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Review

Sources of uncertainty in gas chromatography and high-performance liquid chromatography

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Abstract

Analysts are increasingly being required to evaluate the uncertainty associated with their methods. Indeed, estimating the uncertainty of an analytical result is an essential part of quantitative analysis. The approach of the International Organisation for Standardisation to uncertainty estimation requires the identification of the possible sources of uncertainty for a procedure, followed by the evaluation of their magnitude. A review is presented of the sources of uncertainty associated with analysis by gas chromatography and high-performance liquid chromatography. The review is intended as a source document for analysts evaluating uncertainties for chromatographic procedures. The sources of uncertainty associated with the techniques are presented, and where such data were available, quantitative estimates of their magnitude are given.

Keywords: Reviews; Gas chromatography; Ruggedness; Liquid chromatography; Detection, GC; Detection, LC; Measurement uncertainty; Injection methods

Contents

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1. Introduction

The evaluation of the uncertainty associated with a result is an essential part of quantitative analysis. Without knowledge of the measurement uncertainty the statement of an analytical result cannot be considered complete. The Guide to the Expression of Uncertainty in Measurement [1] published by the International Organisation for Standardisation (ISO) establishes general rules for evaluating and expressing uncertainty for a wide range of measurements. This guide has subsequently been interpreted for analytical chemistry by Eurachem [2]. We have published approaches to the evaluation of measurement uncertainty in analytical chemistry elsewhere [3–5]. In these studies the uncertainty was generally estimated by considering ''whole method'' parameters such as recovery and precision, rather than attempting to evaluate the individual contributions. These measures, if properly assessed, can include contributions to the overall precision and accuracy from many stages of the method. This approach significantly reduces the effort required to obtain an uncertainty estimate, as such information is often available in the form of validation and quality control (QC) data. In our previous studies it was found that the overall method precision and recovery generally accounted for a significant proportion of the uncertainty. The main disadvantage of this approach is that it gives the analyst little or no insight as to the major sources of uncertainty for a method. If the analyst wishes to reduce the uncertainty by improving the method, the dominant sources of uncertainty must be identified so that method development can be concentrated in those areas. The aim of this review was to identify published information on the key parameters that affect analysis by gas and liquid chromatography. Where data on the magnitudes of the effects of the parameters were given, the figures are reported here.

The review was restricted to quantitative analysis by gas chromatography (GC) and high-performance liquid chromatography (HPLC). It does not cover the preparation of samples prior to analysis. The study covered the main detection techniques used with GC and HPLC but excludes mass spectrometry (MS). The review has been subdivided into a number of sections covering different aspects of chromatographic analysis. The section on general chromatography covers parameters such as integration which are applicable to both GC and HPLC. Separate sections on GC and HPLC follow. Each has been further subdivided to cover specific areas such as detectors and injection systems. It should be noted that uncertainty estimation applies only to methods which are under statistical control. It does not include the effects of ''spurious'' errors due to, for example, analyst error or equipment malfunction. Results for which such errors are suspected should be rejected and the analysis repeated. The review has not therefore covered in any detail factors which could cause a method to fall out of statistical control.

The search was primarily based on the *Analytical Abstracts* database compiled by the Royal Society of Chemistry. The database references over 3000 journals, beginning in 1980. The main areas covered by the referenced journals are general analytical chemistry, inorganic chemistry, organic chemistry, biochemistry, pharmaceuticals, food, agriculture, environmental chemistry, apparatus and techniques. Initially the search was aimed at identifying key review papers and books on gas and liquid chromatography. It was then expanded to cover particular aspects of these techniques such as detectors and

Table 1
Summary of factors affecting analysis by GC

Parameter	Factors affecting parameter
Result of analysis	Carrier gas flow-rate [12], column temperature [12], injection temperature [12], sample size [12], split ratio [12,14]
Peak area	Baseline drift [6], carrier gas flow-rate [6,10,16], integrator settings [6,8], noise [6,8], peak tailing [6,7], peak resolution [7,9]
Peak height	Carrier gas flow-rate [6,15,16], column temperature [10,15], detector temperature (temperature dependent detectors) [15], integrator settings [6], repeatability of sample injection [10,15]
Split injection	Carrier gas flow-rate [26], initial column temperature [21,23,26], injection speed/technique [15,21,23,28], injector liner [15,26,28], injector temperature [15,20,26,27], sample discrimination [15,16,21,22,23,24,27], split ratio [15,26,28]
Splitless injection	Carrier gas flow-rate [21,30], column position [20], initial column temperature [15,21,29,30], injection purge time [21], injection speed [15,28,29,30], injector temperature [15,21], injection volume [15,21,30], liner volume [15], needle discrimination [15], needle length [30], needle position [20], sampling time [21]
Temperature programmable injection	Injection technique [31], liner diameter [31]
Cold on-column injection	Carrier gas flow-rate [30], initial column temperature [30], injection speed [30], injection volume [30]
FID	Carrier gas:hydrogen:air flow-rate ratio [12,15,16,21,37,40] detector temperature [12,41]
FPD	Carrier gas flow [42], column temperature [42], detector temperature [40,42,43], oxygen:hydrogen ratio [42,43,44], quenching [45], total gas flow [42,44]
ECD	Carrier gas flow-rate [15,40,46], carrier gas impurities [39,40], detector temperature [15,21,39,40,46,47,48,49], make-up gas flow-rate [46]
TCD	Carrier gas flow-rate [12,40], detector temperature [12,15,39,40,47], wire temperature [12]
TID	Hydrogen flow-rate [15,39], temperature of thermionic source [15,39]
PID	Detector temperature [50]

injection systems. The search was restricted to English language papers. It is interesting to note that an initial search for papers referring specifically to uncertainty yielded only 368 hits from the whole database. A summary of the parameters affecting analysis by GC and HPLC identified in the literature, together with the relevant references, is presented in Tables 1 and 2.

2. Information identified

2.1. Sources of variation affecting analysis by both HPLC and GC

Dyson [6] presents a detailed discussion of integration techniques in which many sources of error and variability associated with peak integration are highlighted. The width of a given peak should remain constant during replicate analyses. Variation in peak widths therefore indicates that column performance or other controlling parameters, such as temperature, are drifting. Changes in peak width can also be caused by the performance of the injection and detection system. Factors identified as affecting the accuracy and precision of peak area measurements include baseline noise which blurs the base of peaks making it difficult for the integrator to identify the beginning and end of peaks; noise at the top of a peak which can cause the integrator to assign a valley thus splitting the peak and integrating the two halves separately; the integrator slope sensitivity parameter which if set too large causes the slope of the peak to be detected late and lost early; baseline drift which causes precision problems in area measurement when it is not constant over a series of runs; and peak tailing which makes it more difficult for the integrator to identify the end of the peak.

The effect of the data sampling frequency on the accuracy of peak area measurements is also discussed. A plot of sample interval versus percentage error in peak area is presented. The percentage error in the area measurement increases with the sampling interval. However, if the sampling interval is too small, unwanted baseline noise will be detected and measured as peaks.

