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Experimental studies of uncertainties associated with chromatographic techniques

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Abstract

The paper describes experiments for the evaluation of uncertainties associated with a number of chromatographic parameters. Studies of the analysis of vitamins by HPLC illustrate the estimation of the uncertainties associated with experimental ''input'' parameters such as the detector wavelength, column temperature and mobile phase flow-rate. Experimental design techniques, which allow the efficient study a number of parameters simultaneously, are described. Multiple linear regression was used to fit response surfaces to the data. The resulting equations were used in the estimation of the uncertainties. Three approaches to uncertainty calculation were compared – Kragten's spreadsheet, symmetric spreadsheet and algebraic differentiation. In cases where non-linearity in the model was significant, agreement between the uncertainty estimates was poor as the spreadsheet approaches do not include second-order uncertainty terms.

Keywords: Uncertainty; Experimental design; Response surface fitting; Central composite design; Regression analysis; Oils; Food analysis; Tocopherols

1. Introduction

The estimation of uncertainties in chemical measurement is considered an important topic and has generated a significant level of interest and discussion [1–5]. It is generally acknowledged that the fitness for purpose of analytical results cannot be assessed without some estimate of the measurement uncertainty to compare with the confidence required. The *Guide to the Expression of Uncertainty in Measurement* (GUM) published by the ISO [6], establishes general rules for evaluating and expressing uncertainty. The guide has been interpreted for analytical chemistry by EURACHEM [7,8]. The approach described in the GUM requires the identification of all possible sources of uncertainty associated with the procedure; the estimation of their magnitude from either experimental or published data; and the combination of these individual uncertainties to give standard and expanded uncertainties for the procedure as a whole. However, the GUM principles are significantly different from the methods currently used in analytical chemistry for estimating uncertainty [4,5,9]. These generally make use of ''whole method'' performance parameters such as precision and recovery, obtained during laboratory validation studies. In much of our previous work on uncertainty estimation for analytical

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methods, we have focused on the use of these performance parameters as the basis of sound uncertainty estimates [10–13]. The advantage of this approach is that it generally allows a rapid evaluation of the uncertainty, often from existing validation or quality control data [14,15]. The main disadvantage of such an approach is that it gives little insight as to where the major sources of uncertainty lie. If the uncertainty is acceptable for the intended use of the method, this will not be a problem. However, if the initial uncertainty estimate requires refinement, or the method needs improvement, the analyst will need to identify the major sources of uncertainty separately. In such cases separate studies of the individual stages of the method will be required. In addition, even with carefully planned precision and recovery experiments, separate evaluation of parameters not adequately covered by these studies may be required to complete the uncertainty budget. There is therefore a need for information on the magnitudes of the uncertainties associated with various stages of analytical methods and where none exists, experimental approaches which can be used in their evaluation are required.

This paper describes two experimental studies undertaken to investigate the uncertainties associated with: (1) the effect of variations in the detector excitation/emission wavelengths on the quantification of α-tocopherol by high-performance liquid chromatography (HPLC) with fluorescence detection; (2) the effect of variations in HPLC operating conditions (flow-rate, column temperature, mobile phase composition) on the determination of all-*trans*-retinol.

In both cases the uncertainties were studied by observing the effect of changes to experimental parameters on output parameters such as peak areas and heights. The experimental parameters were varied about their normal method settings. This variability was significantly greater than that which would be expected during the usual operation of the method, to allow an empirical relationship (e.g., a quadratic function) to be established between the experimental and output parameters. These relationships were then used in the evaluation of the uncertainties. Response surface modelling was used in all cases as it was suspected that there may be some non-linearity in the relationships. It was therefore necessary to evaluate the significance of higher-order terms in the models. In all cases the uncertainties were modelled in absolute units (i.e., peak area units, concentration units, etc). The uncertainties would therefore be included in the overall uncertainty budget for the method as uncertainties associated with a particular parameter in the calculation of the final result (e.g., a peak area) or as a direct effect on the final analytical result.

The paper describes the experimental designs used for the experimental studies, and the various approaches used to estimated the uncertainties.

