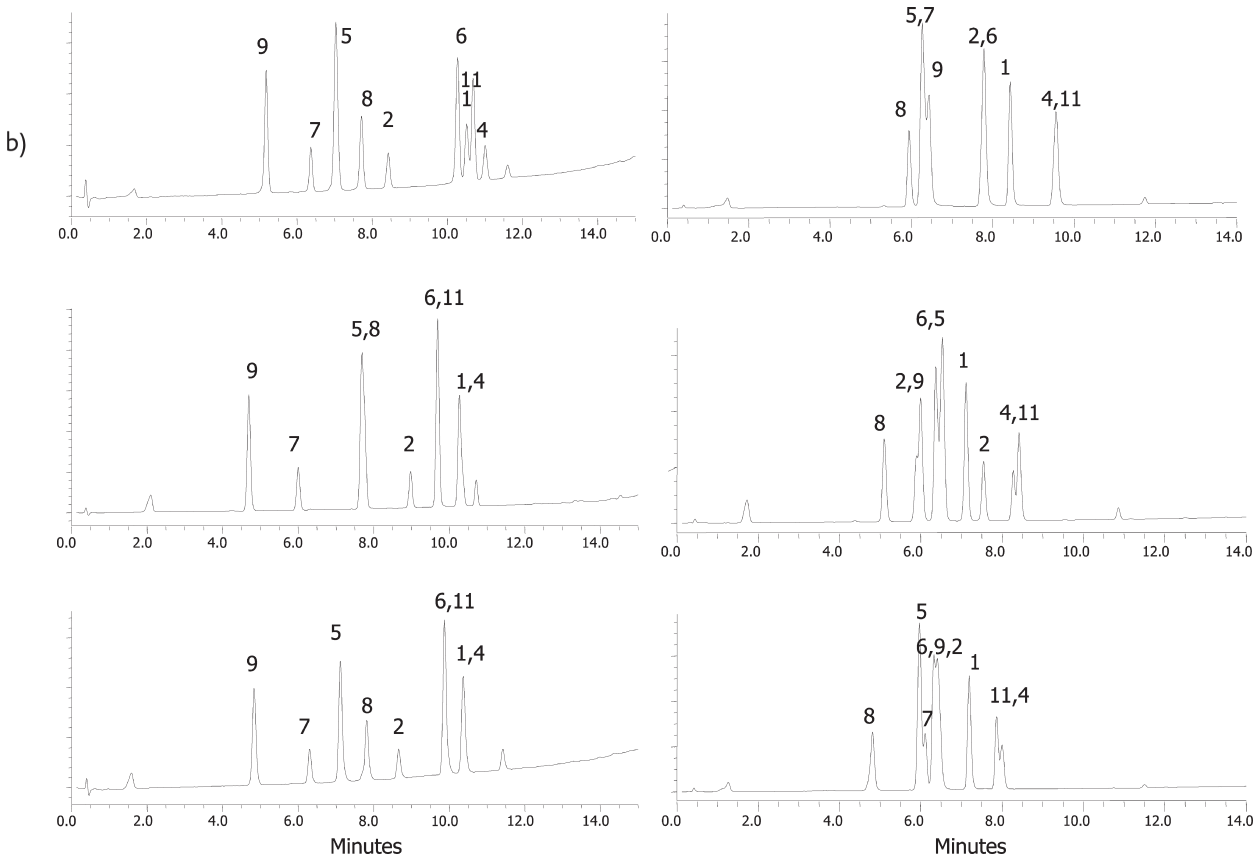

Fig. 9. Gradient separation of nine diuretics with acetonitrile (a) and methanol (b) as organic modifiers.
Left: pH 3.64; right: pH 9.0.
Top: XTerra® MS C_{18}. Middle: XTerra® RP_{18}. Bottom: XTerra® Phenyl.
Samples: bumetanide (1), benzothiazide (2), canrenoic acid (4), althiazide (5), probenecid (6), chlorothalidone (7), furosemide (8), triamterene (9), and ethacrynic acid (11)
(from ref. [11] with permission from Elsevier Science B.V.).

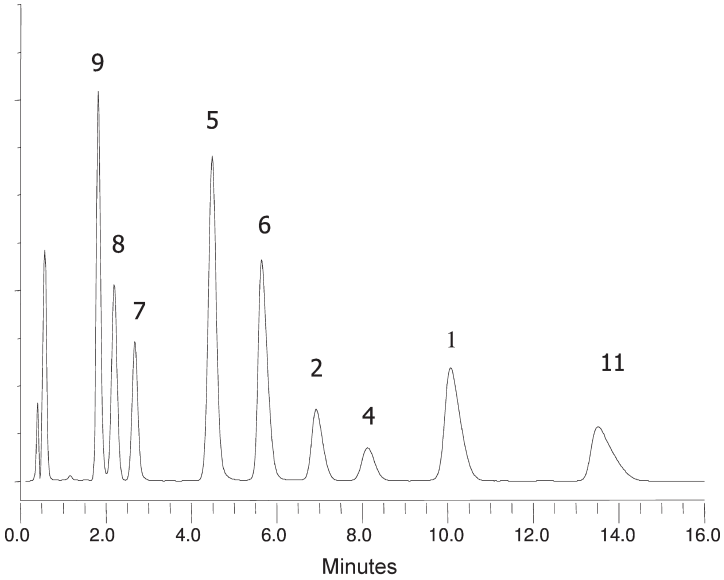

Fig. 10. Isocratic separation of nine diuretics. Column: XTerra® MS C_{18}, 4.6 mm × 50 mm, 3.5 µm. Mobile phase: 13% acetonitrile, 4% methanol, 73% water, 10% 100 mM ammonium formate, pH 9.0. Detection: 254 nm. Peak designation as in Fig. 9 (from ref. [11] with permission from Elsevier Science B.V.).

1.3.4 Troubleshooting

1.3.4.1 Reproducibility Problems

When separating ionizable compounds, new problems emerge that are unknown for simple non-ionic molecules. As shown in this chapter, the retention times of analytes in reversed-phase chromatography change drastically with their level of ionization. In order to obtain reproducible results, it is therefore important to maintain a constant level of ionization. The degree of ionization is stable outside of ± 1.5 pH units around the pK_a of the analyte. If the analytes are simply bases or acids, the best results are obtained at either high or low pH values. Virtually all bases are completely ionized in a phosphate buffer at pH 2. Under the same conditions, most, but not all acids are not ionized. On the other hand, all acids are completely charged in an ammonium hydrogencarbonate buffer at pH 10, while most, but not all bases are uncharged. If the charge of all the compounds is well-defined at the pH value under consideration, then it is often not necessary to control the pH with great precision. As an example, the use of trifluoroacetic acid ($pK_a = 0.5$) is often sufficient to achieve a uniform ionization at acidic pH.

If the analytes contain several ionizable groups, then it is often impossible to find a pH range in which the dissociation does not change upon small changes in the pH value. This is also true for all substances in the intermediate pH range.

Under these circumstances, it is absolutely necessary to have excellent control over the pH value. The consequence of this is that true buffers must be used in order to achieve and control the desired pH value. If this is not done, problems with the reproducibility of retention times are almost inevitable, and often problematic peak-shape phenomena such as fronting or tailing occur as well. In addition to the use of a true buffer, it is also essential to measure and control the pH with high precision to obtain reproducible separation patterns. On the one hand, a change in the pH value is the best tool to manipulate and optimize the separation of ionizable compounds. On the other hand, careful pH control is mandatory.

1.3.4.2 **Buffer Strength and Solubility**

In order to achieve good reproducibility of a separation, it is important that the buffers used have a sufficient buffer capacity. A good buffer concentration is around 50 mM. However, with a high proportion of organic solvent in the mobile phase, such a concentration may result in precipitation of the buffer. Due to the multitude of buffers and solvent compositions, there are no tables that describe the solubility of the buffers used in reversed-phase chromatography. If one needs to work with a new buffer/mobile phase combination, it may be advisable to quickly test its solubility in the presence of the organic solvent. This can be done without difficulties and very rapidly, and it saves a lot of trouble with precipitated salts in the check valves of the pump.

In LC/MS, the typical buffer concentration used today is around 10 mM. This results in a reduced control over retention compared to classical HPLC. At the same time, the specificity of the mass spectrometer compensates for the sloppiness on the chromatography side.

1.3.4.3 **Constant Buffer Concentration**

Today, the HPLC instrument is commonly used to mix the aqueous buffer solution with the organic component of the mobile phase. For an isocratic analysis, this is only a convenient option, but for gradient separation there is no way around this. Under normal circumstances, such a procedure implies that the ionic strength of a buffer changes with the concentration of the aqueous component in the mobile phase. However, there is another way to do this, and with this method the concentration of the buffer can be kept constant over the gradient. The HPLC instrument needs three solvent inlets to the gradient mixing chamber to accomplish this. One of the inlets is connected to water, the second to the organic component of the mobile phase, and the third inlet is used for the buffer. The buffer is then added throughout the gradient at a constant rate of 10% of the flow rate. The buffer concentration is ten times higher than the desired final concentration in the mobile phase. With respect to the organic solvent, the gradient is mixed as usual. Commonly, there are four inlets to the gradient mixing chamber. If one is used for the addition of methanol, and another for the addition of acetonitrile, one has already part of a basic set-up for automated method development.

1.3.5 Summary

The majority of the separation problems in reversed-phase chromatography require buffered mobile phases. We have shown in this chapter the possibilities that exist, and what complications are likely to be encountered. While knowledge about the properties of buffers in the presence of organic solvents has increased in the last decade, one can still expect an improvement of this knowledge in the future. We hope to have pointed out the major problems in this chapter.

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1.4
Selecting the Correct pH Value for HPLC

Michael McBrien

1.4.1
Introduction

Mobile phase pH is one of the most powerful variables for changing selectivity in high-performance liquid chromatography. It is common to observe k changes of an order of magnitude with changes in mobile phase pH. Additionally, pH can have a profound effect on peak broadening and tailing.

The strong influence of pH on chromatographic performance has implications for the robustness of chromatographic methods as well. The sensitivity of retention time to small changes in pH (see the pH region close to the pK_a in Fig. 1) leads to concerns about the reproducibility of separations. Systematic approaches to pH choice are particularly important when stable, reproducible results are sought. Fortunately, the practical range of pH values available to the chromatographer has recently been expanded considerably with the addition of new base-stable stationary phases.

Effective chromatographic pH values can be determined on a purely experimental basis, but this process can be facilitated greatly through the use of any knowledge that the chromatographer has regarding expected structure(s) and/or functional groups of the compounds of interest.

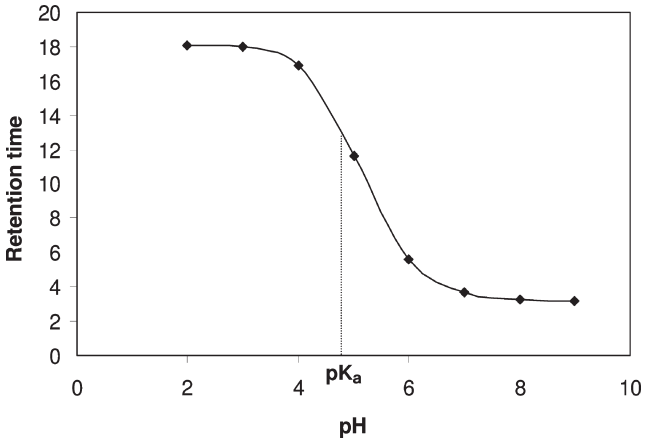

Fig. 1. Retention of an acidic compound as a function of pH in reversed-phase chromatography. At low pH, the neutral, protonated form predominates; the compound has a relatively long retention time. At high pH, the compound is present in ionized form and is weakly retained.

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1.4.2
Typical Approaches to pH Selection

Changes in reversed-phase chromatographic performance as a function of pH can be due to solvation sphere changes, stationary phase modification, or changes of ionization state of the analyte. For the purpose of this chapter, the main focus will be the effects of changes in hydrophobicity due to changes in ionization state. The change in hydrophobicity of a compound as a function of pH can be quite complex depending on the type and number of ionization sites for that compound. For this reason, optimization of pH cannot be treated easily with the same systematic software optimization tools that are commonly applied to optimization of other parameters, such as concentration of organic modifier in the mobile phase and column temperature [1]. Figure 2 shows the change in *k* as a function of pH for norfloxacin, fleroxacin, and ofloxacin. It is clear that a linear (or simple curve-based) relation of pH to retention time will be effective only over very small pH ranges. For this reason, it is wise to consider chemical structures when optimizing separations. These can give a good starting point for method development. If further consideration of pH is desired, the range of optimization can be chosen to be suitably small, enabling accurate experimental optimization.

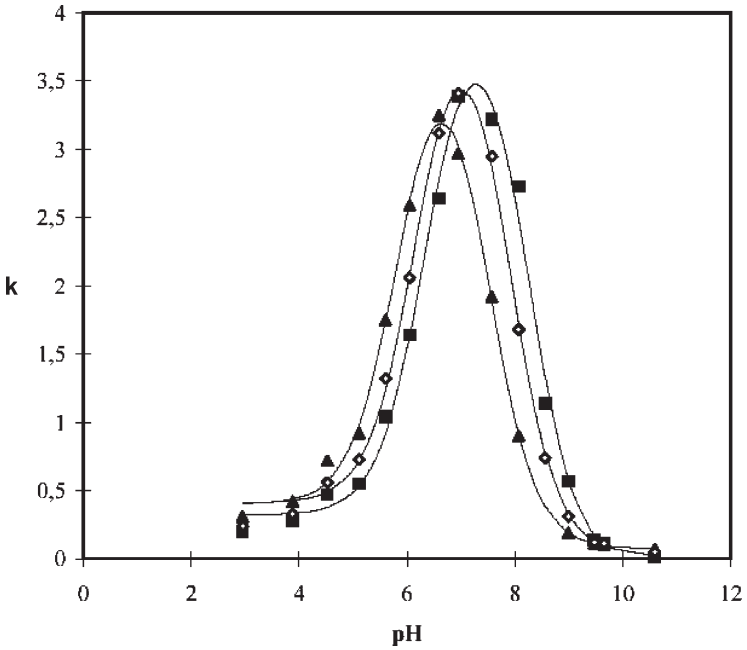

Fig. 2. The change in capacity factor for multiply ionizable species with change in pH: □ norfloxacin, ▲ fleroxacin, ○ ofloxacin [2].

1.4.3 Initial pH Selection

In Chapter 1.3, options for buffer content were discussed. There are a number of ways to utilize any structural information available to assist with selections between available buffers. These can be grouped into two main approaches:

1. Application Databases

The global body of work in chromatography is simply astounding. Recently, there have been a number of efforts to compile at least a subset of this knowledge into a mineable resource [3]. Application databases can offer very fast insights into pH and other method choices, particularly when chemical structures are stored with the applications. Even when only partial structures are known, substructure searches on key (especially ionizable) functional groups give a good estimation of methods that previously worked for similar compounds. A drawback of this approach is that subtleties in the chemical structures can change pK_a values significantly, and invalidate these choices. However, this is a very fast approach to retrieval of reasonable starting points for method development, and this, in conjunction with subsequent optimization over a small pH range, can be effective.

2. pK_a Look-up and Prediction

If we consider Fig. 1 in more detail, it is clear that the largest changes in retention time occur when the pH of the mobile phase is close to the pK_a of the analyte. In addition, the secondary equilibria that result in peak broadening can also be emphasized at this pH. There is a fairly simple solution to this problem: where analyte pK_a values are known, the chromatographer can simply retrieve these values and be sure to work at least 2 pH units above or below the pK_a. Chromatographic mechanisms should be relatively free from secondary equilibria effects in this area; i.e., the analytes will exist in either fully ionized or fully unionized form. This should result in better peak shape and more reproducible chromatography.

In addition to the familiar CRC Handbook [4], there are software-based resources for the retrieval of pK_a values [5]. The advantage of these is that they are typically also substructure-searchable, such that the resource is useful even for novel compounds. Substructure searches for ionizable groups should give a reasonable idea of pK_a values for these groups present in novel species.

Approximate pK_a values for individual functional groups can be obtained from any standard reference system. Chromatographers can, with some confidence, estimate pK_a values for new species based on these compounds. However, errors will arise when considering secondary effects of other functional groups. A more systematic approach is to use prediction software that is now readily available. Predicted pK_a values can be obtained from several resources, including on-line [5], as a standalone package [6], or as part of chromatographic prediction software [7]. All of these resources offer predictions based on an approach similar to the one described below.

1.4.4
Basis of pK_a Prediction

The ACD/pK_a algorithm has not been reported in the literature to date. The general structure of the algorithm is classification followed by a linear free energy (Hammett equation) relationship, using the well-known sigma constants as descriptors of the electron withdrawal and/or donation potency of substituents electronically connected to the ionization center. Several additional complexities need to be considered, however: ionic forms for polyelectrolytes, reference compounds, and transmission effects for subclasses of compounds with distal substituents (see Fig. 3 for an illustration). The classes, subclasses, reference compounds, sigma parameters, and transmission coefficients were derived by study of nearly 16,000 compounds with over 30,000 pK_a values. Advanced topics such as tautomeric equilibria, proton migration, covalent hydration, vinylology, ring-breaking approximations, ring-size correction factors, steric effects, and variable charge effects are also taken into account, but are beyond the scope of this description.

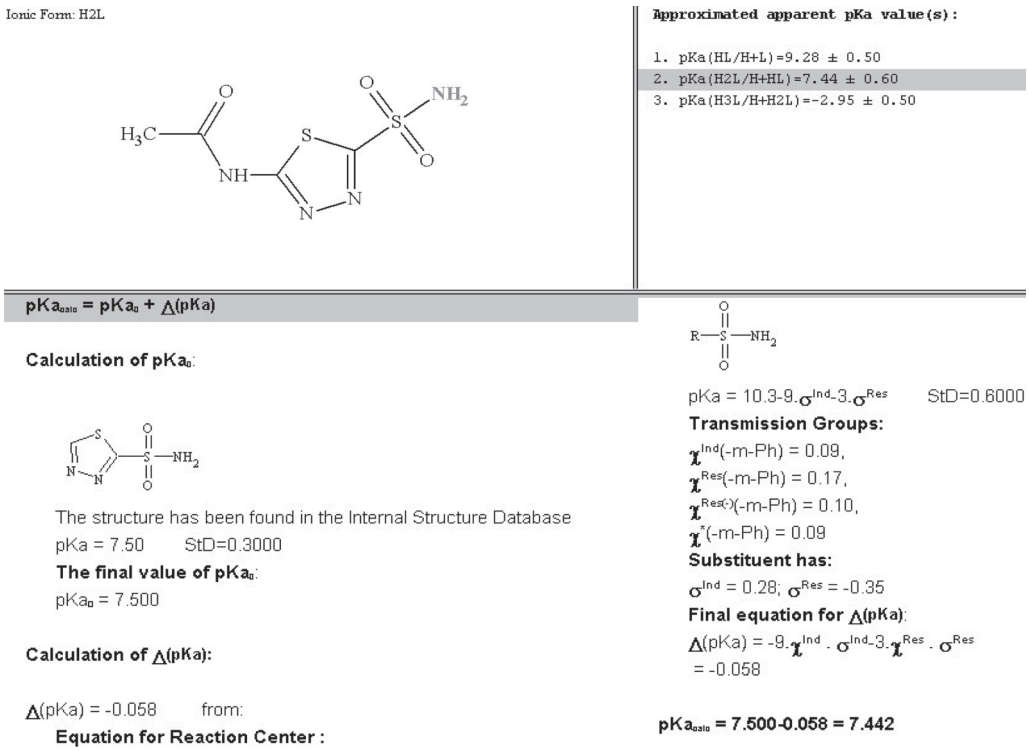

Fig. 3. Illustration of the computation of pK_a for acetazolide; ACD/pK_a DB 8.0.

1.4.5
Correction of pH Based on Organic Content

Organic mobile phase solvents in reversed-phase chromatography influence the selection of pH in two ways. The first is that there are differences in the pK_a values of compounds in the presence of organic solvent as opposed to the pure aqueous phase. The second issue is that the effective pH of the mobile phase changes due to the presence of organic solvent (see also Chapter 1.3). This pH shift is due to a change in the ionization of the buffer, not changes in buffer concentration or buffer capacity. Due to this pH shift, a correction factor should be applied prior to selecting the mobile phase pH based on pK_a values [8, 9]. The correction factor is of the order of 0.2 units per 10% modifier for acetonitrile, and 0.1 units per 10% methanol, depending on the buffer system, and is well-behaved in the range 0–60% in each case.