Table 2
Summary of factors affecting analysis by HPLC

Parameter	Factors affecting parameter
Result of analysis	Column temperature [21], mobile phase composition [21], mobile phase flow-rate [21], sample loop volume [64]
Peak area	Baseline drift [6], column temperature [57], detector wavelength (UV detection) [66,67], injection precision [61,62,68], integrator settings [6,8,47,57], mobile phase composition [57,67,69], mobile phase flow-rate [6,10,47,57,67,69], peak tailing [6,7], overall system precision [47], signal:noise ratio [6,8,47,55], peak resolution [7,9]
Peak height/peak width	Column temperature [10,21,52,67], detector response time [67], detector wavelength (UV detection) [66], integrator settings [6,8], mobile phase composition [10,21,52,66,67,69], mobile phase flow-rate [6,21,52,69], overall system precision [47], repeatability of sample injection [10], signal:noise ratio [6,8,47,55], slope of elution gradient [66]
Capacity factor	Column age [65], column temperature [51,66], mobile phase composition [51,65,66,68], slope of elution gradient [66]
Retention time	Column age [65], column temperature [47,51,52,57,58,60,66,67], integration [6,57], mobile phase composition [47,51,52,57,58,60,65,66,67,68,69], mobile phase flow-rate [52,57,65,67,69], overall system precision [47], signal:noise ratio [47], slope of elution gradient [66]
Resolution	Column age [51,65], column temperature [51,67], mobile phase composition [51,65,67,69], sample volume [63]
Relative retention	Column age [51,65], mobile phase composition [65]
Refractive index detection	Column temperature [71], mobile phase composition [21,71], mobile phase degassing [71], mobile phase flow-rate [47], pressure [21,71], temperature [21,47,71]
Fluorescence detection	Mobile phase composition [21,71,72], temperature [71,72]
UV–Vis detection	Solvent degassing [74,75], wavelength accuracy [73]

Meyer [7] addresses the subject of errors in the area determination of incompletely resolved peaks, integrated using the vertical drop method. This is identified as a major source of uncertainty. A number of cases of unresolved peaks are investigated, including Gaussian peaks and exponentially modified peaks. Area ratios ranged from 1:1 to 10:1. The errors in the peak areas depended on the size ratio, tailing and resolution. In the worst cases, errors of up to $\pm 40\%$ of the true area were observed. Meyer has also considered in detail the sources of error associated with the determination of peak ratios [8]. Parameters associated with peak integration which contribute to the error are identified as the sampling rate, the threshold value and noise.

Papas and Tougas [9] have also considered the problems associated with the integration of overlapping peaks. The accuracy of algorithms used for the deconvolution of skewed peaks were investigated. Using simulated studies based on exponentially modified Gaussian peaks, inaccuracies in the measurement of the areas of unresolved peaks using the tangent-skim and perpendicular drop methods were investigated. Errors of up to 50% are reported.

Dyson [6] and Scott [10] discuss the parameters affecting the response of mass and flow (or concentration) sensitive detectors. In the case of flow sensitive detectors, such as those commonly used in HPLC, the detector output is proportional to the concentration of the solute in the mobile phase. The peak area is proportional to the amount of solute, provided that the flow-rate is held constant. Increasing the flow-rate through a flow sensitive detector will result in a reduction in the peak area. However, the peak height remains approximately constant thus resulting in a narrower peak. A reduction in flow-rate of 1% is reported as producing an increase in the peak area of 1% and an increase in the peak height of less than 0.3% [6]. For peak area measurements with a concentration sensitive detector, a high quality flow controller (for GC) or pump (for HPLC) is therefore required. For mass-sensitive detection methods, such as flame ionisation detection (FID) commonly used in GC, the response is proportional to the mass of solute in the detector but independent of the flow-rate. However, the peak shape will vary as the flow-rate varies. Reducing the flow-rate results in a reduction in the peak height but the peak area

remains constant thus producing a shorter broader peak.

Skoog et al. [11] identify column temperature, flow-rate and rate of sample injection as key parameters which must be controlled when making peak height measurements. The effect of sample injection rate is particularly important for the early peaks in a chromatogram. Relative errors of 5% to 10% due to this cause are reported for syringe injection.

2.2. Factors affecting analyses by GC

An ACOL text on GC [12] provides a good general introduction to the experimental factors that affect gas chromatographic analyses. Important parameters identified include column temperature, carrier gas flow-rate, injection temperature, split ratio and sample size. Another useful general text on error sources in GC is that of Guiochon and Guillemin [13].

Wynia et al. [14] discuss the ruggedness testing of a temperature programmed gas chromatographic method with flame ionisation detection used for the determination of residual solvents in steroids. The effects of variations in the injection temperature, split flow, type of injector liner, concentration of components, rate of temperature change, detector temperature, column flow-rate and sample matrix on the quantitation of methanol, acetone, methylene chloride and ethyl acetate were investigated using a central composite design, with each of the parameters set at three levels. The study found the method to be particularly sensitive to changes in the split flow.

Quantitative analysis by GC is discussed in detail by Bebbrecht [15]. The factors affecting the measured peak heights are considered. As peak heights are inversely related to peak widths, experimental parameters that cause variations in peak widths will also affect peak heights. The column temperature is identified as having an effect on retention time and therefore on the peak width of up to 3% per °C. Similarly the carrier gas flow affects the retention time and consequently the peak height. The detector temperature can also affect the observed peak height if its response is temperature dependent. Peak height reproducibility is also dependent on injection reproducibility. This is especially important for early eluting sharp peaks. A fraction of a second increase in injection time can double the peak width and therefore reduce the peak height by 50%. The injection of the sample is also identified as a great source of error. Even if an internal standard is used to solve the problem of injecting the small volumes required for capillary chromatography with any degree of repeatability, there can still be problems due to sample discrimination. Poor injection technique will affect both the precision and accuracy of the analysis.

A detailed discussion of the application of capillary GC is presented by Grant [16]. Sample introduction, detectors, quantitative analysis and optimisation are all considered.

The factors affecting run to run variations in retention times in GC are discussed by Hinshaw [17]. One cause of retention time uncertainty identified is poor oven temperature repeatability. This can be caused by the initial oven temperature being set too close to room temperature. Even when the oven temperature readout shows that it has reached the required temperature, significant temperature gradients may persist thus causing variations in the temperature experienced by the analytes. Problems can also be caused when insufficient equilibration time is allowed at the end of a temperature programmed run. Poor injection technique is also identified as a cause of retention time variability. This is generally more of a problem for manual injections. In the long term, gradual loss of stationary phase from the column causes a trend towards shorter retention times.

2.2.1. Factors affecting sample introduction in GC

Two general texts covering sample introduction were identified [18,19]. In addition, a number of authors have discussed the critical parameters and potential problems associated with split and splitless injection systems.

Jennings and Mehran [20] identify the critical parameters for split injection as injector temperature and the split ratio. In particular, low split ratios can cause problems by affecting the mixing of the sample with the carrier gas. This can in turn affect the linearity of the split. Poole and Schuette also discuss the factors affecting split injection systems [21]. One of the main problems identified with split

injections is discrimination against high boiling sample components. The problem is also addressed by Hinshaw [22]. Discrimination occurs when differing fractions of a sample, in proportion to their masses or boiling points, pass through the injector onto the column. A sample that spans a carbon number range of greater than 10 carbons may be subject to this effect. The typical sign of mass discrimination is lower than expected peak areas for either the later eluted or the earlier eluted peaks. It is caused by non-linear solute transport through the inlet, and stems from effects such as sample fractionation from a heated syringe needle, or the formation of a mist of sample droplets that sweeps past the split point without entering the column. Other causes of poor injection accuracy, such as selecting a very low split ratio (i.e., less than 1:10) are also considered. Ulberth and Schrammel [23] discuss the problems of sample discrimination in relation to the accurate quantitation of fatty acid methyl esters (FAMEs) by capillary GC with split injection. In theory, the area percentages of the recorded chromatogram should represent the fatty acid composition of the sample. Previous studies had identified sample introduction as one of the major sources of error in FAME analysis, with key factors affecting sample discrimination identified as syringe handling techniques, temperature and the injector design. The authors studied six needle handling techniques and estimated the percentage total error and sample discrimination resulting from each in the analysis of a soya–maize oil blend certified reference material. The discrimination was estimated by comparison with results obtained from on-column injection (OCI). Filled needle injection was identified as being the least accurate technique and also produced the greatest sample discrimination. Solvent flush injection in combination with a hot syringe needle produced the most accurate results. Solvent flush injection with a hot syringe needle and a post injection dwell time of 2 s produced results closest to those observed with OCI. In a second study the effects of varying injector temperature, split-vent flow, speed of needle penetration and injector sleeve design on the analysis of milk fat were investigated. The non-corrected area percentages of nine FAMEs and the percentage discrimination were monitored. The speed of needle insertion was identified as a key variable. The temperature of the injector was also found to have a significant effect.