2. Experimental

2.1. Study 1: The effect of variations in detector excitation and emission wavelengths on the quantification of α-tocopherol

The study used a sample of olive oil containing approximately 360 mg kg^{-1} α-tocopherol. The sample was prepared by dissolving 2.01 g oil in hexane (Rathburn, Walkerburn, UK) and diluting to a volume of 100 ml. The analyses used a normal-phase HPLC system [Jasco PU-1580 pump, Jasco (UK), Great Dunmow, UK] fitted with a 25 cm×4.6 mm I.D. stainless steel column packed with 5 μm LiChrosorb Si60 (Jones Chromatography, Hengoed, UK), maintained at 25°C (Jasco CO-965 column oven). The mobile phase was hexane–propan-2-ol (Rathburn) (99.5:0.5, v/v), with a flow-rate of 1.5 ml min^{-1}. The system was calibrated using a single standard, prepared in the mobile phase, with an α-tocopherol (Merck, Poole, UK) concentration of 7.1 μg ml^{-1}. The sample and standard were injected using an autosampler (Jasco AS 1555) fitted with a 50-μl injection loop. The detector was a Jasco FP-1520 fluorescence detector.

The normal detector conditions for the quantification of α-tocopherol are an excitation wavelength of 290 nm and an emission wavelength of 330 nm. The effect on the determination of α-tocopherol of variations in the excitation/emission wavelengths was investigated using a central composite experimental design (Fig. 1) [16]. The design required nine experiments. In addition, a further five replicates

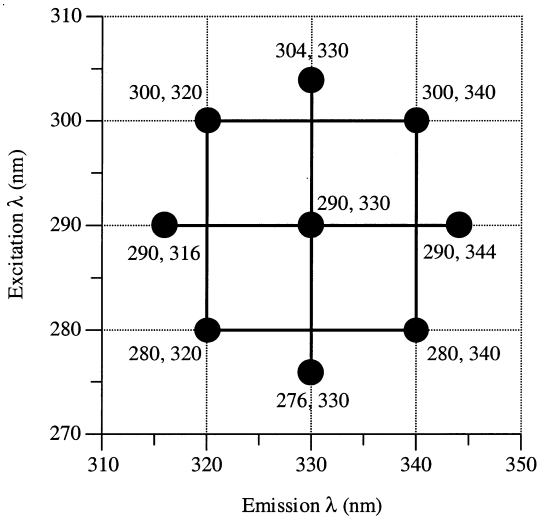

Fig. 1. Central composite experimental design for the study of the effect of variations in detector wavelengths on the determination of α-tocopherol.

were carried out under the normal detector conditions. The first and last experiments in the study were carried out under normal detector conditions. The order of the remaining experiments was randomised to eliminate the effect of any drift in the HPLC system. The sample and standard were analysed in duplicate under each set of detector conditions. The experiments were carried out in a single batch of analyses which took approximately 24 h. During this time, other HPLC parameters were held constant. Peak areas and peak heights were recorded for both the sample and standard for each experiment. The concentration of α-tocopherol in the sample was calculated for each experiment using peak area and peak height measurements from the standard run under the same conditions.

2.2. Study 2: The effect of variations in HPLC conditions on the determination of all-trans-retinol

The study used a sample of cod liver oil containing approximately 700 mg kg^{-1} of all-*trans*-retinol. The sample (4.87 g) was prepared by saponification with ethanolic potassium hydroxide (Rathburn) solution followed by extraction into light petroleum (Rathburn) [17]. The extract was then evaporated to dryness and the residue dissolved in methanol (Rathburn) to a final volume of 40 ml. The all-*trans*-retinol was quantified using a reversed-phase HPLC system (Shimadzu LC-10AD solvent delivery module, Shimadzu, Milton Keynes, UK) fitted with a 25 cm×4.6 mm I.D. stainless steel column packed with Zorbax 5 μm ODS (Jones Chromatography), and connected to a UV–visible detector (Shimadzu SPD 6AV) set at 325 nm. The injection volume was 100 μl, delivered by a Shimadzu SIL-10A automatic sample injector. Calibration was by means of a single standard, prepared in methanol, with an all-*trans*-retinol (Sigma–Aldrich, Poole, UK) concentration of 1.5 μg ml^{-1}. The column temperature was controlled via a Shimadzu CT0-10A column oven.