Correction of pH based on the presence of organic solvent should be done in two simple steps. The first accounts for the effect of the solvent on the buffer. The second accounts for the effect on the ionizable species.

Notice that if the buffer and the analyte are both acid or both base, the terms will cancel out to a large extent. In cases where the analyte is basic and the buffer is acidic, or vice versa, the effect will be very pronounced.

An illustration may be helpful in this case. If we consider an acidic analyte/acidic buffer, we can, in general, use the aqueous pK_a as an indicator of the pH at which to prepare the aqueous buffer. The changes in pK_a for the buffer and analyte

Table 1. Correction factors for acidic buffer, acetonitrile, and mobile phase pH.

Acetonitrile %	*Acid analyte correction factor*	*Base analyte correction factor*	*Acidic buffer correction*	*Total correction factor*
10	+0.2	–	–0.2	0
20	+0.4	–	–0.4	0
30	+0.6	–	–0.6	0
40	+0.8	–	–0.8	0
50	+1.0	–	–1.0	0
60	+1.2	–	–1.2	0
10	–	–0.2	–0.2	–0.4
20	–	–0.4	–0.4	–0.8
30	–	–0.6	–0.6	–1.2
40	–	–0.8	–0.8	–1.6
50	–	–1.0	–1.0	–2.0
60	–	–1.2	–1.2	–2.4

will cancel. We need only make sure that we consider the effective pH gradient that we will see. In the case of a basic compound with an acidic buffer, if the pK_a of the species indicates that a pH range of 3 to 5 is unusable and we anticipate working at 50% acetonitrile, we then must consider the effective change. Looking at Table 1, we can see that the correction factor will be about 2 pH units. This means that our effective unusable pH range is actually 1 to 3 instead. Provided that we prepare the mobile phase with a pH greater than 5, we can expect stable retention times and reasonable peak shapes.

Particularly interesting is the mixture of an acid and a base, wherein we must then explicitly model the change in pK_a of the base and mobile phase. Initially, we consider the "aqueous" systems that are shown in Fig. 4a and b. These are the pH/retention factors for an acid and a base with no acetonitrile. Based on this, the pH choices could be either less than 2.5, or more than 7.5. Most chromatographers would elect to choose a pH of 2.5 or so. However, if we are going to use an appreciable concentration of an organic modifier, the picture changes. If we assume that we are going to prepare an acid-based buffer, the elution profile for the acid remains the same; however, for the base, the pH shift will be in opposite directions for buffer and analyte. The elution profile will shift.

Based on this result, a pH of more than 6.5 is acceptable.

To summarize: The presence of organic modifiers affects the ionization of both analytes and buffers. The effects are documented and well-behaved for acetonitrile and methanol. Mobile phase pH correction is particularly important when analyte and buffer are not of the same type; i.e., when bases are studied with an acidic buffered mobile phase. In these cases, the effective change is twofold, with the pK_a of the base shifting down on the pH scale, and the pK_a of the acid shifting up.

1.4.6
Optimization of Mobile Phase pH Without Chemical Structures

Chromatographers will likely be familiar with standard systematic approaches to optimization of well-behaved variables such as concentration of organic modifier in the mobile phase and column temperature in chromatographic method development. This concept is covered in Chapter 1.1. It is possible to approach the problem of pH optimization in a similar manner, provided that it is done with caution. Figure 2 shows the response of a series of ionizable compounds to changes in mobile phase pH. It is clear that the response of these compounds across the entire pH range cannot be modeled based on a few simple experiments. However, due to the tremendous power of pH in changing the retention times of ionizable compounds, optimization of pH can still be helpful in designing a robust separation. Where methods will be applied repeatedly, such as the quality control laboratory, it is wise for the method development chromatographer to anticipate the needs of validation, and to simply perform an optimization of pH over a narrow range (say, three or four experiments over a single pH unit) to examine the effect on the suitability of the separation.

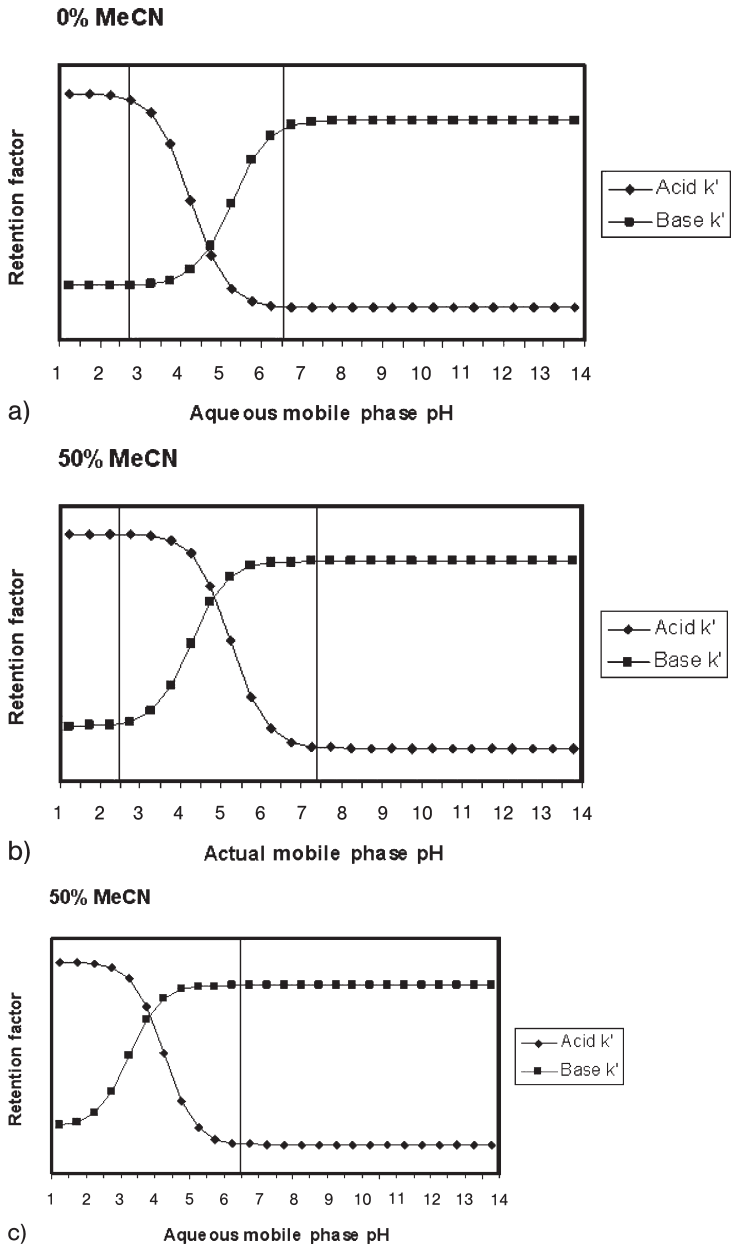

Fig. 4. Retention of acid and base as a function of pH.
(a) pH ranges for robustness and peak shape.
(b) With the addition of acetonitrile as a modifier, the inflection points of the acid and the base are moved in opposite directions. This assumes that the user has prepared a buffer that corrects for organic modifier content.
(c) Assuming that buffers are made up in aqueous solution, this is the profile that would result. The acceptable aqueous range is above pH 6.5.

This non-structural approach provides an effective complement to experimental design based on pK_a values. Solvation spheres and effects of pH on columns will inherently be taken into account, as is the ionization of unknown species. The key to this step is the small pH range; extrapolation over a large range of pH values has little hope of being successful.

1.4.7 A Systematic Approach to pH Selection

It is a relatively simple process to select the pH for analysis of a given sample, even in cases when one or more compounds are not known. In general, it can be expected that compounds for which no functionality is known will be at trace levels, and thus peak shape will be less critical. In addition, unknown compounds will often tend to have similar functionality to other compounds in the sample. For this reason, it is reasonable to use the pK_a values of known compounds as a starting point wherever possible.

An effective approach is as follows:

1. Collect all structural information on the sample in question.
2. Obtain or predict pK_a values for all species. In cases where only partial structures are known, consider ionizable functional groups.
3. Divide the pH scale into areas of "use/don't use" based on keeping each compound in the unionized state. This implies 2 pH units from the pK_a. Where conflicts arise, choose the major components first, and move to areas that push the equilibria for other compounds to be fully ionized where possible. Estimate the solvent strength required and correct pH as detailed in Section 1.4.4. Note that slight adjustments may still need to be performed.
4. Perform a slow gradient experiment at the indicated pH. Estimate the solvent strength at which compounds elute and correct the pH accordingly, if desired.
5. Prepare the new buffer, and re-inject.
6. Where necessary, optimize pH across a small range, and/or perform optimization of other parameters. Note any broad peaks in the separation. If they are part of the critical pair (two closest eluting significant peaks), then this may be a concern. Remember that more than just pH will vary on subsequent separations, and look for a separation that is very stable. If the suitability is sensitive to the pH, consider a slight modification to a more reasonable pH area. If this is not possible, then it may be necessary to control other factors very carefully, including column parameters and temperature.
7. When validating the final method, prepare buffers based on gravimetric preparation rather than adjusting pH using a pH meter and "titration" with acid or base. pH meter maintenance is often neglected, resulting in inconsistencies of measurement.

1.4.8 An Example – Separation of 1,4-Bis[(2-pyridin-2-ylethyl)thio]butane-2,3-diol from its Impurities

An example of this approach to pH selection is shown for an example compound, 1,4-bis[(2-pyridin-2-ylethyl)thio]butane-2,3-diol (see Fig. 5) and three unknown impurities.

The predicted most acidic pK_a of this species is 5.1 (ACD/LC Simulator v8.04, Advanced Chemistry Development, Inc.). It can be concluded that pH ≤ 3.1 should be reasonable for examination of this species. If we knew the structures of impurities we could also predict for these, of course. Our next step is to design a gradient experiment around this pH and inject.

Figure 6 shows the resulting chromatogram. Based on the elution time of the known compound, we could apply pK_a correction factors based on solvent content. For the sake of simplicity, we will not do this here. Note that we have reasonable resolution of the four components, but it is not yet clear if this will be a robust separation or if we can achieve better resolution. Prior to optimization of other parameters, we will study the pH further; we inject at pH 2.5 and 3.8 in order to generate an optimization system. At this stage, we can determine an optimal pH by using experimental optimization software.

Figure 8 shows the resolution map describing the response of the system versus pH. Indeed, despite the fact that we had reasonable resolution of all components in our first experiment (pH 2.9), it is clear that a small change in pH (Fig. 8) would result in co-elution of components 3 and 4. pH 2.6 appears to offer a good solution; we can proceed to further optimization, examining for an isocratic and/or faster separation from here.

This approach has enabled us to target a pH range that is relatively stable to small changes, but it has also speeded the process of optimization while decreasing the likelihood of validation difficulties. Further optimization of other variables should lead to a high quality method.

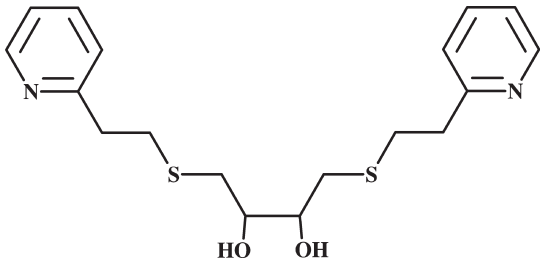

Fig. 5. Chemical structure of 1,4-bis[(2-pyridin-2-ylethyl)thio]butane-2,3-diol.

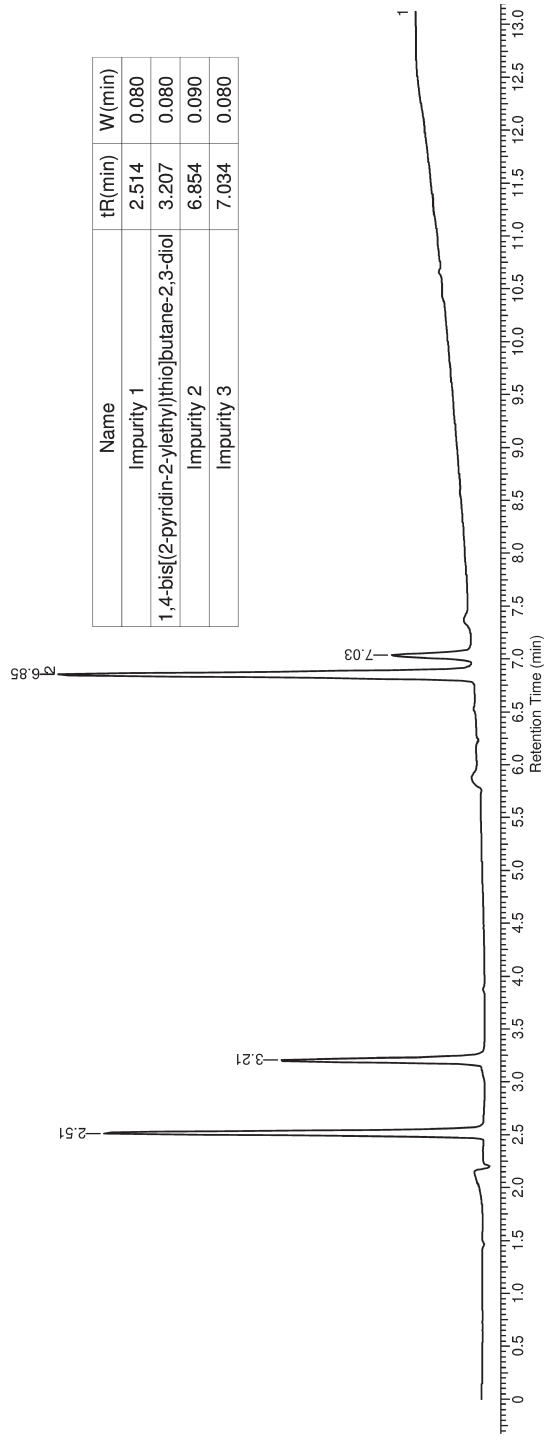

Fig. 6. Initial "scouting gradient" for 1,4-bis[(2-pyridin-2-ylethyl)thio]butane-2,3-diol and three unknown impurities. Conditions: 11 min gradient from 10 to 80% acetonitrile; buffer was 50 mM $HCOOH/HCOONH_4$.

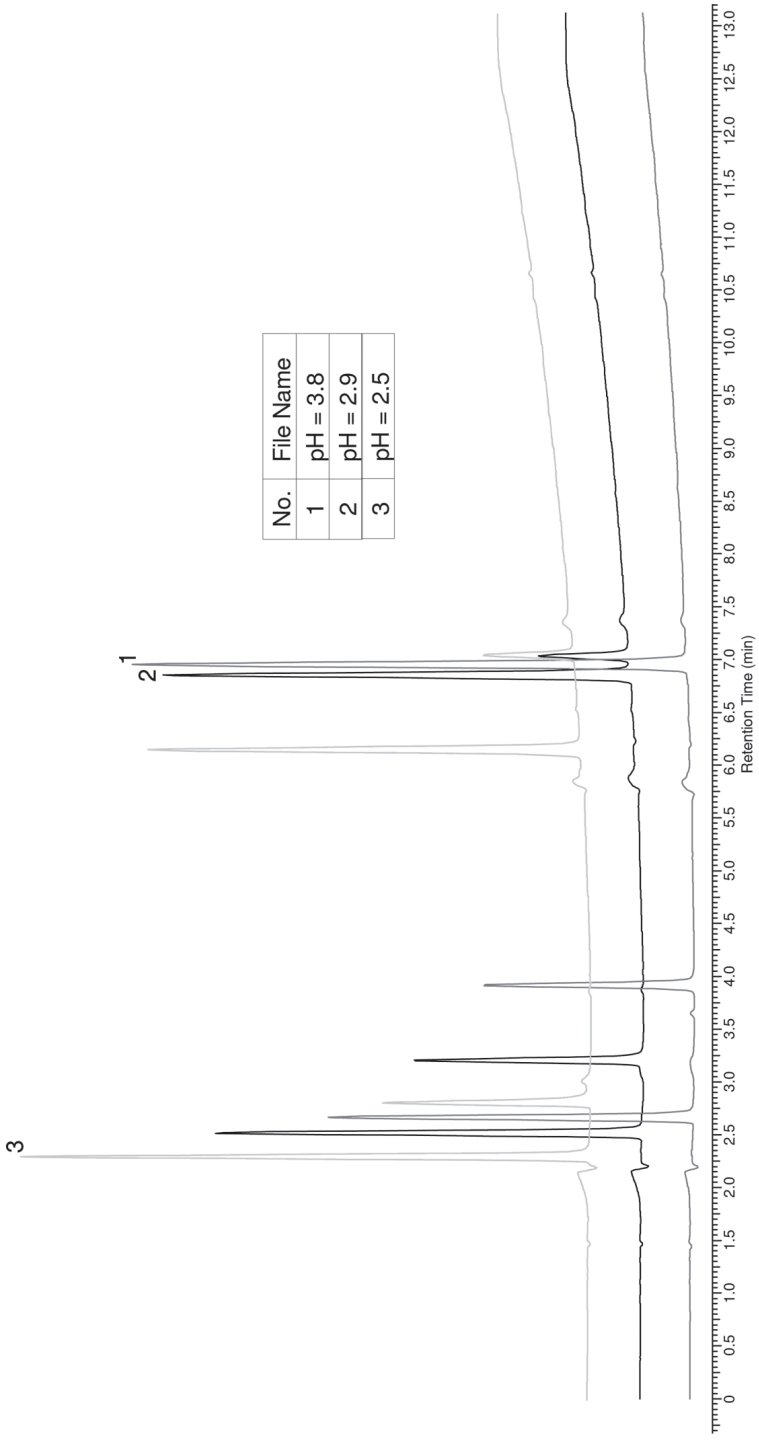

Fig. 7. Comparison of chromatograms with varied pH; sample and other HPLC conditions as noted in Fig. 6.

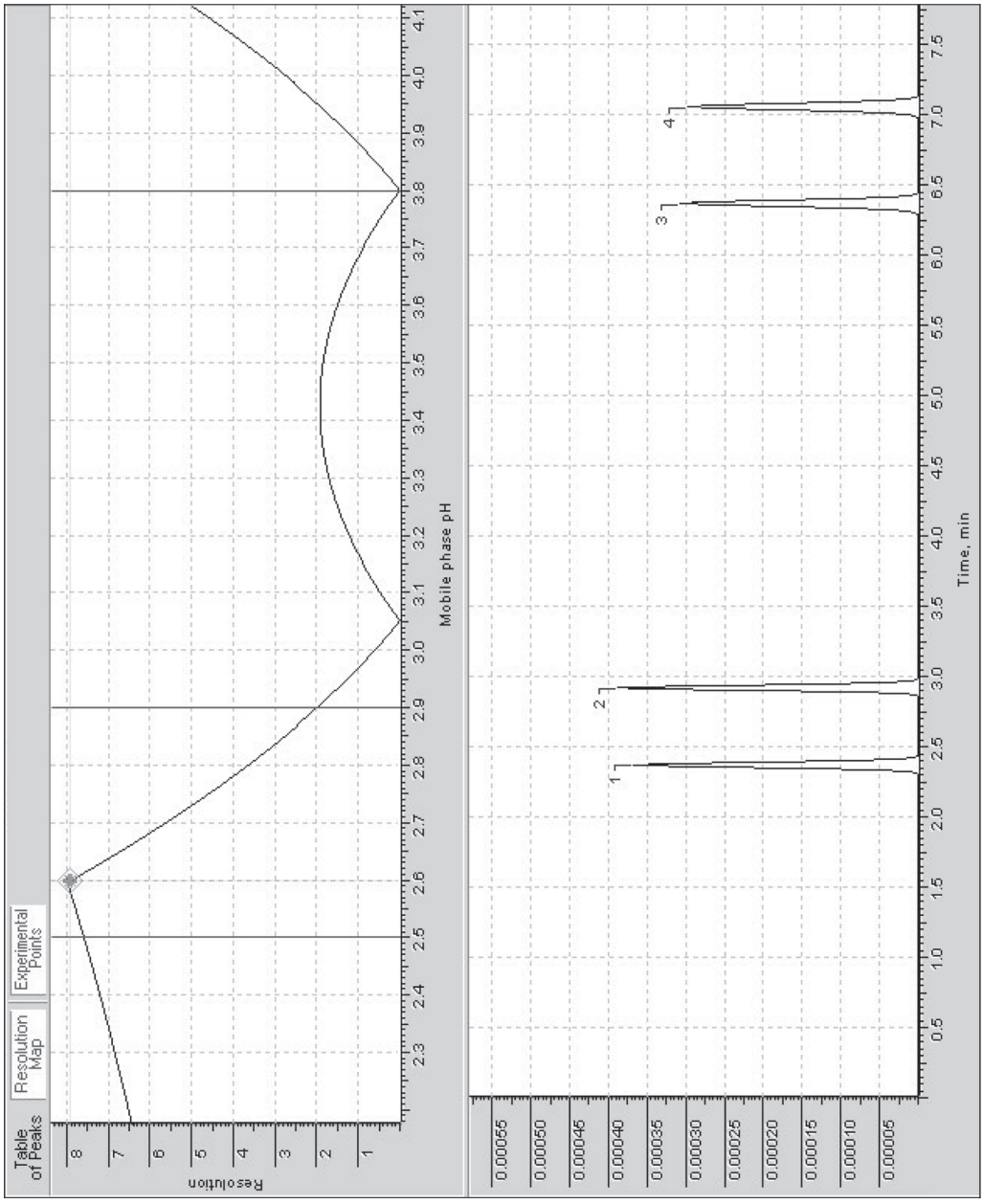

Fig. 8. Optimization of pH for 1,4-bis[(2-pyridin-2-ylethyl)thio]butane-2,3-diol and three unknown impurities; ACD/LC Simulator v8.04, Advanced Chemistry Development, Inc.

