VAM Project 3.2.1 Development and Harmonisation of Measurement Uncertainty Principles

Part (d): Protocol for uncertainty evaluation from validation data

V J Barwick and S L R Ellison

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Protocol for uncertainty evaluation from validation data

V J Barwick
S L R Ellison

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Contact Point:

Vicki Barwick

Tel: 020 8943 7421

Prepared by:

Vicki Barwick

VALID ANALYTICAL MEASUREMENT

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Contents

1. Introduction

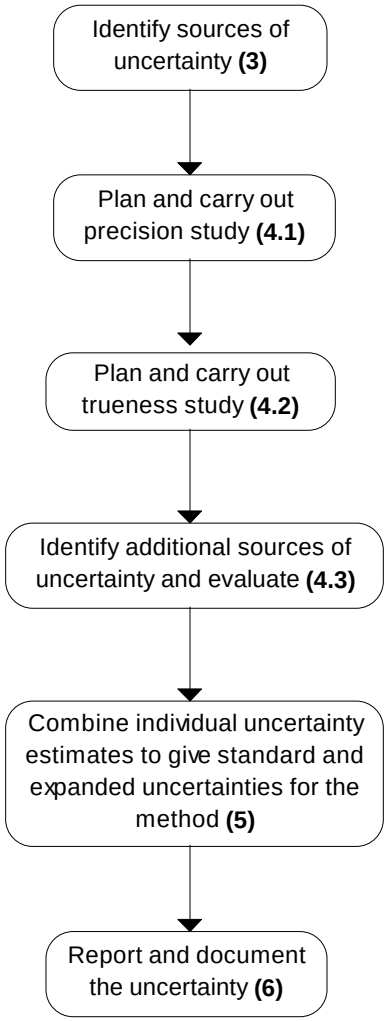
It is now widely recognised that the evaluation of the uncertainty associated with a result is an essential part of any quantitative analysis. Without knowledge of the **measurement uncertainty**†, the statement of an analytical result cannot be considered complete. The "Guide to the Expression of Uncertainty 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. The ISO guide has subsequently been interpreted for analytical chemistry by Eurachem.[2] The Eurachem guide sets out procedures for the evaluation of uncertainty in analytical chemistry. The main stages in the process are identified as:

- specification - write down a clear statement of what is being measured, including the full expression used to calculate the result;
- identify uncertainty sources - produce a list of all the sources of uncertainty associated with the method;
- quantify uncertainty components - measure or estimate the magnitude of the uncertainty associated with each potential source of uncertainty identified;
- calculate total uncertainty - combine the individual uncertainty components, following the appropriate rules, to give the combined **standard uncertainty** for the method; apply the appropriate **coverage factor** to give the **expanded uncertainty**.

This guide focuses on the second and third stages outlined above, and in particular gives guidance on how uncertainty estimates can be obtained from method validation experiments. The guide does not offer definitive guidance on the requirements for method validation, for which other texts are available.[3] Instead, it recognises that key studies routinely undertaken for validation purposes, namely **precision** studies, **trueness** studies and ruggedness tests, can if properly planned and executed, also provide much of the data required to produce an estimate of measurement uncertainty. Figure 1 illustrates the key stages in the uncertainty estimation process. The purpose of this guide is therefore to give guidance on the planning of suitable experiments that will meet the requirements of both method validation and uncertainty estimation. The procedures described are illustrated by worked examples.

† An alphabetical list of definitions is contained in Annex 3. Each term is highlighted in bold upon its first occurrence in the main body of the text.

Figure 1: Flow chart summarising the uncertainty estimation process
(The numbers in parentheses refer to the relevant sections in the guide)



2. Structure of the guide

There are four main stages to calculating uncertainty using validation studies. Each of these is dealt with in a separate section and a flow chart illustrating the process is given in Figure 2. The first stage of the procedure is the identification of sources of uncertainty for the method. This is dealt with in Section 3. Once the sources of uncertainty have been identified they require evaluation. The main tools for doing this are ruggedness, precision and **recovery** studies. How to plan and carry out the necessary experiments is discussed in Section 4. Once the individual uncertainty components for the method have been calculated they must be combined to give standard and expanded uncertainties for the method as a whole. The relevant calculations are given in Section 5. Finally, the

standard and expanded uncertainties need to be reported and documented in an appropriate way. This is discussed in Section 6. In addition to the sections mentioned above, the guide also contains a number of annexes. Guidance on calculating standard uncertainties from various sources of information, for example calibration certificates, is given in Annex 2. Definitions are contained in Annex 3, worked examples are given in Annex 4 and Annex 5 contains a proforma for documenting uncertainties.

Figure 2: Flow chart illustrating the stages in the measurement uncertainty process
(The numbers in parentheses refer to the relevant sections in the guide)

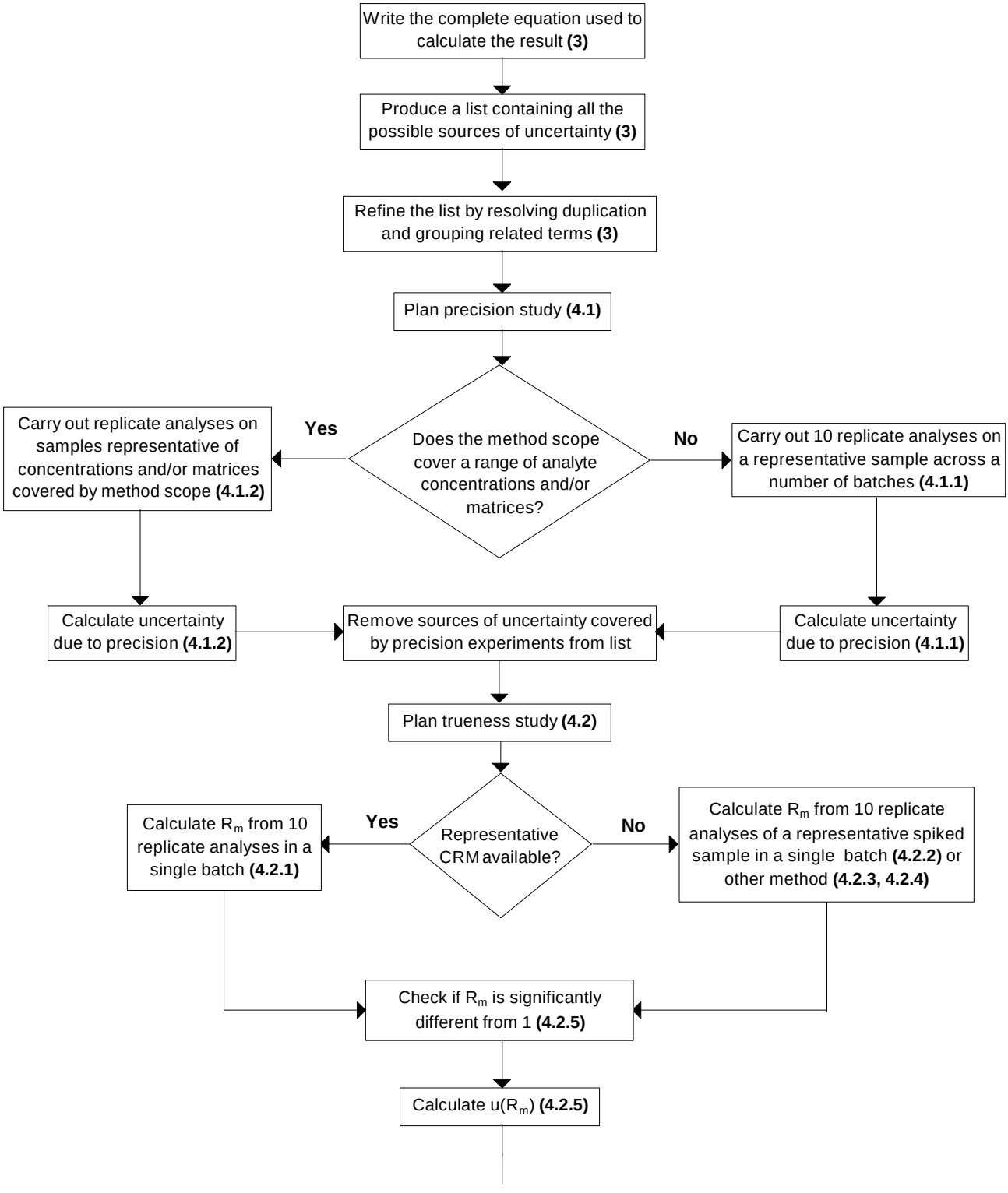
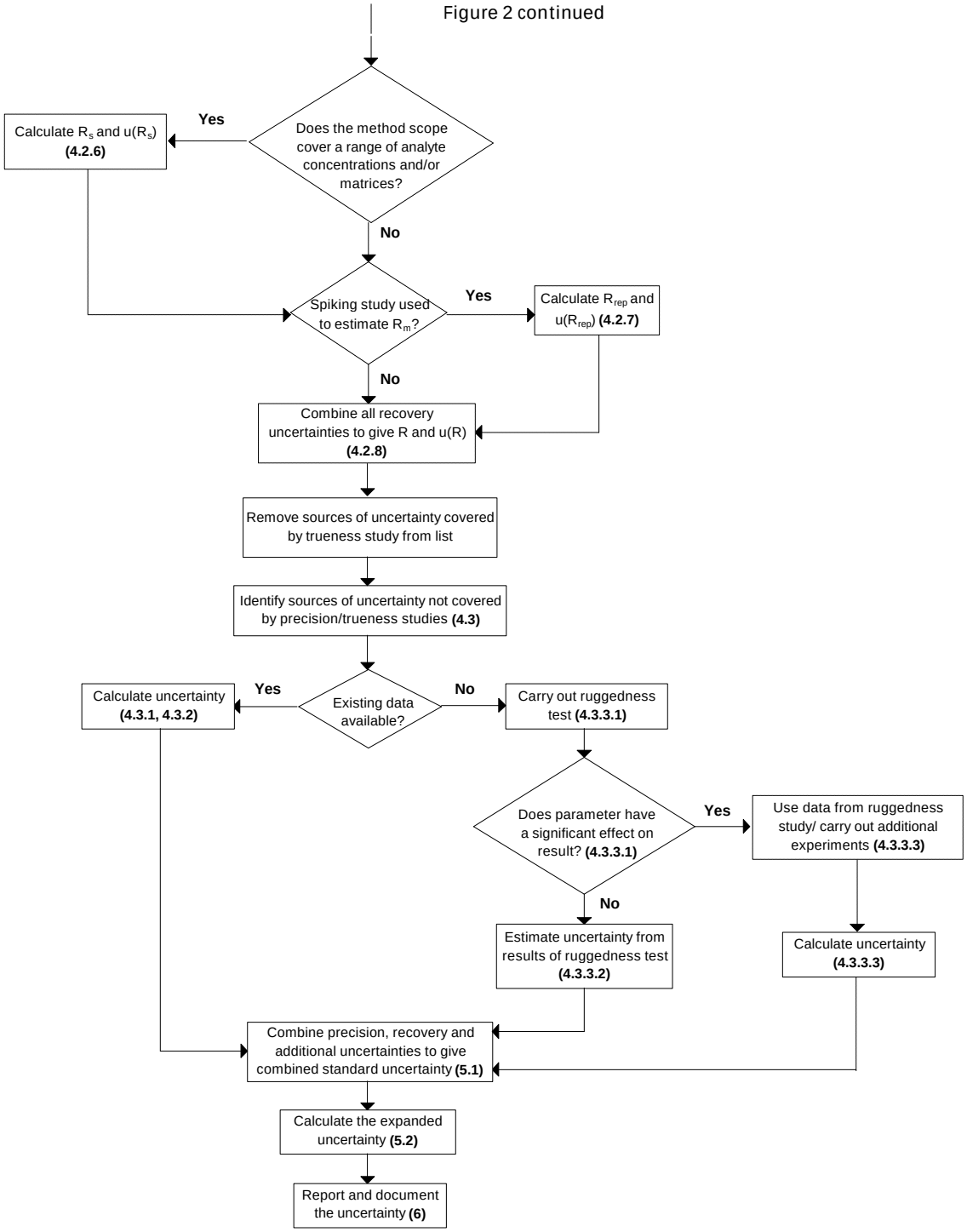