The effect of varying the mobile phase flow-rate, mobile phase composition and column temperature was investigated using a three-dimensional central composite experimental design (Fig. 2). The normal method conditions are a flow-rate of 1 ml min^{-1}, a mobile phase of methanol–water (90:10, v/v) and a column temperature of 35°C. The experimental design required 15 experiments. In addition, a further five replicates were carried out under the normal method conditions. Due to the equilibration time required after each change of mobile phase composition and/or column temperature, it was not possible to fully randomise the order of the experiments as this would have led to a prohibitively long run time. As a compromise, the experiments were arranged to minimise the number of changes in mobile phase and column temperature, whilst ensuring that the various sets of conditions were distributed evenly across the 20 experiments. The sample and standard were analysed under each set of experimental conditions. The experiments were carried out in a single batch of analyses, which took approximately 26 h. For each experiment, peak areas and peak heights were recorded for all-*trans*-retinol in both the sample and standard. In each case, the concentration of all-*trans*-retinol in the sample was calculated using peak area and peak height measurements from the standard run under the same conditions.

2.3. Approaches to measurement uncertainty estimation

Three approaches to estimating the measurement

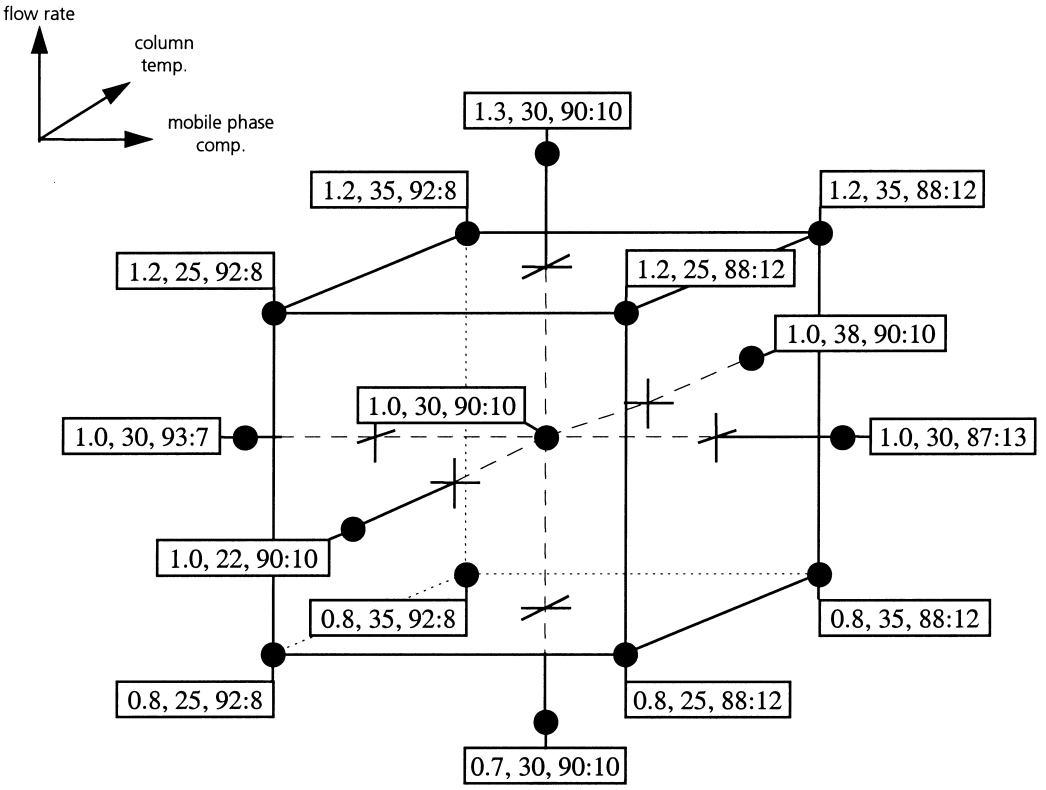

Fig. 2. Central composite experimental design for the study of the effect of variations in HPLC parameters on the determination of retinols.