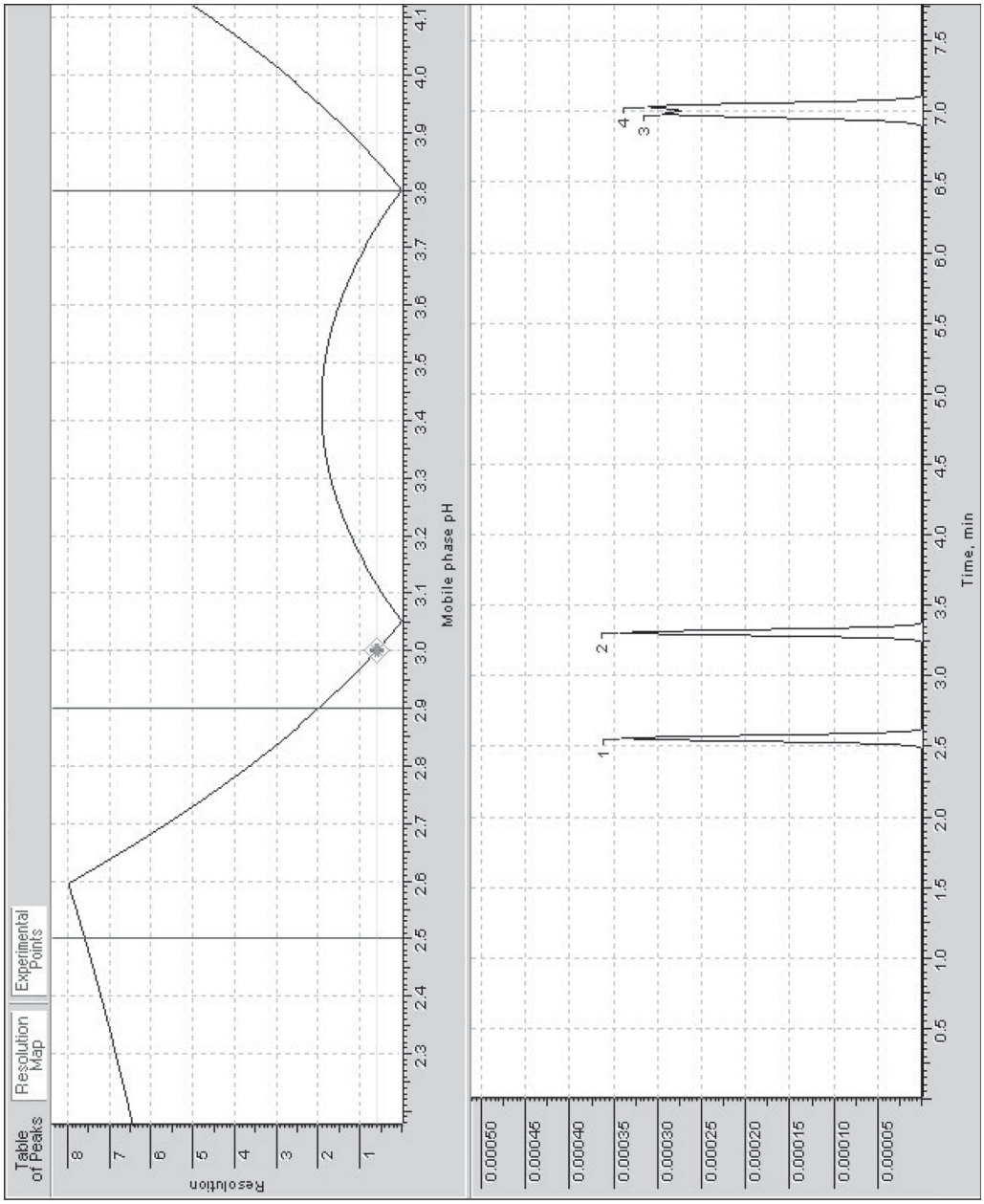

Fig. 9. The predicted response at pH 3.0.
Note that components 3 and 4 can be expected to co-elute.

1.4.9
Troubleshooting Mobile Phase pH

Due to the tremendous sensitivity of selectivity to changes in mobile phase pH, problems with separations are often a result of relatively small changes in effective pH. This may be due to the usual causes, including temperature changes and incorrect buffer preparation, or more subtle effects. The effective design of robust methods should help to alleviate these problems. When the methods break down, it can often be useful to revisit the original development work.

It can be helpful for the troubleshooter to revisit optimizations of pH that were performed during method development. For example, an experimental chromatogram from a failed suitability test can be reconciled with the predicted chromatogram based on, for example, a 0.3 unit difference in pH from the original optimization, leading to the conclusion that mobile phase pH may have been the culprit. Where these optimizations are not available (and where chemical structures *are* available), structure-based systems can be generated quickly, combining the pH/hydrophobicity curves with experimental retention times. The familiar resolution maps contain extrapolated chromatograms that can be quickly examined and reconciled to observed chromatograms. The reader is reminded that small pH changes may alleviate the problem at hand, but this sensitivity should be noted. Again, the addition of column temperature control or gravimetric mobile phase preparation to the method may help to reduce future failures in the method.

1.4.10
The Future

In order to estimate biological activity of given compounds, it is important to consider the ionization state in the medium of interest. Solubility, in particular, is of great interest, and highly dependent on pK_a. For this reason, interest in effective pK_a prediction is increasing in many areas of chemistry. Initiatives are being put in place to measure pK_a values for drug candidates, for example. The pK_a prediction algorithm described in Section 1.4.4 depends in part on the experimental data that forms its basis. The more comprehensive and chemically relevant the measurements are, the better the predictions can be expected to be. It is not unreasonable to expect that these pK_a measurement initiatives will continue. It is possible to make these measurements available to the entire organization, reducing pK_a prediction to "look-ups" in some cases, or in the worst-case scenario, giving prediction algorithms access to experimental values for more relevant compounds, thus increasing accuracy. This accumulation of chemical knowledge both within and outside organizations should be an objective for chemists in the future. In addition, the simple rule-based correction of pH for organic solvent content described in Section 1.4.5 is a problem that should be solvable within pK_a prediction software, particularly software designed for support of chromatographic method development.

1.4.11 Conclusion

Mobile phase pH is one of the most powerful tools for modifying selectivity of compounds in HPLC. A systematic selection process for pH can make the difference between a fast development of a robust method and a long development process that may result in a separation that cannot be reproduced. While it is usually impractical to measure pK_a values of species prior to method development, the combination of literature searches, investigative experiments, and software tools can result in an effective pH choice in a very short time frame. A primary goal in buffer selection should be a successful separation that is stable with respect to small changes in pH. Where this is not possible, developers can explicitly note the sensitivity of the method and create stringent experimental specifications.

References

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1.5
Optimization of the Evaluation in Chromatography

Hans-Joachim Kuss

1.5.1
Evaluation of Chromatographic Data – An Introduction

Chromatography is a very important part of instrumental analytics and has some unique features. Optimization means improvement or best utilization of the given conditions. Thus, one of the aims of an analytical chemist is the successful optimization of a chromatographic technique. For the optimal use of chromatography, it is also necessary to optimize the evaluation so as not to lose any information. Integration software provides a quick and flexible means of evaluating data. However, decisions can only be made by the user; only he or she can assess the method.

The most important information is contained in the chromatogram. It would seem obvious that the person responsible for the analysis of results should inspect each chromatogram. This has to be done by a chromatography specialist, not by a computer specialist or statistician.

Clearly, the potential offered by computer techniques should be exploited. The individual values of the internal standard in a serial analysis should always be visualized graphically, so as to recognize any possible trend. An automated calculation should be used to determine the area/height ratio for each peak and to reassess the (graphically) established extreme values in the chromatogram. If possible, two detection signals should be monitored simultaneously, for example, two UV wavelengths or signals from two independent detectors such as UV and fluorimeter, FID and NFID, two mass traces, etc. The two signals should be estimated independently and it should be assessed whether the results are in a predefined window. This twofold analysis can lead to a marked improvement in the quality of results.

1.5.2
Working Range

The measured signals can only be calculated and assessed after prior calibration. First, the working range *WRx* has to be defined, which is limited by the lower concentration x_L and the upper concentration x_U.

$$WRx = \frac{x_U}{x_L}$$

On the one hand, one wishes to take advantage of the sensitivity of the detector by measuring low concentrations. On the other hand, in a few cases it may be possible that high concentrations are present. A value can then only be quantified

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with much additional and troublesome work because it is above the upper concentration x_U of the working range. If the value for very few samples exceeds x_U, one should consider the possibility of dilution with fresh matrix [1].

Quantification is limited on the lower side by the limit of quantification (LOQ). In the case of very sensitive measurements, one may encounter problems with the LOQ. Therefore, it would seem advantageous to estimate the LOQ early in the development of a method.

$$x_L > LOQ$$

In chromatography, the LOQ can be simply estimated by calculation of the signal-to-noise ratio of a peak with known concentration. The height of the peak H_x corresponds to the signal intensity and will be given directly in µV. One then looks for a region of, for example, one minute in the chromatogram that corresponds to baseline only and measures the noise N in µV as lower apex to upper apex. The LOQ is the concentration with a 10-fold signal-to-noise ratio – in practice, one considers a 20-fold signal-to-noise ratio, in order to compensate for any slight instrumental deteriorations.

$$LOQ = \frac{20 \cdot x \cdot N}{H_X}$$

In principle, any peak with known (preferably low) concentration can be used to estimate the LOQ by calculating the height in relation to the noise. This method should always be used in chromatography, provided that it conforms to existing guidelines.

1.5.3 Internal Standard

In the next step in the preparation of a validation, three (or preferably more) samples with the envisaged lowest concentration x_L after sample preparation considering the estimated recovery rate should be analyzed. If it is at all possible, an internal standard should be added to the samples. A final decision as to whether to use a final method with or without internal standardization can then be taken later. An internal standard is generally the best quality control possibility. Each analysis sample will then be individually controlled with virtually no additional effort.

With three measurements, it is possible to calculate the mean My, the standard deviation SDy, and the coefficient of variation cv(%) using the method of external and internal standards. If linear regression leads to a straight line with an intercept not significantly different from zero – which is normal in chromatography – then the cv(%) also holds for the relative deviation in the concentration axes.

$$\frac{SDy_L}{My_L} = \text{cv}(\%) = \frac{SDx_L}{Mx_L} \approx \frac{MU_L}{1.5 \cdot t}$$

Here, MU_L is the measurement uncertainty at x_L and t is Student's t-factor, which depends on the number of calibration points.

Measurement uncertainty MU_L can be roughly estimated from the product of $1.5 \times t \times$ cv(%). If the governing rules allow a maximal MU_L of 25%, 33% or 50%, the highest tolerated cv(%) can be readily obtained through division by $1.5 \times t$.

For the internal standard method, the areas (heights) of the compound peaks divided by the areas (heights) of the related internal standard peaks in the same chromatogram should be used, instead of using the areas (heights) directly. It can immediately be seen if the internal standard decreases the cv(%), which is the purpose of the exercise.

1.5.4 Calibration

After tentative predefinition, the working range must be verified with several calibration concentrations in order to establish a linear relationship between measurand (y) and concentration (x).

$$y = b \cdot x + a$$

The calculation of the intercept a and slope b for the straight line and the back-calculation of the concentration for the analysis samples according to

$$x = \frac{y - a}{b}$$

is an essential feature of each integration program. It may be the case that some chromatographers use weighted regression giving scant attention to its origin. In the usual integration programs it is possible to weight with $1/y$ or $1/y^2$. This is confirmed by a mouse click; thereafter, subsequent workings are hidden and so this can be forgotten. If the values are carried forward into a validation program – which often does not allow for weighting – anomalies will inevitably be programmed. Using high working ranges, weighting often generates an advantage, but this needs to be demonstrated.

For validation purposes, some further parameters have to be calculated, including measurement uncertainty MU from the calibration line. To date, this has usually been included in validation programs, but not in integration programs. Unfortunately, without a basic understanding, it can be hard to see what the meaning of some calculated values really is. The principle train of thought is simple, but often unnecessarily complicated.

1.5.5 Linear Regression

Linear regression is also called the method of least squares. This refers to the squared residues R, which are the perpendicular (y)-distance of the calibration

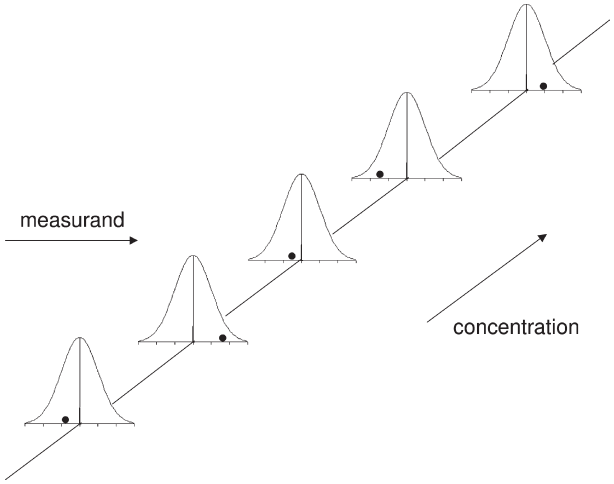

Fig. 1. Linear Regression is based on the assumption of the same standard deviation at all calibration points. Then all deviations are considered to be of the same importance – they have the same weight. The variation in the residues is thought to be random.

points y from the calculated points yx on the straight line. It is assumed that the imprecision in the concentration is sufficiently low that it need not be taken into account when calculating the inaccuracy of the measurand.

$$R = y_k - yx_k \qquad R^2 = \min$$

y_k = measurand at the calibration point k
yx_k = value calculated from the straight line for calibration point k

This calculation relies on the equality of the residues R and assumption of the same standard deviation at different concentrations (Fig. 1).

If this assumption is not fulfilled, the residues with higher standard deviations, due to their higher averaged amount, exert a greater influence on the location of the straight line than do residues at calibration points with lower standard deviations (Fig. 2). In these cases, it is necessary to minimize:

$$\sum \frac{(y_k - yx_k)^2}{SD_k^2} = \sum w(y_k - yx_k)^2$$

This may re-establish the equality of the residues. In the standard form, the residues are individually multiplied by a weighting factor w, which is normally adjusted for $\Sigma w = n$. This can only be done in two steps. First, a temporary weighting g is calculated, which is adjusted using the previously unknown Σg.

$$g = \frac{1}{y^{WE}} \qquad w = \frac{g \cdot n}{\sum g}$$

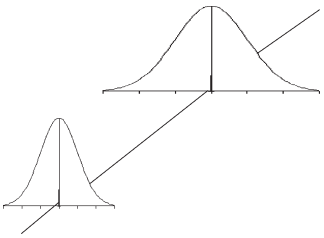

Fig. 2. Knowing from the *f*-test (variance quotient) that the assumption of the same standard deviation does not apply, we have to balance the disequilibrium by a factor – the weighting factor. Otherwise the calculation will be wrong. The 2-fold standard deviation of the upper point averaged induced 2-fold residues. Thus, the straight line will be (2^2) 4-fold more influenced by the location of the upper than by the lower calibration point. Therefore the weighting factor has to weight the variances at the lower point 4-fold.

The calculation method known as linear regression is based on the equality of the standard deviations in the whole working range. In chromatography, we often have the same percentage deviations. In a defined concentration region a method has a precision of a few percent. At lower concentrations, the imprecision will increase to a limit that cannot be tolerated – for instance 33% MU – and the LOQ is undercut.

Let us consider a working range of 100, and assume that the cv in the middle of the working range is 1%. According to the (unweighted) linear regression, the residual standard deviation appears as a nearly constant band spanning both sides of the straight regression line. The cv(%) at x_L must be 10% and cv(%) at x_U must be 0.1%. The cv values should be multiplied by 1.5 times Student's *t*-value to give a rough estimate of the confidence interval. Therefore, 10% is higher than allowed for the LOQ. To reach a cv of 0.1% in chromatography is unrealistic. Often it is hard to reach even 1%. The conclusion that can be drawn is that using a working range higher than 10 in chromatography, linear regression without weighting is a model that is most probably inadequate.

Let us assume that in a chromatographic study with multiple measurements of the same sample the cv(%) is constant in the working range from x_L to x_U. Then:

$$\frac{SDx_k}{My_k} = \text{cv}(\%) = \text{const.}$$

This means that over the whole calibration line there is a constant ratio between the measurand y_K and the related standard deviation SDy_K. It is then possible to replace $w = 1/SD^2$ with $w = 1/y^2$. The standardization on n compensates for differences in the values of y. The unknown standard deviation is estimated from the known measured values.

A weighting with $1/y^2$ is exactly equivalent to the case when the cv is constant in the working range. Experimentally, this may be found only in approximation. Therefore, it is a model assumption. However, a model is necessary to calculate a weight for the measured values of unknown analysis samples. Measuring the

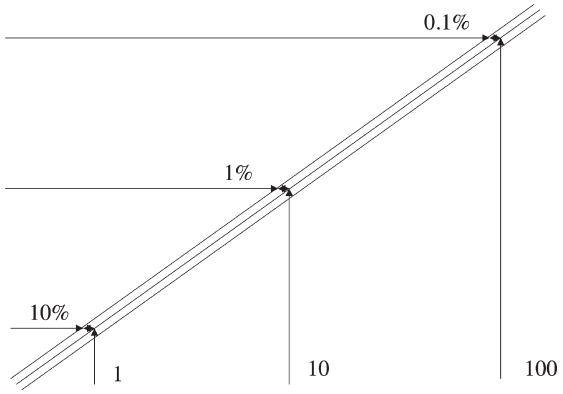

Fig. 3. The same standard deviation at all concentrations (1, 10, 100) leads to great differences in the coefficients of variation (10%, 1%, 0.1%).

standard deviations with great effort at each calibration point only allows interpolation at the measured analysis values with unavoidable inconsistencies.

1.5.6 Weighting Exponent

In $1/y^2$, the weighting exponent WE is 2. With WE = 0, we have the ordinary linear regression because all weighting factors are set to 1 ($y^0 = 1$).

Let us first look at these two extreme cases.

In chromatography, we have on the one hand the "instrumental variance", which is not dependent on the concentration, and which is related only to the noise of the detector or the oscillations of the pump. This variance is the same at high and at low concentrations and is described by WE = 0 ($w = 1$ at all concentrations). On the other hand, we can postulate a "sample preparation variance" with the same percentage deviations at all concentrations (characterized by WE = 2).

The dominating volume effects (the error in volume does not "know" the concentration) of the injection volume variability or of the volume transferring steps in sample preparation have the effect that the standard deviation increases linearly with the concentrations and the measured values (SDy/y = const.). The chromatographic reality can be between the two extremes, depending on the dominant effects. This means that all values between 0 and 2 are possible. Using a very simple sample preparation a WE near 0 can be expected; using multiple volume transfer steps a WE near 2 can be expected.

$$w = \frac{1}{y^{\mathrm{WE}}} \qquad \mathrm{WE} = 0 \text{ to } \mathrm{WE} = 2$$

The broader the working range *ABx*, the earlier the deviation from ordinary linear regression can be detected. The F-test is used to discriminate between homogeneous and heterogeneous variances.