In addition to the problems mentioned above, the split may not be homogenous due to poor mixing of the sample with the carrier gas [21]. The column temperature can also influence the split ratio due to sample recondensation. Recondensation reduces the volume of sample vapour in the cooled column inlet creating a zone of reduced pressure which sucks in more sample vapour. This causes a decrease in the split ratio (i.e., an increase in peak areas) as the column temperature is reduced. This is particularly important when the column temperature is close to the solvent boiling point. It is therefore important that the initial column temperature is reproduced accurately from run to run. The speed of injection of the sample also influences discrimination. A slow injection results in high discrimination. The importance of a reproducible injection technique is stressed. Schomburg et al. [24] also discuss problems associated with split injection procedures. These include selective vaporisation from the syringe needle and after release of the sample into the injection chamber, leading to sample discrimination; aerosol formation during vaporisation of the sample leading to inhomogeneous splitting into the column and splitting flow; and back ejection of parts of the sample from the injection chamber into cold parts of the flow system due to the sudden increase in volume (and therefore pressure) on vaporisation of the sample.

Grant [16] identifies sample introduction as accounting for the majority of analytical error in GC. The problems of sample discrimination associated with flash vaporisation injection techniques are discussed. Vaporisation from the needle on initial entry of the syringe into the injector, before the syringe plunger is depressed, can result in partial loss of high boiling components. When the plunger is depressed, aerosol formation can affect the split ratio thus giving rise to discrimination. Finally, when the needle is withdrawn from the injector, high boiling components may remain in the needle due to fractionation of the sample from the needle. Hot needle injection techniques are reported as giving the lowest sample discrimination.

The various problems associated with vaporising injection systems are also considered by Keele [25].

These include discrimination, degradation and flashback caused by sample overload. If too much sample is injected, the volume of the vaporised sample can exceed that of the inlet. The excess vapours reach the top of the inlet and condense onto the septum, backdiffuse through carrier gas lines and condense on cool surfaces, and/or exit through the septum purge line. This flashback leads to loss of sample, ghosting on subsequent injections, sample discrimination and poor precision. Split inlets are again reported as having a high probability of suffering sample discrimination. It is therefore important to select the appropriate inlet liner, inlet temperature, injection technique (fast autoinjection is recommended), solvent, column installation and split ratio. Factors affecting splitless injection are listed as inlet temperature, column temperature, column head pressure, solvent, injection volume, injection speed, liner volume. Particular problems associated with splitless injection include needle discrimination, inlet discrimination, solvent overload of the column, sample degradation and flashback.

For splitless injections, Jennings and Mehran [20] identify the positioning of the column in the inlet and the positioning of the syringe needle during injection (as well as other aspects of the injection technique) as key factors affecting quantitative analysis. Poole and Schuette [21] identify the key parameters as sample size, sampling time, injection purge time, initial column temperature, injection temperature and carrier gas flow-rate.

Eyem [26] discusses the conditions required for the accurate split injection of samples containing analytes with a wide boiling point range. The effects of injection temperature, carrier gas flow-rate, geometry of the glass liner and column temperature on the accuracy and precision of injection were investigated. Three injection techniques were compared-injection into a hot isothermal injector, isothermal injection with the injector at the solvent boiling point, and programmed temperature injection. The study used a mixture of hydrocarbons (C_{16}, C_{26} and C_{36}) dissolved in xylene. Increasing the injector temperature resulted in an increase in the normalised peak areas. The effect was most significant for C_{16} and least pronounced for C_{36}. The effect of the split flow on the normalised areas was significant for C_{36} but not for C_{18} or C_{26}. The results indicated a strong interaction between the effect of carrier gas flow through the liner and temperature on the normalised areas. The effect of these parameters on accuracy was found to be significant but the precision was unaffected. Four types of liner were investigated. The accuracy and precision were found to vary with the liner. A narrow empty liner gave the most accurate results but the precision was unacceptable for practical application. Packing the liners improved precision but at the expense of accuracy. Factors affecting temperature programmed injection were also investigated. Experiments investing the effect of the injector temperature programme parameters on the accuracy and precision did not show any significant contribution from programme rate or starting temperature (provided it is well below the boiling point of the solvent). However, the carrier gas flow-rate through the injector and the length of the initial column isothermal period were found to be significant.

Grob [27] discusses the problems associated with sample introduction into hot injectors. Accurate injection can be difficult as solutions often start to evaporate in the needle. This can cause more sample to be injected than is read on the barrel of the syringe. Evaporation from the needle is also a cause of sample discrimination. Losses of up to 80% are reported as being fairly common for high boiling sample components. These effects can be minimised by using rapid injection so that the needle is withdrawn before evaporation begins. The temperature in the upper part of the injector, particularly at the septum, is also reported as being important. In some systems the set injector temperature may only be reached in a short section near the centre of the vaporising chamber. The septum may be much cooler which can cause high boiling components to re-condense.

Kane and Rothman [28] discuss the ruggedness testing of a split–splitless injection technique for capillary GC. The authors used a fractional factorial experimental design to investigate the effect of changes in the injection port seal (septum vs. duckbill seal), syringe type (sharp tip vs. cone tip), injection speed, injection depth, air gap between the solvent plug and the sample (no air gap vs. 0.5 μl air gap), needle temperature (hot vs. cold) and the liner. The last parameter allowed the investigation of the

effect of changing from direct injection to splitless injection. The parameters were investigated by monitoring the peak areas obtained for six deuterated semivolatile organic compounds. The critical parameters identified varied with analyte. For [$^2H_{10}$]1,4-dichlorobenzene the only parameter having a significant effect was the injection speed. This parameter was also identified as critical for [$^2H_8$]naphthalene and [$^2H_{10}$]acenaphthalene. The presence or absence of an air gap had a significant effect on the analysis of [$^2H_{10}$]phenanthrene, [$^2H_{12}$]chrysene and [$^2H_{12}$]perylene. The liner type was found to be critical for [$^2H_8$]naphthalene and the seal type for [$^2H_{12}$]chrysene.

Penton [29] compares the performance of a splitless injector and a temperature programmable injector for the determination of pesticides by capillary GC. The injectors were compared for area count precision and for their performance in avoiding discrimination against pesticides of low volatility. The effects of injector temperature, injection speed and post injection needle residence time were investigated for splitless injection. Three injection modes were also evaluated. It was found that a slow injection speed was beneficial in recovering late eluting components. However, this is more difficult to effect reproducibly with manual injection. It was found that the temperature programmable injector gave better precision and recovery of high boiling pesticides than the splitless injector.

An investigation of the parameters affecting the performance of cold on-column and splitless injection systems is reported by Snell et al. [30] The parameters identified for the optimisation of cold on-column injection were solvent, column temperature, flow-rate, injection speed and injection volume. In addition, for splitless injection, injector temperature, valve time and needle length must also be considered. Studies were made of the effects of all these parameters on the performance of the injection systems.

Mol et al. [31] discuss in detail the factors affecting large volume splitless and solvent split programmed temperature vaporising (PTV) injection. In particular, they focus on the effect of the liner diameter and injection technique on the recovery of *n*-alkanes. In addition, the effect of the liner dimensions on the occurrence of thermal degradation during splitless injection is considered.

2.2.2. Factors affecting detection in GC

ASTM have published a series of standards on the evaluation of detector parameters such as drift, noise, sensitivity, linear range, dynamic range etc. [32–36].

Grant has considered the effect of carrier gas flow-rate on detector response [16]. As mentioned earlier, the variation in detector response with flow-rate depends on whether the detector is concentration or mass flow dependent. For concentration dependent detectors (e.g., thermal conductivity detector, photoionisation detector) a decrease in the flow-rate does not affect the peak height, which remains approximately constant. However the peak width, and consequently the peak area, increase. In contrast, for mass flow detection systems (e.g., flame ionisation detection, flame photometric detection, nitrogen–phosphorus detection) the response is inversely proportional to the retention time. Therefore, any change in chromatographic conditions which cause a change in the retention time will also affect the peak height. It follows that a decrease in the flow-rate results in reduced peak heights, however the peak area remains approximately constant.

