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Quantitation of perfectly co-eluting analytes on dual-channel-subtraction chromatograms[1]

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Abstract

A dual-channel aroyl luminescence detector (ALD) was constructed and used as a demonstration system for quantitating two perfectly co-eluting model analytes (fluorobenzaldehyde isomers) from subtraction chromatograms. The linear part of the calibration curve of either *ortho*-, or *meta*-, or *para*-fluorobenzaldehyde remained the same in the presence of 0.3- to 3-fold amounts of a co-eluting isomer. The accuracy of individual quantitation for two completely overlapping peaks was similar to that for a single peak: the error band started to increase only when the interfering isomer was present in more than a 10-fold excess. Formulas were developed, and experimentally confirmed, that allowed easy calculation of analyte peak size and detection limit in a subtraction chromatogram from corresponding single-channel data. The simple technique of resolving two peaks of identical retention into two quantifiable subtraction chromatograms is not restricted to the ALD, but can be carried out on various types of dual-channel detectors. A similar technique can be employed to check the purity of analyte peaks.

Keywords: Detectors, GC; Luminescence detector; Dual-channel luminescence detector; Fluorobenzaldehydes

1. Introduction

Some chromatographic detectors, particularly spectral ones, can be upgraded from being inherently selective to being tunably specific by a change from single-channel to dual-channel operation [1–3]. Tunable specificity implies that overlapping peaks can be quantified as if they were completely resolved, even if their retention times are identical. Technically, quantitation is obtained from two subtraction chromatograms, each of which shows only one of the two co-eluting compounds. Many different pairs of overlapping analytes (or analytes/interferents) can thus be algorithmically resolved from a single chromatographic run stored in computer memory.

In the case of *spectral* detection, the obvious condition for quantitating a pair of completely overlapping analytes is that their dual-channel response ratios at the two chosen wavelengths must be sufficiently different (and, of course, known prior to analysis). Similar arguments can be made for two different detector types mounted in series or parallel; they can also be made for multichannel detectors, say a diode array or a multi-electrode redox system. To quantitate three overlapping analytes requires three channels and three suitably different response

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ratios; four overlapping peaks require four detector dimensions and so forth.

We might add that, in unrelated studies from our own group, a "TRIDEC" system [4] combining in one physical unit an electron-capture detector, two optical channels of a reactive-flow detector [5], and a flame ionization sensor [6] has been successfully used for correlation chromatography [4]. It obviously could have served as well for a quantitation study of the type reported here (or even for a study based on three or four channels). So could have the recently developed "true" triple-channel [7], or "multiplexed" ten-channel [8] FPDs. More important than these studies is, however, the fact that the literature abounds with examples of suitable detector combinations from simple to hyphenated: any of these could be used as long as they would function with no (or negligible, or correctable) discrepancies in retention time and peak shape between the output channels.

However, the raison d'être for systems with more than two readouts is often other than chromatographic quantitation: the occurrence of triple and quadruple peaks of identical retention time becomes exponentially less probable. Therefore, only the case of two overlapping analytes in a dual-channel detector will be probed in this study.

Conventional (mathematical) deconvolution of overlapping peaks from a single-channel detector has been the subject of much research over the years (see Refs. [9] and [10] for two of several recent examples). There, as well as in this study, quantitation of deconvoluted peaks is the major concern. Clearly, however, purely mathematical deconvolution is not possible in the case of perfectly co-eluting peaks. Yet, the analytical need for quantitating such peaks is both strong and widespread.

We thought it therefore worthwhile to demonstrate the use of correlation chromatography for just such a case, simple though it may be in both principle and practice. In principle, the approach represents the gas chromatographic equivalent of the old two-component problem in solution spectrophotometry. In practice, the quantitation will be carried out, and its accuracy checked, on two differential chromatograms obtained from a dual-channel GC detector. To our knowledge, no explicit study of this kind exists in the literature, despite the ample presence of GC detectors suited to the task.