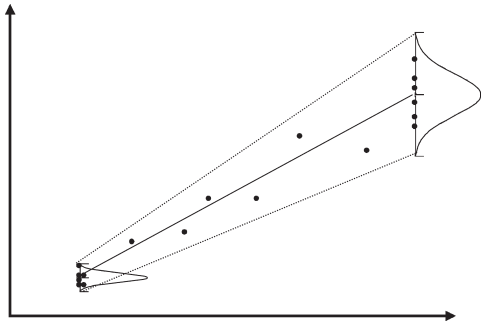

Fig. 4. The weighting factor interpolates the standard deviations between the measured values at the lower and the upper ends of the working range.

$$F = \frac{SD_U^2}{SD_L^2}$$

It is possible to weight variances in such a way that F will be 1. Therefore, WE must be calculated from:

$$WE = \frac{\log F}{\log AB\gamma} = \frac{\log SD_U^2 - \log SD_L^2}{\log \gamma_U - \log \gamma_L}$$

The quotient of the variances is used for this calculation. Therefore, the method might be called weighting by variance ratio.

1.5.7
In Real Practise

In quality control, it is usual to use a working range of 2. Assuming the same cv(%) over this range, the variance at the upper calibration point is $2^2 = 4$ times the variance at the lower calibration point. Measuring these two concentrations six times, no significance is reached at 95% confidence. This means that the differences in such a small working range are too low to be of practical importance.

In a working range of 10 and with the assumption of the same cv(%), F will be calculated as 100, which means heterogeneity of variances. In DIN 32645 [4] it is pointed out that in a working range of 10 homogeneity of variances can be expected. In chromatography, this is not normally the case. As ever, theory must bow to reality. If heterogeneous variances are identified, it is necessary to calculate them by weighting variances or to decrease the working range.

1.5.8
Drug Analysis

When measuring drug concentrations, a working range of 10 is normally too small. Often, a factor of 100 is necessary. Scattering of the calibration points at equal

distances is then not possible. It is unclear as to whether in some governing rules a logarithmic arrangement of concentration steps is forbidden, even if this makes common chemical sense. This does not influence the calculated reference values, with the exception of the process coefficient of variation (*Mx* is lowered; see below).

In reality, the very powerful chromatographic methods can reach a working range of 100 or 1000. The equations of LOQ, *PI*, and *MU* include the RSD and, without weighting, give far too high values in the lower concentration range. These complicated equations give the impression of high precision, but they are only valid under defined preconditions.

The FDA guidelines for bioanalytical method validation [5] not only point out a minimal precision of 15%, but also a sensible choice for maximal deviation RE(%) of the back-calculated calibration concentrations of 15%. This level of accuracy is not normally obtainable in chromatography with a working range above 10 without weighting, because the lower calibration points, due to their low weight, often show systematic deviations. The FDA guideline accepts the use of a weighting, assuming that an explanation is given.

$$\mathrm{RE}(\%) = \frac{x_{\mathrm{calc}} - x_{\mathrm{k}}}{x_{\mathrm{k}}}$$

It has been suggested [6] that one should create a diagram showing the relative percentage deviation RE(%) plotted against the logarithm of the concentration. This prevents the low concentrations from collapsing graphically into one point by taking into account the constancy of the relative and not the absolute deviations typically found in chromatography. In this way, the possible advantage of weighted regression can be illustrated.

1.5.9 Measurement Uncertainty

By measuring usually six equal samples at x_{U} and x_{L}, the measurement uncertainty at these concentrations will be known. By interpolation between these two concentrations, a reasonable estimate of the measurement uncertainty is possible. This estimate is certainly better than sticking with a constant RSD and *MU* over the whole working range, as in the case of ordinary linear regression, when the readings (significant F-test) show otherwise.

MU at x_{L} can be used to estimate LOQ: For *MU* below 33%, x_{L} is above the LOQ. Essentially no more information is necessary. The statistical considerations for LOD, identification limit, and LOQ are partly inconsistent and may create irritation and confusion.

It seems doubtful whether neologisms such as "uncertainty manager" and "budget of measurement uncertainty" will be able to increase quality in daily practice. Analytical jargon contains too much theory and formalism [7].

Using the linear regression equation, it is simple to calculate *yx*-values at the calibration concentrations. The residues *R* are the differences of measurand *y* and calculated *yx*.

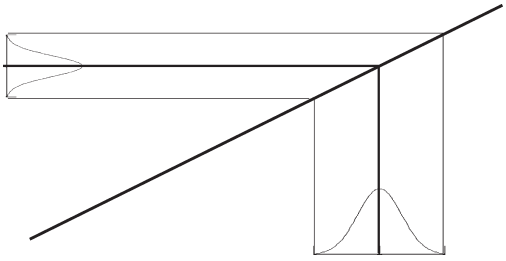

Fig. 5. The measurand of an analysis sample has a 99% prediction interval, which is reflected through the straight line in a 99% measurement uncertainty at the concentration axis.

$$\mathrm{RSD} = \sqrt{\frac{\sum R^2}{n-2}}$$

The sum of the squared residues increases with the number n of calibration points. The square root of the sum of the squared residues divided by $n-2$ is called the residual standard deviation RSD, which represents a mean value for the residuals. Using ordinary linear regression, the RSD nestles at both sides against the straight line as a constant band, according to a random scatter of the residues.

More useful than the deviation in measurand direction is the directly resulting deviation of the concentrations. Graphically, this is a view on the same RSD band vertically or horizontally. The standard deviation on the concentration axis is the process standard deviation VSD, which is calculated by division of RSD by the slope b. VSD is the central goodness criterion [3], which can be used to calculate the process coefficient of variation pcv(%).

$$\mathrm{VSD} = \frac{\mathrm{RSD}}{b} \qquad \mathrm{pcv}(\%) = \frac{\mathrm{VSD}}{Mx}$$

So far, we have only considered standard deviations, which means mean deviations containing in a normal distribution 66% of the individual values. To get the 95% confidence interval of the y-values (PI) or the x-values (MU), it must be multiplied by Student's t-factor.

Prediction interval: $\pm\mathrm{PI}(95\%) = t(95\%) \cdot \mathrm{RSD} \cdot \sqrt{1}$

Measurement uncertainty: $\pm\mathrm{MU}(95\%) = t(95\%) \cdot \mathrm{VSD} \cdot \sqrt{2}$

The complete (shown in the following inclusive of weighting) equations can be found in Ref. [8] or papers cited therein. Here, the two square roots should be replaced by a factor of 1.2, which leads (without weighting) to the approximation of two constant confidence lines parallel to the straight line. Visualized, these confidence lines would be elastically fixed at the ends and slightly pressed in the middle. This is exactly the effect of the real square-root term, for which using ordinary linear regression w_A (w_K) must be set to 1 and $\Sigma w = n$.

$$\sqrt{}_1 = \sqrt{\frac{1}{w_K} + \frac{1}{\sum w} + \frac{(x_K - Mxw)^2}{\sum w \cdot (x - Mxw)^2}}$$

$$\sqrt{}_2 = \sqrt{\frac{1}{w_A} + \frac{1}{\sum w} + \frac{(y_A - Myw)^2}{b_w^2 \sum (x - Mxw)^2}}$$

Limit of Quantification (LOQ). The LOD according to DIN 32645 can be estimated as 4 × VSD, because Student's *t*-value for eight degrees of freedom and 1% error probability is 3.35 and the square root can be approximated by 1.2. The LOQ is approximately three times the LOD.

$$\text{LOD} = t \cdot \text{VSD} \cdot \sqrt{1 + \frac{1}{n} + \frac{Mx^2}{\sum (x - Mx)^2}}$$

$$\text{LOQ} \approx 3 \cdot \text{LOD} \approx 12 \cdot \text{VSD} \qquad \frac{\text{VSD}}{\text{LOQ}} \approx 8\%$$

Therefore, cv(%) must be below 8%. Let us assume a constant VSD in the working range (homogeneous variances). Then, VSD > SDx_L, because the location of Mx_L is not exactly on the straight line and there is an additional scatter about the straight line. Let us additionally assume that VSD decreases from x_U to x_L (variance heterogeneity). Then VSD must be much greater than SDx_L. Because VSD > SDx_L, it has realistically to be expected that cv(%) values above 5% are due to the LOQ being exceeded. In the case of variance heterogeneity this can occur much earlier (perhaps 1%). This can lead to serious problems in trace analysis.

The obvious solution would be to use SDx_L directly. The best estimate of the standard deviation at x_L is a direct measurement, rather than a complicated calculation. Dividing the maximum tolerated deviation of 33% by Student's *t*-value of 3.3 gives a maximum tolerated cv(%) of 10%. A method can break down as a result of the difficulty of obtaining a cv(%) of 5% instead of 10%.

To estimate the LOQ according to DIN 32645, a linear regression with ten concentrations in the region of the LOQ needs to be performed; the price is thus a very considerable working effort. In chromatography, the integration systems are unfortunately unable to give area values for peaks at concentrations near the LOQ. Therefore, this method must be skipped. The calibration method should only be used if neither the signal noise nor the blank sample method are possible.

Great calculation expenditure gives an impression of increased security. The problem is not the increased (computer) effort, but the unnecessary intransparency in using complicated equations. Each quality demand is useless when it is suffocated in formalism. It is carried out because rules have to be fulfilled. It is well meant, but intransparent perfectionism can be demoralizing.

1.5.10
Calibration Line Through the Origin

What advantage can be gained in chromatography by using a calibration line with an intercept? In a blank sample, no peak should appear in the chromatogram at the retention time of the substance to be analyzed. If at this stage no baseline is found, the method would seem to be inadequate. In each chromatographic analysis series, some blank samples should be included to establish this. In this way, it is possible to recognize memory effects, but the possibility of interfering underlying peaks at the same retention time in a chromatogram cannot be excluded. Fitting a straight line with an intercept does not prevent this. Only measurements with two different detectors can largely exclude the occurrence of interferences.

For non-chromatographic analyses with a measurable blank value, a straight line with an intercept is unavoidable. In cases where the intercept has a physical importance, results are invalidated by neglecting the intercept.

Conversely, results are also invalidated by considering an (insignificant) intercept in chromatography, even though it has no importance either physically or statistically. The intercept corresponds to a systematic deviation that must be considered (especially important at low concentrations) in the calculation, although by chance it can take positive or negative values. The back calculation of the signal values for the calibration concentrations clearly shows this.

An analytical chemist who is able to optimize his or her sample preparation and HPLC analysis can very well decide whether to use an internal standard, whether to calculate the straight line with or without weighting, and whether or not to consider an intercept. Obviously, it is mandatory that one can give reasons for these decisions.

The rapid advent of LCMS(MS) has changed liquid chromatography. It is regrettable that changes in the governing rules have lagged behind for a decade. Let us hope that future guidelines will take into account the idea of giving the practitioner real help and will allow the necessary flexibility in the different analytical applications.

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1.6
Calibration Characteristics and Uncertainty – Indicating Starting Points to Optimize Methods

Stefan Schömer

1.6.1
Optimizing Calibration – What is the Objective?

The goal of any optimization is the most efficient use of the available resources (time, devices, staff). For calibration, this may mean:

- minimizing efforts in measuring, without compromising in terms of precision or accuracy, or
- improving precision and/or accuracy, without increasing measurement efforts.

In this chapter, six issues are addressed with a view to providing guidelines for the optimization of calibration procedures, as well as of routine analytical methods in general:

1. Does enhanced sensitivity mean improved methods?
2. A constant variation coefficient – is it good, poor or just an inevitable characteristic of method performance?
3. How to prove effects due to matrices – may the recovery function be replaced?
4. Having established matrix effects – does spiking prove necessary in every case?
5. Testing linearity – does a calibration really need to fit a straight line?
6. Enhancing accuracy – obtaining 'robust' calibration functions with weighting.

Considerable potential for optimization will arise just by analyzing routine procedures. The essential objective of any calibration, which is clearly ensuring the accuracy of results, should always be kept in mind. Hence, the expenditure of some effort in calibrating a method is undoubtedly unavoidable.

Appraising trueness and accuracy has to proceed with an evaluation of precision in order to finally ensure and prove the traceability of analytical results to known standards. Thus, in the examples given herein, the focus is on calibration data and characteristics in order to derive an accurate estimate of the precision and trueness of the method.

Why should we optimize the calibration of a method?
What benefit should we expect from optimizing calibration, in view of the relatively small error ranges due to calibration that arise in routine analytical methods? To answer simply, we look for improvements in precision and accuracy to enable efficient use of resources; this holds true not only for calibrating, but also more generally with regard to efficiency for daily routine analysis. Clearly, we have to consider different starting points for optimization and to look for the optimal balance between individual, possibly conflicting, objectives.

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1.6.2 The Essential Performance Characteristic of Calibration

The method's standard deviation (DIN 32645, DIN 38402 T51) represents the key parameter of performance. It is calculated by dividing the residual standard deviation by the sensitivity of the method and it essentially determines the maximum precision for evaluation of analytical results. The standard deviation of a method provides the scale to estimate the accuracy of a method early on the basis of its calibration characteristics.

Due to the software that is widely available for evaluating calibration characteristics, calibration formulas are not explicitly written out here. Instead, we refer to plots of the calibration function and especially of the residuals, which provide a suitable basis for discussion by transparently showing the expected range of possible but still reliable results. More so than the confidence range of the calibration function, the prediction interval enables an evaluation of the precision and trueness. For this reason, we included the prediction limits in the residuals plot in a manner first published in Ref. [1].

1.6.3 Examples

1.6.3.1 Does Enhanced Sensitivity Improve Methods?

At first sight, striving for enhanced sensitivity seems to be worthwhile in any case to achieve optimization. A better response, or more exactly an increased change in signals as a result of changes in concentrations, should enable more precise measurements. For example, changing reagents to obtain a more intense color, exchanging detector systems or choosing an alternative detection wavelength are possible courses of action.

We wish to optimize the detecting components of an established HPLC method. Which parameter will provide a reliable characterization of the improvements achieved?

The example will give us the opportunity to introduce reliable characteristic data as well as some criteria for visually evaluating the calibration function and the respective, specially designed residuals plot [1, 2]. We will be able to recognize an efficient improvement at one glimpse.

The measurements of the calibration series to be considered are given in Table 1. The response of method 2 clearly seems to be the better one. The detector used was based on additional electronic amplification of input signals.

Sensitivity is given by the slope of the calibration function applied for the desired operating point. Because we are also going to consider nonlinear calibrations, with their sensitivity being dependent on the operating point, we focus on the middle of the working range. This will enable sensitivity data to be compared numerically and visually for any calibration function that may apply.

The characteristics and especially the ranges of uncertainty that prove approximately equal for the concentration evaluated, are given numerically as well as

Table 1. Measurements provided in two calibration series by methods of different sensitivity.

No.	*Values 'x'* concentration [mmol]	*Method #1 values 'y'* signal [arb.]	*Method #2 values 'y'* signal [arb.]
1	0,15	9,17	22,32
2	0,30	20,75	44,21
3	0,45	31,58	61,43
4	0,60	39,09	85,19
5	0,75	52,50	101,64
6	0,90	59,54	124,98
7	1,05	69,82	142,47
8	1,20	82,11	163,00
9	1,35	88,66	175,61
10	1,50	100,21	198,70

Table 2. Some essential method characteristics of the two calibration series.

	Method 1	*Method 2*
Sensitivity [arb./mmol]	66.53	129.71
Method standard deviation [mmol]	0.0214	0.01980
Uncertainty at the operating point [mmol]	0.825 ± 0.0753	0.825 ± 0.0700

graphically. Hence, efficiently increased sensitivity does not automatically ensure better analytical results. The residuals plot shows the sensitivity enhanced by a factor of about two. However, this is in fact compensated by an equally increased sensitivity for interference effects. The range of random errors increases by a factor of two on the signal scale as well. The two effects cancel each other out almost completely.

Thus, we should avoid interference effects and any errors additionally arising in the optimization of detector systems. Our example proves amplifying as a stand-out measure to fail the desired improvement of methods.

Conclusion

Increasing response does not automatically lead to better analytical methods. This fact is surely well-known in practice. Nevertheless, most laboratories cannot help but take immense efforts in measuring series of results for repeatability and reproducibility in the belief of verifying actual improvements. Such effort may be confidently left out.

Calibration, which is anyway available and required for both methods, provides objective evidence through the standard deviation of methods, which will indicate the method of significantly lower standard deviation to be the better one. Also, the coefficient of variation of the method (method 1 at 2.59%, method 2 at 2.41%) may help to verify that an improvement has actually been achieved.

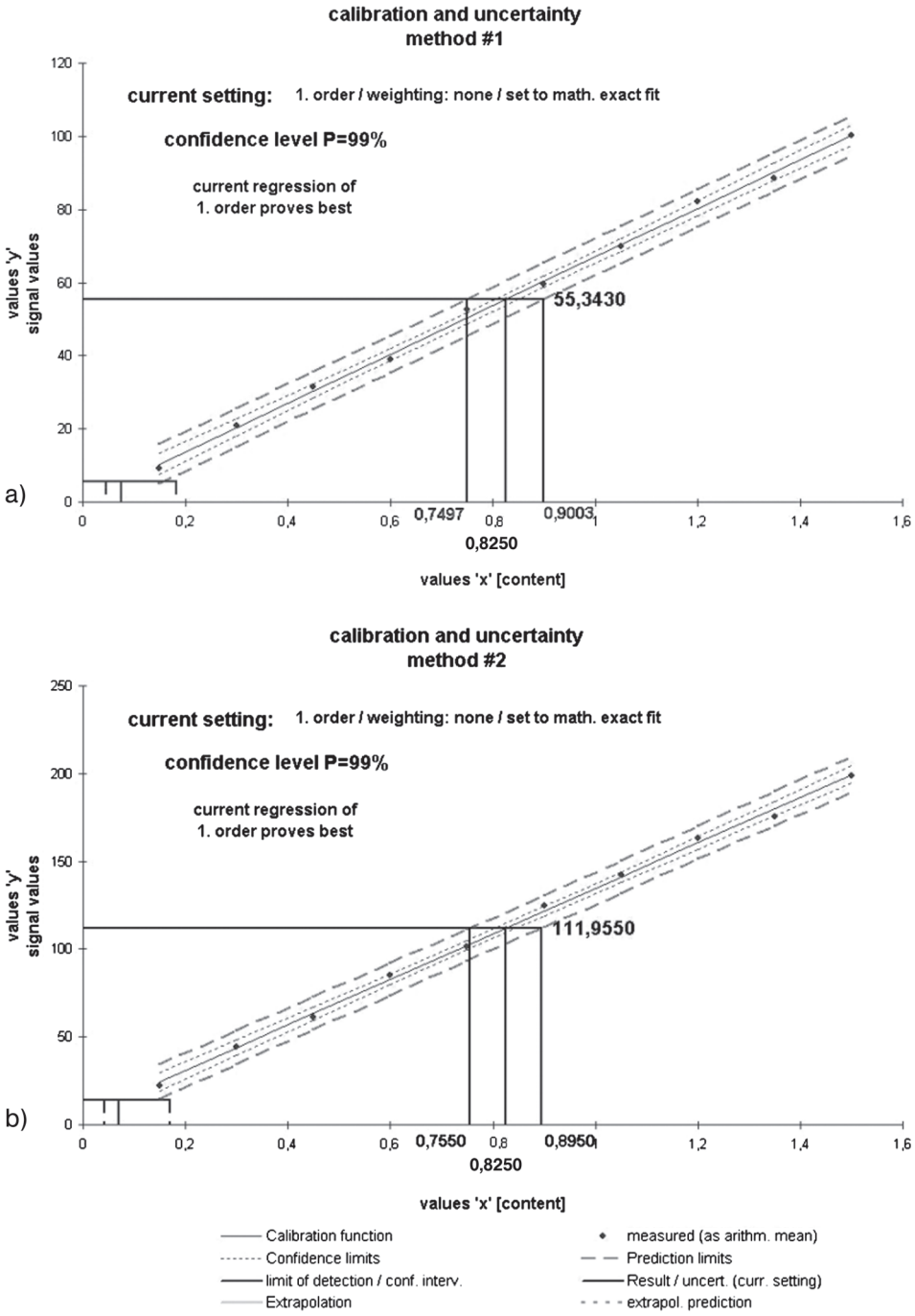


Fig. 1. Comparison of calibration functions and their residuals;
uncertainty of measurements as a criterion for success in optimizations.

