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Towards a chromatographic quantitator

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SUMMARY

A specification is outlined for a quantitation system which takes chromatographic signals from one or more single or multichannel detectors, records more than one chromatogram, and uses objective chromatographic information provided by the operator. This information will help estimate the comparative concentration, and its uncertainty of selected components. The advantages of this approach are compared to the limitations of conventional integration of single chromatograms. Some tentative suggestions for implementation are given.

INTRODUCTION

Chromatography is the source of many quantitative analytical results and the quantitation of chromatograms is a vital part of an analyst's professional tools. Analytical results are almost always referenced to *some* standard material: whether it is yesterday's batch or National Bureau of Standards certified Standardised Reference Material, the answers are relative. Current commercial systems concentrate on integration of individual chromatograms. They fail to meet the two real needs of analysts: the comparison of unknown and standard samples yielding an estimate of comparative concentrations of components, and an estimate of their uncertainty.

These simply stated requirements are not met by any commercial systems, nor described in the chromatographic literature. The history of quantitative chromatography is refinement of single chromatogram integration, a difficult task constrained by very wide dynamic range signals, and until recently, too little storage space to keep even one whole chromatogram in fast access memory. The hardware no longer constrains us. This paper proposes a new approach to the whole problem and outlines a specification. Its objective is to encourage discussion and refinement of the specification by analysts, and commercial implementation. Some research, and considerable development, will be needed to implement systems of this sort. They will be large software systems requiring professional production tools such as high level languages of certified compliance to international standards. They should be independently validated to public standards. For brevity a system of this class will be called a chromatographic quantitator. (It is worth noting that the historical record in

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the chromatographic literature[1–14] is sparse compared to the proprietary information guarded by integrator vendors, and in contrast to the importance of these unpublished algorithms to analysts using them. With so much secrecy, thorough validation is impossible.)

SCOPE

The scope of the quantitator concept is all separation systems using elution from a column with serial detectors, for example gas and liquid chromatography, ion chromatography, amino acid analysers with all detectors, and detectors scanning a sorbent like thin-layer chromatography. The quantitator software might be built into a chromatograph, perhaps in ROM (Read Only Memory firmware), externally as part of a data system, or a combination. The principles apply whether the detector(s) is (are) single- or multi-channel though the implementation problems are quite different if more than a few channels are involved. The UV diode-array detector is the most important multi-channel detector. It is suggested that all implementations cater for at least two channels, and solve the problem of synchronisation of detectors in series when one will lag by a constant time. Beyond a few channels, different computational techniques will be needed[15,16]. In the following discussion it is assumed that the quantitator has some control over the chromatograph. If automatic control is not implemented, this may require the analyst to alter settings manually.

WHY DO INTEGRATORS FAIL TO QUANTITATE RELIABLY?

There can be few users of chromatography who have not seen integrations which even novice chromatographers can dismiss as nonsense. Worse still is that integrators rarely warn when results are unreliable, even when this is obvious to the human eye. I submit that this is because the chromatographer has information which has not been given to the integrator, and without which integrators are doomed to unreliability. Moreover, until algorithms for processing digitised analog voltages are given, and can accept, all the available information, they can never produce the best possible estimates of quantities, nor useful estimates of uncertainty. Even if information can be inferred from closer examination of the data, reliability and credibility will be improved if it can be checked against operator input information. For example, it may be possible to deduce the unretained peak time from a chromatogram, but it is still worth checking that this agrees with an operator input value and thus compute confidence limits for this time. It will probably be more accurate to use a column bore input by an operator, but useful to cross check that this information is not grossly wrong, for example 0.46 instead of 4.6 mm. Apart from some assumptions, usually rather weak, about peak shape made by integration algorithms, the only other source of information is the so-called integration parameters.

INTEGRATION PARAMETERS

Chester and Cram[1] in 1971 showed that "...the limits of integration have an astounding effect on the accuracy...". Many current problems with reliability of integration are still rooted in the need for integration parameters from the operator. It

is undesirable that the adjustment of integration parameters provides the user with a way of manipulating the results and not consistent with Good Laboratory Practice or quality standards like ISO 9000. Integration parameters are idiosyncratic and not portable.