In some of our earlier studies [1–3], which could be considered 'precursors' to this one, a commercial dual-channel flame photometric detector (FPD) was used. While the different responses of various elements in this detector would seem to offer easy testing, we did not consider the system challenging enough. Highly similar types of spectra, such as UV–Vis spectra of positional isomers, would offer a much more discriminating model. Furthermore, positional isomers often co-elute, thereby providing both analytically relevant and experimentally convenient test compounds.

Some time ago our group described a detector based on monitoring the phosphorescence of certain types of aroyl compounds (benzaldehyde, benzophenone, anthraquinone, and the like) in excited nitrogen, with detection limits in the picogram range [11–14]. Gas-phase phosphorescence spectra of aroyl compounds are, of course, broad and fairly similar in shape and wavelength range – particularly so in the case of positional isomers. Thus they seemed to offer the perfect test case. (Another, similarly demanding test would have been to use the UV absorption spectra of various isomeric aromatics, cf. Ref. [15]).

2. Experimental

For this test, a newly designed [16] aroyl luminescence detector (ALD) was converted from single- to dual-channel operation. The vertical wedge-type beam splitter resembled one used to adapt a conventional single-channel FPD to triple-channel operation [7]. Fig. 1 gives a schematic representation of the dual-channel ALD. The two channels used R-268 photomultiplier tubes behind a 420-nm shortpass and a 530-nm longpass interference filter, respectively (i.e. a rather crude optical arrangement that left much of the luminescence unused).

The response ratio (the ratio of peak height or peak area in the two optical channels) was predetermined for each pure fluorobenzaldehyde isomer. It served as the algorithmic "magnification factor" that allowed a particular isomer to be deducted.

When an isomer mixture was chromatographed, the simultaneous outputs from the two ALD channels were transferred to computer memory. The second

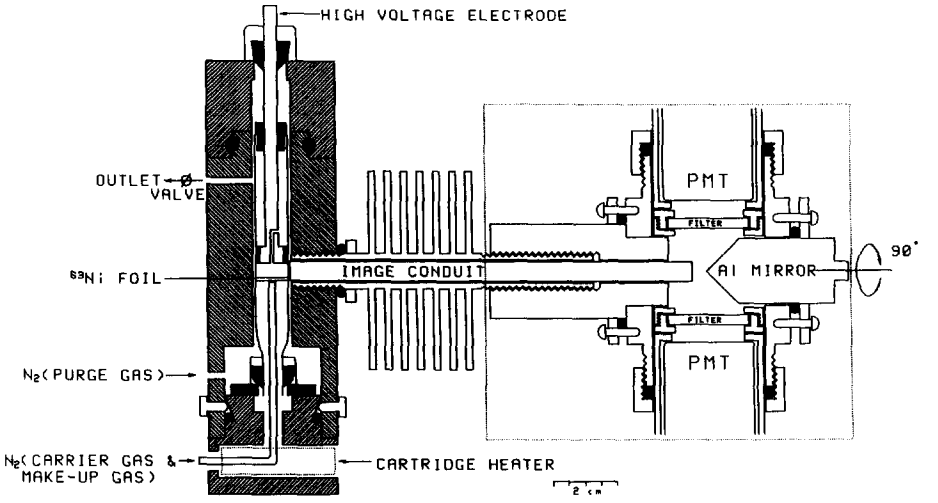

Fig. 1. Schematic of the dual-channel aroyl luminescence detector.

channel was scaled by the predetermined magnification factor (the response ratio of the interferent). Its subtraction from the first channel then annulled the respective isomer in the differential (subtraction) chromatogram. This procedure was repeated (still on the same data file) by using the magnification ratio of the other isomer. In essence, this switches the roles of analyte and interferent.

For a comparison of detection limits, the same file again was subjected to a software-based "channel swap". In essence, this switches the roles of the two channels. The whole process described above was then repeated. The necessary computer interface and algorithms are simple and have been described earlier [1].

Solutions were prepared of 3-fluoro- and 4-fluorobenzaldehyde, and of 2-fluoro- and 4-fluorobenzaldehyde, either by themselves or in mixtures of weight ratios 1:3, 1:1 and 3:1, such that the data points would be conveniently spaced on two composite calibration curves for each pair of positional isomers. In a separate experiment, the same amount of one isomer was repeatedly injected in the presence of varying amounts of the other.