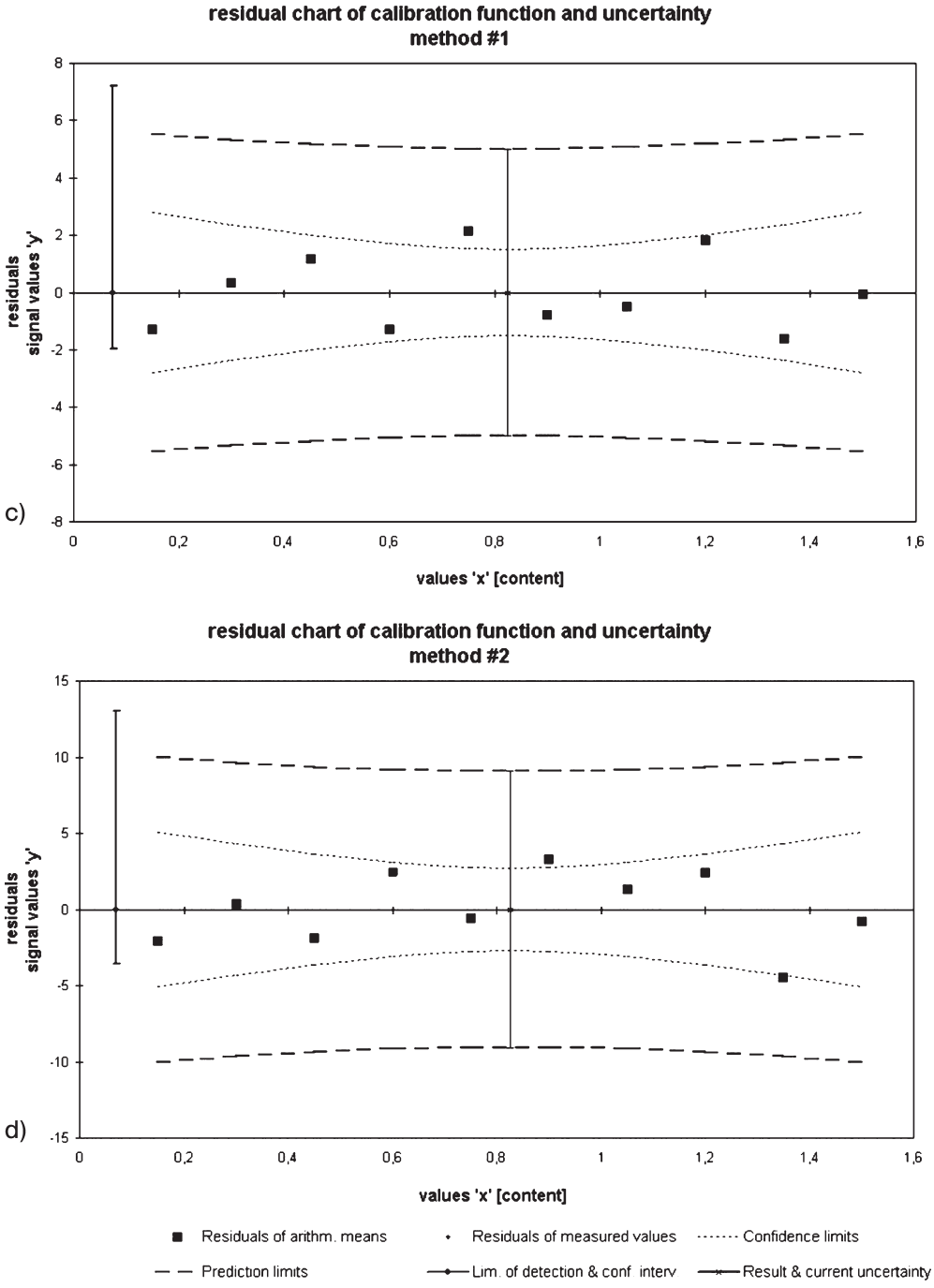


Fig. 1. (continued)

However, variation coefficients only provide valid results for comparisons of methods that both refer to the same concentration range, thus applying the same middle of the operating range as reference to calculate this parameter.

Alternatively, simply by asking one's supplier, one should obtain sufficient information about sensitivity and signal precision of devices to be applied for the purpose of comparison. Even if the manufacturer does not apply the same analytical method for qualification purposes of devices, the performance characteristic will nevertheless provide criteria suitable for your decision and will help to avoid calibration efforts of your own. Again, one should be careful when relying on information about variation coefficients only, since any reported percentage portion is easily changed to the desired value just by referring to a higher concentration.

Additional extensive test series to complete one's own calibration characteristics will provide no further essential information to assist in correctly choosing the "better" method. This effort is only suitable for validation purposes, in order to provide the most realistic information about the performance of the method of one's choice to be established in a routine application.

1.6.3.2 A Constant Variation Coefficient – Is it Good, Poor or Just an Inevitable Characteristic of Method Performance?

As introduced in the previous example, the standard deviation of the method evaluated on the basis of calibration data already provides sufficient information to describe the expected range of random error. It should apply to the whole operating range considered.

Recording additional measurements of repeatability is absolutely no longer required for this purpose.

In chromatographic practice, we expect variation coefficients (relative standard deviations) to be constant in the operating range of concentration. Thus, the uncertainty of measurements, scaled in absolute concentration units, will increase directly proportionally with increasing concentration of samples. What are the consequences of the variation coefficients being constant, if we want to apply calibration characteristics in order to recognize some starting point for optimization as early as possible? In the following example, the aim of optimization is an expansion of the intended operating range by about one order of magnitude of concentrations.

At first sight, the calibrating function appears suitable for the purpose of quantifying samples in the desired operating range. However, in particular those points labeled by their residual values in the residuals plot give an indication that the standard deviation of the method provided by the calibration data is no longer able to fit the uncertainty that is actually involved. The precision that actually applies proves to increase with concentration. Thus, we are faced with an increasing risk of measurements exceeding prediction limits provided by calibration characteristics. Referring to the so-called conventional true result, the method increasingly encounters systematic errors as we approach the upper operating range. Consequently, quantifying samples will sometimes result in too low or too high a concentration. The risk of encountering systematic errors that prove

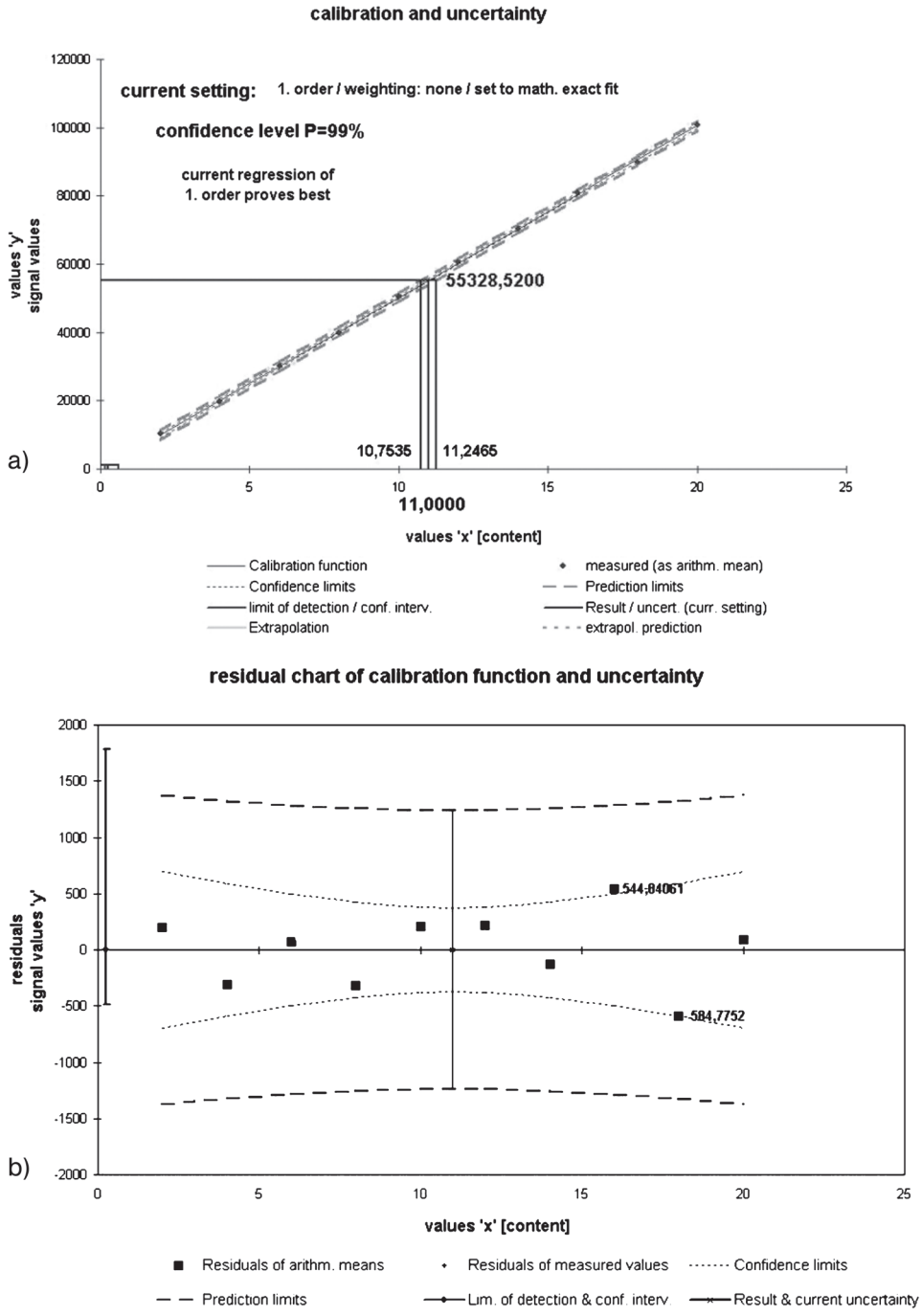


Fig. 2. Ten points of calibration at constant variation coefficient, single measurements at each concentration.

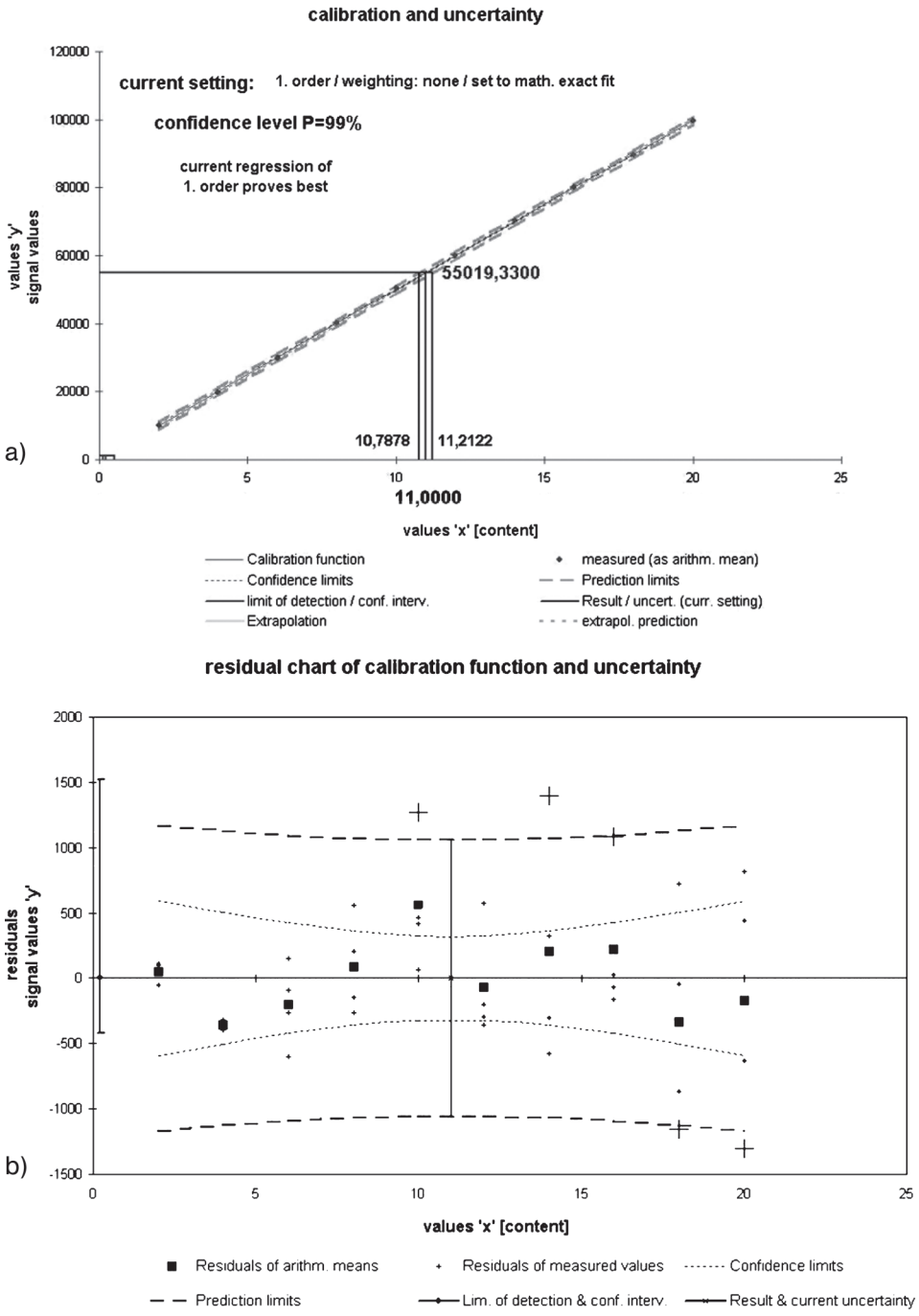


Fig. 3. Ten points of calibration, four repeated measurements at each standard, assuming a constant variation coefficient.

to be caused by random but underestimated effects may easily escape one's attention if only single measurements are applied [3], as is surely commonly the case.

This behavior of a method at constant variation coefficient becomes clearer if we recalibrate the method, this time applying fourfold repeated measurements at each standard concentration. The result is shown in Fig. 3.

The five values marked in the residuals plot evidently reveal the risk of wrong quantification, even for the same calibration standards treated as if they were unknown samples.

Keeping in mind our original purpose of minimizing measuring efforts, the following examples will go on expanding the operating range up to four orders of magnitude of concentrations. Choosing such a widely spanned operating range is quite common practice in testing the purity of products, applying one chromatographic run to simultaneously scan for the target substance (content set to 100%) as well as for impurities (down to 0.01%) [4].

Operating Range 0.01–100% with Homogeneous Variances

Even with the calibration spanning four orders of magnitude of concentration, Fig. 4 evidently shows the calibration data to provide valid performance characteristics. The actual behavior of measurements will be correctly described by the calibration. The obtained standard deviation of the method applies to the whole operating range, as long as we can ensure that the variances are homogeneous. The residuals reveal that just one single result (out of 60 measured) exceeds the estimated limits of prediction (i.e., results just meet the initially set error level and thus prove statistically acceptable).

As can be seen, it is simply impossible to introduce an optimization strategy that should be applicable for the entire desired operating range. We do not have to refer to statistics or calibration characteristics. Looking at Fig. 4, the question obviously arises as to why anyone should calibrate by further diluting standards to below 1% anyway.

One does not need to be an expert to recognize the obvious way of proceeding with the optimization, considering the multitude of highly condensed overlaying calibration points in the range below 1%.

Applying considerably fewer calibration measurements, i.e. one single concentration measured below 1%, we may instantly introduce a 50% reduction in measuring efforts without any loss of quality. With one single calibration standard remaining in this range – let us choose 0.5% for example – we will of course no longer be able to quantify samples at concentrations of less than 1% at the precision required. To reach this conclusion is just a matter of common sense and is not affected by any restricted boundary conditions given by guidelines or regression statistics, as some authors continue to claim. Sometimes even a semi-logarithmic scale to plot calibration data is recommended in order to conceal the facts that we have discussed. However, we feel responsible to serve our reader's interests by clearly rejecting to open some boxes of tricks or to introduce possible conversions. These are nothing more than fudge factors. In the final analysis, moreover, concentrations will not be reported on a logarithmic scale either.

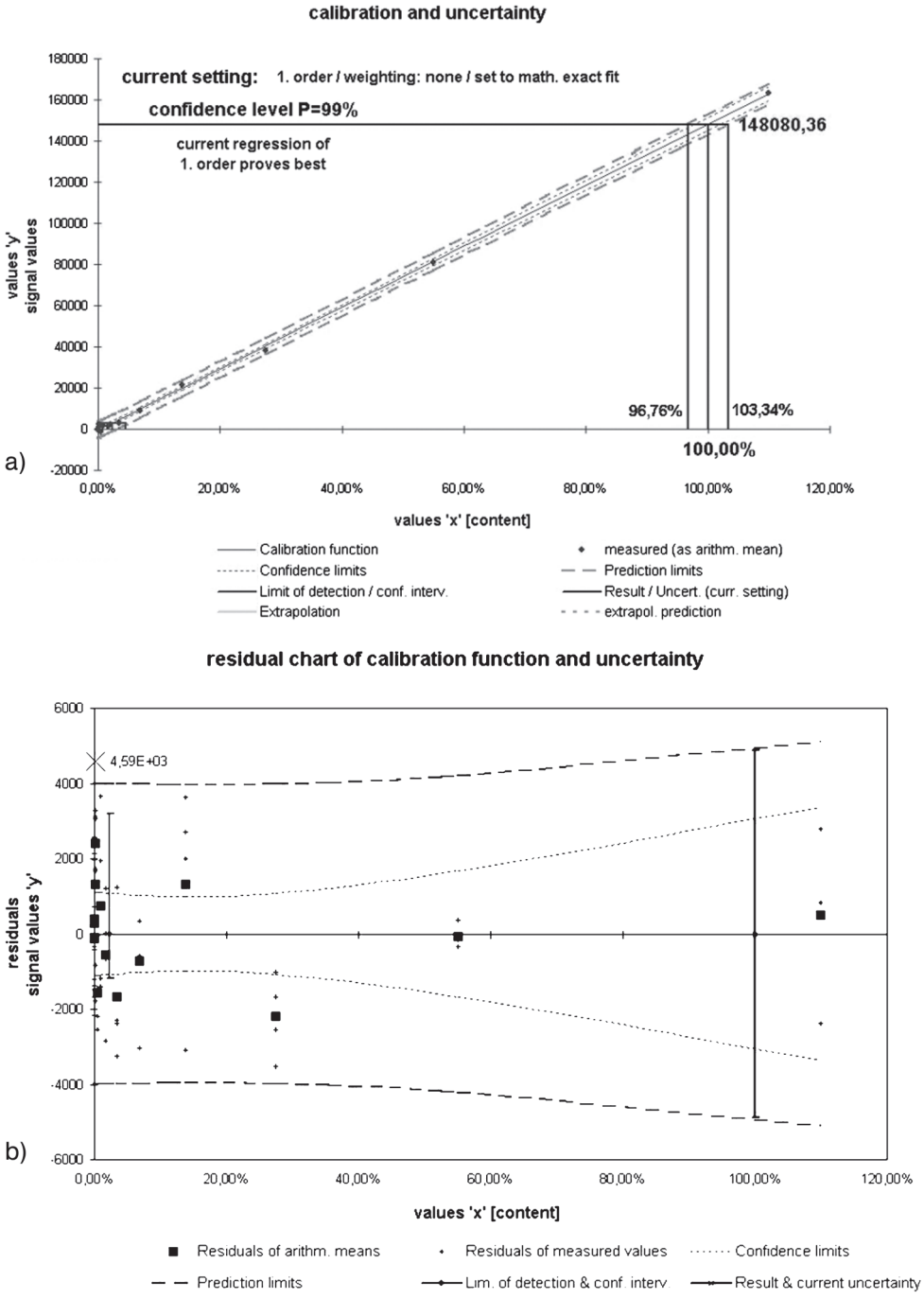


Fig. 4. Homogeneous variances in the operating range 0.01% to 100%.

The uncertainty in the operating point at 100%, as is clear from Fig. 4, is in fact unsymmetrical and correctly indicated [1, 5]. The formulas given in standards and guidelines (i.e., ISO 8466, DIN 38402, DIN 32654) to calculate uncertainties in the concentration scale show deviations hereafter and are no longer strictly valid.