Of course, the integrator needs to have any information that cannot be inferred from the chromatogram, such as whether isothermal or isocratic conditions apply, or whether any switching takes place, and when negative peaks are reasonable. For example, when a quantitator has assessed sample chromatogram(s), it should be much better than the operator at assessing the effect of different analog to digital sampling rates. If the sampling rate is controlled by hardware, a change in rate might necessitate reinjection of some or all samples.

Although many commercial systems have progressed in reducing the number of integration parameters required from the operator, a quantitator must not require, nor even permit, any at all. The input must be objective, observable and thus portable between systems, for example column length or flow-rate.

INFORMATION AVAILABLE TO A QUANTITATOR

To quantitate, information can be obtained from the operator, calculated from the basic theories of chromatography, deduced from the analog signal, extrapolated from previous chromatograms using the same column or previous injections of the same and other samples, and confirmed from expectations of results.

(1) The operator might tell, for example, the column size, flow-rate, isocratic or gradient elution, size and type of packing material.

(2) From chromatographic theory for liquid chromatography the algorithm might calculate, for example, the elution volume of unretained substances and the monotonic change in peak width under isocratic elution. Appropriate theory for the type of chromatography, gas or liquid, eluted or developed like thin layer chromatography, must be used of course.

(3) From the analog signal the algorithm might deduce, for example, the character of detector noise and analog-to-digital quantisation[17] and the effect this will have on peak start and end detection and thus the uncertainty of peak area measurement[18–22]. Because of the changing nature of noise and the large number of measurements needed to determine noise, it is impractical to ask the operator to estimate this information. However, it would be useful to ask for an expectation, perhaps from the instrument specification or previous performance, to allow a check for satisfactory operation, for example checking that the deuterium lamp does not need replacing.

(4) Previous injections will hold information about the actual peak shape for this column, and previous chromatograms of the same sample will allow extrapolation to predict the peak shape expected for a particular component for future injections. Peaks with un-chromatographic shape, for example, with a flat top (not detected at all by integrators), must have a very high uncertainty.

Pattern matching by using peak shape must surely be the most sensitive and reliable method[23–25].

(5) Informed expectations, for example that the sample will be 0.95 to 1.05 times the standard, are an important and underused source of information.

ANALOG SIGNALS

The obvious input to a quantitator is the raw analog detector signal. This must be converted to digital form either by sampling very frequently using an auto-ranging high-speed analog-to-digital converter (typically sampled at line frequency or integrated by counting pulses from a voltage-to-pulse converter less frequently, clearing at each time slice).

Because analog signals are degraded the longer the transmission distances, it is almost certainly best to place the analog-to-digital converter inside the chromatograph. (It can never be best, as many diode detectors are linked at present, to convert to a digital signal and then back to an analog signal which is fed to an analog-to-digital converter in the integrator.) Whatever the mechanism, these data must be transferred to the quantitator automatically (and error free). Data compression algorithms can be used to reduce the volume without any information loss, that is the original data can be obtained exactly by expanding the compressed data. Digital filtering can also reduce the data volume, with negligible loss of information if applied correctly. Combining too many adjacent time slices, for example, would materially reduce the chromatographic information.

There is a continuous spectrum of rawness of data from 100 μs analog-to-digital samples at line frequency, through integrated time slices of about a second, up to the final peak areas and uncertainties. The only information output really required is component concentrations and their uncertainty[20]. Only when a quantitator is proven can we safely regard this information as the rawest data that need to be stored permanently to conform to "good laboratory practice". This validation will not be a trivial task. Meanwhile the arid debates on "what are raw data?" will no doubt continue.

Comparison with a modern balance may be helpful. After a sample is placed on the pan and released, weights are taken many times per second. Only when the variance of weight reaches a target value (usually implicitly plus or minus about the least significant digit) is a weight actually recorded, for example in a laboratory information management system (LIMS). Because the algorithm for weighing is simple, and can be tested simply by placing known weights on the balance, we regard it as well-proven (perhaps naively). We would not consider storing all the weighings taken: nor should we need to for chromatography.