The emission spectra of the three fluorobenzaldehyde isomers were determined by replacing the image conduit shown in Fig. 1 by a quartz rod, and the wedge-mirror/PMT arrangement by a quarter-meter monochromator (Jarrel-Ash 82-415) with an 1180 lines/mm grating of 500 nm blaze, 2-mm slits (corresponding to a 6.7-nm bandpass) and an R-374 photomultiplier tube. The spectral intensity was measured as the peak height of repeated injections, to ensure that it was not disturbed by background emissions.

3. Results and discussion

For this study, a large number of well-responding and overlapping analyte pairs was available. The simplest compound that responds well in the ALD is benzaldehyde. If all three isomers of a given benzaldehyde monosubstituent are to be used, the choice narrows to those whose bond energy to the aromatic ring is not too low and which do not contain hydrogen on the no. 1 carbon of an *ortho* substituent. (Such configurations, similar to well-known examples from fluorescence spectroscopy [17,18], show reduced or no phosphorescence.)

The three monofluoro isomers of benzaldehyde did, among many other compounds, conform to these conditions. They were selected for their conceptual simplicity, good response, easy availability, reasonable chromatography, and adequate stability. As is common among substituted aromatics, the *ortho* isomer eluted first. It can, if desired, be easily separated from the closely eluting *meta/para* pair.

For the current study, however, a column of very low resolution was deliberately used. Furthermore, the chromatographic separation was "detuned" by using a column temperature significantly higher, and a column flow significantly lower than that required by the resolution optimum. Under these conditions, the three isomers merged.

Fig. 2 shows as chromatographic example the 1:1 mixture of *meta*- and *para*-fluorobenzaldehyde, first as it appears in the two single channels, then as the individual components appear in the two subtraction chromatograms. Since both isomers phosphoresce with substantial intensity within both wavelength windows, and since the contribution of one isomer has been subtracted, it is not surprising that the individual subtraction peaks, as well as their sum, should be considerably smaller than the composite ones. (This, however, is not always the case. As will be demonstrated in a quantitative manner later, an inverted analyte peak in the subtraction chromatogram can actually be larger than either of the corresponding single-channel peaks.)

As befits an exacting test system, the difference in response ratios among the three isomers is not overly large. The phosphorescence spectra (obtained with a quartz rod as light conduit) explain why. They are shown in Fig. 3.

The next three figures – Fig. 4, Fig. 5 and Fig. 6 – display the composite calibration curves of the three isomers. Within linear range – the line is always drawn at a slope of precisely unity – the four calibration curves in each plot are identical. Since in each case one of them is that of the pure analyte, while the other three represent analyte in the presence of varying amounts of the overlapping isomer, it is obvious that the subtraction procedure provides accurate quantitation throughout the linear range. (For sake of brevity, some confirmatory calibration

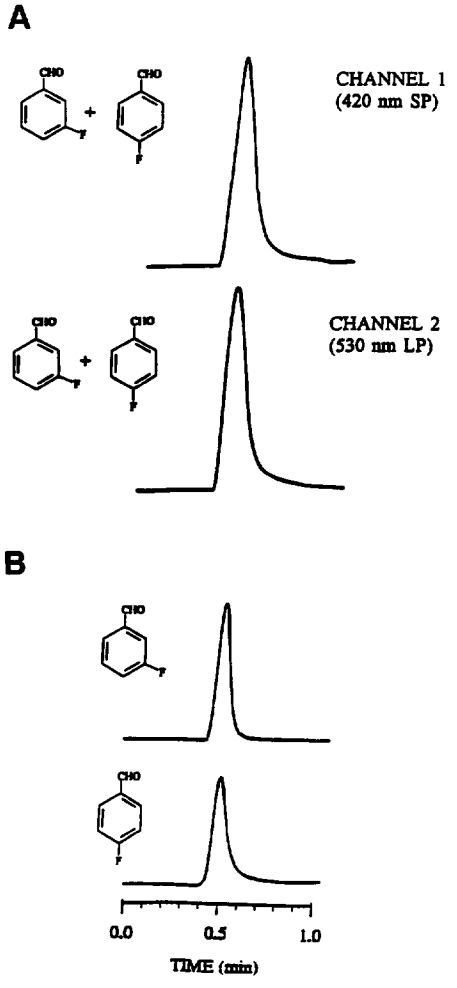

Fig. 2. Typical peak shapes of an overlapping pair of fluorobenzaldehyde isomers, 20 ng each, appearing together in the two single channels (A), and appearing separated in the two subtraction chromatograms (B).