Operating Range 0.01–100% with a Constant Variation Coefficient

The effects and risks involved as previously discussed increase dramatically if we take variation coefficient to be constant in the operating range. Obtaining a valid statement in quantifying samples on the basis of a calibration spanning four orders of magnitude of concentration is associated with a serious and great risk of encountering systematic errors. In principle, it is impossible that an analytical assay, whatever clever and optimized procedure is applied, will enable valid characterization of such wide spanning operating ranges by calibration, in spite of constant variation coefficients occurring at the same time. We also feel responsible to reveal and not to conceal limiting conditions for optimization strategies and requests that sometimes may exceed even the capabilities of modern powerful analytical assays. The result of a calibration applying these boundary conditions is illustrated in Fig. 5.

No regression model in the world will be able to accurately describe the behavior of an analytical assay spanning more than four orders of magnitude, nor will any calculation provide valid and realistic characteristics. The reason is simple and easy to understand. Any evaluation of calibration characteristics is designed to provide one single parameter for precision, i.e. one single residual standard deviation and hence one single standard deviation of the method. In mathematical terms, the specified purpose definitely requires the homogeneity of normal distribution applying to the desired operating range of the calibration as a whole, whereas taking a variation coefficient to be constant means just the opposite.

Trying to ignore these requirements will cause various problems in the practice of future routine analysis. Here, it should be mentioned that we are going to encounter "out of specification" (OOS) results again and again. These are only due to random effects, and only seldom are they ascribed to the real reason of precision being estimated too optimistically.

Fortunately, this fact is of little consequences in practice, because common operating ranges are limited to span only one order of magnitude of concentration. In this case, the precision will be estimated quite correctly, especially if we consider the middle of the operating range.

Evaluating the following example demonstrates that the weighting of the calibration function does not provide a valid solution, but increases the problem. Any basics required for calculating are stressed over their limits.

The correct estimation of performance characteristics, i.e. the standard deviation of the method, will be further distorted by any weighting applied. Consequently, false analytical results are unavoidable, occurring at higher frequency than valid results. More than three-quarters of the operating range show this effect. Focussing on concentrations between 0.01% and 1% in Fig. 6 d), one can see that one runs

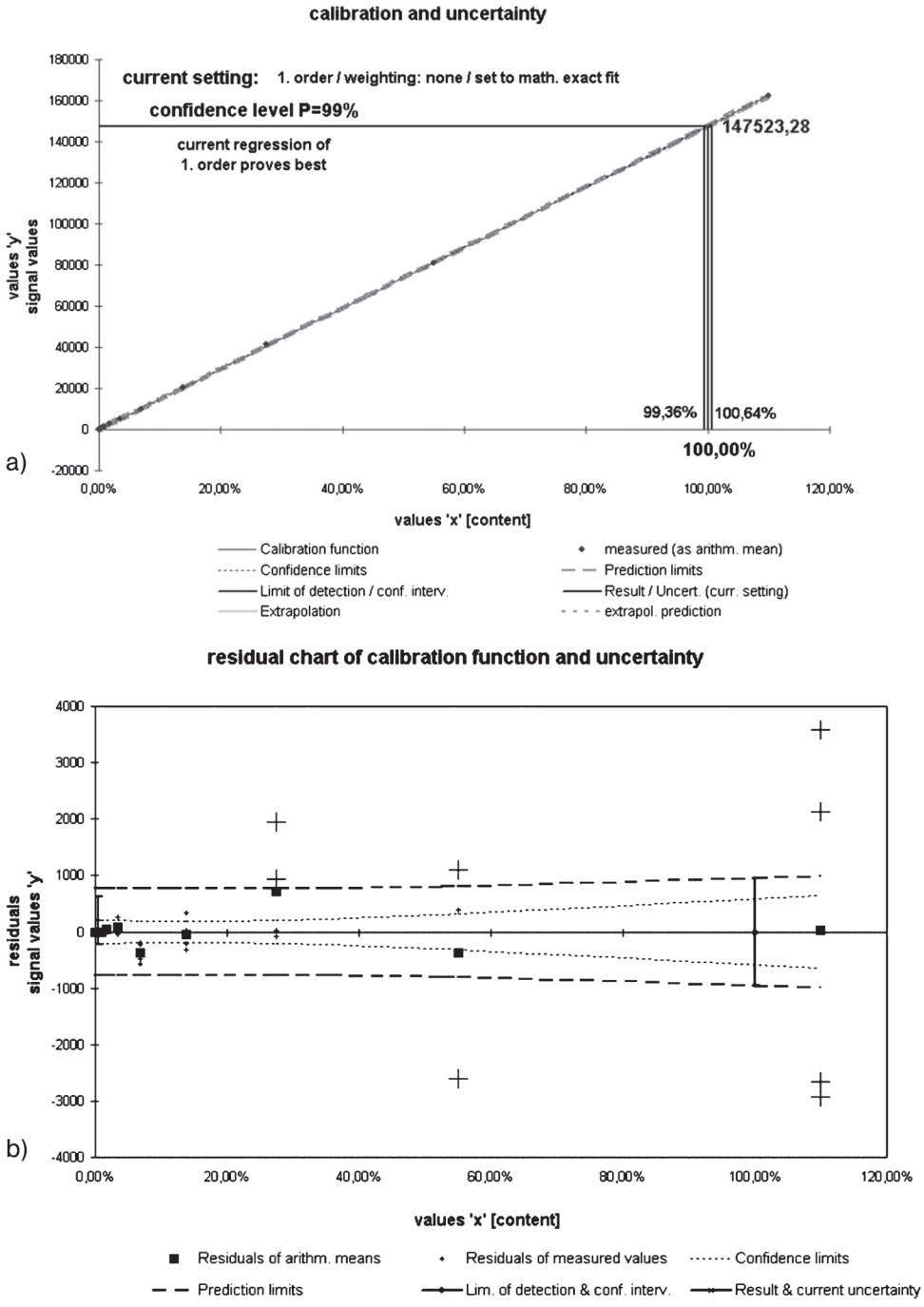


Fig. 5. Constant variation coefficient in the operation range 0.01% to 100%.

the risk of obtaining false analytical results. Even quantifying the calibration standard at concentration 0.27% fails in the case of three out of four measurements.

Solely a purely empirical model, introduced by Kuss [6], applying set points of precision at the boundaries of the operation range, enables adjustment of the calculation and evaluation to the set points of the precision specified but requires the precision at each of the relevant concentrations to be known already. Calibration, which we formerly expected to provide this information, in fact fails. Hence, additional repeated measurements are required, contrary to our original objective, which was to allow less effort in terms of measurements. Unfortunately, the calibration model provides no reliable precision characteristics, but in this case it is no longer required.

Conclusion

Constancy of the variation coefficient for chromatographic assays is equivalent to the standard deviation of the method, depending on the concentration. Consequently, the standard deviation of the method provided by the calibration model does not apply. By introducing this performance parameter as essential for our optimization strategies, we actually prove that optimizing an analytical assay, which appears with constant variation coefficients, will never apply to the operating range as a whole. By the way, this is state-of-the-art and has always been reflected in various guidelines and technical standards.

In any case, one should define exactly the section of one's operating range to be optimized in order to evaluate a standard deviation that will really apply for this section. Only repeated measurements will provide the precision data depending on concentration that will actually apply at each respective operating point. Any further calculation as well as weighting has to refer to these repeatability precision measurements and to take into account the measured precision data as boundary conditions in order to provide reliable and realistic confidence limits of analytical results as a function of concentration. Without any knowledge provided by repeated measurements, evaluation of pure calibration characteristics can easily yield arbitrary and even wrong results. The possibilities for optimizing such methods are considerably restricted, since calibration data alone are unable to provide valid precision characteristics.

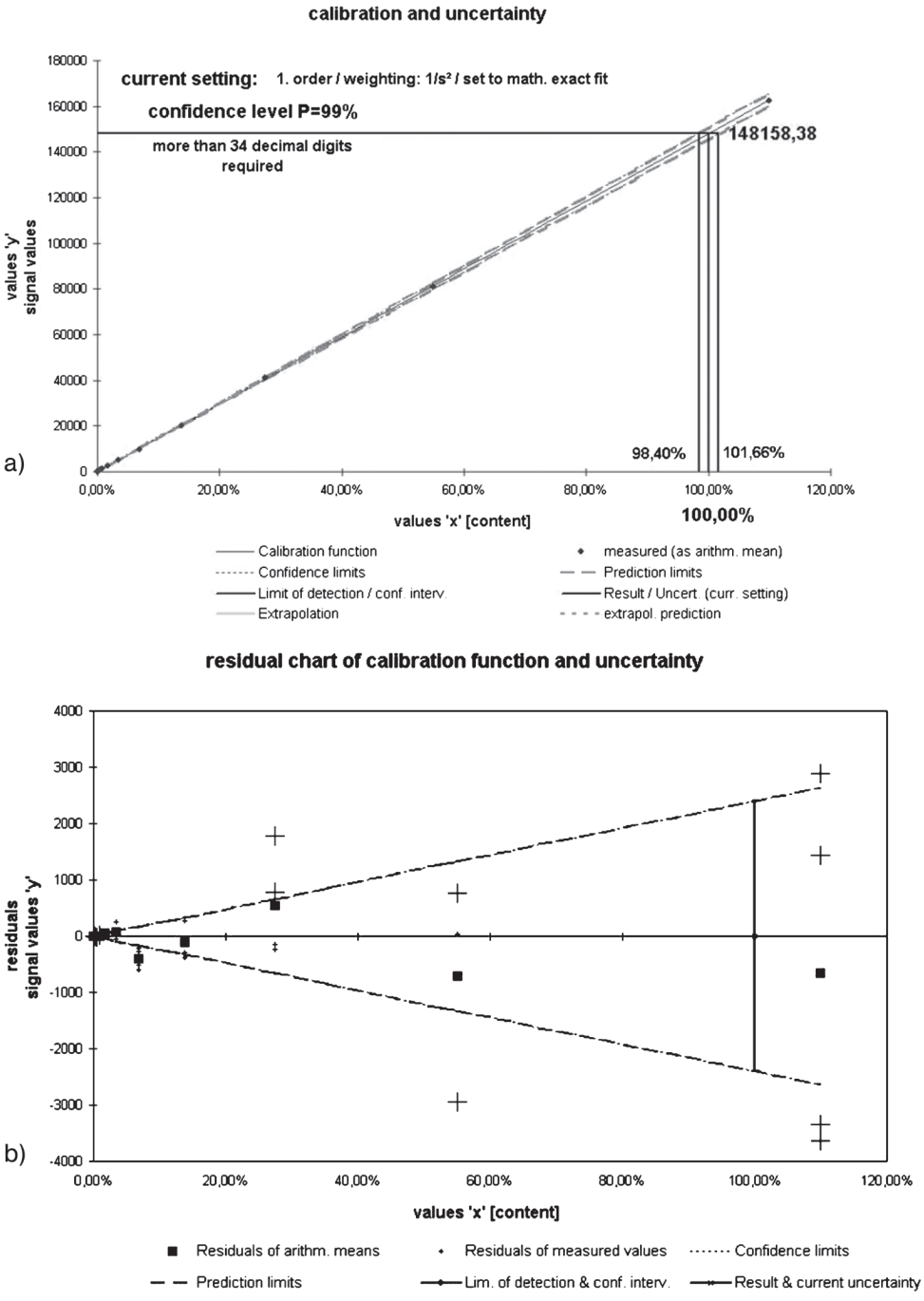
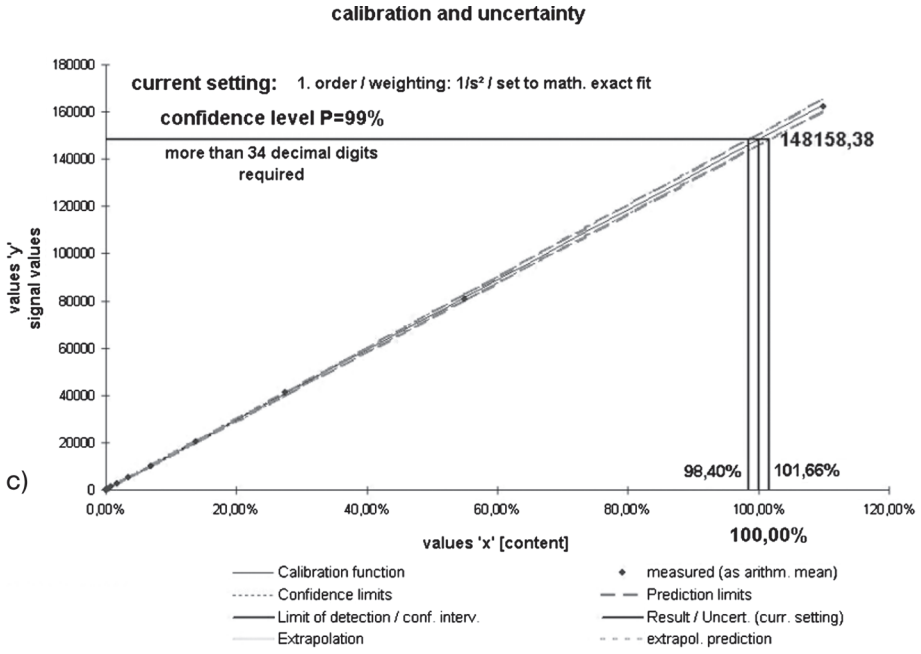
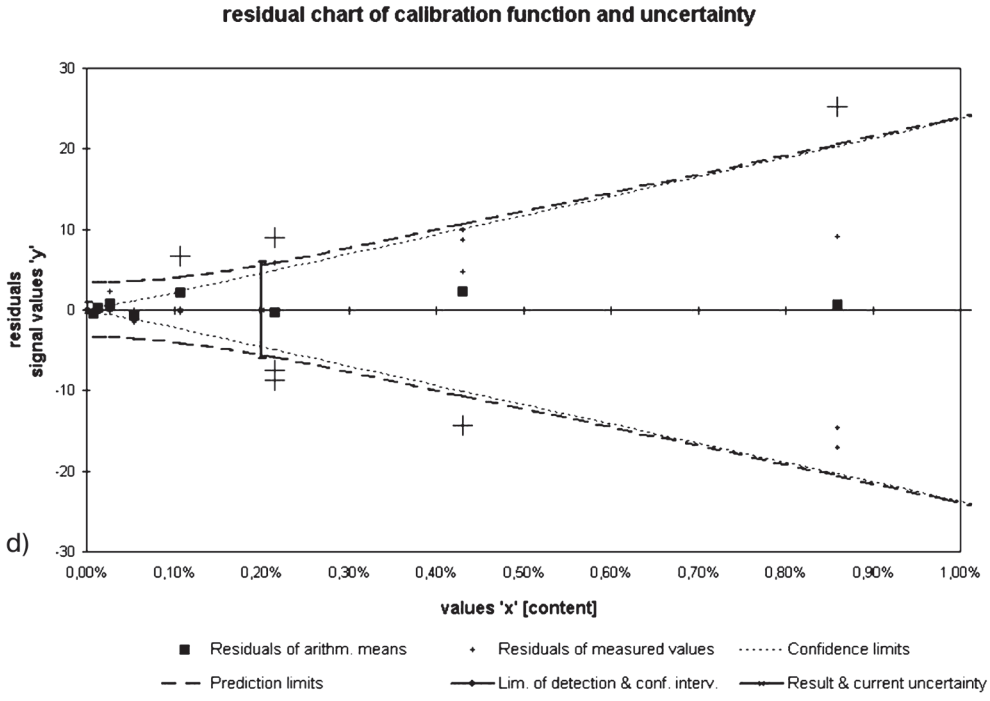


Fig. 6. Weighted calibration; weighting $1/s^2$ applies standard deviations s proportional to concentration; the average variation coefficient of the method is 1.88%.



c)



d)

Fig. 6. (continued)

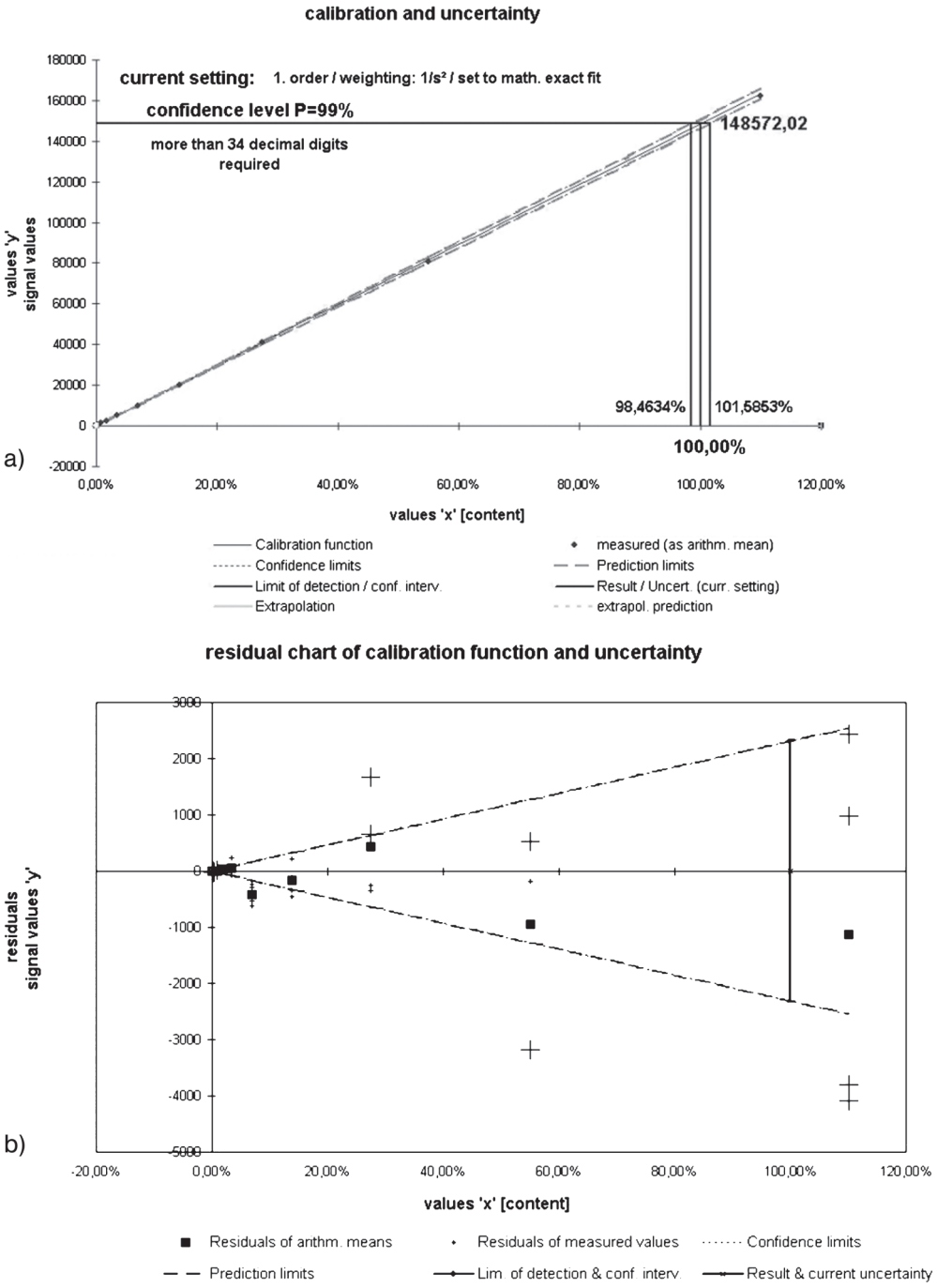


Fig. 7. Weighting with concentrations '*x*' applies $1/x^2$ with absolute units of concentration portion '*x*'. The result does not really differ from the one in Fig. 6.

1.6.3.3 How to Prove Effects Due to Matrices – May the Recovery Function be Replaced?

Determining the recovery function surely requires some additional measurement efforts. Table 3 sets out some possible ways of proceeding following procedure A or procedure B.

Procedure B already reduces measurement efforts down to two steps. To achieve this 33,3% reduction, the recovery function, i.e. plotting the concentration of individually prepared standards measured in the matrix against the known concentration, is required to reveal no significant deviations from expected slope '1' and intercept '0'. If significant differences are detected, even procedure B leads to a third step. It is then necessary to prepare recalibration standards in the matrix, since the standard sample applied for calibration should be prepared individually and separately. The existing control samples should not be used for this purpose.