IMPLEMENTATION

The implementation problem of how to get and store this information will not be discussed further, except to note that it would be too tedious if the operator had to answer questions on all the conceivable conditions for every chromatogram. A store of data will be needed, filled initially with suitable default values, for example with 4.6 mm bore for liquid chromatography but 0.25 mm for capillary gas chromatography. It must be possible to notify a quantitator of changes, in the flow-rate, for example, perhaps automatically if the quantitator can communicate with the pump. As other information becomes relevant, a quantitator must be able to ask for it, and to resolve conflicts between information supplied and that deduced from the analog record. Implementors will have ample chance to show their user-friendliness, but automatic interrogation of the hardware will be even more friendly and reliable.

It is not yet clear how much information should be input (and output) interactively with the operator, and how transfers to and from any LIMS should be done. For example, final results need to be stored in a LIMS, methods loaded from LIMS, and sample sequence information sent to a quantitator. It may prove impractical or undesirable to store standard chromatograms and extensive history of results in a quantitator (it might imply disc storage) but it is essential to implement a portable interface between LIMS and quantitator so that the quantitator can ask LIMS to store or provide data. When results validation is considered there are some tests that quantitator cannot reasonably perform. For example, checking against an expected value, and upper and lower limits should be carried out by quantitator but assessing the rate of product degradation can only reasonably be carried out by a LIMS. Uncertainty estimates will surely be stored with each result.

PORTABILITY

An important implementation objective is that the information, and thus the method of analysis, shall be portable. This means that if the same input data, for example both digitised analog data and chromatographic data like flow-rate, are fed to more than one quantitator, the results should not differ more than the (un)certainty or confidence limits. (This does not mean that one quantitator cannot be better than another in being able to justify smaller uncertainty estimates, perhaps at the expense of computation time or memory size).

At present, if an analysis is carried out by using one chromatography data system, we cannot be confident that the same results will be obtained from another type of data system, even after choosing integration parameters to suit the new system. In practice, the work involved in re-validating the method is so great that it is skimped. Because standards may change, for example over the decades of life of a pharmaceutical compound, even if all the validation work is repeated, comparisons are not without risk. Without uncertainty estimates, the comparison of results from different systems always carries some risk.

SAMPLES AND THE INFORMATION THEY MAY YIELD

It may be helpful to classify, at least roughly, samples which may be available, and the information a quantitator may derive from them.

Reference samples

These are often the purest available, with information on purity, and impurities if known from other analytical methods, and an uncertainty estimate for this additional information. Classification as reference or standard is, of course, an arbitrary decision made by the analyst.

Typical samples

These will contain more than one component, but not contain more impurities than most of the samples to be analysed.

Control samples

These are synthetic mixtures of standards and the matrix. They should reveal the effect of the matrix on the analysis.

Atypical samples

These will contain unusual concentrations of components. For example, the liquor after crystallisation will often contain much of the impurities in the purified sample and these may be the purest sample of the impurities that can be obtained. The retention time, and perhaps spectrum or relative response in ultraviolet and refractive index detection, can be best assessed from these type of sample. This information can be used to estimate how well they can be measured in more typical samples.

Pure samples of impurities

Sometimes quite pure samples of some impurities are available. Occasionally, chemists can be persuaded to synthesise these specially!

Internal standards

These may show the effect of sample preparation and variation in injection volume. They may also provide useful markers for retention times.

Blanks

Samples in which some standard(s) have zero concentration.

REPLICATE RESULTS

In estimating uncertainty, the number of replicates of integrations of injections are usually too few (often two or three) to be statistically interesting compared to the peaks measured with hundreds of rather precise digitised voltages (better than 12 bit or 1 in 4096 relative to a nearby reading). [Manufacturers' claims of over 20 bits refer more to the range of voltages that can be measured and obscure the precision and/or accuracy with which any particular voltage can be measured, usually between 12 bits (1 in 4096) and 16 bits (1 in 65538)]. Digitisation can cause computational noise[17].

Results from replicate injections also contain the confounding influence of the many operating conditions that will vary between injections.

EXPECTED RESULTS

The quantitator might be able to use any prior knowledge about expected results. For example, if we are measuring the degradation of a batch of material then any significant increase in the concentration of the main component is not plausible. We can extrapolate from measurements on previous batches of the same material, and measurements on this batch at earlier times to give an expected degradation (and an uncertainty estimate). Armed with the data from previous analyses, a quantitator could assess the significance of differences between expectations and result, and determine the number of replications necessary to achieve a arbitrary level of confidence, or advise the level of confidence as a function of the number of replicates. This would follow the current practice of repeating suspect results without sinking to repeating the analysis until the expected result is obtained!