Figure 2 continued



3. Identification of sources of uncertainty

One of the critical stages of any uncertainty study is the identification of all the possible sources of uncertainty. The aim is to produce a list containing all the possible sources of uncertainty for the method. At this stage it is not necessary to be concerned about the quantification of the individual components; the aim is to be clear about what needs to be considered in the **uncertainty budget**. The Eurachem Guide discusses the process of identifying sources of uncertainty and a number of typical sources of uncertainty are given, including:

- Sampling - where sampling forms part of the procedure, effects such as random variations between different samples and any **bias** in the sampling procedure need to be considered.
- Instrument bias - *e.g.*, calibration of analytical balances.
- Reagent purity - *e.g.*, the purity of reagents used to prepare calibration standards will contribute to the uncertainty in the concentration of the standards.
- Measurement conditions - *e.g.*, volumetric glassware may be used at temperatures different from that at which it was calibrated.
- Sample effects - The recovery of an analyte from the sample matrix, or an instrument response, may be affected by other components in the matrix. When a spike is used to estimate recovery, the recovery of the analyte from the sample may differ from the recovery of the spike, introducing an additional source of uncertainty.
- Computational effects - *e.g.*, using an inappropriate calibration model.
- Random effects - random effects contribute to the uncertainty associated with all stages of a procedure and should be included in the list as a matter of course.

A simple approach to identifying the sources of uncertainty is as follows:

3.1 Write down the complete calculation involved in obtaining the result, including all intermediate measurements. List the parameters involved.

3.2 Study the method, step by step, and identify any other factors acting on the result. Add these to the list. For example, ambient conditions such as temperature and pressure affect many results.

3.3 Consider factors which will affect the parameters identified in 3.1 and 3.2 and add them to the list. Continue the process until the effects become too remote to be worth consideration.

3.4 Resolve any duplicate entries in the list. Listing uncertainty contributions separately for every input parameter will result in duplications in the list. Three cases arise and the following rules should be applied to resolve duplication:

3.4.1 Cancelling effects: remove both instances from the list. For example, in a weight by difference, two weights are determined and both are subject to the balance zero bias. This bias will cancel out of the weight by difference calculation and can therefore be removed from the list.

3.4.2 Similar effects, same time: combine into a single input. For example, run-to-run variation on a number of operations can be combined into an overall "precision" term representing the run-to-run variability of the method as a whole. This is particularly useful as the uncertainty estimate is based in part on estimates of the precision for the complete method (see Section 4.1).

3.4.3 Similar effects, different instances: re-label. It is common to find similarly named parameters in the list which actually represent different instances of similar effects. These must be clearly distinguished before proceeding. For example, there may be several instances of parameters such as "pipette calibration" which refer to the calibration uncertainties associated with different pipettes. It is important to be clear about which stages in the method these parameters refer to.

The result of the above should be a structured list of all the possible sources of uncertainty for the method.

An alternative method for producing the required list of uncertainty components for a method is cause and effect analysis. This approach uses a cause and effect diagram (sometimes known as an Ishikawa or "fishbone" diagram) to help identify effects in a structured way. The advantages of this approach are that it allows the analyst to clearly identify the relationship between sources of uncertainty, thus avoiding the possibility of double-counting of effects in the uncertainty budget. Examination of the diagram generally leads to considerable simplification either by grouping of sources of uncertainty which can be evaluated in a single set of experiments, or by removing duplicated terms. The simplified diagram can then be used as a checklist to ensure that all the sources of uncertainty have been accounted for. The construction of cause and effect diagrams for uncertainty estimation is discussed in detail in Annex 1.

4. Quantification of uncertainty contributions

The next stage in the process is the planning of experiments which will provide the information required to obtain an estimate of the combined uncertainty for the method. Initially, two sets of experiments are carried out - a precision study and a trueness study. These experiments should be planned in such a way that as many of the sources of uncertainty identified in the list obtained in Section 3 as possible are covered. The contributions covered by the precision and trueness studies can then be removed from the list. Those parameters not adequately covered by these experiments are evaluated separately. This section discusses the types of experiments required and gives details of how the data obtained are used to calculate uncertainty. The results from such studies will also be required as part of a validation exercise. However, the experiments given here do not necessarily form a complete validation study and further experiments may be required, for example, linearity or detection limit studies.[3]

The stages in the quantification of measurement uncertainty are as follows:

- precision study;
- trueness study;
- identification of other uncertainty contributions not adequately covered by the precision and trueness studies;
- evaluation of the other uncertainty contributions.

Each of these stages is discussed below. The simplest case is the estimation of precision from the replicate analysis of a single typical sample and the estimation of trueness from the analysis of a representative certified reference material (CRM). The appropriate experiments are described in 4.1.1 and 4.2.1 below. However, for many methods the situation will be more complex than this and the procedures for dealing with a number of common situations are outlined in Sections 4.1, 4.2 and 4.3.

When deciding which experiments are appropriate for the evaluation of a particular method, it is important to keep in mind one of the key principles of method validation and uncertainty estimation: the studies must be representative of normal operation of the method. That is the studies must cover the complete method, a representative range of sample matrices and a representative range of analyte concentrations. In other words, they must cover the full scope of the method.

4.1 Precision study

The experiments required to obtain an estimate of the method precision depend on the scope of the method. The simplest case is when the method is used for the analysis of a single matrix type with the analyte at a single concentration (see Section 4.1.1). The situation is more complicated when the method scope covers a range of sample matrices and/or analyte concentrations (see Section 4.1.2). The flow diagram presented in Figure 3 and the information in Table 1, will help with the identification of suitable precision experiments.

4.1.1 Single typical sample

If the method scope covers only a single sample matrix type and a single analyte concentration, the precision can be estimated from the replicate analysis of a single typical sample. Identify a suitable sample with a matrix and analyte concentration typical of those which will routinely be analysed using the method. Carry out a minimum of 10 analyses of the material. Each analysis must represent a complete application of the method, including sample preparation steps. It is not sufficient to simply carry out ten determinations on a single batch of extracted sample. The analyses should be spread over several different batches, and between batches as many of the method parameters as possible should be varied.

Parameters to consider include:

- calibration: the study should cover different calibrations prepared with different batches of calibration solutions (including stock solutions used to prepare the calibration solutions);
- reagents: different batches of reagents should be prepared;
- analyst: if the method will routinely be used by a number of different analysts then more than one analyst should take part in the precision study.

Refer to the list of effects produced for the method (see Section 3) and cross off the parameters which have been varied representatively during the precision study. No further study of the contribution to the uncertainty by these parameters is required.

It may not be necessary to carry out separate experiments specifically to obtain a precision estimate. If the method is in regular use, the samples for the precision study can be included in routine batches of analyses.

The uncertainty due to the method precision, $u(P)$, is the **sample standard deviation**, s, of the results of the precision study. To convert to a **relative standard deviation**, *i.e.*, $u(P)/P$, divide the sample standard deviation by the mean of the results of the precision study.

4.1.2 Range of samples covering the method scope

In many cases a method will be used for the determination of an analyte at a range of concentrations in a range of matrices. In such cases the precision study must consider a range of representative samples. It may be possible to use a single uncertainty estimate that covers all the sample types specified in the method scope, if there is evidence to suggest that the uncertainties are comparable. However, it may be found that different sample matrices and/or analyte concentrations behave differently so in some cases separate uncertainty estimates will be required.

4.1.2.1 Single analyte concentration, range of sample matrices

Identify samples representative of each of the matrices covered by the method scope. If the method has a broad scope it may not be practical to analyse, in replicate, a sample of each matrix type. In such cases it is up to the analyst to decide on the appropriate number and type of samples to be analysed. Analyse each sample in replicate. Ideally, at least 10 replicate analyses should be carried out for each sample. However, if a large number of matrices are being investigated this may be impractical, depending on the method. A minimum of four replicates per sample is therefore recommended. The replicates for each sample should be spread across different batches of analyses (see Section 4.1.1).

Calculate the standard deviation of the results obtained for each sample. If the standard deviations are not significantly different, they can be pooled to give a single estimate of precision which can be applied to all the matrices covered by the precision study (see Eq. 4.1). However, if one or more of the matrices produce standard deviations which are very different, it will be necessary to calculate separate uncertainty budgets for these matrices.

Deciding whether or not there is a "significant" difference between the standard deviations obtained for each sample is ultimately up to the analyst. Statistical tests can be used but their value depends very much on the number of results available for each sample. If 10 or more replicates have been made for each sample, the standard deviations can be compared using **F-tests**[4]. If a smaller number of results are available for each sample then it is more appropriate for the analyst to decide whether to pool the standard deviations or not. In making this decision, the contribution of the uncertainty due to precision to the combined uncertainty for the method as whole should be borne in mind. If the precision uncertainty is a dominant contribution it follows that the estimate used will have a significant effect on the combined uncertainty. Therefore, pooling precision estimates which cover a wide range of values may lead to a substantial underestimate in the combined uncertainty for some matrices and an overestimate for others. If, however, the precision uncertainty is not a major component of the combined uncertainty, its value will have less of an impact. Therefore, pooling the precision estimates should not lead to a significant over or underestimate of the combined uncertainty for a particular matrix.

To pool standard deviations use the following equation:

$$s_{pool} = \sqrt{\left(\frac{(n_1 - 1) \times s_1^2 + (n_2 - 1) \times s_2^2 + \ldots..}{(n_1 - 1) + (n_2 - 1) + \ldots..} \right)} \qquad \text{Eq. 4.1}$$

where s_1 is the standard deviation calculated for matrix 1, n_1 is the number of replicates for matrix 1, *etc*.

4.1.2.2 Single sample matrix, range of analyte concentrations

The precision should be investigated at concentrations covering the full range specified in the method scope. If samples containing appropriate concentrations of the analyte are not available then spiked samples should be prepared. It is recommended that at least three concentrations are investigated (*e.g.*, low, medium and high) with at least four replicates at each concentration. As discussed in Section 4.1.1, the replicates for each sample should be spread across different batches.

Calculate the standard deviation and relative standard deviation of the results obtained for each sample. If there is no significant difference between the relative standard deviations for each sample this indicates that the precision is proportional to the analyte concentration. In such a case the relative standard deviations can be pooled to give a single estimate which can be applied to the concentration range covered by the precision study (see Eq. 4.2). However, it is not unusual to find that the precision is not proportional to concentration over the entire range specified in the method scope, especially if that range is wide. It may therefore be necessary to calculate separate uncertainty estimates for certain concentrations, for example at very low concentrations, where proportionality may be lost.