Possibilities for Optimization with a Specified Degree of Reduction of Efforts

In our example, measurement efforts are minimized to a maximum of two calibration series. If the recovery function is not explicitly required by guidelines that apply to one's practice, one may skip the preparation and independent measurement of series of control samples. An instant comparison of calibrations in the matrix and in pure solvent will suffice. The objective evidence of this statistically sound comparison proves absolutely equivalent to evaluating the recovery function.

Result of the Graphical Comparison

If the calibration function of the pure solvent without matrix (lower residual standard deviation expected) does not intersect the confidence limits of the calibration function applied in blank matrix spiked with standards of known concentration (higher residual standard deviation expected), or completely exceeds

Table 3. Efforts to evaluate the recovery function applying procedure A or procedure B.

Procedure A (admittedly, a somewhat circumstantial way!)	***Procedure B*** (established, quick, and at first sight efficient)
1. Calibrate in pure solvent	1. Calibrate in pure solvent
2. Calibrate including matrix	2. Control samples, prepared independently from calibration samples, evaluated based on the current calibration function
3. Control samples: samples in matrix, prepared expressly independently from calibration standards, are evaluated applying the respective calibration function in each case	3. Calibrate including matrix (only required if the recovery does not meet the slope '1' and intercept '0')

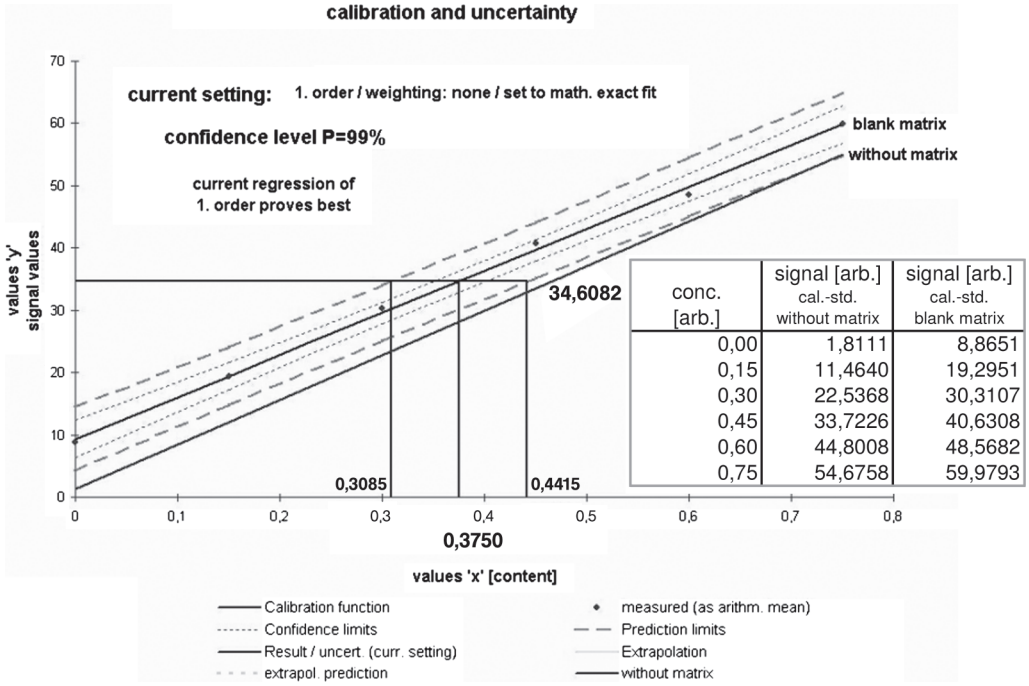


Fig. 8. Exemplary data and graphic of a calibration – a constant matrix effect is evident and we include a blank sample for clarity.

the confidence ranges, an effect caused by the matrix is significant. Figure 8 illustrates this result graphically. If no blank matrix is available, one may also apply the same principle to a reference matrix (sample matrix), as long as the effect due to the pure blank matrix is known. It should be explicitly pointed out that including a blank sample in a calibration series does not comply with common guidelines relating to calibration, but we apply a blank here to emphasize transparency of results.

The sum of squared differences of each standard to the middle of the operating range (Qxx) and the squared standards (Sxx), both given on the concentration scale, are the only parameters that were additionally required to proceed with comparing linear calibration functions directly [5]. All other input refers to common calibration characteristics. On the important left-hand-side of the spreadsheet, one can see the calculation proceeding step-by-step in the way which is commonly known in chemical laboratories for validation purposes [7], i.e. explicitly comparing precision data and mean values applied to two measured series under repeatable conditions (F-test, t-test).

Some software, such as validation or chromatographic software, enables t-testing of the intercept or the slope of the recovery function compared to theoretically expected set points '0' or '1'. However, this strategy for a statistical test procedure does not apply for our purposes and results may be unreliable.

Table 4. Results – statistically sound comparison of linear calibration functions [7].

Wshstve3.xls [Schreibgeschützt]

Comparing two Calibrations (applies to straight lines only, regression of 1st. order)

values given in units [x]	calibration series #			
characteristics	1st. calibration		2nd. calibration	
number of cal.-points:	6		6	
	slope	intercept	slope	intercept
coefficient:	71,52757 [y/x]	1,34568 [y]	67,37339 [y/x]	9,34317 [y]
standard deviation:	0,83061 [y/x]	0,37722 [y]	1,42809 [y/x]	0,64856 [y]
residual std.-deviation:	0,5212 [y]		0,89612 [y]	
sum of squares Q_x:	0,39375 [x]²		0,39375 [x]²	
sum of squares S_{xx}:	1,2375 [x]²		1,2375 [x]²	
df of calibation:	4		4	

conf. level P=99,00%	comparing precisions / testing for homogeneous variances		
F-test:	residual variance	precision of the slope	precision of the intercept
s^2_{max}:	0,80303 [y]²	2,03944 [y/x]²	0,42063 [y]²
s^2_{min}:	0,27165 [y]²	0,14229 [y/x]²	0,14229 [y]²
df (s_{max}):	4	4	4
df (s_{min}):	4	4	4
$F_{crit.}$:	15,97709343	15,97709343	15,97709343
F_{test}:	2,956081711	14,33251739	2,956081711
result:	variances prove homogeneous (ok)	proved homogeneous (ok)	proved homogeneous (ok)
approval:	Res. std.-dev. homogeneous ◉ Yes ○ No	s² homogeneous (slopes) ◉ Yes ○ No	s² homogeneous (intercept) ◉ Yes ○ No
Comparing coefficients requires homogeneous residual variances!	F-test passed	F-test passed	F-test passed

conf. level P=99,00%	comparing coefficients / testing homogeneity	
t-test:	slope	intercept
	only applies for homogeneous variances	
df_{test}:	8	
$s^2_d = s^2_{test.}$:	2,72935 [y/x]²	0,56293 [y]²
$t_{crit.}$:	3,355380613	
t_{test}:	2,514524209	10,65926659
result:	slopes prove the same	intercepts differ
approval:	homogeneous slopes ◉ Yes ○ No	homogeneous intercepts ○ Yes ◉ No
	t-test passed	t-test: homogeneity rejected

Conclusion

Recording a maximum of two calibration series enables proof of a matrix effect being significant and at the same time provides a calibration series in the matrix should this be required. This is further discussed and verified in the following example.

Measuring and evaluating a recovery function does not provide any further information. Measuring efforts to determine the recovery function may be cut in this step of the procedure without replacement.

Further proof of the previous statements will be obtained by submitting control samples (low, medium, high concentrations) to medium- and long-term validations or by evaluating long-term observations supported by control charts.

In summary, our aim is to apply to the full the existing elements that quality management provides anyway and to combine the information so as to succeed in optimizing, i.e. reducing efforts in measurements to the minimum required to obtain objective evidence.

1.6.3.4 Having Established Matrix Effects – Does Spiking Prove Necessary in Every Case?

In the previous example, we discussed a deviation of the calibration function, which was proved to be constant and due to an interfering matrix effect. Of course, proportional deviations may occur, either alone or in addition to existing deviations. The procedure introduced also applies in all these cases. The actual uncertainty proves relevant in reporting analytical results and again provides a valid criterion for decision. Applied to some boundary case, one will achieve the goal of cutting the immense efforts of spiking and even gain improved precision and accuracy of the method.

Figure 9 illustrates the calibration function of the spiking experiment shifted to a higher signal level and involving an enhanced uncertainty compared to the calibration applying a blank matrix shown in Fig. 8. This result is expected due to the finite but unknown concentration of the sample. We note an additional uncertainty in Fig. 9, which is solely due to the evaluation of the spiking series. Evaluating needs extrapolation exceeding the actual operating range of concentrations of the spiked standards.

Extrapolation is allowed here by referring to the exact regression formulas [1, 5]. Applying exact calculation formulas ensures compliance to existing standards and is required by extrapolating, whereas evaluations exceeding the operating range have to be rejected if we refer to calibration formulas given by standards. This is because these formulas represent approximations that are restricted to the actual operating range only and will no longer be valid as we exceed the operating range. From the residuals plot of Fig. 9, one obtains the overall combined uncertainty in units of measured signals at a variation coefficient of 7.73%.

In spite of the asymmetric uncertainty on the concentration scale, the uncertainty in signal units is sure to still provide symmetrical confidence limits.

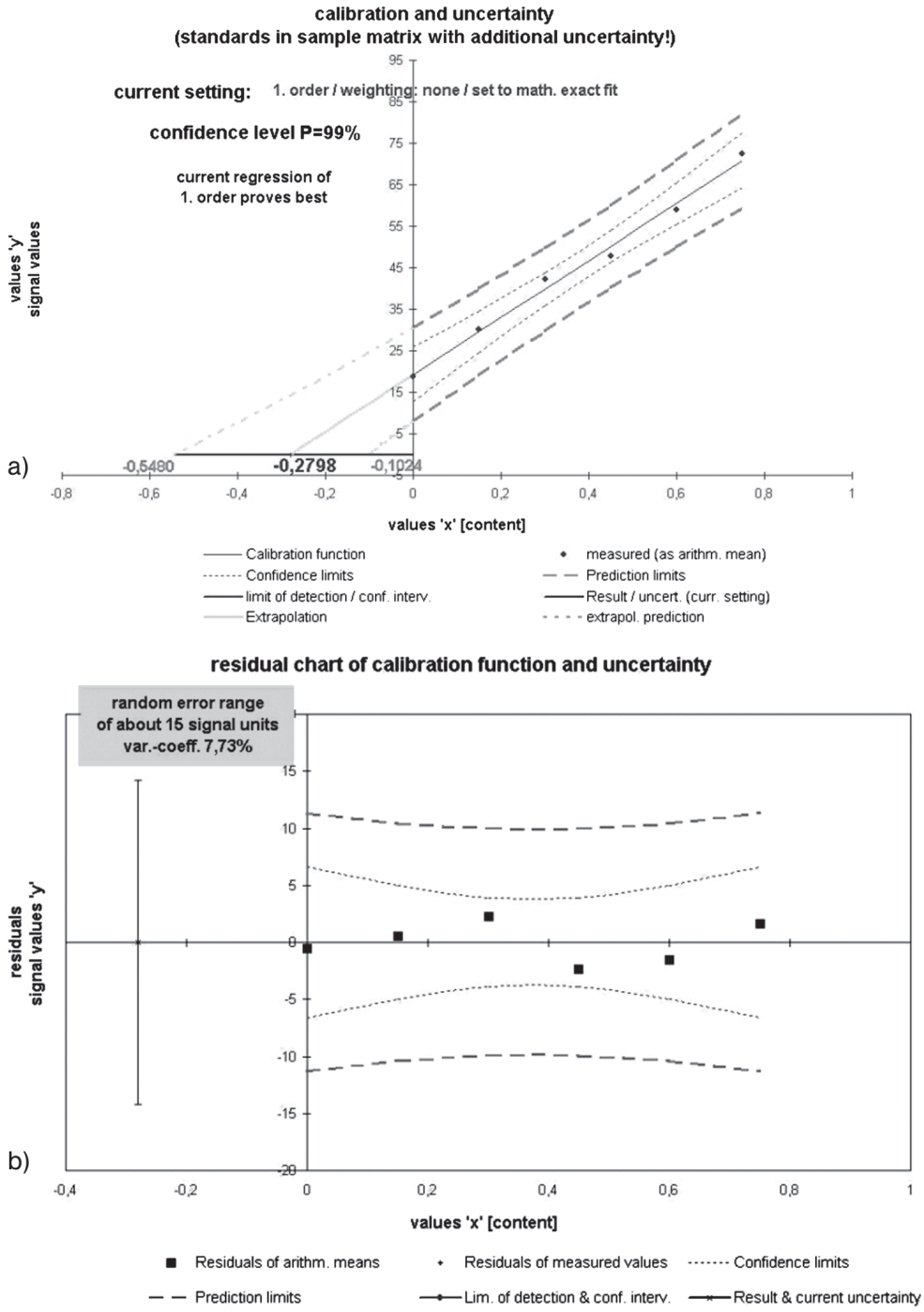


Fig. 9. Calibration by spiking procedure (standard addition method).

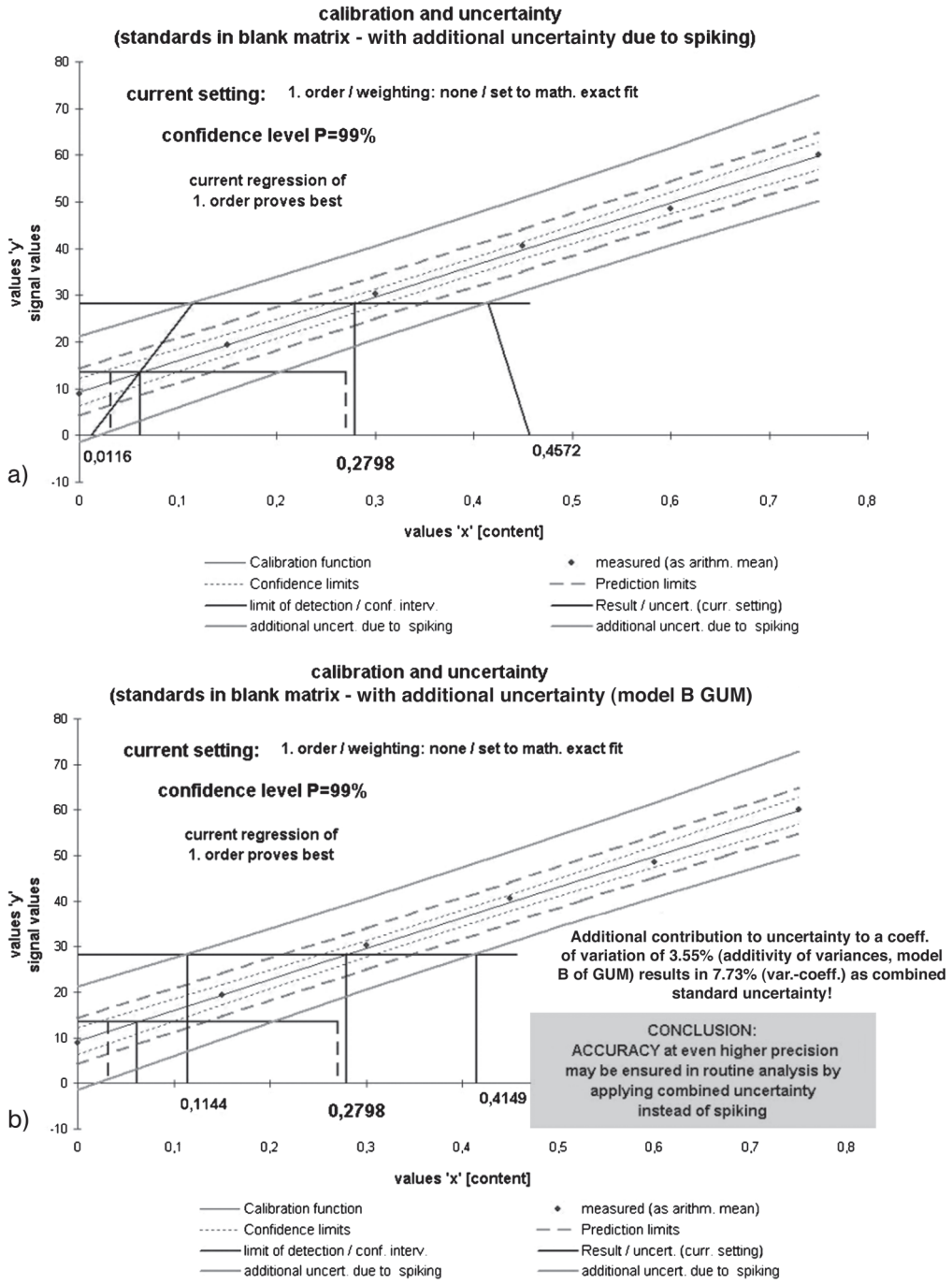


Fig. 10. Calibrations in a blank matrix including enhanced uncertainty given by one single and initial spiking experiment.

We just need to proceed with one single spiking series to get information about the actual uncertainty involved in order to apply this enhanced uncertainty level to any further calibration. Hence, routine calibration requires only a blank matrix solvent and calibration will be possible without spiking.

The enhanced uncertainty due to extrapolation is illustrated in the left plot of Fig. 10 for comparison purposes. In fact, we do not need to consider this additional component of uncertainty (trapezium). We thus get results of higher precision because future evaluations do no longer need extrapolations according to DIN 32633 to calculate the analytical result.

Conclusion

In our example, spiking is no longer required in spite of matrix effects being evident. The additional effort would be nothing but a waste of resources. By comparing calibrations and their respective uncertainty shown in the two plots of Fig. 10, we even improve the quality of our analytical result in spite of reduced measurement efforts. The trueness of the result remains unchanged, but we are able to report an improved precision.

The described strategy provides an even better method at essentially reduced measurement effort. In this way, we even succeed in the simultaneous realization of two competitive and conflicting objectives in optimization (precision versus number of measurements).

1.6.3.5 Testing Linearity – Does a Calibration Really Need to Fit a Straight Line?

Testing the linearity of a measured calibration is an established part of any calibrating. Software provided by modern instrumental analytical methods, such as HPLC, of course supports evaluation of nonlinear calibration. Unfortunately, only a few standards and guidelines deal with basic requirements to evaluate such calibration functions. In principle, there is no reason to deny nonlinear functions. Even the limits of detection and of quantification may be provided by nonlinear calibration and will comply with the sense of existing standards. As long as the analytical assay is not applied to quantify concentrations approaching the limit of detection, the limits of detection and of quantification that result on the basis of nonlinear calibration functions will surely provide sufficient evidence to prove fitness for use, i.e. to meet requirements in validation. Although the focus of standards has hitherto been in terms of straight-line calibration, we refer, for example, to ICH guidelines, which explicitly allow the application of alternative and further calculations, as long as they prove statistically sound.

In our example, we will take this proposal for granted. Again, we focus attention on the expected uncertainty of analytical results to reach our objective in optimizing methods.

We suppose that the method is going to be used for purposes of monitoring and approval at given limits of specifications. The task in optimizing is to reach the best precision available. Thus, when specified limits are approached, we will provide enhanced margins for decision thresholds at an unchanged significance level. The calibration data are presented in Table 5.

Table 5. Calibration measurements provided to test linearity.

Values 'x' (initially set)	***Measurand values 'y'*** arith. means
0,15	0,0448
0,3	0,0886
0,45	0,1317
0,6	0,1716
0,75	0,2179
0,9	0,2556
1,05	0,2939
1,2	0,3388
1,35	0,3764
1,5	0,4124

Tests for linearity are designed to find a calibration function that fits best, but at the same time it should be as simple as possible. Although commonly applied, neither correlation coefficients nor residual standard deviations provide valid criteria to choose the optimum order of a calibration function. We will apply comparison of F-statistics of both regressions involved to provide results that exactly meet the sometimes better known Mandel test. This test supports practical help for decision-making. The result for the data considered suggests the application of a linear regression of first order. However, merely by visually checking the residuals it can be quickly realized that we may just as well choose a calibration function of the second order and ignore the results of numerical linearity testing.

To the trained eye, the clearly bent course of the residuals is sufficient reason to doubt the results of linearity testing, and to proceed to look for an essentially enhanced precision of future analytical results. As Fig. 12 illustrates, we reach a considerably improved precision simply by applying a squared function for calibration.