uncertainty in the measured responses (i.e., peak area, peak height or concentration) were used. In all cases, the first stage was to establish the relationship between the response and the experimental parameters. Multiple linear regression (MLR) was used to fit a quadratic function of the general form:

$$y = B_0 + \sum_i B_i x_i + \sum_i \sum_{j \geq i} B_{ij} x_i x_j \quad (1)$$

where y is the response, B_0, B_i and B_{ij} are constants, and x_i and x_j represent the values of the experimental parameters. The constants were calculated using the Statistica software package [v5.1, StatSoft (1996), Tulsa, OK, USA]. Once the relationship between the response and the experimental parameters had been established, three approaches to calculating the uncertainty were used: (1) Kragten spreadsheet method; (2) symmetric spreadsheet method; (3) algebraic differentiation.

2.3.1. Kragten spreadsheet method

The uncertainty in the response y, due to uncertainties in the input parameters $x_1 \ldots x_n$ was calculated initially using the spreadsheet method described by Kragten [7,18].

2.3.2. Symmetric spreadsheet method

The symmetric spreadsheet estimates $\partial y / \partial x_i u(x_i)$ from:

$$\frac{\partial y}{\partial x_i} u(x_i) \approx \frac{y[x_i + u(x_i)] - y[x_i - u(x_i)]}{2} \quad (2)$$

which is based on the usual numerical estimate of $\partial y / \partial x_i$.

2.3.3. Algebraic differentiation

For comparison with the spreadsheet approaches, which rely on certain approximations, the uncertainties were also calculated using the formal ISO

approach [6]. When the non-linearity in the relationship between y and the input parameters x_i is significant, an additional term must be included in the expression for the uncertainty in y, $u(y)$. This is given by the second- and third-order differentials in Eq. (3). The recommended uncertainty propagation equation based on the Taylor expansion for independent variables is:

$$u(y)^2 = \sum_i \left(\frac{\partial y}{\partial x_i}\right)^2 \cdot u(x_i)^2 + \sum_{i=1}\sum_{j=1} \left[\frac{1}{2}\cdot\left(\frac{\partial^2 y}{\partial x_i \partial x_j}\right)^2 + \frac{\partial y}{\partial x_i}\cdot\frac{\partial^3 y}{\partial x_i \partial x_j^2}\right] \cdot u(x_i)^2 u(x_j)^2 \quad (3)$$

Evaluation of the uncertainty requires differentiation of Eq. (1):

$$\frac{\partial y}{\partial x_i} = B_i + 2B_{ii}x_i + \sum_{j>i} B_{ij}x_j \quad (4a)$$

$$\frac{\partial^2 y}{\partial x_i \partial x_i} = \frac{\partial^2 y}{\partial x_i^2} = 2B_{ii} \quad (4b)$$

$$\frac{\partial^2 y}{\partial x_i \partial x_j} = B_{ij} \quad (4c)$$

$$\frac{\partial^3 y}{\partial x_i \partial x_j^2} = 0 \quad (4d)$$

Note that centring on the experimental domain would lead to some simplification, since the gradient at the centre point is then equal to the first coefficient for the centred model; in many cases this renders the higher-order terms unnecessary. Thus, centring can be recommended where the dependence is approximately linear in the region of interest, even where the second-order term is significant. The general treatment is shown here, first, because it does not assume approximate linearity in the experimental region; second, because in practice modelling is generally done with software, and centring the data first adds an operation which does not affect the outcome; and third, the equations given can be applied whether the model is centred or not – only the coefficients change.