As discussed in Section 4.1.2.1, the decision on whether there is a difference between the relative standard deviations calculated at each level rests with the analyst. If at least 5 concentration levels have been investigated, a plot of the standard deviation of the results obtained at each level against concentration will give an indication of the relationship between precision and concentration. If the plot is approximately linear, indicating that precision is proportional to level, the relative standard deviations can be pooled. If fewer than 5 levels have been investigated, the analyst should use his or her judgement to decide whether or not any differences in the relative standard deviations observed at the various concentrations are "significant". Factors to be considered when making this decision are discussed in Section 4.1.2.1.

An estimate of the pooled relative standard deviation, RSD_{pool} is obtained using the following equation:

$$RSD_{pool} = \sqrt{\left(\frac{(n_1 - 1) \times RSD_1^2 + (n_2 - 1) \times RSD_2^2 + \ldots..}{(n_1 - 1) + (n_2 - 1) + \ldots..} \right)} \qquad \text{Eq. 4.2}$$

where RSD_1 is the relative standard deviation calculated for the sample at concentration level 1, n_1 is the number of replicates for that sample, *etc*.

4.1.2.3 Range of sample matrices, range of analyte concentrations

If the method is used for the analysis of a range of sample matrices and analyte concentrations, it may not be possible to use a single estimate of the uncertainty due to precision (and consequently obtain a single estimate of the combined uncertainty) for all the samples covered by the method scope. The precision may not be proportional to the analyte level over the entire concentration range and/or the magnitude of the precision may vary from matrix to matrix. Guidelines for assessing the relationship between precision, sample matrix and analyte concentration were given in Sections 4.1.2.1 and 4.1.2.2. The experiments described are also useful in this case. For example, if there is a sample matrix in which the analyte is typically found at the range of concentrations specified in the method scope, carry out an experiment of the type described in Section 4.1.2.2. This will give an indication of the relationship between precision and analyte concentration. If the concentration range is similar for all sample matrices then a study of the type described in Section 4.1.2.1 can be carried out to investigate the effect of matrix on precision.

As discussed in previous sections, the replicates for each particular sample should be spread across different batches of analyses.

It may be necessary to perform additional experiments, for example if the concentration of the analyte varies substantially from matrix to matrix. As always with uncertainty estimation, it is important to remember that the estimate must be representative of the method scope. For methods with a broad scope (*i.e.*, wide range of sample matrices and analyte concentrations), it is strongly recommended that advice on planning suitable experiments is sought from a statistician or someone with experience of experimental design. This will help to ensure that the required information is obtained, whilst avoiding unnecessary experimental work.

Whichever experiments are carried out, the result will be a range of precision estimates representing different sample matrices and analyte concentrations. These estimates should be compared to determine whether pooling of some or all of them is appropriate. A number of possible cases arise.

1. The precision is proportional to the analyte level across the entire concentration range (see Section 4.1.2.2), and is independent of the sample matrix. In this case the precision estimate for each sample studied should be converted to a relative standard deviation and pooled using Eq. 4.2. This will give an estimate of the uncertainty due to precision, as a relative standard deviation (*i.e.*, $u(P)/P$), which can be applied to all the samples covered by the method scope.

2. The precision is proportional to the analyte level across the entire concentration range, but the magnitude varies from matrix to matrix. In this case the relative standard deviations calculated at the different concentrations for the individual matrices can be pooled (using Eq. 4.2) to give separate estimates of precision for each matrix type.

This will in turn lead to separate estimates of the combined uncertainty for each matrix.

3. The precision is not proportional to the analyte level over the entire concentration range. The experimental studies may show that the precision is proportional to the concentration over only a limited concentration range, or not at all. In addition, this may vary from matrix to matrix. In such cases it may be possible to pool some of the precision estimates to give estimates of $u(P)$ for particular groups of sample matrices and/or concentration ranges. For example, for a particular method it was observed that at low concentrations the standard deviation was similar for a range of sample matrices and remained fixed over a narrow concentration range. This indicates that the precision is independent of both the matrix and analyte level for the concentration range studied. In such a case the standard deviations observed for each sample can be pooled using Eq. 4.1 to give a single estimate of precision that can be applied to that particular group of samples.

In summary, when the method scope covers a range of sample matrices and analyte concentrations, the aim should be to pool precision estimates were appropriate. If the precision is proportional to the concentration of the analyte the precision estimates should be pooled as relative standard deviations using Eq. 4.2. If the precision is found to be independent of the analyte concentration then the estimates should be pooled as standard deviations using Eq. 4.2. As discussed in Sections 4.1.2.1 and 4.1.2.2, the decision on whether or not it is appropriate to pool precision estimates rests with the analyst. Factors to be considered and suitable statistical tests are given in these sections.

4.2 Trueness study

In this protocol, trueness is estimated in terms of overall recovery, *i.e.*, the ratio of the observed value to the expected value. The closer the ratio is to 1, the smaller the bias in the method. Recovery can be evaluated in a number of ways, for example the analysis of certified reference materials (CRMs) or spiked samples. The experiments required to evaluate recovery and its uncertainty will depend on the scope of the method and the availability, or otherwise, of suitable CRMs. The recovery for a particular sample, R, can be considered as comprising three components:

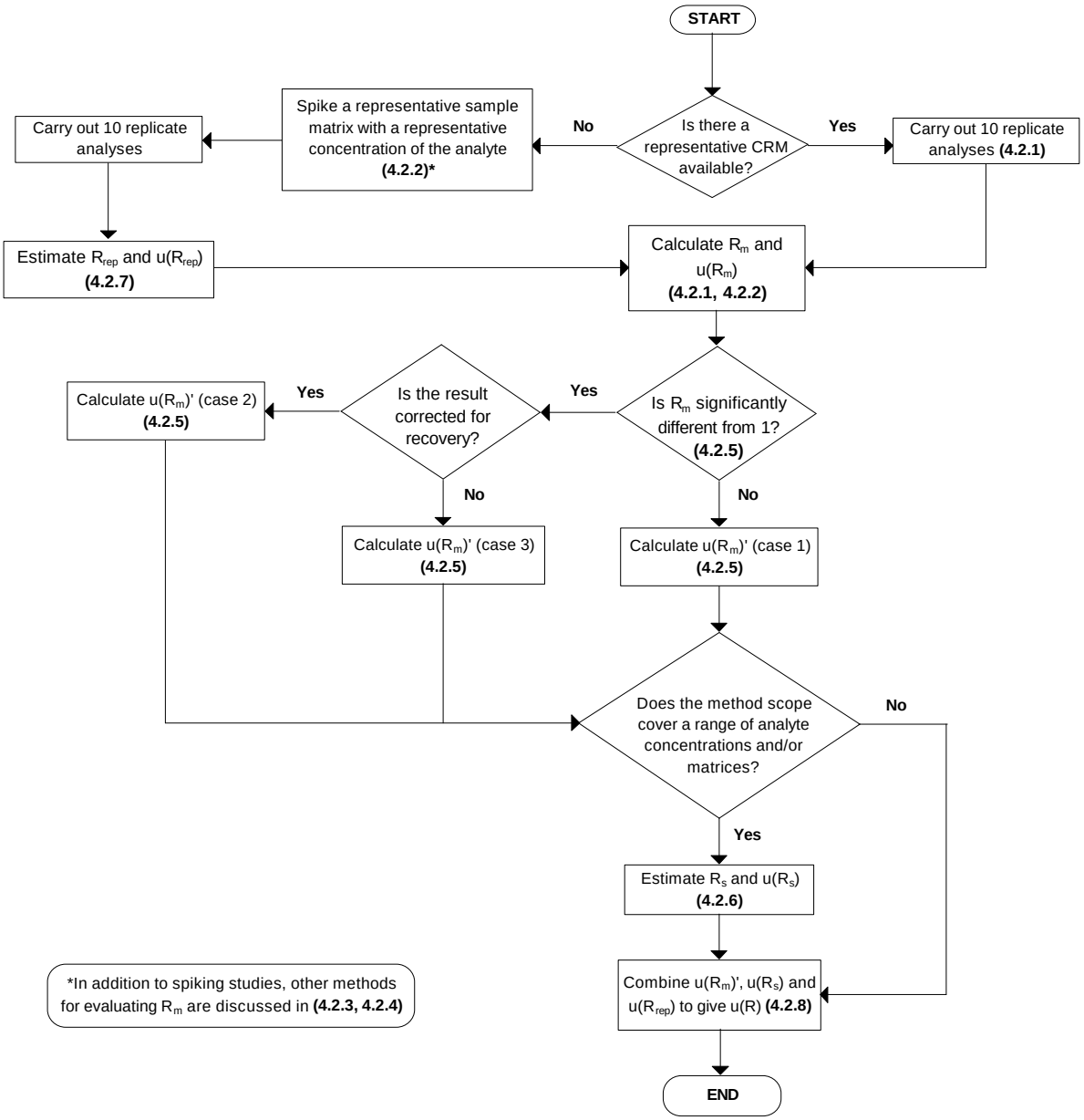
- $\overline{R}_m$ is an estimate of the mean method recovery obtained from, for example, the analysis of a CRM or a spiked sample. The uncertainty in $\overline{R}_m$ is composed of the uncertainty in the reference value (*e.g.*, the uncertainty in the certified value of a reference material) and the uncertainty in the observed value (*e.g.*, the standard deviation of the mean of replicate analyses). The contribution of $\overline{R}_m$ to the overall uncertainty of the method depends on whether it is significantly different from 1, and if so, whether a correction is applied.

- R_s is a correction factor to take account of differences in the recovery for a particular sample compared to the recovery observed for the material used to estimate $\overline{R}_m$.
- R_{rep} is a correction factor to take account of the fact that a spiked sample may behave differently to a real sample with incurred analyte.

These three elements are combined multiplicatively to give an estimate of the recovery for a particular sample, *i.e.*, $R = \overline{R}_m \times R_s \times R_{rep}$. It therefore follows that the uncertainty in R, $u(R)$, will have contributions from $u(\overline{R}_m)$, $u(R_s)$ and $u(R_{rep})$. How each of these components and their uncertainties are evaluated will depend on the method scope and the availability of reference materials. The various options are summarised in Figure 4 and Table 2.

Approaches to estimating recovery, together with worked examples, are discussed in more detail elsewhere.[5, 6]

Figure 4: Flow chart illustrating the stages in estimating the uncertainty associated with recovery
(The numbers in parentheses refer to the relevant sections in the guide)



4.2.1 Estimating $\overline{R}_m$ and $u(\overline{R}_m)$ using a representative certified reference material

Identify a certified reference material with a matrix and analyte concentration representative of those which will routinely be analysed using the method. Analyse at least 10 portions of the reference material in a single batch.[‡] Each portion must be taken through the entire analytical procedure. Calculate the mean recovery, $\overline{R}_m$, as follows:

$$\overline{R}_m = \frac{\overline{C}_{obs}}{C_{CRM}} \qquad \text{Eq. 4.3}$$

where $\overline{C}_{obs}$ is the mean of the replicate analyses of the CRM and C_{CRM} is the certified value for the CRM.