Conclusion

Simply by applying a calibration function of second order, we enable optimization of the method. The improvement of precision of the analytical assay reaches considerable 35.2% and provides enlarged margins of uncertainties to be tolerated in routine production in our example. In boundary cases, only the precision improved in the way that we have described will enable validation and prove the method's fitness for use, whereas controlling of given specification limits with the poorer precision associated with a first-order calibration will fail. In fact, we enable validation of the method: Even with respect to the demanding requirements of high precision, some extended margins as action limits at specified tolerances are opened up.

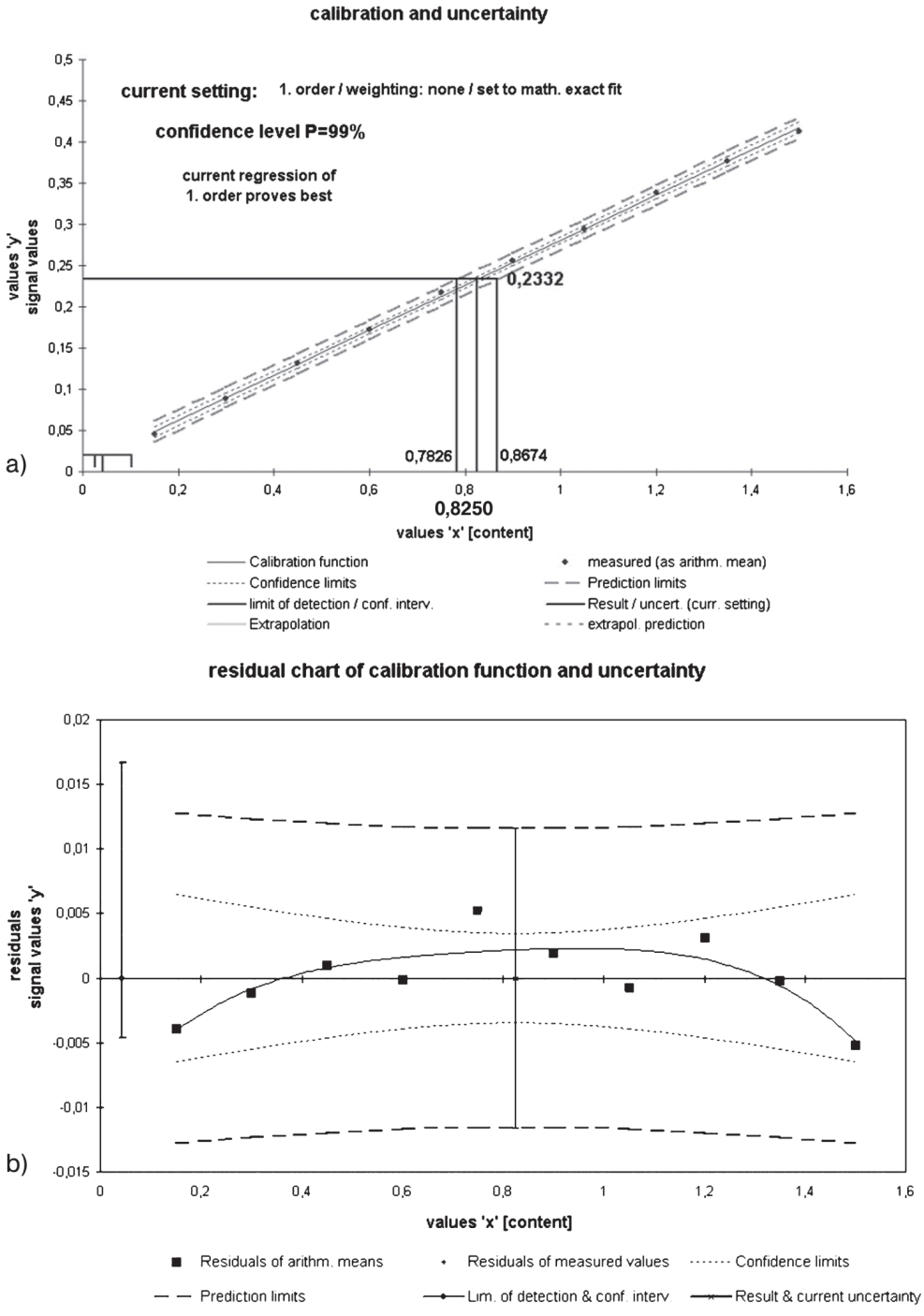


Fig. 11. Calibration of first order, including residuals.

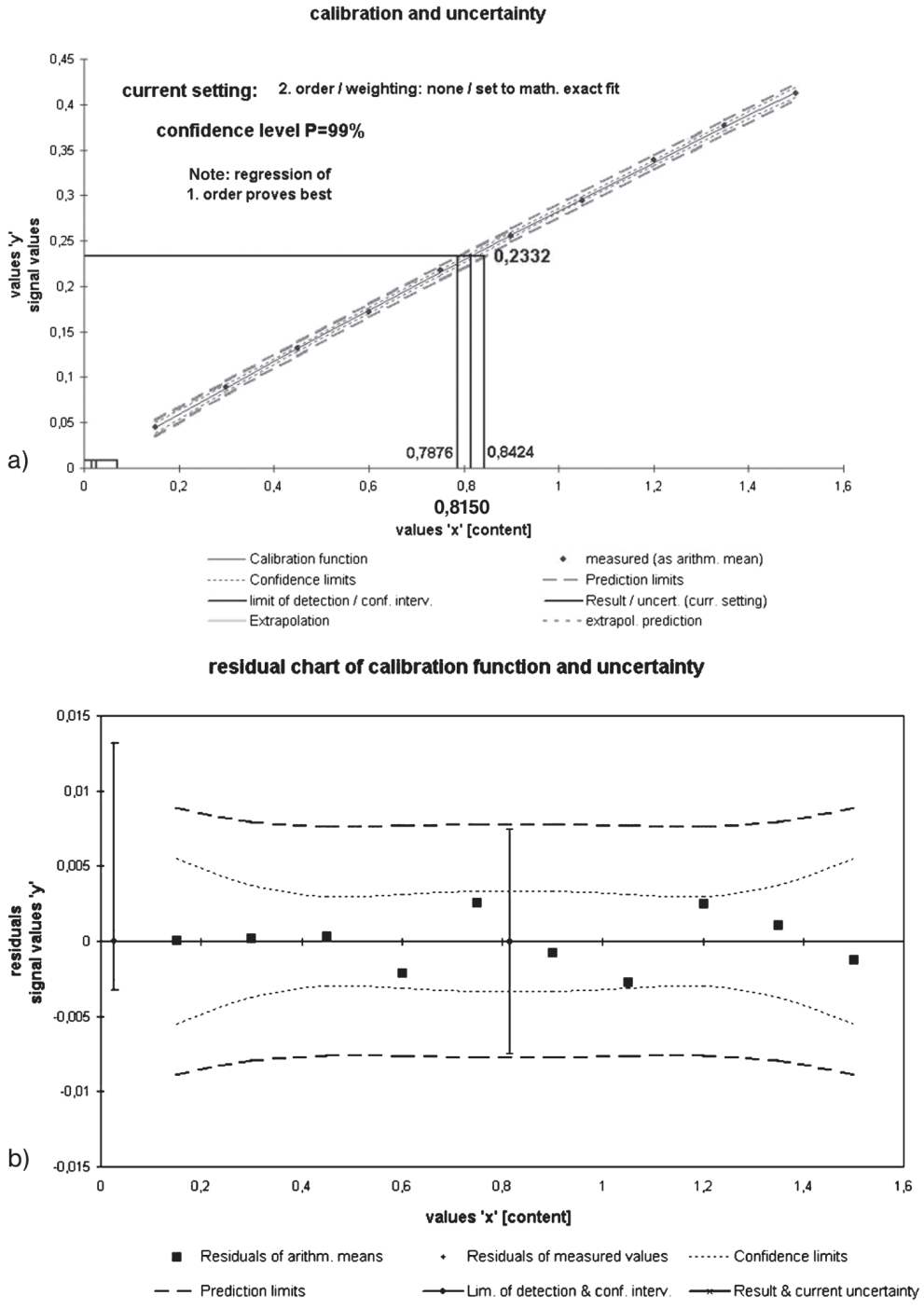


Fig. 12. Calibration of second order, including residuals.

1.6.3.6 Enhancing Accuracy – Obtaining 'Robust' Calibration Functions with Weighting

Calibration guarantees the trueness and ultimately the traceability of analytical results. Errors encountered in calibration give rise to a risk of affecting all future results through a systematic propagation of errors (biases). For this reason, the German DEV[1]-39, 1997 "Strategien für die Wasseranalytik" recommends replacement of the total calibration if outliers of regression are evidently encountered.

Following this recommendation would necessitate a considerably enhanced effort in calibrating. It is surely preferable to check for reasons beforehand and to avoid them in future routine chemical analysis. However, we have to be aware that high sensitivity of methods often implies low robustness at the same time and such methods are not immune from being sensitively affected by interference effects. Whether interference effects are due to the sample matrix itself or to a varying handling of analytical procedures is immaterial.

Especially if we consider the development of methods, it is of no use to track down suspicious results already in the first development cycles. The same applies to wasting time recalibrating again and again until no further suspicious calibration point occurs. After all, it is an essential task to identify possible failure modes and effects as development progresses so that significant errors can be avoided in routine analysis later on. Nevertheless, we should in any case avoid the effect of suspicious calibration points that may result in wrong calibration functions and hence in biasing of results.

Even the question as to whether calibration points outside of the expected range are best eliminated or left unchanged will impede any progress in development the same way as recalibrating does for regression outliers.

Table 6. Calibration data, residuals, and weighting factors (method development).

Values 'x' (initially set)	***Measurand values 'y'*** arith. means	***Weighting factor*** $1/s^2$	***Residuals*** unweighted
0,15	10,92	1	2,21
0,3	18,81	1	–0,76
0,45	31,67	1	1,25
0,6	40,38	1	–0,89
0,75	51,6	1	–0,52
0,9	60,43	1	–2,54
1,05	77,58	3,76	3,76
1,2	79,36	1	–5,31
1,35	90,41	1	–5,12
1,5	114,3	7,92	7,92

[1] DEV denotes "Deutsche Einheitsverfahren", i.e. "German Standard Procedures", dedicated to the chemical analysis of water, waste water, and sludge; the relevant paper is entitled "Strategies for chem. analysis of water".

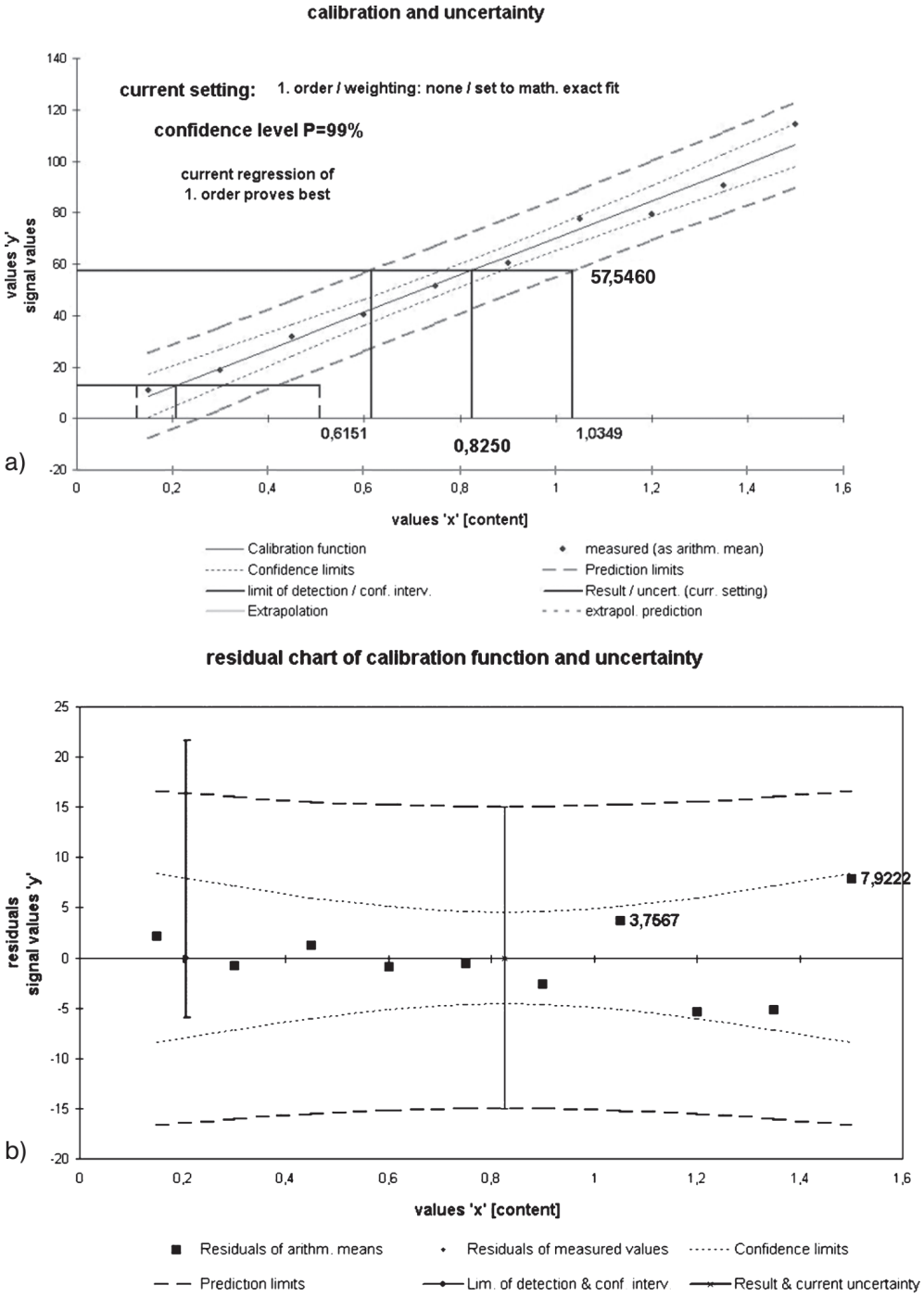


Fig. 13. Unweighted calibration including suspicious regression outliers.

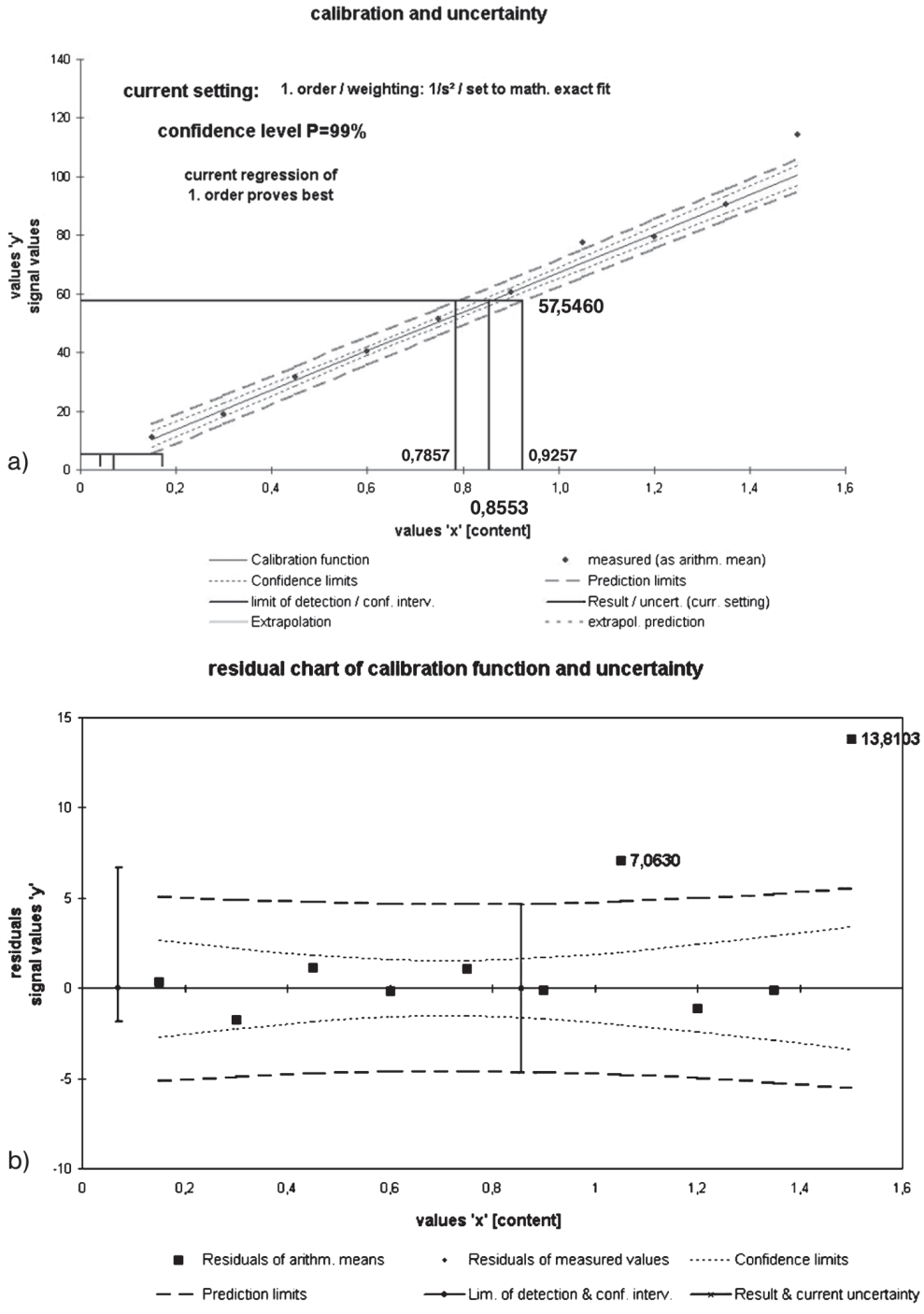


Fig. 14. Weighted calibration (weighting factors are 1/(squared distance)).

Therefore, a weighting of regression is applied in our final example to ensure accuracy of the calibration. Weighting will ensure that calibration is no longer affected by outliers.

We quickly realize that the uncertainty of the calibration is quite high and unsatisfactory, but typical in such cases. The residuals of Fig. 13 clearly reveal the calibration points #7 and #10 to be suspicious stragglers. Both prove to be outliers by testing for regression outliers according to DEV. The residuals of the eight remaining calibration points are supportive assuming the slope of the "true" calibration to be essentially lower.

Instead of eliminating the straggling values, we proceed by applying "weighted regression" [5, 8]. With the current calibration data that lack any individual precision information, only the residuals provide a suitable measure of distances, which we apply as reciprocal squares for weighting. The calibration points that remain unsuspicious are each assigned a distance of "1" for weighting, which corresponds to no weighting and results in normal unweighted regression for these values. Any weighting other than the reciprocal squares of distances should be rejected in principle, since the units of calculation only remain consistent in this way. This helpful principle is worth remembering for any scientific calculation. Besides the sound theoretical background, this principle has considerable practical consequences. If we should ignore it, we easily run the risk of encountering some arbitrary results in formally applied calculations.

Conclusion

Weighted regression proves to be robust and is not affected by suspicious calibration outliers. Weighting ensures valid evaluation of the "true" calibration function, also providing the "true" precision as well. "Weighting out" suspicious calibration points leads to a reduction in uncertainty by a factor of three, which represents a 300% improvement in precision. The measurement of repeatability standard deviations will confirm the better precision, once the method is validated and established for routine analysis. Again, we emphasize the requirement cited in the primary literature [5], to apply squared distances for any weighting. As already mentioned, all other weighting factors run the risk of providing nothing more than arbitrary results. In particular, weighting that refers to concentrations may be ascribed to misunderstanding, since the abbreviation assigned to the weighting factors in primary literature is "c", which in no case means concentration, but is well defined and applied as $1/c = 1/s^2$, with s assigned to standard deviations. Hence, any estimates of s by other measures of distance will be adequate.

Once development and validation of the method has been completed, most effects that triggered regression outliers will have been eliminated anyway. Hence, the profit we obtain by weighted regression is a reliable and true operating calibration provided early in the first steps of development. We avoid posing the question of elimination of calibration points too early as we do not need to recalibrate too often. We thereby proceed to develop the method without any disturbance. The strategy will apply systematically when searching for and recognizing undesired influences that still affect the method. Finally, in the

development of methods one should not waste time in eliminating individual measurements but efficiently achieve the goal of establishing a valid method that is fit for use by efficiently eliminating interference effects.

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