3. Results

3.1. Study 1

MLR was used to fit Eq. (5) to the peak area and peak height data obtained for α-tocopherol in both the sample and standard:

$$y = B_0 + B_1x_1 + B_2x_2 + B_{11}x_1^2 + B_{22}x_2^2 + B_{12}x_1x_2 \quad (5)$$

where x_1 and x_2 represent the excitation and emission wavelengths, respectively. All the terms, except for B_{12}, were significant at the 95% confidence level. The model was therefore refitted excluding that term. As an example, the relevant equations and their correlation coefficients, r^2, for the peak area data are given in Table 1. The fit of the peak area and peak height data to the equations was good, with r^2 values generally greater than 0.90. The relationship between the peak area of α-tocopherol in the sample and the detector wavelengths is illustrated in Fig. 3. When the concentration data were analysed, the fit of Eq. (5) was poor ($r^2 \approx 0.25$). This is because the changes in magnitude of the peak areas (or heights) were broadly similar for the standards and samples, and in the same direction. The changes in peak area therefore cancel, so that the final result (in terms of analyte concentration) is not significantly affected by changes in the wavelengths.

To calculate the uncertainty in peak areas and heights due to changes in detector settings, an estimate of the uncertainty in the detector wavelengths is required. The manufacturer of the detector quoted a wavelength accuracy of ±2 nm and a repeatability of ±0.3 nm. The accuracy estimate was assumed to be a rectangular distribution, and was converted to a standard uncertainty by dividing by $\sqrt{3}$ [7]. This was combined with the repeatability estimate (assumed to be a standard deviation) to give a standard uncertainty in the excitation and emission wavelengths of 1.19 nm (approximately 0.4%). Note that this uncertainty relates to the possible variation across instruments; in normal practice the sample and standard are measured on the same instrument. This correlation is dealt with separately below. The uncertainties in the peak areas and heights recorded for the sample and standard solutions, due to varia-

Table 1
Results from study 1 – uncertainties associated with changes in excitation and emission wavelengths

	Value x_i	Uncertainty $u(x_i)$	Numerical		Algebraic		
			Kragten spreadsheet	Symmetric spreadsheet	Linear $\frac{\partial y}{\partial x_i}u(x_i)$	Combined non-linear	Combined algebraic[a]
Sample peak area							
Model: $y = -7.2787 \cdot 10^6 + 29\,722x_1 + 18\,309x_2 - 51.357x_1^2 - 27.935x_2^2$, $r^2 = 0.962$							
Excitation wavelength (x_1) (nm)	290	1.19	−150	−77.4	−77.4	−103	129
Emission wavelength (x_2) (nm)	330	1.19	−192	−152	−152	−55.9	162
Response y[b]	21 530		**244**[c]	**171**	**171**	**117**	**207**
Standard peak area							
Model: $y = -6.3306 \cdot 10^6 + 27\,606x_1 + 14\,402x_2 - 47.693x_1^2 - 22.005x_2^2$, $r^2 = 0.906$							
Excitation wavelength (x_1) (nm)	290	1.19	−134	−66.1	−66.2	−95.5	116
Emission wavelength (x_2) (nm)	330	1.19	−175	−144	−144	−44.1	150
Response y	20 803		**220**	**158**	**158**	**105**	**190**

[a] The ''combined algebraic'' column shows the combination of first- and higher-order algebraic terms.
[b] Value of the response under normal operating conditions, calculated from MLR equations.
[c] The values in bold show the combination of excitation and emission wavelength effects.