Calculate the uncertainty in the recovery, $u(\overline{R}_m)$, using:

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\left(\frac{s_{obs}^2}{n \times \overline{C}_{obs}^2}\right) + \left(\frac{u(C_{CRM})}{C_{CRM}}\right)^2} \qquad \text{Eq. 4.4}$$

where s_{obs} is the standard deviation of the results from the replicate analyses of the CRM, n is the number of replicates and $u(C_{CRM})$ is the standard uncertainty in the certified value for the CRM. See Annex 2 for information on calculating standard uncertainties from reference material certificates.

The above calculation provides an estimate of the mean method recovery and its uncertainty. The contribution of recovery and its uncertainty to the combined uncertainty for the method depends on whether the recovery is significantly different from 1, and if so, whether or not a correction is made. Section 4.2.5 gives guidance on estimating the contribution of recovery to the combined uncertainty.

4.2.2 Estimating $\overline{R}_m$ and $u(\overline{R}_m)$ from a spiking study at a single concentration on a single matrix

If there is no appropriate CRM available then $\overline{R}_m$ and $u(\overline{R}_m)$ can be estimated from a spiking study, *i.e.*, the addition of the analyte to a previously studied material. The spiked sample should be prepared in such a way as to represent a test sample as closely as possible. There are several approaches to this, depending on whether a "blank" sample matrix, free from the analyte of interest is available.

[‡] If it is impractical to carry out 10 analyses in a single batch, the replicates should be analysed in the minimum number of batches possible over a short period of time.

4.2.2.1 Spiking a bulk sample of a "blank" matrix

Spike a bulk sample of a suitable sample matrix known to be free from the analyte of interest with an appropriate concentration of the analyte. Analyse at least 10 portions of the bulk spiked sample. $\overline{R}_m$ is given by:

$$\overline{R}_m = \frac{\overline{C}_{obs}}{C_{spike}} \qquad \text{Eq. 4.5}$$

where $\overline{C}_{obs}$ is the mean of the replicate analyses of the spiked sample and C_{spike} is the concentration of the spiked sample. The uncertainty in $\overline{R}_m$ is given by:

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\left(\frac{s_{obs}^2}{n \times \overline{C}_{obs}^2}\right) + \left(\frac{u(C_{spike})}{C_{spike}}\right)^2} \qquad \text{Eq. 4.6}$$

where s_{obs} is the standard deviation of the results from the replicate analyses of the spiked sample, n is the number of replicates and $u(C_{spike})$ is the standard uncertainty in the concentration of the spiked sample.

4.2.2.2 Spiking a bulk sample of a matrix containing the analyte

If no blank sample matrix is available, prepare a bulk spiked sample from a matrix which contains the analyte. Analyse the spiked sample in replicate. $\overline{R}_m$ is given by:

$$\overline{R}_m = \frac{\overline{C}_{obs} - \overline{C}_{native}}{C_{spike}} \qquad \text{Eq. 4.7}$$

where $\overline{C}_{native}$ is the concentration of the analyte in the unspiked sample. Note that since we are concerned only with the difference between the spiked and unspiked concentrations, $\overline{C}_{native}$ does not have to represent the "true" value of the analyte concentration in the unspiked matrix. The uncertainty is given by:

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\frac{s_{obs}^2 / n + s_{native}^2}{\left(\overline{C}_{obs} - \overline{C}_{native}\right)^2} + \left(\frac{u(C_{spike})}{C_{spike}}\right)^2} \qquad \text{Eq. 4.8}$$

where s_{native} is the standard deviation of the mean of the results of repeat analyses of the unspiked matrix.

4.2.2.3 Spiking individual portions of a "blank" matrix

If it is impractical to prepare a homogeneous bulk spiked sample for sub-sampling, then individual spiked samples are prepared. Prepare the spiked samples from approximately the same weight of a blank sample matrix, and add the same weight of spike to each sample. It is recommended that at least 10 samples are analysed. The recovery is given by:

$$\overline{R}_m = \frac{\overline{m}_{obs}}{m_{spike}} \qquad \text{Eq. 4.9}$$

where $\overline{m}_{obs}$ is the mean weight of the spike recovered from the samples and m_{spike} is the weight of the spike added to each sample. $u(\overline{R}_m)$ is given by:

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\left(\frac{s^2_{m_{obs}}}{n \times \overline{m}^2_{obs}}\right) + \left(\frac{u(m_{spike})}{m_{spike}}\right)^2} \qquad \text{Eq. 4.10}$$

where $s_{m_{obs}}$ is the standard deviation of the results obtained from the spiked samples, n is the number of spiked samples analysed and $u(m_{spike})$ is the uncertainty in the amount of spike added to each sample.

4.2.2.4 Spiking individual portions of a matrix containing the analyte

If the spiked samples are prepared from a sample matrix which contains the analyte the situation is somewhat more complex. The recovery for each sample, $R_{m(i)}$, is given by:

$$R_{m(i)} = \frac{C_{obs(i)} - C_{native}}{C_{spike(i)}} \qquad \text{Eq. 4.11}$$

where $C_{obs(i)}$ is the concentration of the analyte observed for sample i and $C_{spike(i)}$ is the concentration of the spike added to sample i.

The mean recovery, $\overline{R}_m$, is given by:

$$\overline{R}_m = \frac{1}{n}\sum_{i=1}^{n}\frac{C_{obs(i)} - C_{native}}{C_{spike(i)}} \qquad \text{Eq. 4.12}$$

The uncertainty, $u(\overline{R}_m)$, is given by:

$$u(\overline{R}_m)^2 = \sum_{i=1}^{n}\left[\frac{1}{n}\times\frac{1}{C_{spike(i)}}\times u(C_{obs(i)})\right]^2 + \left[\frac{1}{n}\sum_{i=1}^{n}\frac{1}{C_{spike(i)}}\right]^2 \times u(C_{native})^2 + \sum_{i=1}^{n}\left[\frac{1}{n}\times\frac{\left(C_{obs(i)} - C_{native}\right)}{C^2_{spike(i)}}\times u(C_{spike(i)})\right]^2 \qquad \text{Eq. 4.13}$$

However, if the following conditions are met, Eq. 4.13 simplifies and Eq. 4.14 can be used:

- $u(C_{spike(i)})$ is much smaller than $u(C_{obs(i)})$ and $u(C_{native})$. This is often the case, as spiking is generally achieved by adding an aliquot of a solution or a known weight of the analyte. The uncertainties associated with such operations are usually small compared to the uncertainties associated with the observation of the amount of the analyte in a sample.

- The standard deviation of the $C_{spike(i)}$ values is small compared to the mean of the $C_{spike(i)}$ values. If this condition is met, the mean of the $C_{spike(i)}$ values, $\overline{C}_{spike}$ is used in the calculation. This is likely to be the case in recovery studies at a single level using similar quantities of the sample matrix in the preparation of each spiked sample.
- The estimates of $u(C_{obs(i)})$ are all similar. In such cases the mean, $\overline{u}(C_{obs(i)})$ can be used. Again, this is likely to be the case when each sample is spiked at the same concentration so that all the $C_{obs(i)}$ values are of a similar order of magnitude.

$$u(\overline{R}_m) = \frac{1}{\overline{C}_{spike}} \sqrt{\frac{\overline{u}(C_{obs(i)})^2}{n} + u(C_{native})^2} \qquad \text{Eq. 4.14}$$

4.2.3 Estimating $\overline{R}_m$ and $u(\overline{R}_m)$ by comparison with a standard method

$\overline{R}_m$ can be evaluated by analysing a typical sample using the method under evaluation and an alternative standard technique for which the uncertainty is known. It is recommended that at least five determinations are made for each method. $\overline{R}_m$ is given by:

$$\overline{R}_m = \frac{\overline{C}_{method}}{\overline{C}_{standard}} \qquad \text{Eq. 4.15}$$

where $\overline{C}_{method}$ is the mean of the results obtained using the method under consideration and $\overline{C}_{standard}$ is the mean of the results obtained using the standard method. The uncertainty in the recovery, $u(\overline{R}_m)$, is given by:

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\left(\frac{s^2_{method}}{n \times \overline{C}^2_{method}}\right) + \left(\frac{u(C_{standard})}{\overline{C}_{standard}}\right)^2} \qquad \text{Eq. 4.16}$$

where s_{method} is the standard deviation of the results obtained using the method, n is the number of replicates and $u(C_{standard})$ is the standard uncertainty associated with the standard method.

4.2.4 Other methods for estimating $\overline{R}_m$ and $u(\overline{R}_m)$

If there are no CRMs or standard methods available, and spiking samples is impractical, alternative methods of investigating the recovery are required. However, such techniques generally require an element of judgement on the part of the analyst and can only be used as an initial indication of the uncertainty associated with method recovery. If the results of such a study indicate that the uncertainties associated with recovery are a significant contribution to the uncertainty budget, further investigation will be required to obtain a better estimate. Techniques include:

4.2.4.1 Investigating extraction behaviour

One technique is the re-extraction of samples either under the same experimental conditions, or preferably with a more vigorous extraction system (*e.g.*, a stronger extraction solvent). The amount of analyte extracted under the normal method conditions is compared with the total amount extracted (amount extracted initially plus the amount extracted by subsequent re-extractions). $\overline{R}_m$ is the ratio of these estimates. If re-extraction was achieved using the same conditions as the initial extraction, the difference between the true recovery and the assumed value of 1 is known to be at least $1-\overline{R}_m$. The difference could be greater, as repeated extractions under the same experimental conditions may not quantitatively recover all of the analyte from the sample. It is therefore recommended that that uncertainty, $u(\overline{R}_m)$, associated with the assumed value of $\overline{R}_m = 1$ be estimated as $(1-\overline{R}_m)$. If repeat extractions were carried out using a more vigorous extraction system, the confidence about the difference between $\overline{R}_m$ and the assumed value of 1 is greater, as it is more likely that repeat extractions will have quantitatively recovered the analyte. In such cases it is recommended that $u(\overline{R}_m)$ is estimated as $(1-\overline{R}_m)/k$, where k is the coverage factor that will be used to calculate the expanded uncertainty.

In these cases it is already assumed that $\overline{R}_m$ is equal to 1 and the uncertainty has been estimated accordingly. There is therefore no need to follow the procedures outlined in Section 4.2.5.

Another technique involves monitoring the extraction procedure with time and using the information to predict how much of the analyte present has been extracted. This technique is described in detail elsewhere.[5, 7]

4.2.4.2 Analysis of a "worst case" CRM

If a CRM is available which has a matrix known to be more difficult to extract the analyte from compared to test samples, the recovery observed for the CRM can provide a worst case estimate on which to base the recovery for real samples. If the matrix is known to be extreme, compared to test samples, it is reasonable to assume that recoveries for test samples are more likely to be closer to 1 than to R_{CRM}, the recovery for the CRM. It is therefore appropriate to take R_{CRM} as representing the lower limit of a triangular distribution. As a first estimate, $\overline{R}_m$ is assumed to equal 1, with an uncertainty, $u(\overline{R}_m)$, of:

$$u(\overline{R}_m) = \frac{1-R_{CRM}}{\sqrt{6}} \qquad \text{Eq. 4.17}$$

Note that if there is no evidence to suggest where in the range $1-R_{CRM}$ the recovery for test samples is likely to lie, a rectangular distribution should be assumed. $u(\overline{R}_m)$ is therefore given by $1-\overline{R}_m/\sqrt{3}$.

Since assumptions about $\overline{R}_m$ and $u(\overline{R}_m)$ have been made at this stage, there is no need to follow the procedures outlined in Section 4.2.5.