2.2.2.1. Flame ionisation detection

Detector temperature and the relative flow-rates of the carrier gas, hydrogen and air into the detector are identified as key operating parameters [12,16,21,37]. It is noted that when the carrier gas:hydrogen:air flow ratios exceed 1:1:10, effects of changes in the flows become less noticeable [12]. Grob, Jr. [37] focuses on the effect on the detector sensitivity of variations in the carrier gas (in this case hydrogen) flow. The most common changes in the carrier gas flow-rate occur during temperature programs with a pressure controlled gas supply. A plot of changes in flow-rate with temperature is given. For example, a hydrogen flow of 8 ml min^{-1} at 25 °C dropped by approximately 3 ml min^{-1} at 350°C. It was found that variations in the carrier gas flow-rate of 1 ml min^{-1} caused changes in the FID sensitivity of between 1% and 5%, depending on the fuel gas supply. Grant [16] presents plots illustrating the effect of air and hydrogen flow-rates on detector response. FID is also discussed by Henrich [38]. Maximum sensitivity is reported at a particular ratio of carrier gas (or carrier and make-up gas) flow to hydrogen flow. Fluctuations in the detector temperature are reported as having a slight effect on the

response. It is also stated that the area response for a compound does not change with small variations in carrier gas flow, such as those observed during temperature programming. IUPAC [39] also report that the FID response is virtually unaffected by changes in carrier gas flow-rate, pressure or temperature. However, it is recognised that the response is dependent on the hydrogen:air ratio which must be carefully controlled.

In his book on GC detectors, Ševcík discusses the operation of the FID system [40]. A list of experimental factors that affect the detector response is given. The major factors identified are the flow-rate of hydrogen to the flame and the carrier gas flow-rate. The flow-rate of air into the flame has little effect as it is always in a large excess. The FID response increases as the carrier gas flow-rate increases, reaches a maximum and then decreases. However, it is noted that increases in response obtained by increasing the hydrogen and carrier gas flow-rates generally result in an increase in the noise.

Dressler and Cigánek [41] have studied the effect of detector temperature on the FID response. The changes in the peak areas with detector temperature for a mixture of alkanes (C_9–C_{12}), *n*-octanol and dimethylphenol were studied for four different detector designs. In all cases the temperature was found to affect the response but the nature of the variation depended on the detector design. For two of the detectors the actual temperature at different positions inside the detector body was measured. It was found that the real temperature was always lower than the temperature set. The higher the set temperature, the greater the relative differences.

2.2.2.2. Flame photometric detection

The effects of experimental conditions such as gas flow-rates, detector temperature and column temperature on the response of a flame photometric detector (FPD) operating in the sulphur mode, with a packed column, are discussed by Quincoces and González [42]. The FPD response is reported as being affected by the oxygen:hydrogen ratio in the detector and the total gas flow. Plots are presented illustrating the effect of variations in the hydrogen and air flows, and the oxygen:hydrogen flow ratio on detector sensitivity. An optimum oxygen:hydrogen ratio of 0.35 was identified but the maximum detector response did not significantly change in the oxygen:hydrogen range of 0.2 to 0.3. At lower and higher values the sensitivity of the detector decreased. At constant oxygen:hydrogen values the detector sensitivity was also influenced by the total gas flow supplied to the detector. A plot of variation in the FPD sensitivity with the total gas flow is given. The FPD response was also found to be dependent on the carrier gas flow (nitrogen in this case). An increase in the nitrogen flow resulted in a significant decrease in the detector response. The effect of column and detector temperatures on the detector response was also investigated. The influence of column temperature was investigated between 80°C and 140°C with the gas flow-rate and detector temperature held constant. It was observed that the response decreased as the column temperature increased (plot given). The effect of varying the detector temperature between 130°C and 190°C was also investigated. In this case the response also decreased as the temperature increased. Ševcík [40] also discusses the effect of the detector temperature on the FPD response. It is reported that increasing the detector temperature from 100°C to 160°C resulted in a halving of the response to hydrogen sulphide and sulphur dioxide.

A detailed study of the effect of temperature on the behaviour of the FPD system has been undertaken by Dressler [43]. It is reported that the peak heights observed for sulphur containing compounds decreased as the detector temperature increased from 80°C to approximately 160°C. As the temperature increased from 160°C to 200°C the peak height started to increase gradually. It was found that the exact nature of the relationship between peak height and detector temperature depended on the air flow-rate and the structure of the compound. A plot is given illustrating the relationship between peak height and temperature for two sulphur compounds at four different air flow-rates. It was also observed that detector noise and background current increased with increasing temperature. The peak heights for phosphorus containing compounds were found to increase linearly over the temperature range 80 to 200°C. As in the case of sulphur compounds, an increase in the detector noise was observed. The dependence of peak height, h, on the amount of sulphur compound, c, is given by the relationship $h=ac^n$ where a and n are constants depending on the flame conditions. In theory $n=2$, however, it was

observed that n decreased with increasing detector temperature. The rate of decrease was found to vary, depending on the nature of the sulphur compound. As the response of the sulphur mode is second-order rather than linear, variations in experimental parameters will have a greater effect on response than in the phosphorus mode.

The optimisation of an FPD system operating in sulphur and phosphorous modes is discussed by Cardwell and Marriott [44]. A number of experiments evaluating the detector response under various conditions are reported. In the first study the flow-rates for an air–hydrogen flame were optimised. Hydrogen flow-rates were increased from low to high for each of a number of air flow settings. The air flow was then varied at a number of hydrogen flow settings. Plots are given of the detector response to sulphur dioxide and triphenylorthophosphate at the various hydrogen and air flow-rates. For changing gas flows it was observed that the detector background increased with increasing hydrogen and/or air flow. For each of the maxima identified in the plots of response vs. gas flow, the oxygen:hydrogen ratio was calculated. It was found that as the total flow-rate increased, the oxygen:hydrogen ratios at the response maxima decreased. Maximum responses for sulphur dioxide were observed when the oxygen:hydrogen ratio was in the range 0.29 to 0.33. In addition, it was observed that at constant oxygen:hydrogen ratios the response increased as the total flow increased. For the phosphorus compound the maximum responses occurred at oxygen:hydrogen ratios in the range 0.32 to 0.38.

The effects of hydrocarbon and water quenching on the FPD response have been studied by Dressler [45]. In the cool flame of an FPD system, the decomposition of sulphur or phosphorus containing substances produces excited S_2^* and HPO* species. As these species revert to the ground state they emit radiation at characteristic wavelengths. The presence of other compounds in the flame, for example hydrocarbons, causes a decrease in the detector response. The magnitude of the quenching effect depends on the detector design, the oxygen:hydrogen flow ratios and the concentration of the quenching compound. It is known that the response of the detector is affected by its temperature [43]. The purpose of this study was to determine whether the quenching effect was also temperature dependent. Using cyclohexane as the quencher it was shown that the intensity of the quenching effect varied with temperature. Without cyclohexane present, the peak height observed for thiophene with a detector temperature of 100°C was 151 mm. With a cyclohexane concentration of $4.67 \cdot 10^{-6}$ g μl^{-1}, the peak height was reduced to 8.6 mm. At a detector temperature of 190°C the peak height was 67.5 mm in the absence of cyclohexane and 6.8 mm with cyclohexane added. Investigations of the effect of water on the sulphur and phosphorus response found no quenching effect.

2.2.2.3. Electron-capture detection

A number of publications addressing the key experimental parameters associated with electron-capture detection (ECD) were identified [15,40,46–49]. Poole [46] has published a review of the use of ECD in capillary column GC. An equation is given relating the detector signal measured at the peak maximum, $S_{x_{max}}$, to various experimental parameters:

$$S_{x_{max}} = \frac{AKM_x}{(u + f_m)t_R} \cdot \sqrt{\frac{N}{2\pi}} \quad (1)$$

where A is a proportionality constant to account for amplification factors, etc., K is the electron-capture coefficient, M_x is the number of moles of substance x, N is the number of column theoretical plates, u is the carrier gas flow-rate, t_R is the retention time of substance x and f_m is the flow-rate of the make-up gas. The detector response therefore depends on a number of experimental factors. The detector response is maximised for columns with a large number of theoretical plates operated at low carrier and make-up gas flow-rates, and with separation conditions optimised to minimise retention. The electron-capture coefficient, K, is a temperature dependent constant. Consequently, the detector response is also temperature dependent. The detector temperature can have a significant effect on the response. It is reported that a change in temperature of 100°C may result in variations in response of two- or three-orders of magnitude. A number of other papers identified temperature as a critical experimental parameter. Ševcík [40] recommends that the detector temperature is maintained within ± 0.1°C for quantitative analysis as does Henrich [15]. Poole and

Schuette [21] also note that the maximum ECD response to different compounds is temperature dependent. They report that a 100°C change in the detector temperature can lead to a change in response of 100- to 1000-fold. Uden [47] presents a plot of detector temperature vs. response for bromobenzene, benzaldehyde and nitrobenzene. The ECD response is reported as being highly temperature dependent. Peltonen [48] discusses the temperature optimisation of ECD systems in GC. Again, temperature optimisation is identified as a key factor affecting the ECD sensitivity. It is reported that the relative response of the detector can vary by more than two-orders of magnitude over the useful detector temperature range of 50°C to 350°C. The effect of temperature can be calculated by plotting the response of a standard solution against detector temperature. The ECD response to carbon disulphide at varying temperatures was studied and a plot is given. The detector was operated between 40°C and 260°C. Maximum sensitivity was obtained at a detector temperature of 40°C. As the temperature was increased the response decreased. Cigánek et al. [49] consider the influence of detector temperature, with reference to the responses of selected polychlorinated biphenyl congeners. It is reported that a 3% change in temperature can result in a 10% error in the evaluation of the electron-capture coefficient. The effect of temperature on nine congeners was investigated. In all case the temperature affected the response, but the nature of the effect depended on the individual congener. Plots are presented of response vs. temperature for each of the congeners.