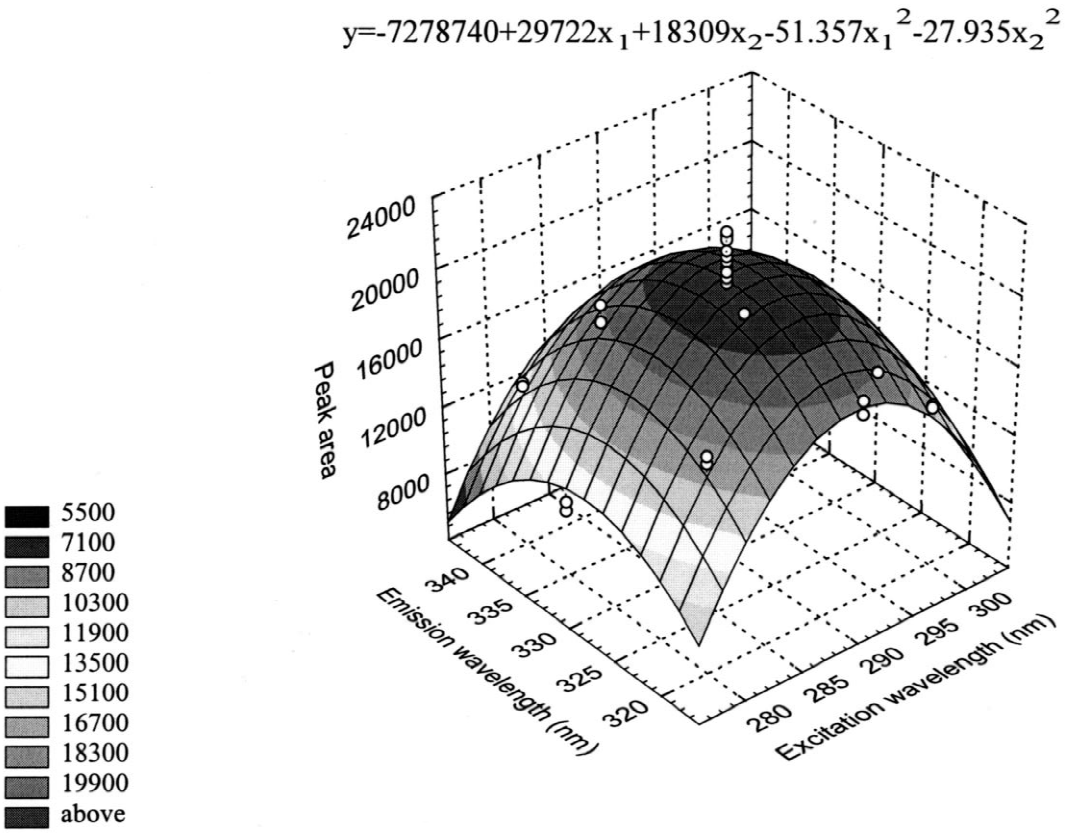

Fig. 3. Illustration of the relationship between the peak area of α-tocopherol in the sample solution and the detector wavelengths.

tions in the detector wavelengths, were calculated by the three methods described previously using the regression constants calculated from MLR and the estimated uncertainty in the detector settings. The results for the standard and sample peak area measurements are presented in Table 1.

3.2. Study 2

MLR was used to fit Eq. (6) to the peak area and peak height data obtained for all-*trans*-retinol in both the sample and the standard:

$$y = B_0 + B_1x_1 + B_2x_2 + B_3x_3 + B_{11}x_1^2 + B_{22}x_2^2 + B_{33}x_3^2 + B_{12}x_1x_2 + B_{13}x_1x_3 + B_{23}x_2x_3 \quad (6)$$

where x_1, x_2 and x_3 represent the flow-rate, mobile phase composition (expressed as the percentage of methanol in the mobile phase) and column temperature, respectively. In each case, a number of the constants were not statistically significant at the 95% confidence level. The equations were therefore refitted until only the statistically significant constants remained. The resulting equations for the peak area data, and their correlation coefficients, are given in Table 2.

The uncertainties in experimental parameters studied were estimated from the manufacturers' specifications for the equipment used. Where statements of accuracy were given, a rectangular distribution was assumed for conversion to standard uncertainties [7]. Where a precision value was quoted, it was assumed to be a standard deviation. The accuracy of the flow-rate was quoted as ±2% with a precision of ±0.3%. Combining the two contributions gave an uncertainty in a flow-rate 1 ml min^{-1} of 0.0119 ml min^{-1}. The accuracy of the mixing of the mobile phase components was quoted as ±1%. This corresponds to an uncertainty in the amount of water in the mobile phase of 0.0577, for a mobile phase containing methanol–water (90:10, v/v). The accuracy of the column temperature control was given as ±0.1°C, which corresponds to a standard uncertainty of 0.0577°C. Using these uncertainty estimates and the calculated MLR constants, uncertainties were calculated for the various responses using the approaches discussed previously. The results for the peak area data are presented in Table 2.