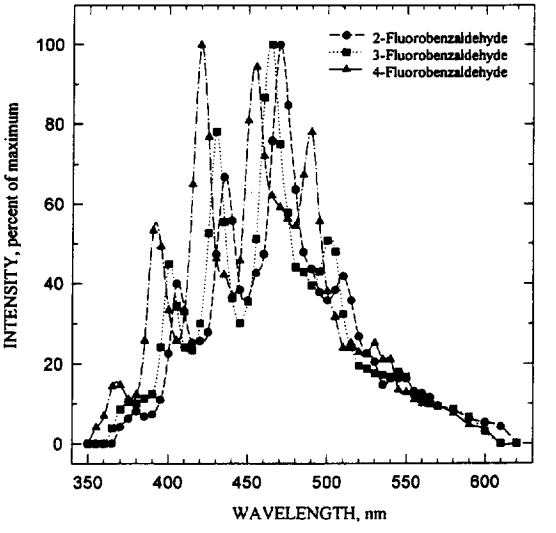

Fig. 3. Normalized emission spectra of the three fluorobenzaldehydes in excited nitrogen, under normal detector operating conditions but with quartz rod used as light guide. Bandpass ca. 6.7 nm.

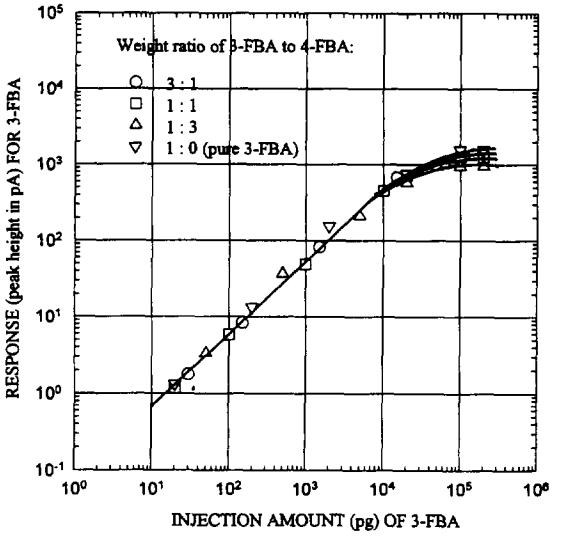

Fig. 4. Composite calibration curve of 3-fluorobenzaldehyde, pure and in the presence of 0.3, 1 and 3 times the amount of the 4-fluorobenzaldehyde isomer, as measured from peak heights in subtraction chromatograms. The straight line is drawn at unity slope.

curves are not shown here, e.g. those of the *para* isomer from the *para/ortho* mixture.)

Beyond the linear range, the calibration curves

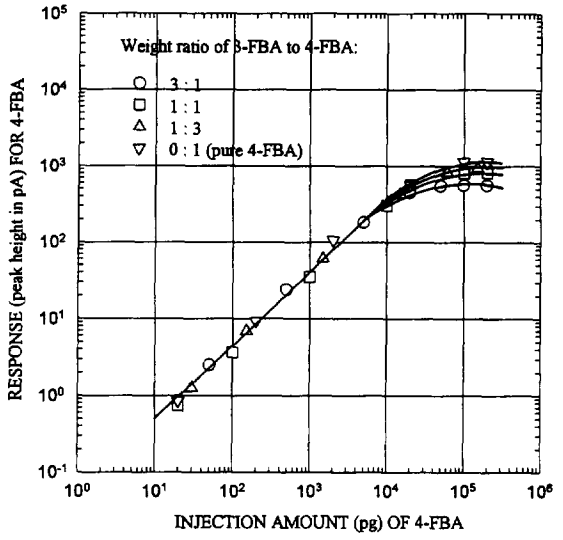

Fig. 5. Composite calibration curve of 4-fluorobenzaldehyde, pure and in the presence of 3-fluorobenzaldehyde. Otherwise similar to Fig. 4.