Pool [46] identifies the flow-rate of the make-up case as another important parameter. Its influence on column efficiency, peak skewness and detector sensitivity is illustrated on two graphs. In the analysis of four pesticides an increase in the make-up gas flow from approximately 15 to 65 ml min^{-1} produced a decrease in the observed peak area. In the case of lindane the peak area decreased from 9 to 3. For aldrin the area decreased from 8 to 1.5. When the flow was increased from 65 to 75 ml min^{-1} the peak areas showed a small increase. Ševcík [40] and Henrich [15] highlight the carrier gas flow-rate as having an effect on the detector response. Constant flow control is recommended with capillary columns [15]. If pressure control is used, the flow-rate decreases as the temperature increases, for example during a temperature programmed analysis. However, this effect can be minimised by using a make-up gas.

2.2.2.4. Thermal conductivity detection

The ACOL text [12] identifies the temperature of the wire (i.e., the current flowing through it), the detector temperature and the carrier gas flow-rate as key experimental parameters affecting the thermal conductivity detection (TCD) response. Ševcík [40] identifies the key factors affecting the detector response as temperature and carrier gas flow-rate. Henrich [15] identifies control of the temperature of the detector block as critical if noise and drift are to be prevented. Temperature control is also identified as critical by Uden [47]. Temperature control to within 0.1°C and the absence of extraneous temperature gradients are cited as critical. IUPAC [39] note that TCD is very susceptible to environmental fluctuations and that a reference cell is essential for a stable baseline.

2.2.2.5. Thermionic detection

Thermionic detection (TID) is also referred to as nitrogen–phosphorus detection (NPD). The key factors affecting the response of this detector are identified as the hydrogen flow-rate to the detector and the magnitude of the heating current to the thermionic source [15]. These parameters affect both the sensitivity and specificity of the detector. A plot of the effect of the hydrogen flow-rate on the detector selectivity is presented. IUPAC [39] also identify the temperature of the alkali source and the flow-rate of hydrogen as key experimental factors.

2.2.2.6. Photoionisation detection

Adamia et al. [50] have investigated the effect of temperature on the photoionisation detection (PID) sensitivity. Chromatograms of a mixture of hydrocarbons dissolved in *n*-octane were obtained with the detector temperature in the range 48°C to 200°C. The peak height response for each component was plotted against detector temperature. It was found that in the temperature range 70°C to 160°C the responses of all the components decreased. For example, for hexane the peak height decreased from 120 mm to 65 mm.

In the range 150°C to 200°C the responses all remained approximately constant.

2.3. Factors affecting analyses by HPLC

Poole and Schuette [21] discuss a number of key factors affecting HPLC. The column temperature is reported as affecting sample solubility, solute diffusion and mobile phase viscosity such that the retention times decrease as temperature increases. Variability in retention volumes and peak widths observed in gradient elutions are considered to be due primarily to the limits of reproducibility of the mobile phase composition, flow-rate and column temperature. The most important of these are identified as the mobile phase composition and flow-rate. Random and or systematic deviations from pre-set mobile phase composition and flow-rate are caused by imperfect functioning of mechanical parts of the pump or electronic parts of the system.

A number of factors affecting HPLC analysis are discussed by Snyder et al. [51] The strength of the mobile phase affects the capacity factor, k', for each band. As the solvent strength (i.e., the percentage of the organic component for reversed-phase chromatography) increases the capacity factor decreases. An increase in the percentage of the organic solvent present of 10% typically results in the reduction of k' for each band by a factor of 2 to 3. Compared to manual preparation, on-line mixing of the mobile phase is reported as minimising problems associated with variations in mobile phase composition. However, if the mobile phase contains less than 10% of any one solvent, and especially if low flow-rates are being used, manual preparation can be more accurate. The selectivity of the mobile phase affects the separation factor α. α is also affected by the pH of the mobile phase, which can have a significant effect for samples containing acid or basic components; variations in the percentage of the organic solvent in the mobile phase; and the temperature of the column. Temperature also affects the column plate number, N. As the temperature increases so does N. The column also changes during use. Temperature changes are a common cause of varying retention, especially for ionic or ionisable compounds. Ideally the column should be thermostatted to ± 0.2°C. In ion-pair HPLC retention depends on the relative ionisation of each solute. This can change with temperature. However, when a sample compound is ionised (or non-ionised) to the extent of 95% or more, a moderate change in temperature (e.g., of 20°C) should have only a small effect on relative ionisation. Changes in band spacing as a result of temperature changes are most likely for compounds which have pK_a values within ± 1 unit of the mobile phase pH. In addition to the above, the column ages during use which can affect k', α and N. Disturbances in the column packing affect N, loss of the bonded phase affects k' and α, dissolution of the silica support affects N, and build-up of non-eluted material affects k' and N.

Jandera et al. [52] have considered the sources of error in gradient elution chromatography. If the sample size and the chromatographic system are appropriately chosen and sufficiently stable, the errors in retention volumes and peak widths are reported as being due primarily to the limits of precision of three chromatographic variables, namely, the mobile phase composition, the flow-rate and the temperature (with their relative importance decreasing in that order). In gradient elution the actual flow-rate and the profile of the gradient may deviate from the required pre-set values for a number of reasons. A solvent demixing effect occurs when the more efficient eluting component of the mobile phase is preferentially retained on the column, thus decreasing its content in the mobile phase. The gradient profile therefore deviates from its pre-set value. This effect is much more significant in adsorption than in reversed-phase chromatography, and increases with the difference in polarities between the components of the mobile phase. In addition, imperfect functioning of the mechanical parts of the pumps (plungers, valves, seals) or electronic parts of the system also causes variability.

Engelhardt and Siffrin [53] discuss system suitability tests plus procedures for evaluating the precision of pumps. By repeated analysis of a standard solution the repeatability of the injection system and the short term flow accuracy (during the elution of the peaks) can be estimated. At least six consecutive injections are recommended. The constancy of the retention time of the last eluting peak gives a measure of the long term flow accuracy. The average value of the peak areas for each component,

and their standard deviations, are a measure of the short term flow accuracy of the pump. This is a very important parameter as it is directly related to the accuracy of quantitative analysis when concentration sensitive detectors are used (as is the case for the majority of HPLC detectors). As far as the performance of the pump is concerned, the accuracy of the flow (i.e., how well the delivered volume corresponds to the set value) can easily be determined volumetrically. However, measuring the consistency of the flow-rate over a period of time is less straightforward – a method is proposed. Methods for estimating the linear and dynamic ranges, the noise and the wavelength accuracy of detectors are also described.