Table 2
Results from study 2 – uncertainties associated with changes in chromatographic conditions

	Value x_i	Uncertainty $u(x_i)$	Numerical		Algebraic		
			Kragten spreadsheet	Symmetric spreadsheet	Linear $\frac{\partial y}{\partial x_i}u(x_i)$	Combined non-linear	Combined algebraic[a]
Sample peak area							
Model: $y = 6.3924\cdot10^6 - 7.6192\cdot10^6x_1 + 98\,999x_2 - 15\,722x_3 + 2.8318\cdot10^6x_1^2$, $r^2 = 0.920$							
Flow-rate (x_1) (ml min^{-1})	1	0.0119	−22 871	−23 272	−23 272	567	23 279
Mobile phase composition (x_2) (water, %, v/v)	10	0.0577	5712	5712	5712	–[b]	5712
Column temperature (x_3) (°C)	30	0.0577	−907	−907	−907	–	907
Response y[c]	2 123 299		**23 591**[d]	**23 980**	**23 980**	**567**	**23 987**
Standard peak area							
Model: $y = 1.27271\cdot10^5 - 1.46156\cdot10^5x_1 + 54\,713x_1^2$, $r^2 = 0.958$							
Flow-rate (x_1) (ml min^{-1})	1	0.0119	−429	−437	−437	11.0	437
Mobile phase composition (x_2) (water, %, v/v)	10	0.0577	–	–	–	–	
Column temperature (x_3) (°C)	30	0.0577	–	–	–	–	
Response y	35 828		**429**	**437**	**437**	**11.0**	**437**

[a] The ''combined algebraic'' column shows the combination of first- and higher-order algebraic terms.
[b] Indicates that the term was insignificant in the MLR.
[c] Value of the response under normal operating conditions, calculated from MLR equations.
[d] Values in bold show the combination of flow-rate, mobile phase and column temperature effects.

4. Discussion

In study 1, the uncertainty estimates obtained by the different approaches did not agree. The symmetric spreadsheet gave, as expected, uncertainties in close agreement with the first-order uncertainty term calculated via differentiation. The Kragten method gave uncertainties greater than the total uncertainty calculated by differentiation. The variability of uncertainty estimates from one estimation method to another arises from the significant non-linearity present. Neither spreadsheet includes the second-order uncertainty terms. The model used here and illustrated in Fig. 3, provides an extreme example of this behaviour if optimal (maximum peak height/area) settings are chosen for the emission and excitation wavelengths. The setting is at the maximum of a parabolic model; at this point, the partial differentials $\partial y/\partial x_i$ are zero. First-order uncertainty estimation then yields a zero uncertainty estimate. Allowance for non-linearity is essential in these circumstances.

A secondary issue is that the errors induced by the non-linear terms are all negative. The result is that the mean error in peak height/area across the input uncertainty range is not zero, but significantly negative; the uncertainty has introduced a bias. Formally, the bias should be calculated and corrected for, and its uncertainty (which is smaller than the uncertainty estimates calculated so far) should replace the estimates shown above. Using the method described by Ellison et al. [19] we obtained a bias in the area recorded for the standard of -68 and -31 for the excitation and emission wavelengths, respectively. These estimates are, in this instance, significant when compared to the uncertainty contributions calculated for the excitation and emission wavelengths. However, it will be seen that they remain insignificant when compared to the repeatability.

Similar issues regarding bias arise, in principle, where the optimum is at zero gradient but at a saddle point (i.e., where the second-order derivatives for different variables have different signs). However, inspection of the dispersion of possible results round a typical saddle point shows that the problem is much reduced; deviations in y are expected in both directions, making a negligible bias more likely and leading back to the relatively simple symmetric treatment of Eq. (3) and Eq. (5).

The manufacturer's specification for the detector indicated uncertainties of approximately 1.19 nm in the excitation and emission wavelengths. This resulted in uncertainties in the peak areas and heights of approximately 0.9% of the value obtained under normal method conditions, for both the standard and the sample. These uncertainties are substantially smaller than the precision observed for the measurements made under the normal detector conditions. For example, the uncertainty calculated for the sample peak area was 207 peak area units (obtained via algebraic differentiation) whilst the standard deviation of the 12 peak areas recorded for the sample under the normal method conditions was 1000 (4.7% RSD). Unless the uncertainty associated with the detector wavelengths has been seriously underestimated, it appears that the main sources of variability must arise from sources other than the wavelength settings (e.g., variability in HPLC conditions, peak integration).