UNEXPECTED RESULTS

A quantitator might also respond specially to results outside expected limits, for example outside 95 to 105% of a nominally pure standard. The principle proposed is that the risk of accepting a result is lower the more nearly it meets expectations.

UNCERTAINTY ESTIMATES

Errors can be absolute inaccuracy of concentration if standards containing known concentration (and known inaccuracy of concentration) are available. If standards are not available, then uncertainty must be expressed as precision of peak area, in, say, mV · s. For this reason the term "uncertainty estimate" is used in this paper. As usual, the error will vary with concentration or peak size, height and/or area, and only variation of the concentration of component(s) in standard chromatogram(s) will allow this to be explored in detail[26,27].

CONFIDENCE

An indicator of the confidence we can place in the uncertainty estimate would also be useful. The simplest is the number of measurements, related to degrees of freedom in the jargon of statistics, but it might be helpful in view of the complexity of combined uncertainty estimation to compute an explicit estimate of the uncertainty in the uncertainty estimate —"a variance of the variance". If it is based on only a few chromatograms this value will be rather rough, but for hundreds of samples both the uncertainty estimate and its variance will become quite well defined, correctly reflecting the real situation. Confidence could also be expressed by upper and lower confidence limits with some arbitrary probability.

POSSIBLE METHODS OF PEAK AREA UNCERTAINTY ESTIMATION

Detector noise and pump flow-rate variation can probably be considered separately. Liquid flow-rate imprecision has always been impossible to measure well enough at high enough speed. Retention time is a highly integrated value and its variation only places very wide limits on the short-term variations. The detector noise is easier to tackle, and may well dominate errors. The most serious uncertainties in peak area assessment arise from uncertainties about peak start and stop points, especially on long tails, of course. For many chromatograms (especially isothermal and isocratic) the noise on the baseline can be studied in detail and its frequency distribution[18] assessed quite well, if necessary, by monitoring for quite long periods without injection. There are usually some quiet sections of undoubted baseline before injection or during chromatograms, for example before the first peak is eluted.

A naive method of establishing error limits would be to establish a point at which the peak-start criterion is not yet met, and another at which the peak has undoubtedly started, the peak-start point normally used lying somewhere midway. Similarly peak-end limits could be calculated (and would usually be wider apart because of tailing). The peak noise might be added to or subtracted from the observed reading to estimate these points. The baseline and peak area could then be calculated assuming

the first start and last end point, and then the last start and first end, and thus the uncertainty estimated from their differences from the most probable estimate from midway start and end points. For groups of peaks, the computations are more complex, but in principle the valley positions suffer a similar uncertainty.

More sophisticated curve fitting and deconvolution methods[23,25,28–51] might well lead to better estimates. Deconvolution would achieve more acceptance by users if they knew how reliable its results were. Estimation of uncertainty must correctly reflect the reality that measurement of small peaks before large ones is much better than measuring on the tail of the large peak. This has important advantages in the use of the uncertainty as a criterion for optimisation[52].

PEAK HOMOGENEITY

The reliability, or accuracy, of chromatography is most seriously compromised when a peak represents more than one component and this is not sensed.

Our most precise detectors, the flame-ionisation detector and the single-wavelength ultraviolet detector, are non-identifying[53]. They provide no information about peak homogeneity, apart from peak shape. Only by *comparison* of chromatograms can we assess the limits on homogeneity. As more chromatograms are compared, a better assessment can be made. For example, if a series of samples, perhaps interposed with standards, are being run and the column performance is steadily being degraded by sample debris or column decay, it is possible to predict the most likely and worst likely peak shape for the next sample, If the next sample produces a wider peak, then we must suspect a hidden peak. A quantitator algorithm would probably request a standard chromatogram or two which would confirm or deny the diagnosis: rejection of the sample result might be premature.

Detectors combining techniques (so-called hyphenated) are usually much less reliable than chromatography (the liquid branch of which has an unenviable reputation itself) but they, and all multichannel detection, dramatically improve the chance of sensing hidden peaks.