Grize et al. [54] discuss the effect of integration parameters on method development and validation in HPLC. In an earlier study, the importance of threshold, area reject and minimum peak width had been studied. It was found that the threshold (i.e., the minimum peak height detected by the integrator) was the most influential of the three. This parameter was therefore made the focus of this study. The effect of changing the threshold setting was monitored by recording the number of peaks detected in a complex mixture. As expected, the number of peaks detected decreased as the threshold value was increased (plots given). However, the change was not linear. The size of the threshold effect was also found to be clearly distinct from the repeatability. The effect of the threshold setting on robustness analysis of an HPLC procedure was also investigated. The parameters investigated in the robustness test were the amount of ion pair reagent in the mobile phase, the amount of methanol in the mobile phase, the pH of the mobile phase and the type of analytical column. The effect of these parameters was assessed using a full factorial design. Each of the chromatograms obtained was processed for each threshold value between -12 and 4 and the number of peaks recorded. It was found that the significance or otherwise of the chromatographic parameters varied with the threshold value. The authors conclude that the effects of column type and mobile phase composition depend critically on the value of the threshold parameter.

The precision of HPLC measurements at low signal to noise ratios is discussed by Pauls et al. [55]. Three aspects were considered: the variation in precision with peak size; a comparison of the precision of peak height and peak area measurements; and a comparison of the precision obtained with fixed and variable wavelength UV detectors. The study was based on the analysis, by a number of laboratories, of solutions of caffeine with concentrations in the range 1 to 0.05 $\mu g\ ml^{-1}$. At these low concentrations the precision of peak height measurements was found to be better than the precision of peak area measurements. A plot of relative reproducibility against concentration is presented. The relative reproducibility became worse as the concentration decreased. When the results obtained with fixed and variable-wavelength UV were compared, no significant difference in the precision of peak height measurements was observed. In contrast, the precision of peak area measurements obtained using fixed wavelength detectors was significantly better than that obtained using variable wavelength detectors. Over the concentration range studied no significant systematic bias in the measurements was observed.

In addition, Meyer [56] has published a detailed text on sources of error associated HPLC analysis.

2.3.1. Factors affecting retention times in HPLC

Grushka and Zamir present a discussion on precision in HPLC analysis [57]. The factors identified as affecting the precision of retention times are mobile phase flow-rate, column temperature, mobile phase composition and integration. Fluctuations in the flow-rate are caused by fluctuations in the pump operation. Manufacturers usually report the relative error in the flow-rate delivered by the pump; a typical value is given as 0.3%. The relative error in the retention time due to fluctuations in the flow-rate will therefore also be $\pm 0.3\%$. Small variations in the column temperature can have a significant effect on the retention time. In unthermostatted systems the variation in retention time due to temperature fluctuations will be greater than the variation due to flow-rate changes. If the variation in temperature is $\pm 3°C$ (at a nominal temperature of 25°C) the corresponding relative variation in retention time is estimated as 1.5%. However, if the temperature is controlled to $\pm 0.1°C$, the relative error in retention time is estimated as only 0.2%. It is concluded that if high precision in retention time measurements is

required, the temperature should be controlled to within ±0.05°C. As the capacity factor varies exponentially with changes in the mobile phase composition, a relatively small change in the mobile phase composition can have a large effect on the retention time. For isocratic elution it is estimated that the mobile phase composition typically varies by ±1%. This corresponds to relative errors in the retention time of between 0.4 and 0.7%. For gradient elution the reproducibility of the mobile phase composition from run-to-run is likely to be worse than for isocratic analysis. It is therefore to be expected that variations in retention time between runs will be larger. The integration system can also contribute to variation in the measurement of retention times, although the effect is unlikely to be large. For example, in the case of a digital integrator with a sampling rate of 100 ms, the time recorded for the peak maximum could be out by a maximum of ±0.05 s. Excessively noisy signals can also affect the recording of the retention times as the noise causes problems with the identification of peak maxima.

Scott [47,58,59] and Scott and Reese [60] also discuss the factors affecting retention time. Temperature is identified as key parameter and it is estimated that to control the retention time to ±1% it is necessary to control the temperature to ±0.35°C. To control the retention time to within 0.1% the temperature must be controlled to within ±0.04°C. A plot is given of the effect of column temperature on the retention of three solutes. The average decrease in retention volume as the temperature increased was 2.20% per °C. The effect of mobile phase composition on retention time is also discussed. It is estimated that for a 1% precision in retention time the mobile phase composition must be kept constant to within 0.1%. For 0.1% precision the mobile phase precision must therefore be 0.01%. A plot of the change in retention time with solvent composition is given for three solutes. The precision of the retention time in a typical HPLC analysis was investigated by recording the retention times for 12 replicate injections of a solution containing three components [47]. For a peak with a retention time of 6.283 min the standard deviation was 0.38 s (0.1% relative standard deviation; RSD), for a retention time of 8.119 min the standard deviation was 0.20 s (0.04% RSD) whilst for a retention time of 20.421 min the standard deviation was 0.46 s (0.04% RSD).

2.3.2. Factors affecting peak areas and heights in HPLC

Grushka and Zamir [57] consider the factors affecting peak area measurements. For concentration sensitive detectors, the peak area (A) is related to the detector response (S_c), the weight of solute injected (w) and the flow-rate (F) by the equation:

$$A = \frac{S_c w}{F} \tag{2}$$

Fluctuations in the flow-rate will therefore introduce variation into the measured areas. A variation in the flow-rate of 0.3% will lead to a corresponding variation in the peak areas. As mentioned earlier, the temperature of the column has an effect on the retention time. It will therefore also affect peak areas. The relative error in the area of a peak due to changes in temperature will be equivalent to those observed for the retention time. The effect of mobile phase composition on retention times was also discussed previously. Variations in retention time due to fluctuations in the mobile phase composition will lead to comparable variations in the measured peak area.

The performance of the integration system will contribute to variation in measured peak areas [59]. There are a number of possible sources of uncertainty, including noise and the identification of the beginning and end of peaks. The relative error in peak areas is inversely proportional to the signal-to-noise ratio. The higher the noise, the poorer the precision of the measured area. The relative error in the area due to noise is also proportional to the width of the peak and inversely proportional to the number of data points recorded across a peak. The decision by the integration system of when to start and stop the integration will obviously affect the recorded area. The error can be systematic if the integration parameters are set incorrectly so that integration begins or stops well inside or outside the peak. Variations in the identification of the beginning and end of peaks from run-to-run will contribute to the precision of the area measurements. An excessively noisy signal will adversely affect the ability of the

integrator to recognise the beginning and end of peaks.

Scott [47] discusses factors affecting quantitative analysis by LC. He identifies a major source of error in peak height and peak area measurements as detector noise due to flow-rate fluctuations. The precision of a typical HPLC analysis was investigated by recording peak heights and peak areas for 12 replicate injections of a solution containing three components. The first peak had a retention time of 6.283 min, a mean peak height of 1.937 with a standard deviation of 0.0465 (2.4% RSD) and a mean peak area of 0.633 with a standard deviation of 0.032 (5.1% RSD). The second peak had a retention time of 8.119 min, a mean peak height of 16.491 with a standard deviation of 0.121 (0.74% RSD) and a mean peak area of 7.486 with a standard deviation 0.072 (0.96% RSD). The third peak had a retention time of 20.42 min, a mean peak height of 81.574 with a standard deviation of 0.148 (0.18% RSD) and a mean peak area of 91.884 with a standard deviation of 0.0823 (0.090% RSD).

2.3.3. Factors affecting sample injection in HPLC

Problems of poor peak area reproducibility associated with using an autosampler are discussed by Dolan [61,62]. Several potential problems at the sample vial level are identified [61]. Obviously the sample must be homogeneous for good peak area reproducibility. Sample matrices containing high concentrations of salts can cause layering in the vials, as can poorly mixed samples that have been frozen. A sample vial that is too full can cause variable results if the seal is too tight. As the sample is withdrawn a slight vacuum can form making it more difficult to withdraw the sample. This can increase sample size variability. Poor sealing of the sample vial can cause problems if the sample solvent is sufficiently volatile. Evaporative loss of the solvent can cause the sample concentration to change between injections. General problems associated with autosampler operation are also discussed [62]. One of the sources of variability discussed is the syringe. When a 10-μl sample was injected with a 10-ml syringe in the autosampler the variation in peak areas across six injections was 12% (RSD). With a 100-μl syringe the RSD was 4%. When 50-μl injections were made the RSD was the same in both cases (approximately 1.5%). The following explanation is given. Whenever the syringe plunger is moved by the autosampler motor there is an inherent variability in the positioning of the plunger. This translates into variation in the volume delivered and hence in the observed peak areas. After injecting 50 μl, the positioning variability is small compared with the total movement of the plunger for both syringes. However, when 10 μl was injected, the positioning variability for the 10-ml syringe is large compared to the total movement of the plunger leading to the larger observed variability in peak areas. It is therefore important to select a syringe of appropriate volume otherwise the injection system could contribute significantly to the uncertainty in measured peak areas.