In study 2, the agreement between the uncertainty estimates obtained from the three techniques was good. This is because the effect of non-linearity in the model is much less significant than in the case of the detector study. In many cases the non-linear terms in the original quadratic fits of the data were not significant. They were therefore removed and the data refitted (Table 2). Where non-linear terms were retained, their contributions to the measurement uncertainty, when calculated via differentiation, were insignificant (Table 2).

In study 1, it was found that the effect on the standard and sample peak areas and heights of changing detector wavelengths were similar, leading to no net change in the calculated concentrations. In the study of HPLC parameters this was not the case. Changes in flow-rate produced similar changes in peak areas and heights for the sample and standard. However, changing the mobile phase composition had a significantly different effect on the standard and sample. The effect of column temperature also differed between sample and standard, but to a lesser extent. The differing effects of these parameters on the sample and standard resulted in variations in the calculated concentrations, compared to those observed under normal method conditions. This was reflected in the r^2 values obtained when quadratic models were fitted to the concentration data (e.g., $r^2 = 0.781$ for all-*trans*-retinol concentrations calcu-

lated from peak area data). It was therefore possible to calculate the uncertainty in the observed all-*trans*-retinol concentration due to changes in the HPLC conditions. However, the uncertainties were small compared to the standard deviation of the six results obtained under the normal experimental conditions. For example, for a concentration of 730 mg kg^{-1} calculated from peak area data, the standard deviation of the results obtained under normal method conditions was 37 mg kg^{-1} whereas the uncertainty calculated via numerical differentiation was 1.8 mg kg^{-1}. This was also the case for the uncertainties calculated for the peak areas and heights. For example, the uncertainty calculated by algebraic differentiation for the sample peak area was 23 987, compared to a standard deviation of results obtained under normal method conditions of 118 277. Therefore, unless the performance of the HPLC system is significantly poorer than that specified by the manufacturer, there must be other significant parameters (e.g., peak integration) contributing to the variability in results.

In both study 1 and study 2, uncertainties were calculated independently for both the samples and the standards. However, during the analysis of test samples, it is the ratio of the sample and standard peak areas (or heights) that is used in the calculation of the final result. In calculating the uncertainty in the test result, it is therefore the uncertainty in the ratio that is required. Calculating this by combining the individual uncertainties for the sample and the standard will lead to an over estimate as the uncertainties are correlated. For illustration, the uncertainties in the ratios of the peak areas and heights of the sample and standard used in study 1 were calculated using the symmetric spreadsheet approach. The relative uncertainties were 0.045% for the area ratio and 0.065% for the height ratio. Calculating the uncertainties by combining the individual estimates obtained from the symmetric spreadsheet for the sample and the standard gave 1.1% for both peak area and peak height ratios.

5. Conclusions

The experimental studies reported illustrate various approaches to estimating measurement uncertainty. The studies of the HPLC analysis of vitamins describe an approach to investigating the uncertainty associated with different ''input'' parameters, i.e., experimental parameters under direct control of the analyst. In HPLC studies, experimental design techniques were used to plan efficient experiments which allow the simultaneous evaluation of a number of parameters. The data generated were used to establish the relationship between the experimental parameters and the ''outputs'' such as peak area, analyte concentration and retention time. In both studies, response surfaces based on quadratic equations were found to fit the data well. Three approaches to calculating the uncertainty were compared – Kragten's spreadsheet, symmetric spreadsheet and algebraic differentiation. In the cases where non-linearity was significant (i.e., the study of detector wavelength settings), the three approaches showed poor agreement. This is because the spreadsheet approaches do not take account of the non-linearity. In such cases, allowance must be made for the second-order uncertainty terms. Estimates of these terms were obtained via algebraic differentiation. In the second HPLC study the non-linear terms in the models were of much less importance. Consequently the uncertainty estimates obtained from the different approaches showed much better agreement.

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