These approaches to estimating $\overline{R}_m$ are discussed in more detail elsewhere.[5, 6]

4.2.5 Estimating the contribution of $\overline{R}_m$ to $u(R)$

Assuming an estimate of the recovery $\overline{R}_m$ and its uncertainty $u(\overline{R}_m)$ has been obtained using one of the procedures outlined in Sections 4.2.1 to 4.2.4, three possible cases arise:[8, 9]

1. $\overline{R}_m$, taking into account $u(\overline{R}_m)$, is not significantly different from 1 so results are not corrected for recovery.
2. $\overline{R}_m$, taking into account $u(\overline{R}_m)$, is significantly different from 1 and results are corrected for recovery.
3. $\overline{R}_m$, taking into account $u(\overline{R}_m)$, is significantly different from 1 but a correction is not applied.

To determine whether the recovery is significantly different from 1 a significance test is used.[4] Calculate the test statistic *t* using the following equation:

$$t = \frac{\left|1 - \overline{R}_m\right|}{u(\overline{R}_m)} \qquad \text{Eq. 4.18}$$

If the **degrees of freedom** associated with $u(\overline{R}_m)$ are known, compare *t* with the 2-tailed critical value, t_{crit}, for the appropriate number of degrees of freedom at 95% confidence. If *t* is less than the critical value then $\overline{R}_m$ is not significantly different from 1.

If the degrees of freedom associated with $u(\overline{R}_m)$ are unknown, for example if there is a contribution from the uncertainty in the certified value of a reference material, compare *t* with *k*, the coverage factor that will be used in the calculation of the expanded uncertainty (see Section 5.2 for guidance on selecting an appropriate value for *k*).

If $\left|1 - \overline{R}_m\right| / u(\overline{R}_m) < k$ the recovery is not significantly different from 1.

If $\left|1 - \overline{R}_m\right| / u(\overline{R}_m) > k$ the recovery is significantly different from 1.

Case 1

The significance test indicates that the recovery is not significantly different from 1 so there is no reason to correct analytical results for recovery. However, there is still an uncertainty associated with the estimate of $\overline{R}_m$ as the significance test could not distinguish between a range of values about 1.0. If the test statistic was compared with t_{crit} the range is $1 \pm t_{crit}\, u(\overline{R}_m)$. The uncertainty associated with recovery in this case, $u(\overline{R}_m)'$, is given by:

$$u(\overline{R}_m)' = \frac{t_{crit} \times u(R_m)}{1.96} \qquad \text{Eq. 4.19}$$

If the test statistic was compared with the coverage factor k, the range is $1 \pm k \times u(\overline{R}_m)$. In this case the uncertainty associated with $\overline{R}_m$ is taken as $u(\overline{R}_m)$.

To convert to a relative standard deviation divide $u(\overline{R}_m)$ or $u(\overline{R}_m)'$ by the assumed value of $\overline{R}_m$. In this case $\overline{R}_m = 1$ so the standard deviation is equivalent to the relative standard deviation.

Case 2

As a correction factor is being applied, $\overline{R}_m$ is explicitly included in the calculation of the result. $u(\overline{R}_m)$ is therefore included in the overall uncertainty calculation as the term $u(\overline{R}_m) / \overline{R}_m$ since the correction is multiplicative (see Section 5).

Case 3

In this case the recovery is statistically significantly different from 1, but in the normal application of the method no correction is applied (*i.e.*, $\overline{R}_m$ is assumed to equal 1). The uncertainty must be increased to take account of the fact that the recovery has not been corrected for. The increased uncertainty, $u(\overline{R}_m)''$, is given by:

$$u(\overline{R}_m)'' = \sqrt{\left(\frac{1-\overline{R}_m}{k}\right)^2 + u(\overline{R}_m)^2} \qquad \text{Eq. 4.20}$$

where k is the coverage factor which will be used in the calculation of the expanded uncertainty.

$u(\overline{R}_m)''$ is expressed as a relative standard deviation by dividing by the assumed value of $\overline{R}_m$ as in case 1.

4.2.6 Estimating R_s and $u(R_s)$ from spiking studies

Where the method scope covers a range of sample matrices and/or analyte concentrations an additional uncertainty term is required to take account of differences in the recovery of a particular sample type, compared to the material used to estimate $\overline{R}_m$. This is can be evaluated by analysing a representative range of spiked samples, covering typical matrices and analyte concentrations, in replicate. The number of matrices and levels examined, and the number of replicates for each sample will depend on the method scope. The same guidelines apply as for the precision studies discussed in Section 4.1. Calculate the mean recovery for each sample (see Eq. 4.12). R_s is assumed to be equal to 1.0, however there will be an uncertainty in this assumption. This appears in the spread of mean recoveries observed for the different spiked samples. The uncertainty, $u(R_s)$, is therefore the standard deviation of the mean recoveries for each sample type.

4.2.7 Estimating R_{rep} and $u(R_{rep})$

R_{rep} is generally assumed to equal one, indicating that the recovery from a spiked sample represents perfectly the recovery observed for incurred analyte. The uncertainty $u(R_{rep})$ is a measure of the uncertainty associated with that assumption, *i.e.*, how different R_{rep} might be from the assumed value of 1.

The complexity of evaluating how representative a spike is of the behaviour of native material varies from matrix to matrix and with the method being studied. In some cases it can be argued that a spike is a good representation of a real sample, for example in liquid samples where the analyte is simply dissolved in the matrix. In addition, if the method involves total dissolution or destruction of the matrix, for example by ashing, there may be no reason to believe that a spike would behave any differently from the incurred analyte. However, problems arise for more complex matrices and where the method involves extraction rather than total destruction or dissolution. Possible approaches to investigating the performance of spiked versus real samples include monitoring the extraction of spiked and native analytes with time, and comparison of spiked recovery with the recovery from a less representative CRM. However, these may not be appropriate in all cases. If experimental evidence on the appropriateness of spiking cannot be obtained, then judgements and/or assumptions have to be made. Ideally, R_{rep} should be evaluated by the analysis of a reference material (even if it is not directly comparable to the test samples) and comparing the recovery obtained with those observed from the spiking studies. The uncertainty $u(R_{rep})$ is then estimated as:

$$u(R_{rep}) = \sqrt{\left(\frac{1-R_{rep}}{k}\right)^2 + \left(u(R_{rep})'\right)^2} \qquad \text{Eq. 4.21}$$

where k is the coverage factor which will be used to calculate the expanded uncertainty and $u(R_{rep})'$ is the uncertainty associated with the estimate of R_{rep}. The most straightforward approach is to spike the CRM and compare the recovery observed with that observed from the analysis of the unspiked reference material. In such cases R_{rep} is given by:

$$R_{rep} = \frac{\overline{C}_{obs(\,spike\,)} - \overline{C}_{obs(\,CRM\,)}}{C_{spike}} \times \frac{C_{CRM}}{\overline{C}_{obs(\,CRM\,)}} \qquad \text{Eq. 4.22}$$

where $\overline{C}_{obs(spike)}$ is the mean concentration observed from replicate analyses of the spiked CRM, $\overline{C}_{obs(CRM)}$ is the mean concentration observed from replicate analyses of the unspiked CRM, C_{spike} is the concentration of the spike added and C_{CRM} is the certified concentration of the reference material. The uncertainty, $u(R_{rep})'$, obtained by differentiating Eq. 4.22:

$$u(R_{rep})' = R_{rep} \times \sqrt{\left(\frac{u(\overline{C}_{obs(spike)})}{\overline{C}_{obs(spike)} - \overline{C}_{obs(CRM)}}\right)^2 + \left(\frac{u(\overline{C}_{obs(CRM)}) \times u(\overline{C}_{obs(spike)})}{\overline{C}_{obs(CRM)} \times \left(\overline{C}_{obs(CRM)} - \overline{C}_{obs(spike)}\right)}\right)^2 + \left(\frac{u(C_{CRM})}{C_{CRM}}\right)^2 + \left(\frac{u(C_{spike})}{C_{spike}}\right)^2} \qquad \text{Eq. 4.23}$$

In this case, we are only interested in the dispersion of results obtained for the mean values $C_{obs(spike)}$ and $\overline{C}_{obs(CRM)}$. The corresponding uncertainties are therefore given by the standard deviation of the mean of the observed concentrations in each case. Note that the above equation holds if the spiking study was based on the replicate analysis of a singled spiked sample of the CRM. If the study was based on the analysis of a number of individual portions of the CRM (of similar weight) all spiked at a similar concentration (see Section 4.2.2.4), Eq. 4.22 and Eq. 4.23 are modified slightly:

$$R_{rep} = \frac{\overline{C}_{obs(spike)} - \overline{C}_{obs(CRM)}}{\overline{C}_{spike}} \times \frac{C_{CRM}}{\overline{C}_{obs(CRM)}} \qquad \text{Eq. 4.24}$$

$$u(R_{rep})' = R_{rep} \times \sqrt{\left(\frac{\overline{u}(C_{obs(spike)})}{\overline{C}_{obs(spike)} - \overline{C}_{obs(CRM)}}\right)^2 + \left(\frac{u(\overline{C}_{obs(CRM)}) \times u(\overline{C}_{obs(spike)})}{\overline{C}_{obs(CRM)} \times (\overline{C}_{obs(CRM)} - \overline{C}_{obs(spike)})}\right)^2 + \left(\frac{u(C_{CRM})}{C_{CRM}}\right)^2 + \left(\frac{\overline{u}(C_{spike})}{\overline{C}_{spike}}\right)^2} \qquad \text{Eq. 4.25}$$

where $\overline{u}(C_{obs(spike)})$ is the average of the uncertainties associated with each of the $C_{obs(spike)}$ values divided by the square root of the number of determinations of $C_{obs(spike)}$, $\overline{C}_{spike}$ is the average of the concentrations of the spike added to each sample and $\overline{u}(C_{spike})$ is the average of the uncertainties associated with each of the C_{spike} values.

If there is no CRM available then judgements will have to made based on whatever information is available, for example, published studies. The estimation of R_{rep} is discussed in more detail elsewhere.[5, 6]

4.2.8 Calculating *R* and *u(R)*

The recovery for a particular sample, *R*, is given by $R = \overline{R}_m \times R_s \times R_{rep}$. However, since R_s and R_{rep} are generally assumed to equal 1, $R = \overline{R}_m$. The values of $\overline{R}_m$ and $u(\overline{R}_m)$ used depend on whether or not $\overline{R}_m$ is significantly different from 1, and if so, whether a correction to the result for a particular sample is applied. This was discussed in Section 4.2.5. The uncertainty associated with *R*, *u*(*R*) is given by:

$$u(R) = R \times \sqrt{\left(\frac{u(\overline{R}_m)}{R_m}\right)^2 + \left(\frac{u(R_s)}{R_s}\right)^2 + \left(\frac{u(R_{rep})}{R_{rep}}\right)^2} \qquad \text{Eq. 4.26}$$

However, if $R_s = R_{rep} = 1$, Eq. 4.26 simplifies to:

$$u(R) = \overline{R}_m \times \sqrt{\left(\frac{u(\overline{R}_m)}{R_m}\right)^2 + u(R)_s^{\,2} + u(R_{rep})^2} \qquad \text{Eq. 4.27}$$

4.3 Evaluation of other sources of uncertainty

Sources of uncertainty not adequately covered by the precision and trueness experiments require separate evaluation. For example, a method may require that the sample is heated to a particular temperature. The method may specify a permitted range about this temperature, *e.g.*, heat to 100±5 °C. During the precision study the temperature may not have been varied sufficiently to cover the full range of temperatures permitted in the method specification. Alternatively there may be no method specification for a particular parameter. In both cases the effect of changes in the parameter on the result of the analysis needs to be evaluated. Other sources of uncertainty which may require evaluation include:

- purity of standards, *e.g.*, if a single batch of material has been used to prepare all the standards used during the precision study;
- calibration of glassware, *e.g.*, if a single pipette was used throughout the precision study;
- calibration *e.g.*, if the same set of calibration standards was used throughout the precision studies.