During the next half decade, the diode array detector will gain enough computer power to give useful information on peak non-homogeneity[54], but this will compound the quantitation problem because the area (or volume) integrating role will become embedded into the factor analysing function. It alone promises to be no less reliable than current single-wavelength detectors.

TARGET UNCERTAINTY

Many chromatographic values can change: baseline, retention time, peak shape. The key criterion for whether these changes are important is whether they increase the uncertainty of the result too much. For example, in estimating a main component we often require an accuracy, relative to a "standard", of a few percents or better, whereas for impurities we may be unconcerned at tens of percent relative imprecision. To judge whether a chromatogram is acceptable, we clearly need an estimate of uncertainty and the quantitation process must provide this. The quantitator will advise for all items quantified, if appropriate or requested, the estimates of uncertainty and an indicator of confidence in the uncertainty estimates, and minimum detectable

quantities[18,20,23,55–71]. After one or more chromatograms have been run, the analyst will be able to set target uncertainties for all peaks detected. These may range from infinity (for peaks which are of no interest) downwards. The units may be absolute weights, or concentration, or relative to other components. By comparing the estimated uncertainty with the analyst's target, the chromatograms can be judged acceptable or not. If unacceptable, more injections may be needed, or more drastic action such as instrument repair or method change. If replication is indicated, then it should be possible to predict how certainty will improve as more injections are made.

Target uncertainty is the only input to a quantitator which may be arbitrary and not objective (though of course target uncertainty should be the analyst's objective). It makes a real "system suitability test" possible, rather than indirect ones, for example plate height or resolution targets, used at present. Even more important, the target uncertainty provides an ideal criterion for optimisation of chromatographic conditions[72]. It allows the user to specify in non-chromatographic measurement terms what he wants from the analytical procedure. If other constraints need also to be imposed, for example, maximum time, a quantitator should be able to predict uncertainties. (Most separation quality measures used by optimisation methods, for example resolution, are only one of many factors controlling the uncertainty of measurement).

Finally, because an estimate of uncertainty is available, the result (and the uncertainty estimate, of course) are suitable for storage automatically in a LIMS as raw data.

EXAMPLE OF AN APPLICATION OF A QUANTITATOR

In the analysis of pure chemicals, such as an active drug, a quantitator might guide the operator in the following sequence of measurements on various samples.

Preliminary method development is usually based on known chemical data and this is used to select gradient conditions for scouting chromatograms. At first a quantitator has little additional information apart from the column size and which theory applies. However, it can make estimates of the uncertainty in area measurement from possible baseline and separation degree, and assess the errors caused by sampling rates. As more chromatograms are run, expected peak shapes become more defined.

These experiments should establish chromatographic conditions, perhaps now isocratic, that elute the main component with reasonable retention and specificity. Chromatograms are run for the quantitator until the confidence in uncertainties estimated is high enough. Chromatographic conditions may need altering. The number and retention, and uncertainty estimates, of impurities can now be assessed in more detail, especially by running atypical sample(s). These will need to be analysed more than once, and with standards interposed to compensate for drift of retention. Chromatographic conditions may need refining to improve separation.

When the typical sample(s) are run, the impurities in atypical samples (and standards too, for single peak chromatograms are virtually unknown) can be identified by retention time and peak shape, and perhaps confirmed by other techniques. Initial estimates of uncertainty can be refined. Some strongly overlapped components may be revealed: it will also be possible to estimate from the number and density of impurity peaks, the risks or probability that other underlying peaks will be undetected[73–75].

For example, in a complex and variable matrix like urine, there is a high risk that

a change in diet or metabolism will lead to a new peak overlapping that from an analyte under study. In a pure chemical the risk is statistically much lower. Although the variance of risk calculations is high, it can be used to refine the uncertainty estimate and suggest when more resolution (which only gives a modest improvement to risk) or multichannel detection (which has generally better specificity) is needed to give credible results, for example for forensic evidence.

As more typical samples are analysed, interspersed with standards at intervals determined dynamically by the quantitator, the uncertainty estimates will be continuously refined, in particular, the distribution of errors. A Gaussian distribution is abnormal in analytical chemistry[76]. Baseline noise and drift, and retention drift norms will also be established.

OPTIMISATION

A method of quantitation is now quite well defined. At this point, if not before, optimisation of chromatographic conditions may be appropriate. An important objective of optimisation is to ensure robustness[77] so that small changes in conditions do not lead to sharp increases in uncertainty. For example, does a likely change in column temperature or solvent composition change uncertainty significantly?