The relationship between sample volume and the chromatographic performance factors of detection sensitivity and resolution is discussed by Bakalyar et al. [63]. Columns of 1.0, 2.1 and 4.6 mm internal diameter were studied with sample volumes ranging from 0.4 μl to 2 ml depending on the column and the elution mode. The amount of performance loss, due to dispersion (band broadening) from the injector, depends on the magnitude of this dispersion relative to the magnitude of the dispersion caused by the column and other components in the system. The dispersion can be quantified using the peak variance, σ^2. The total peak variance, σ^2_{tot}, will have contributions from the variances due to the column, injector, tubing, detector and electronic time constant distortions, i.e., $\sigma^2_{tot} = \sigma^2_{col} + \sigma^2_{inj} + \sigma^2_{tub} + \sigma^2_{det} + \sigma^2_{time}$. The reasons for injector dispersion are discussed. Chromatograms illustrating the effect of different sample volumes on different diameter columns are presented and guidelines for selecting appropriate sample volumes are given.

Coburn [64] has investigated the true volumes of sample loops. The actual volumes of two nominally 500-μl loops were found to be almost 700 μl. The manufacturers' stated a tolerance of ±20% on the volumes of the loops.

2.3.4. Ruggedness testing of HPLC methods

A number of papers describing the ruggedness testing of HPLC procedures were identified. These give an insight into the significant sources of uncer-

tainty and also describe experimental designs which could be applied to other methods.

Vander Heyden et al. [65] describe ruggedness testing of the United States Pharmacopeia XXII HPLC method for the assay of tetracycline hydrochloride (TC) and its degradation products 4-epitetracycline (ETC), 4-epianhydrotetracycline (EATC), and anhydrotetracycline (ATC). Six experimental factors were examined using both Plackett–Burman and fractional factorial experimental designs. The experiments were carried out using both C_8 and C_{18} columns. The factors examined were the ionic strength of the mobile phase, the concentration of dimethylformamide (DMF) in the mobile phase, the pH of the mobile phase, the mobile phase flow-rate, the integration parameter signal-to-noise ratio and the age of the column. The latter was only evaluated for the C_8 column. The effects of changes in the parameters were monitored by recording the capacity factors, resolution between consecutive peaks, retention times and relative retention times with reference to ETC. In general, the same parameters were identified as significant regardless of the experimental design used. The ageing of the column was identified as having the greatest effect on the capacity factors. The amount of DMF in the mobile phase had a smaller influence but this was on the limit of significance for both the C_8 and the C_{18} columns. The other parameters did not have a significant effect on the capacity factors, except in the case of the effect of mobile phase pH on the capacity factor of TC on the C_{18} column. A higher pH value was found to increase the capacity factor. The same conclusions were drawn for the retention times as for the capacity factors. The only difference was that, as expected, the mobile phase flow-rate had a significant effect. Changing the flow-rate from 1.1 ml min^{-1} to 0.9 ml min^{-1} caused an increase in the retention times of approximately 20%. The largest influence on resolution was identified as the age of the column. The pH also had a significant effect on the resolution of EATC and TC on both the C_8 and C_{18} columns, and on TC and ATC on the C_8 column. The other parameters did not have a significant effect. The ageing of the column caused a significant decrease in the relative retentions of EATC, TC and ATC on the C_8 column. The pH had a significant effect on the relative retention of TC on both the C_8 and C_{18} columns although the effect was larger on the latter.

A ruggedness test of a gradient elution HPLC procedure for pharmaceutical analysis is described by Vander Heyden et al. [66] The method is used for the assay of a drug substance, DS, and its two known by-products, BP1 and BP2. The ruggedness test was performed using three versions of Plackett–Burman experimental designs. The parameters examined were the pH of the mobile phase, the volume of triethylamine (TEA) in the mobile phase, the column temperature, the slope of the elution gradient, the detection wavelength and the source of the column. Three C_8 columns were investigated. Two were from different batches supplied by the same manufacturer, whilst the third was from a different manufacturer. The effects of the different parameters on the retention time, the capacity factor, the peak asymmetry factor and the peak area and height for DS, and the resolution of DS and BP2, were investigated. In general, the same conclusions were drawn regardless of the experimental design used. All the factors except the detector wavelength showed a significant effect on retention time. Similar conclusions were drawn for the capacity factor. The chromatographic column was the only factor identified as having a significant effect on the peak asymmetry factor. The column also had a significant effect on the resolution of the DS and BP2 peaks. The peak area was significantly affected by the detector wavelength, whereas the peak height was significantly affected by the concentration of TEA in the mobile phase, the slope of the gradient elution, the detector wavelength and the chromatographic column.

Mulholland and Waterhouse [67] also discuss the ruggedness testing of HPLC procedures using Plackett–Burman experimental designs. The method studied was the determination of aspirin and salicylic acid by reversed-phase chromatography with UV–Vis detection. The parameters investigated were the concentration of acetonitrile in the mobile phase, the acid used for controlling the pH of the mobile phase, the flow-rate, the column temperature, the detector wavelength and the detector response time. The chromatographic parameters monitored to evaluate the effects of changes in the method conditions were the concentration of the analytes calculated from

peak areas and peak heights, retention time, peak areas and heights, number of theoretical plates (N), resolution and peak symmetry. Changing the acetonitrile concentration reduced the retention time for both components as the solvent strength increased. The peak heights increased accordingly. The resolution was slightly affected but not enough to change the concentration calculations. Changing the acid used to control the mobile phase pH caused significant changes in retention, resolution, peak heights and peak areas. The spectra for both aspirin and salicylic acid also showed bathochromic shifts. Increasing the column temperature reduced retention and N. This caused a decrease in resolution and an increase in peak heights. An increase in the flow-rate reduced retention and peak areas. Changing the detector wavelength produced the most dramatic effects of all the parameters tested. Although the peak areas were changed by up to 100%, the concentration values remained the same. However, the detection limits would be seriously affected. In the case of aspirin, decreasing the detector response time resulted in an increase in peak height.

The validation of a method for the assay and purity evaluation of a novel drug compound by reversed-phase HPLC is discussed by Colgan et al. [68]. The effect of the mobile phase pH on the retention of the compound and three potential impurities was investigated. The biggest effect was observed for the compound, where increasing the pH from 4 to 5 increased k' from approximately 4.2 to 12. Additional studies were undertaken to investigate the effect on the retention of the compound of varying the mobile phase percentages of methanol and acetonitrile. It was found that small changes in the concentration of acetonitrile had a much larger effect on retention than similar changes in the methanol content. For example, increasing the percentage of acetonitrile from 2 to 3% (whilst holding the methanol concentration constant) reduced k' from approximately 45 to 25. The study also investigated the reproducibility of the method. The precision of injection for each of the possible impurities was also studied using a solution of the compounds at their target levels. The target concentrations were 0.002 mg ml^{-1} for two of the compounds and 0.005 mg ml^{-1} for the third. The RSDs of the peak areas for replicate injections were 0.5% at 0.002 mg ml^{-1} and 0.6% at 0.005 mg ml^{-1}. The number of injections and whether they were intra- or inter-batch is not reported.

Factors affecting the HPLC method for the assay of monensin and narasin have been investigated by Coleman et al. [69] The method involves reversed-phase HPLC and post-column derivatisation with a vanillin reagent (methanol–H_2SO_4–vanillin, 95:2:3, v/v/w) followed by UV detection. The chromatographic parameters evaluated were the water and methanol content of the mobile phase, the temperature of the reaction chamber used for the derivatisation reaction, and the flow-rates delivered by the mobile phase and vanillin reagent pumps. Each of the parameters was evaluated separately. The results obtained under each set of conditions were compared with the resolution, retention time and tailing factor acceptance limits set for the method. It was found that if the reaction temperature was reduced from 98°C to 95°C or 90°C, the control parameters were met but the peak areas decreased (especially for narasin), resulting in reduced sensitivity. An increase in the methanol content of the mobile phase with a decrease in the water content resulted in decreased retention time, increased peak areas and a decreased resolution factor. A decrease in the methanol content with an increase in the water content increased the retention time, peak width and resolution factor but decreased the peak area. The retention time, peak width and peak areas were all affected by variations in the flow-rates of the mobile phase and vanillin reagent.