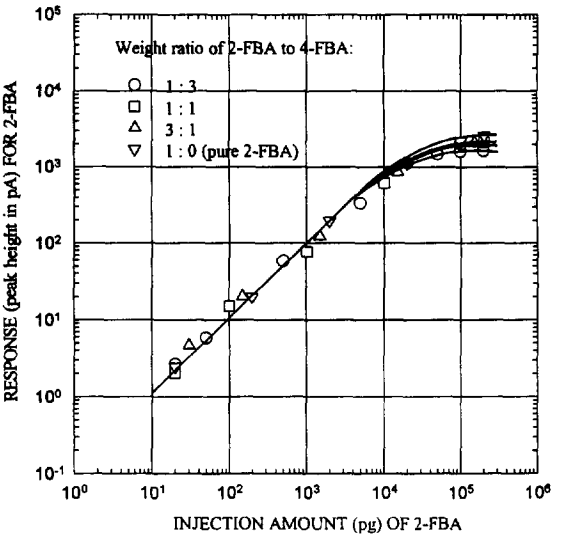

Fig. 6. Composite calibration curve of 2-fluorobenzaldehyde, pure and in the presence of 4-fluorobenzaldehyde. Otherwise similar to Fig. 4.

diverge. They have to: deviation from linearity is caused by *total* sample load. The linear range of the pure analyte appears therefore longest, next comes the analyte with the least amount of co-eluting isomer added, and so on. Conventional quantitation traditionally avoids non-linear behaviour anyway: in the case of correlational quantitation, an additional caution zone, representing the maximum expected amount of overlapping material, should be established. This would not unduly limit analytical applicability.

The lower end of the linear range, i.e. the detection limit, also differs somewhat among calibrations based on single-channel and subtraction chromatograms. It is easy to see why.

Let $R_A(1)$ be the response of the analyte in channel 1 and $R_A(S)$ its response in the subtraction chromatogram. With the response ratio RR between channels 1 and 2 defined as $RR_A = R_A(1)/R_A(2)$ for the analyte and $RR_I = R_I(1)/R_I(2)$ for the interferent, it is easy to show that

$$R_A(S) = RA(1)\left(1 - \frac{RR_I}{RR_A}\right) \quad (1)$$

In other words, if RR_I is smaller than RR_A, the

response of the analyte in the subtraction chromatogram will be positive (a regular peak) and smaller than its response in the first-channel chromatogram, $R_A(1)$, would have been. If RR_I is larger than RR_A, however, $R_A(S)$ will be negative (an inverted peak). In absolute terms, the subtraction response $R_A(S)$ can then exceed the first-channel response $R_A(1)$.

If negative response per se should prove undesirable, it can be prevented by a software-based channel swap. Aside from the direction of the peak, the trace analyst may find the detection limit an important parameter in the decision to swap or not to swap. Which of the two alternatives produces the lower detection limit depends not only on the peak heights but also on the noise levels.

In the aroyl luminescence detector, baseline noise is fundamental in nature [16], i.e. it conforms to a calculation (cf. [19]) based on the molecular randomness of chemiluminescent events, and is numerically approximated by the square root of the photoelectron emission rate. The baseline noise of the subtraction chromatogram, $N(S)$, is therefore larger than the noise of the first channel, $N(1)$:

$$N(S) = N(1)\sqrt{1 + \left(\frac{N(2) \cdot RR_I}{N(1)}\right)^2} \quad (2)$$

The detection limit of the pure analyte in the subtraction channel, $DL(S)$, can hence be calculated from its detection limit in the first channel, $DL(1)$.

$$DL(S) = DL(1)\frac{\sqrt{1 + \left(\frac{N(2) \cdot RR_I}{N(1)}\right)^2}}{\left|1 - \frac{RR_I}{RR_A}\right|} \quad (3)$$

(Note that, if the analyte response in the subtraction chromatogram is negative, $DL(S)$ would turn out to be negative as well. To avoid obtaining a negative value for the detection limit, the denominator is written as an absolute number).