Of course repetition of all the previous experiments to establish the change in uncertainty estimates is hardly necessary. The quantitator might also guide the user in the number of analyses required to achieve an arbitrary level of confidence and indicate the predicted confidence for a given number of analyses. (To have confidence in standard deviations within 20% needs about 50 replicate observations, 10% needs 250, 5% needs 1000 and 1% needs 20 000[78,79].)

Finally, in routine use analysing many typical samples a quantitator should continue to refine the uncertainty estimates showing this through an increased confidence. The distribution of errors will become more defined. This might be used to reject certain results. For example, some instrumental feature, like air bubbles, might cause some results to be more often in error in one direction giving a bimodel error distribution. If analyses are done in triplicate and two results are close and the third is different by the observed bimodal difference, it might be more reasonable to ignore the third result and return the mean of the other two as the best answer. (The third result could still be used to refine the bimodal error distribution.)

The quantitator should advise the error distribution, and any sudden changes to it, as an aid to instrument fault diagnosis and maintenance[80]. For example, the appearance of a bimodal error distribution might signal a leaking injection valve. The Kalman filter, recently reviewed by Brown[81], has been shown useful in this situation[82–86].

METHOD VALIDATION

It follows that the validation of a method, now established by a limited number of initial experiments, should be continuously refined[87–93], narrowing if possible the uncertainty estimate, as more standards and samples are measured. This should lead to best use of the data, neither overoptimistic neglecting, for example, degradation of the column with many chromatograms, nor pessimistic because too few samples are

analysed exploring too many variables. In principle, all previous chromatograms have some relevance to any particular chromatogram, but in practice only a few will prove relevant enough to be considered individually (though the quantitator must be able to incorporate a summary of information from previous chromatograms, for example using the Kalman filter concept).

Many methods are used to analyse hundreds of similar samples. In this case it should be possible to deduce quite a lot about the distribution of errors, especially if replicates are done. For example, do air bubbles in an injection loop cause the largest errors always to be in one direction giving a biased error distribution rather than Gaussian? This would not be obvious from a dozen chromatograms run for uncertainty estimation. Could the risk of outliers from abnormal factors be quantified well enough to justify reducing the replication and thus reduce the cost?

If the column performance declines as more samples are done, an objective, and cheapest, decision to replace it with a new one can be taken if the uncertainty estimate becomes worse than the target uncertainty. The quality of the data is much better assured, and costs should be less than replacing the column after an arbitrary number of analyses.

POTENTIAL BENEFITS FROM USING A CHROMATOGRAPHIC QUANTITATOR

(1) Removal of arbitrary "integration parameters".

(2) Ideal specification of analytical performance for legal, regulatory and commercial purposes because only the target uncertainty need be specified.

(3) Improvement in reliability of results, and an estimate of the reliability achieved.

(4) Direct estimate of uncertainty on each peak.

(5) Explicit determination of the error caused by interfering substances.

(6) Less risk of undetected peaks hidden under others.

(7) Meeting uncertainty targets is the ideal criterion for optimisation of chromatographic conditions.

(8) Placing entire responsibility for the estimation of uncertainty in quantitation on the manufacturer, who cannot claim that user has selected wrong integration parameters.

(9) Easier validation of the quantitation system with real samples rather than artificial tests or inspection of program code.

(10) Elimination of much unnecessary column testing and unnecessary control of column parameters. A system is suitable if the uncertainty estimates are better than specified limits.

(11) Elimination of the need to check chromatograms by eye and the cost of hardware and software to aid this.

(12) Reduced volume of raw data which must be stored.

(13) Far greater tolerance to retention drift and peak shape change.

(14) Reduction in analysis time and solvent use by reducing unnecessary resolution.

(15) Reduction in number of replicates required (or warning that more replicates are required to achieve the target uncertainty).

(16) Less injections of standards without reducing confidence.

(17) Increased column lifetime by only replacing when error rises too high.

(18) Better diagnostic information reducing instrument down time.

(19) Using computing (which is getting cheaper) instead of using chromatography (which is getting more expensive). Helping development of better chromatography which can solve more difficult analytical problems.

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