2.3.5. Factors affecting detection in HPLC

2.3.5.1. Refractive index detection

Poole and Schuette [21] discuss the factors affecting the performance of refractive index (RI) detectors. The background noise of the detector is influenced by changes in solvent composition, pressure and temperature. A concentration of 1 ppm corresponds to a refractive index change of approximately 10^{-7} RI units. This could be exceeded by fluctuations in the mobile phase composition. Temperature can also have a dramatic effect on the detector response. It is reported that a 1°C change in temperature can cause a change of $6 \cdot 10^{-4}$ RI units. RI detectors are reported as being sensitive to the

mobile phase flow-rate and temperature fluctuations by Ogan [70].

Munk [71] also considers the main parameters that affect the response of the RI detector. As identified in other papers, the detector is very sensitive to changes in temperature and pressure. These parameters change the refractive index by changing the density of the liquid. A table showing the temperature dependence of the RI for seven solvents is presented. On average, the RI of organic solvents changes by 0.00045 RI units per °C. The RI of water changes by approximately 0.00009 units per °C. To obtain a baseline which is stable to $4.5 \cdot 10^{-8}$ RI units the temperature of the flow cell must therefore be controlled to within 0.0001°C for organic solvents and 0.0005°C for water. A table illustrating the rate of change of RI with pressure for the same seven solvents is also given. For water, the change in RI is $1.7 \cdot 10^{-5}$ atm^{-1} and for organic solvents (except glycerol) the change is, on average, $5.43 \cdot 10^{-5}$ atm^{-1} (1 atm = 101 325 Pa). It can be seen that temperature has a much greater effect on refractive index than pressure. A 1°C change in temperature has the same effect on the RI of water as a 5.2 atm change in pressure. However, it is reported that even the relatively small influence of pressure can limit baseline stability.

The detector is also very sensitive to changes in the mobile phase composition [71]. Such changes can be due to non-uniformly mixed solvents or changing levels of solvent contamination during the course of an analysis. The performance of premixed vs. pump mixed mobile phases is compared. HPLC pumps typically have a specified composition accuracy of approximately 1%. Even the small non-uniformity in solvent composition delivered by such pumps can limit the performance of an RI detector. A typical change in baseline due to pump variability is quoted as $1 \cdot 10^{-5}$ RI units. Premixed mobile phases do not suffer from this problem. This is therefore the preferred method of mobile phase preparation when using RI detection.

The effect of column temperature on the baseline response of the detector is also considered. For a change in column temperature of 5.2°C a change in baseline of $6.6 \cdot 10^{-6}$ was observed. The amount of gas dissolved in the mobile phase also affects the detector response. A change in the baseline of $2.7 \cdot 10^{-6}$ RI units was observed for degassed water compared to air saturated water. In the case of methanol the observed change was $25.9 \cdot 10^{-6}$. Changes in the dissolved gas content of the mobile phase during a run usually show up as baseline drift.

2.3.5.2. Fluorescence detection

Poole and Schuette [21] discuss the effect of mobile phase composition on the response of fluorescence detectors. In the worst cases, variations in the consistency with which the pump mixes and delivers the mobile phase may influence detector sensitivity and reproducibility more than fluctuations in the detector operating system. The pH of the mobile phase is an important factor. Both the emission wavelength and fluorescence intensity of ionisable aromatic compounds are critically dependent on pH. The responses of many compounds to the detector also show a marked temperature dependence, causing a decrease in intensity of 1–2% per °C. At high concentrations fluorescence emission becomes non-linear. High fluorescence intensity may overload the photomultiplier tube which then returns slowly to its normal operating conditions and misrepresents the actual fluorescence signal until it has restabilised. A detailed discussion of fluorescence detection in HPLC, with over 600 references, is presented by Lingeman et al. [72]. Factors influencing fluorescence are discussed. Solvent composition (including oxygen content) and pH are identified as having an effect on the fluorescence intensity, as is temperature. For most compounds, as mentioned above, a 1 to 2% decrease in fluorescence with a 1°C increase in temperature can be expected. However in some cases the decrease can be as high as 10%.

2.3.5.3. UV–visible detection

Esquivel [73] proposes a straightforward technique for testing the wavelength accuracy of UV–visible detectors. The procedure was applied to a number of commercial detectors and involves recording the maximum absorbancies for solutions of erbium perchlorate and terbium perchlorate. The maxima observed for these solutions cover a wide range of wavelengths (from 218.5 nm to 652.5 nm). The solutions were used to check the wavelength accura-

cy of 11 commercial detectors. The errors observed depended on the wavelength being tested. Errors greater than the manufacturers' specifications were observed for five of the detectors. In general the errors were found to increase in the higher wavelength region.

Brown et al. [74] have investigated the effect of solvent degassing on the stability of a UV detector. Gases dissolved in the mobile phase can affect the UV absorption, causing baseline drift and random noise. The presence of oxygen in the mobile phase was found to cause high background readings. The effect was particularly noticeable at wavelengths below 230 nm. However, replacing the oxygen with helium or nitrogen reduced the absorbance by half. It was found that the absorbance of a methanol mobile phase was critically dependent on its dissolved oxygen content. Any change in oxygen content will cause detector baseline drifts. For example, at 210 nm a 1% change in dissolved oxygen caused a $4 \cdot 10^{-3}$ AU change in absorbance for methanol. The effect of dissolved air in the mobile phase and sample solutions is also discussed by Egi and Ueyanagi [75]. In the case of UV absorbance detection, dissolved oxygen in the sample can cause ghost peaks. The absorbance spectra of aerated and degassed methanol are compared. Degassed methanol exhibits a lower absorbance but the difference depends on the wavelength. At 210 nm the degassed and aerated solvents differ by more than 300 mAU but at 254 nm the difference is only 10 mAU.

3. Conclusions

The aim of this study was to identify the main sources of measurement uncertainty associated with analyses by gas and liquid chromatography. A search of the literature on uncertainty and chromatography yielded relatively little information. However, there is a large amount of published information, in both books and journals, on the development, optimisation and validation of chromatographic methods. This provided the main source of information on the key parameters controlling the performance of various aspects of chromatographic systems. In addition to general textbooks on chromatography, texts specifically covering detection, sample introduction and integration were identified.

In a number of papers estimates of the effects of changes in various parameters on, for example peak heights and areas, and retention times have been given. The effect of these variations on a quantitative analysis will depend on the method of calibration used and whether the effect is of a similar magnitude for both samples and standards. Variations in retention can cause problems in quantitative analysis if it leads to poor resolution or mis-identification of peaks. A number of ruggedness studies on chromatographic procedures have been published. These identify the critical parameters for particular methods and also give examples of experimental designs which can be used to identify the key parameters for other methods. There was a substantial amount of information available on the parameters affecting the performance of common GC detection methods such as FID, FPD and ECD. Information on HPLC detection methods was more limited. Injection systems were identified as a major source of uncertainty for GC analysis. This is due in part to the problems associated with reproducibly injecting the small sample volumes required for capillary GC. A number of papers addressing the problems associated with split/splitless injection were identified.

Individual components of HPLC systems (e.g., injection and detection systems) appear to have been less well studied than for GC. With the exception of refractive index detectors, compared to GC there was relatively little detailed information on the key parameters controlling these components. However, there was a substantial amount of information available on the factors affecting the main ''outputs'' of HPLC analysis, namely peak areas, peak heights and retention times. In addition, a number of published ruggedness tests of HPLC procedures (mainly in the area of pharmaceuticals analysis) were identified.

The information provided in this review should prove a valuable starting point for the analyst faced with calculating an uncertainty budget for a chromatographic procedure. The various sections give guidance as to the areas which should be considered in an uncertainty evaluation. In some cases estimates of the magnitude of the uncertainty components are given thus saving the analyst from having to evaluate them separately.

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