Eq. 3 can be used to determine whether the two channels should be used as filed in memory or – if the analysis comes close to the detection limit – if they should be swapped before one is scaled and deducted from the other.

The aroyl luminescence detector, with its closely overlapping phosphorescence spectra, makes a good test case as far as changes (usually increases) in detection limit from single to subtraction channels are concerned. Table 1 lists the calculated detection limits for the three cases shown in Figs. 4–6, as well as the detection limits for the same cases subsequent to a channel swap. Of the total number of thus scrutinized subtraction peaks, half are inverted (as they must be).

In Table 1, the lower detection limits are associated with inverted peaks. This stems from the fact that (compared on the basis of similar-sized analyte peaks) one of the channels happens to display only about a tenth the noise level. If this channel is used as the "second channel", and if it happens to be multiplied by a relatively large magnification ratio, it will produce a fairly large negative peak in, but add only a small amount of noise to, the subtraction chromatogram. Thus it is possible, although rare, that the detection limit of the subtraction channel can actually be better than that of the first channel ($DL(S)/DL(1) < 1$). One such case is shown in Table 1.

The detection limits of all calculated cases were also experimentally determined. The measurements agreed, within the error limit typical of such single-file processes, with the calculated data of Table 1. It should be noted in this context that conventional detection limits, by virtue of their definition, admit to only one significant digit. The calculated detection limit ratios of Table 1, and the corresponding experimentally determined values, use two.

This is both numerically justified and statistically

Table 1
A comparison of calculated detection limits

Analyte	$DL(S)/DL(1)$	
(interferent)	No channel swap	Channel swap
2-FBA (4-FBA)	0.90*	1.9
3-FBA (4-FBA)	1.5*	2.6
4-FBA (2-FBA)	2.5	1.6*

*=inverted (negative) peak.
FBA=fluorobenzaldehyde.

permissible, because both procedures are based on a single computer file (i.e. they are derived from a single injection). Such "single-file detection limits" [20], while appropriate to the current context, should however not be confused with detection limits resulting from multiple injections of the same standard solution, or from a series of injections of different standard solutions, or, most demanding, from a series of independent, real-life multiple samples, each of which has undergone the complete analytical process from collection and extraction to determination and data interpretation.

The illustrative experiments of the current study are based on the aroyl luminescence detector. If they had relied instead on the flame photometric detector, whose spectra usually differ much more extensively from one another, typical detection limit ratios would have been similar to or better than those of Table 1 (given similar noise levels in the two channels). The same would apply to a variety of other dual-channel and dual-detector systems.

While the log–log plots of Figs. 4–6 illustrate well the overall range and general accuracy of quantitation by correlational chromatography, they are less well suited to depict small possible differences between the overlapping analyte calibration curves (which differ only in the amount of interferent).

Therefore, to explore analyte accuracy and precision over a wider range of such interference, and to check for the potential presence of systematic bias (as could, for instance, result from a slightly inaccurate value of the response ratio used to suppress the interferent), a constant amount of analyte was repeatedly measured in the presence of increasing amounts of overlapping isomer.

Fig. 7 shows this experiment, replete with conventional error bars. It appears that, at least up to a ten-fold excess of overlapping isomer, the analyte is accurately measured. The precision (and accuracy) remains essentially that of the pure analyte (which could undoubtedly be improved by the use of an automatic injector, or an internal standard, or both). Thus, as expected, correlational chromatography can provide *accurate quantitation of completely overlapping peaks over a wide range of conditions*. It should also have significant analytical applicability.