There are three main sources of data: calibration certificates and manufacturers' specifications; data published in the literature; specially designed experimental studies. Each of these is discussed below.

4.3.1 Calibration certificates and manufacturers' specifications

For many sources of uncertainty, calibration certificates or suppliers' catalogues provide the required information:

- Tolerances for volumetric glassware can be obtained from catalogues or the literature supplied with the item.
- Data on the purity of standards and other reagents can be obtained from the supplier.
- Calibration uncertainties for balances can be obtained from the calibration certificate.

Note that the information presented on calibration certificates, *etc.* may not be in the form of a standard uncertainty and must therefore be converted before combining with other uncertainty estimates. Details of how to do this are given in Annex 2.

4.3.2 Published data

As the aim of this guide is to give guidance on obtaining uncertainty estimates during the method validation stage, it might be expected that very little published data would be available for a new procedure. This obviously depends on the method, but it may be that

particular stages of the method have been investigated in the context of other methods so some data may be available. For example, a sample pre-treatment step such a grinding or extraction may be common to a number of different methods; or the stability of samples or reagents used in a procedure may already have been investigated.

4.3.3 Experimental studies

If no existing data are available then the uncertainty must be investigated experimentally. As part of method development and validation, key method parameters are studied to determine the effect of variations in them on the outcome of the analysis. If a significant effect is observed for a particular parameter, appropriate control limits are set such that variations within the limits will not have a significant effect on the outcome of the analysis. Alternatively, the method is improved by concentrating on the stages of the method identified as critical. A common method of identifying the critical method parameters is the ruggedness test. This involves making deliberate variations in the method and investigating the effect on the result. An established technique for ruggedness testing is described by the AOAC.[10] Such a test is also useful for uncertainty estimation. The results from ruggedness studies can be used in the evaluation of uncertainties associated with method parameters not adequately covered by the precision and trueness studies. Such studies can also be used to identify significant sources of uncertainty which require further study. Suggested procedures for estimating uncertainty via ruggedness testing are given in the following sections.

4.3.3.1 Designing a ruggedness test

The ruggedness testing procedure described by the AOAC[10] uses Plackett-Burman experimental designs.[11] Such designs allow the investigation of a number of method parameters in a limited number of experiments. This section focuses on the experimental design used for investigating seven experimental parameters.

Each of the seven parameters are investigated at two levels. Let *A*, *B*, *C*, *D*, *E*, *F*, *G* represent one set of values for the parameters under investigation. Let *a*, *b*, *c*, *d*, *e*, *f*, *g* represent the alternative values for the parameters. If control limits have been set in the method for a parameter (*e.g.*, heat at 100±5 °C) the parameter should be investigated at the extremes of the permitted range (*i.e.*, 95 °C and 105 °C in the example given). If no control limits have been specified it is up to the analyst to choose suitable values for the ruggedness test. This can be based on knowledge gained from similar methods or during the development of the method being studied, or from knowledge of the normal variation of the parameter. For example, the method may require that the sample is left to stand at ambient temperature. The analyst knows that the maximum variation in the laboratory temperature is 20±5 °C. In the ruggedness test the sample would be left to stand at 15 °C or 25 °C as required by the experimental design.

Variables such as temperature and time are known as continuous variables. Ruggedness tests can also be used to evaluate the effects of changes in non-continuous variables such as the type of HPLC column used (*e.g.*, C_8 vs.C_{18}).

The ruggedness test should be carried out on a representative sample. If it is suspected that particular types of sample may behave in different ways, for example from data collected in the recovery and precision studies or during the development of the method, they should be investigated separately and, if possible, the reasons for the differing response identified.

To investigate seven parameters, a minimum of 8 experiments are required using the following experimental design:

Parameter value	Determination number							
	1	2	3	4	5	6	7	8
A or *a*	*A*	*A*	*A*	*A*	*a*	*a*	*a*	*a*
B or *b*	*B*	*B*	*b*	*b*	*B*	*B*	*b*	*b*
C or *c*	*C*	*c*	*C*	*c*	*C*	*c*	*C*	*c*
D or *d*	*D*	*D*	*d*	*d*	*d*	*d*	*D*	*D*
E or *e*	*E*	*e*	*E*	*e*	*e*	*E*	*e*	*E*
F or *f*	*F*	*f*	*f*	*F*	*F*	*f*	*f*	*F*
G or *g*	*G*	*g*	*g*	*G*	*g*	*G*	*G*	*g*
Observed result	**s**	**t**	**u**	**v**	**w**	**x**	**y**	**z**

The effect of a particular parameter is estimated by subtracting the mean of the results obtained with the parameter at the alternative value from the mean of the results obtained with it at the initial value. For example, for parameter *A* the difference, Dx_A, is calculated from:

$$Dx_A = \frac{(\mathbf{s}+\mathbf{t}+\mathbf{u}+\mathbf{v})}{4} - \frac{(\mathbf{w}+\mathbf{x}+\mathbf{y}+\mathbf{z})}{4} \qquad \text{Eq. 4.28}$$

Calculate the differences for all seven parameters and list them in order of magnitude. Note that the signs of the differences are unimportant. If variations in one or two parameters are affecting the analysis then their differences will be substantially larger than those for the other parameters. To determine whether variations in a parameter have a significant effect on the result, a significance test is used to determine if the difference calculated above is significantly different from zero. The procedure is as follows:

1. Obtain an estimate of the within batch method precision, as a standard deviation, from replicate analysis of a representative sample over a short period of time.
2. Calculate the test statistic *t*:[12]

$$t = \frac{\sqrt{n} \times |Dx_i|}{\sqrt{2} \times s} \qquad \text{Eq. 4.29}$$

where s is the estimate of the method precision calculated in (1) above, n is the number of experiments carried out at each level for each parameter ($n = 4$ for the design given above), and D_{x_i} is the difference calculated for parameter x_i.

Compare t with the 2-tailed critical value, t_{crit}, for N-1 degrees of freedom at 95% confidence, where N is the number of determinations used in the estimation of s.

Case 1: If t is less than t_{crit} the difference is not significantly different from zero. Therefore, variations in the parameter do not have a significant effect on the method performance.

Case 2: If t is greater than t_{crit} the difference is significantly different from zero. Therefore, variations in the parameter have a significant effect on the method performance.

In both cases there is an uncertainty associated with the parameter. Procedures for estimating the uncertainties are given in the following sections.

4.3.3.2 Calculating uncertainties for case 1 parameters

Although the ruggedness study indicated that variations in the parameter do not significantly affect the method (*i.e.*, the change in results on varying the parameter is not significantly different from zero), the significance test could not have distinguished between values in the range $0 \pm (\sqrt{2} \times t_{crit} \times s)/\sqrt{n}$). The uncertainty associated with the final result y due to parameter x_i, is given by:

$$u(y(x_i)) = \frac{\sqrt{2} \times t_{crit} \times s}{\sqrt{n} \times 1.96} \times \frac{\delta_{real}}{\delta_{test}} \qquad \text{Eq. 4.30}$$

where δ_{real} is the change in the parameter which would be expected when the method is operating under control in routine use and δ_{test} is the change in parameter that was specified in the ruggedness test. This term is required to take account of the fact that the change in a parameter used in the ruggedness test may be much greater than that observed during normal operation of the method.

If the effect is proportional to the analyte concentration then the uncertainty should be converted to a relative standard deviation by dividing by an estimate of the mean obtained from replicate analysis of the sample used in the study under normal conditions, or if this is not available, by the average of the eight results obtained in the ruggedness test. If, however, the effect is independent of analyte concentration the uncertainty should be expressed as a standard deviation (see Section 5).

4.3.3.3 Calculating uncertainties for case 2 parameters

To calculate the uncertainty for a particular parameter, x_i, an estimate of the **sensitivity coefficient**, c_i, and the uncertainty in the parameter, $u(x_i)$, is required.

4.3.3.3.1 Estimating $u(x_i)$: For example, a method states that the sample must be distilled for 120 minutes. The analyst estimates that the variation in the distillation time in routine application of the method will be ±5 minutes. The uncertainty in the distillation time is therefore 2.9 minutes (see Annex 2 for information on calculating standard uncertainties). Alternatively, control limits can be set for the parameter to ensure that the resulting contribution to the overall uncertainty is acceptable. If a parameter is already controlled by specification (such as specifying a temperature as 4 ± 1°C), the specification limit represents the relevant uncertainty in the parameter, and should be converted to a standard deviation.

4.3.3.3.2 Estimating c_i: If the ruggedness test indicates that the parameter has a significant effect on the result, the sensitivity coefficient can be estimated from the results of the study:

$$c_i = \frac{Observed\ change\ in\ result}{Change\ in\ parameter} \qquad \text{Eq. 4.31}$$

If the parameter is found to be a significant source of uncertainty, or if a better estimate of the effect of the parameter on the result is required, further experimental study is needed. Evaluate the rate of change of the result with changes in the parameter by carrying out a number of experiments with the parameter at a range of different values. Plot a graph of the result versus the value of the parameter. If the relationship is approximately linear, the sensitivity coefficient is equivalent to the gradient of the best fit line.

4.3.3.3.3 Calculate the uncertainty in the final result due to parameter x_i, $u(y(x_i))$, using:

$$u(y(x_i)) = u(x_i) \times c_i \qquad \text{Eq. 4.32}$$

If the effect is proportional to analyte concentration, convert the uncertainty to a relative standard deviation by dividing $u(y(x_i))$ by y, where y is the result obtained with the parameter at the value specified in the method.