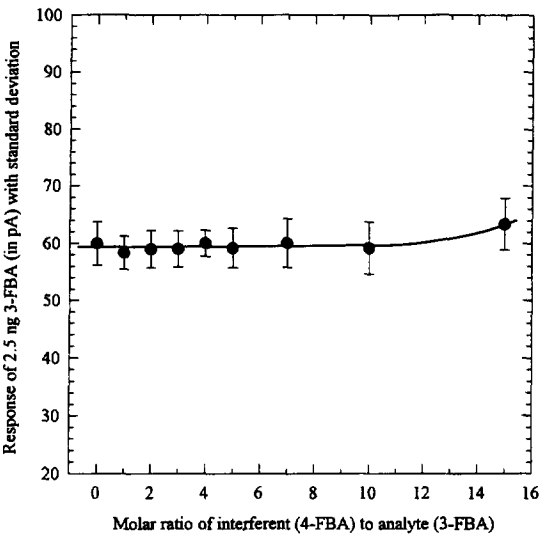

Fig. 7. Replicate (5×) quantitation with standard deviation bars of 2.5 ng 3-fluorobenzaldehyde, in the presence of 0 to 37.5 ng 4-fluorobenzaldehyde.

3.1. Other conditions, systems and operational modes

The applicability of correlational chromatography is limited only by some (fairly obvious) conditions. Clearly, the dual-channel response ratios of the two co-eluting compounds must differ significantly. (If the response ratios are equal, the method does not work. If the response ratios are zero or infinity, i.e. if each compound responds in only one of the channels, the method is not needed.)

A phase shift between the two channels could cause problems, depending on the type of detector(s), amplifiers and software. Normally, electronically caused phase shifts [21] should be too small to matter, particularly if commensurate gain and/or attenuation settings are used on same-type electrometers (i.e. if the RC characteristics of the two amplifying/filtering circuits are similar). Chromatographically caused phase shifts, e.g. slight time delays from sequential detectors, could also cause problems. However, if the peak shape is congruent in the two detectors, an easily installed temporal delay routine can bring the two channels back to synchronicity.

If, however, the peaks have significantly different shapes (response profiles) in the two sequential detectors, a subtraction chromatogram will no longer produce a reasonably looking peak. (While it is possible to adjust the chromatographic dispersion by software means, this is easy to do only with peaks that conform to the same general shape, e.g. a pure or exponentially modified Gaussian. In most cases such problems are better taken care of by other means.)

Even in these extreme cases it would be possible to obtain a number (though not a peak) for the pure analyte. To wit, if the peaks on both channels are integrated – which is possible to carry out by conventional methodology and instrumentation – their area ratios can be used in the same manner as described above for the simultaneous-signal ratios. In this case, care should be exercised that the start and stop commands for peak integration occur at commensurate locations on the concentration profile – although some of the likely arising discrepancies could no doubt be ameliorated by the empirical adjustment of response ratios and calibration curves. But that would still impose a serious handicap on the analyst who, now lacking the richness of perceptional information offered by a chromatogram (cf. [20]), has to make do with mere numbers.

Still, it is obvious that dual-channel subtraction methodology can be used in a wide variety of detector systems. Such methodology requires, of course, that a pure authentic interferent is available for the measurement of its response ratio (which is needed as scaling factor for the second channel). It also presumes, in case of an actual sample, that the expected interferent is, indeed, the one – and the only one – that overlaps the analyte peak. (For the much less probable case of *two* substances co-eluting with the analyte peak, three channels would be needed for complete resolution.)

The general approach of dual-channel deduction methodology can be used in other ways as well. An example may be the chromatogram-based purity check of a presumably interference-free analyte peak. Since the response ratio of the analyte is known, its peak can, in an algorithmic role reversal, be made to vanish in the subtraction chromatogram. Any peak still persisting in its place would therefore have to be that of a previously unrecognized interferent. Similar purity tests of the analyte, based on the complete disappearance of its peak in subtraction chromatograms, have been carried out with transition-metal compounds in a dual-channel flame photometric detector [1].

There exist, no doubt, several more analytical ends to which the means of a simple dual-channel detector and deduction algorithm can be put. Although the underlying principles are so well-known as to be trivial, the application of correlational chromatography can still bring significant advantages to the analytical laboratory.

Acknowledgments

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