5. Calculation of combined standard and expanded uncertainties

5.1 Combined standard uncertainty

Following the estimation of the individual components of the uncertainty using the procedures outlined in Section 4, the next stage is to combine the standard uncertainties to give a combined standard uncertainty. How the individual uncertainty components are combined depends on whether or not they are proportional to the analyte concentration. If the uncertainty component is proportional to the analyte concentration then it can be treated as a relative standard deviation. If, however, the uncertainty is fixed regardless of the analyte concentration then it should be treated as a standard deviation. This leads to two possible cases:

5.1.1 All sources of uncertainty are proportional to the analyte concentration

In this case all the individual uncertainty components should be converted to relative standard deviations. For a result y which is affected by the parameters p, q, r ..... which each have uncertainties $u(p)$, $u(q)$, $u(r)$ ..... the uncertainty in y, $u(y)$ is given by:

$$\frac{u(y)}{y} = \sqrt{\left(\frac{u(p)}{p}\right)^2 + \left(\frac{u(q)}{q}\right)^2 + \left(\frac{u(r)}{r}\right)^2 + ...} \qquad \text{Eq. 5.1}$$

5.1.2 Some sources of uncertainty are independent of analyte concentration

In such cases the uncertainty components that are independent of the analyte concentration must be combined as standard deviations. The uncertainty in the result due to parameters that are not concentration dependent, $u(y)'$, is given by:

$$u(y)' = \sqrt{u(p)^2 + u(q)^2 + u(r)^2 + ...} \qquad \text{Eq. 5.2}$$

To calculate the combined uncertainty in the result, $u(y')$ at an analyte concentration y', the concentration dependent and concentration independent uncertainties are combined as follows:

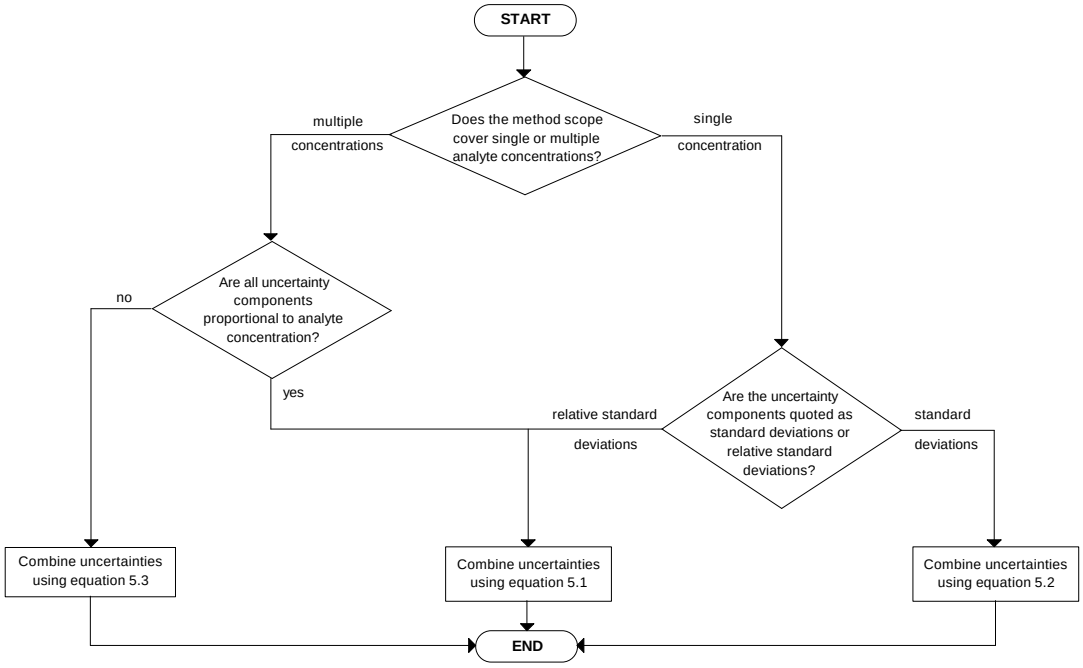
$$u(y') = \sqrt{\left(u(y)'\right)^2 + \left(y' \times \frac{u(y)}{y}\right)^2} \qquad \text{Eq. 5.3}$$

where $u(y)$ is the combined concentration dependent uncertainties calculated using Eq. 5.1 and $u(y)'$ is the combined concentration independent uncertainty calculated using Eq. 5.2.

Note that when the uncertainty estimate is required for a single analyte concentration, the uncertainty components can be combined as either standard deviations or relative standard deviations; it will make no difference to the final answer.

The flow chart in Figure 5 illustrates the different options for combining uncertainties.

Figure 5: Flow chart illustrating the options for combining uncertainty components



5.2 Expanded uncertainty

The combined standard uncertainty obtained in Section 5.1 must be multiplied by an appropriate coverage factor, k, to give the expanded uncertainty. The expanded uncertainty is an interval which is expected to include a large fraction of the distribution of values reasonably attributable to the measurand. For a combined standard uncertainty $u(y)$, the expanded uncertainty $U(y)$ is given by:

$$U(y) = k \times u(y) \qquad \text{Eq. 5.4}$$

The choice of coverage factor depends on knowledge of the use to which the result is put, the degree of confidence required and knowledge of the degrees of freedom associated with individual uncertainty components. For most purposes a coverage factor of $k = 2$ is recommended (however, see note below). For a normal distribution a coverage factor of 2 gives an interval containing approximately 95% of the distribution of values.

For a higher level of confidence, k is chosen as 3. For a normal distribution a coverage factor of 3 gives an interval containing over 99.6% of the distribution of values.

Note: The use of coverage factors of 2 and 3 to give levels of confidence of approximately 95% and 99.6% respectively assumes that there are a reasonable

number of degrees of freedom associated with the estimates of the major contributions to the uncertainty budget. In this guide it is recommended that at least 10 determinations are carried out in the precision and trueness studies. If it is not possible to obtain this many replicates and either of these factors dominate the uncertainty budget, the coverage factor should be obtained from the table of critical values for the Student *t* test. For example, if the dominant contribution to the uncertainty budget was based on only 4 determinations this would give 3 degrees of freedom. The 2-tailed t_{crit} value at the 95% confidence level is 3.182. It can therefore be seen that using uncertainty estimates based on only a small number of determinations will have a significant effect on the coverage factor and hence on the expanded uncertainty.

6. Reporting uncertainty

6.1 Reporting expanded uncertainty

The Eurachem Guide[2] gives the following guidance:

Unless otherwise required, the result y should be stated together with the expanded uncertainty, $U(y)$, calculated using a coverage factor of $k = 2$ (or other appropriate coverage factor, see Section 5.2). The following form is recommended:

"(result): $y \pm U(y)$ (units)

[where] the reported uncertainty is [an expanded uncertainty as defined in the International Vocabulary of Basic and General terms in Metrology, 2nd, ed., ISO, 1993] calculated using a coverage factor of 2, [which gives a level of confidence of approximately 95%.]"

Terms in [ ] may be omitted or abbreviated as appropriate.

6.2 Reporting standard uncertainty

The Eurachem Guide[2] gives the following guidance:

When uncertainty is expressed as the combined standard uncertainty $u(y)$ the following form is recommended:

"(result): y (units) [with a] standard uncertainty of $u(y)$ (units) [where standard uncertainty is defined in the International Vocabulary of Basic and General terms in Metrology, 2nd, ed., ISO, 1993 and corresponds to one standard deviation.]"

The use of the symbol ± is not recommended when using standard uncertainty.

6.3 Documentation

A simple proforma for summarising uncertainty budgets is given in Annex 5. This can be used to summarise details of the sources of uncertainty included in the budget, how they were estimated and their magnitudes. There is also a section for the combined standard and expanded uncertainty.

Annex 1: Cause and Effect Analysis

As discussed in Section 3, uncertainty estimation requires the production of a structured list of possible sources of uncertainty. One way of producing such a list is cause and effect analysis.[13] The principles of the construction of cause and effect diagrams are described fully elsewhere,[13] and detailed discussions of their application to uncertainty estimation, with examples, have been published.[14, 15] The use of cause and effect diagrams has three main stages: construction, refinement and experimental design (also called reconciliation). An outline of the process is presented below.

A1.1 Construction of a cause and effect diagram

A cause and effect diagram consists of a hierarchical structure of "causes" which culminate in a single outcome or "effect". A typical diagram for an analytical method is given in Figure A1.1. In terms of uncertainty estimation, the "effect" is the result obtained from the analysis. The main branches feeding into it represent the parameters used in the calculation of the result. Combining the uncertainties associated with these parameters will give the uncertainty in the final result. The stages in the construction are:

A1.1.1 Write the complete equation used to calculate the result, including any intermediate calculations. The parameters in the equation form the main branches of the diagram. It is almost always necessary to add a main branch representing overall bias, usually as recovery.

A1.1.2 Consider each branch in turn and add additional branches representing effects which will contribute to the uncertainties in the parameters identified in A1.1.1. For example, the uncertainty in the weight of sample taken for analysis will have contributions from the balance precision and calibration. Branches representing these terms should therefore feed into the main branch representing the sample weight.

A1.1.3 For each branch added in A1.1.2, add further branches representing any additional contributory factors. Continue the process until the effects become sufficiently remote.

A1.2 Refinement of the cause and effect diagram

Refinement of the diagram involves the resolution of duplicate terms and rearrangement of the branches to clarify contributions and group related causes. This process results in a simplified cause and effect diagram which can be used as a checklist to ensure that all sources of uncertainty have been considered in the uncertainty budget. Duplications of terms in the diagram are resolved using the rules given in Section 3.4.

Figure A1.2 shows a typical cause and effect diagram after refinement.

A1.3 Experimental design

The final stage in the process is the planning of experiments which will provide the information required to obtain an estimate of the combined uncertainty for the method. Initially, two sets of experiments are carried out - a precision study and a trueness study. These experiments are

planned in such a way that as many of the sources of uncertainty identified in the cause and effect diagram as possible are covered. The diagram is used as a checklist, and those parameters not adequately covered by the precision and trueness experiments are evaluated separately. Section 4 discusses the types of experiments required and gives details of how the data obtained are used to calculate uncertainty.

Key to Figure A1.1 and Figure A1.2

Equation used to calculate the concentration of all-trans retinol, $C_{all-trans}$ in $\mu g\ 100\ g^{-1}$, in a sample of infant formula:

$$C_{all-trans} = \frac{A_S \times V_F \times C_{STD}}{A_{STD} \times W_S}$$

where:

A_S is the peak area recorded for the sample solution;

A_{STD} is the peak area recorded for the standard solution;

V_F is the final volume of the sample solution (ml);

W_S is the weight of sample taken for analysis (g);

C_{STD} is the concentration of the standard solution ($\mu g\ ml^{-1}$).

In the cause and effect diagrams:

C flask/pipette calibration;

T temperature effects;

B_C balance calibration;

L balance linearity.

Annex 2: Calculating standard uncertainties

When estimating uncertainty, a variety of existing information may be available, *e.g.*, calibration certificates. This information may not be expressed in the form of a standard deviation and must therefore be converted before it can be combined with other standard uncertainties. Some common cases are given below.

1. The uncertainty is expressed as a confidence interval with a given level of confidence. For example, a certificate states that the concentration of an analyte in a certified reference material is 100±0.5 mg kg^{-1} with 95% confidence.

   To convert to a standard uncertainty, divide by the appropriate percentage point of the Normal distribution for the level of confidence given. For 95% confidence divide by 1.96.

2. The uncertainty is expressed as an expanded uncertainty calculated using a given coverage factor. For example, documentation supplied with a solution states that its concentration is 1000±3 mg l^{-1} where the reported uncertainty is an expanded uncertainty calculated using a coverage factor $k = 2$ which gives a level of confidence of approximately 95%.

   To convert to a standard uncertainty, divide by the stated coverage factor.

3. Limits of $\pm x$ are given without a confidence level or coverage factor. For example, the manufacturer's specification for the stated volume of a 100 ml volumetric flask is quoted as ±0.08 ml. It is normally appropriate to assume a rectangular distribution with a standard deviation of $x/\sqrt{3}$.

   To convert to a standard uncertainty, divide by $\sqrt{3}$.

Annex 3: Definitions

Bias

The difference between the expectation of the test results and an accepted reference value.[16]

Note: Bias is the total systematic error as contrasted to random error. There may be one or more systematic error components contributing to the bias. A larger systematic difference from the accepted reference value is reflected by a larger bias value.

Coverage factor

The numerical factor, *k*, used as a multiplier of the combined standard uncertainty in order to obtain an expanded uncertainty.[1]

Note: A coverage factor is typically in the range 2 to 3. A coverage factor of 2 gives a level of confidence of approximately 95%.

Critical value

The critical value (*e.g.* t_{crit} in a *t*-test) is the tabulated value of the test statistic obtained in a significance test. The critical value is obtained from statistical tables by reading the value given for the appropriate number of degrees of freedom and the desired level of confidence.[4] In this guide, two-tailed t_{crit} values at the 95% confidence level are always used.

Degrees of freedom

The term *n*-1 in the expression for the sample standard deviation, *s*, is called the number of degrees of freedom (abbreviated to dof or ν in statistical tables). In general it is the number of data points, *n*, less the number of parameters already estimated from the data. In the expression for the sample standard deviation the number of degrees of freedom is *n*-1 as the mean has already been estimated from the data. Knowledge of the number of degrees of freedom is needed for obtaining the appropriate critical value from statistical tables for significance tests. In general it is equivalent to *n*-1.

Expanded uncertainty

Quantity defining an interval about the result of a measurement that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand.[1] The expanded uncertainty, $U(y)$, is calculated from the combined standard uncertainty $u(y)$ and a coverage factor k using:

$$U(y) = k \times u(y)$$

F-test

The **F**-test is a significance test used to compare two variances, s_1^2 and s_2^2, where s is the standard deviation. Calculate the test statistic:

$$\mathbf{F} = \frac{s_1^2}{s_2^2}$$

where s_1^2 is the larger of the two variances (this will guarantee that $\mathbf{F} \geq 1$). Compare **F** with the appropriate critical value, $\mathbf{F}_{crit}$, at n_1-1 and n_2-1 degrees of freedom. If **F** is less than $\mathbf{F}_{crit}$ there is no significant difference between the variances. For a 2-tailed test at the 95% confidence level, the appropriate critical value is obtained from statistical tables for the 97.5% confidence level.[4]

Measurement uncertainty

Parameter associated with the result of a measurement, that characterises the dispersion of values that could reasonably be attributed to the measurand.[17]

Note: The parameter may be, for example, a standard deviation (or given multiple of it), or the width of a confidence interval. Uncertainty of measurement comprises, in general, many components. Some of these components may be evaluated from the distribution of the results of a series of measurements and can be characterised by experimental standard deviations. The other components, which can also be characterised by standard deviations, are evaluated from assumed probability distributions based on experience or other information. It is understood that the result of the measurement is the best estimate of the value of the measurand and that all components of uncertainty, including those arising from systematic effects, contribute to the dispersion.

Precision

The closeness of agreement between independent test results obtained under stipulated conditions.[16]

Note: Precision depends only on the distribution of random errors and does not relate to the true value or specified value. The measure of precision is usually expressed in terms of imprecision and calculated as a standard deviation of the test results.

Recovery

The ratio of the observed value to the expected value.

Note: The expected value can be either the certified value of a reference material or the concentration of a fortified or spiked sample. In analytical chemistry, recovery is often used as a measure of the trueness of a method.

Relative standard deviation

The relative standard deviation, *RSD*, of a set of results with standard deviation *s* and mean $\bar{x}$ is given by:

$$RSD = \frac{s}{\bar{x}}$$

Sample standard deviation

The standard deviation of *n* results with a mean $\bar{x}$ is given by:

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$$

Sensitivity coefficient

The sensitivity coefficient describes how the observed output varies with changes in the input parameter. For example, for a result *y* and a parameter x_i, the sensitivity coefficient c_i describing the effect of changes in x_i on *y* is given by:

$$c_i = \frac{Observed\ change\ in\ y}{Change\ in\ x_i}$$

Significance test

A statistical test used to decide whether there are significant differences between the properties of different sets of data at a given level of confidence. A test statistic is calculated and compared with a tabulated critical value. If the test statistic is greater than the critical value this indicates a significant difference between the properties being compared. Examples of significance tests include the *t*-test which can be used to compare the mean of a set of results with a stated value, or to compare the means of two independent sets of results; and the **F**-test which is used to compare the variances of two sets of data.[4]

Standard deviation of the mean

The standard deviation of the mean, $s_{\bar{x}}$, of n samples with standard deviation s is given by:

$$s_{\bar{x}} = \frac{s}{\sqrt{n}}$$

Standard uncertainty

Uncertainty of the result of a measurement expressed as a standard deviation.[1] For a result x_i, the standard uncertainty is $u(x_i)$.

Trueness

The closeness of agreement between the average value obtained from a large set of test results and an accepted reference value.[16]

Note: The measure of trueness is normally expressed in terms of bias.

Uncertainty budget

List of parameters and their associated uncertainties which contribute to the uncertainty for the method as a whole. The individual uncertainties in the uncertainty budget are combined to give the standard and expanded uncertainties for the method.

Annex 4: Worked examples

A4.1 The determination of vitamin A and vitamin E in infant formula

A4.1.1 Outline of method

The method was developed for the determination of tocopherol, retinol and carotene isomers in a range of food stuffs. This example considers the validation and calculation of an uncertainty estimate specifically for the determination of all-trans retinol (vitamin A) and α-tocopherol (vitamin E) in infant formula. The homogenised sample is hydrolysed to release the retinol and tocopherol isomers. These are then extracted into mixed ethers and the extract concentrated. Portions of the extract are chromatographed on different HPLC systems to separate and quantify the isomers required. A normal phase system is used for the determination of α-tocopherol whilst a reversed phase system is used for the determination of all-trans retinol. In each case calibration is by means of a single standard prepared by serial dilution of a stock solution. The concentration of the analyte in a sample, C_{vit}, is given by:

$$C_{vit} = \frac{A_S \times V_F \times C_{STD}}{A_{STD} \times W_S}$$

where:

A_S is the peak area recorded for the sample solution;

A_{STD} is the peak area recorded for the standard solution;

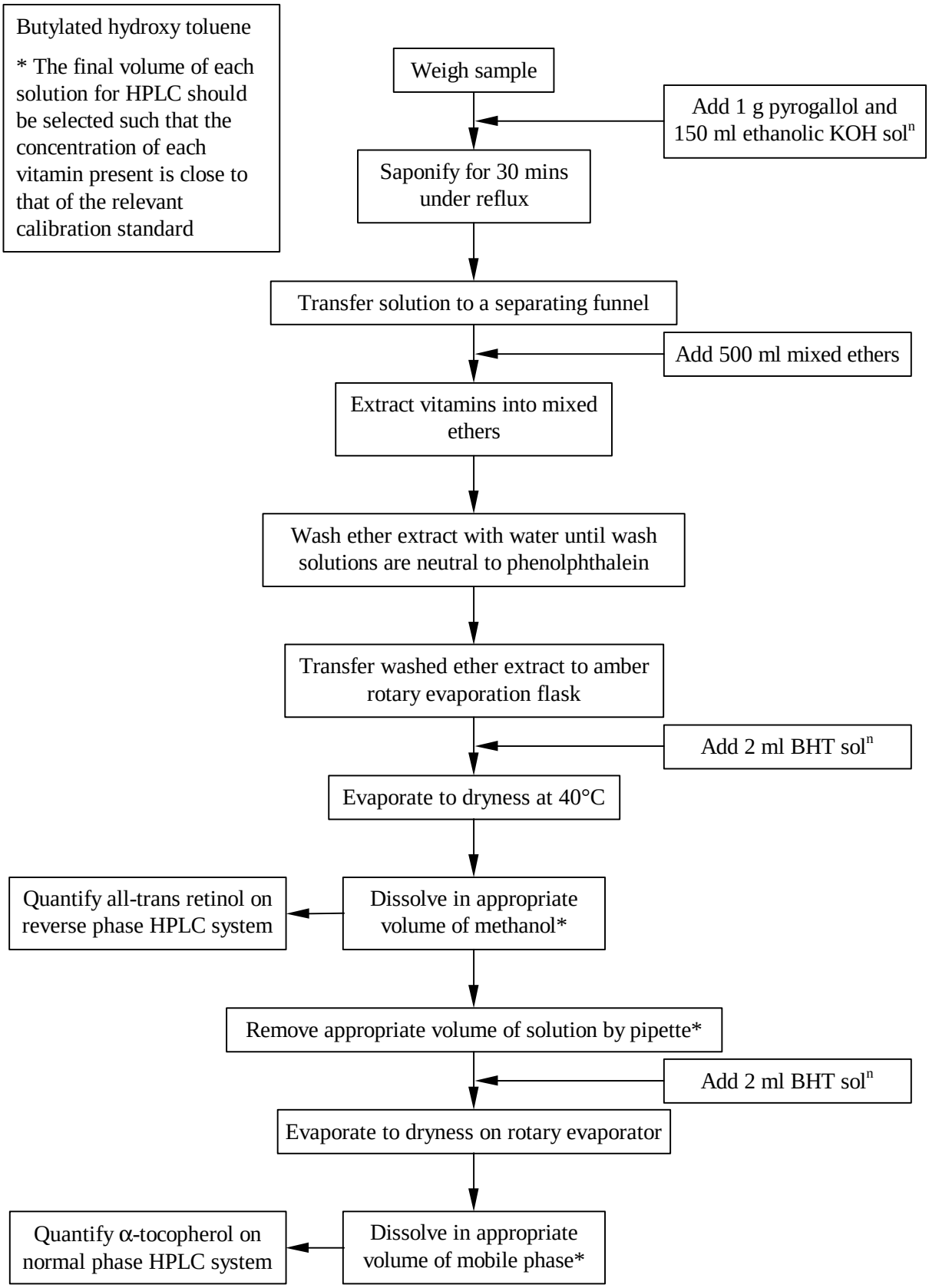
V_F is the final volume of the sample solution (ml);

W_S is the weight of sample taken for analysis (g);

C_{STD} is the concentration of the standard solution (μg ml^{-1}).

A flow diagram illustrating the stages in the method is presented in Figure A4.1 and cause and effect diagrams are given in Annex 1.

Figure A4.1: Flow diagram illustrating stages in the method for the determination of all-trans retinol and tocopherols in infant formula



A4.1.2 Precision study

A survey of the nutritional labelling of commercially available infant formula indicated that that they were all of broadly comparable composition (fat, protein and carbohydrate levels) and contained similar concentrations of vitamin A and vitamin E. Three samples were chosen to cover the widest possible range of vitamin concentrations. Each sample was analysed a total of four times, in four separate extraction and HPLC runs. For each HPLC run fresh calibration standards and mobile phase were prepared. In addition, a certified reference material (SRM 1846) was analysed in replicate for the estimation of $\overline{R}_m$ (see below). These results were included in the precision study. The results are summarised in Table A4.1.[§]

Table A4.1: Summary of results from precision studies on the determination of all-trans retinol and a-tocopherol

Sample	all-trans retinol				a-tocopherol			
	mean (mg kg^{-1})	standard deviation (mg kg^{-1})	relative standard deviation	***n***	mean (mg kg^{-1})	standard deviation (mg kg^{-1})	relative standard deviation	***n***
Infant formula A	9.67	0.146	0.0151	4	64.08	2.411	0.0376	4
Infant formula B	10.40	0.247	0.0238	4	69.05	2.156	0.0312	4
Infant formula C	6.67	0.217	0.0325	4	171.93	7.959	0.0463	4
SRM 1846	5.32	0.285	0.0536	6	278.51	10.663	0.0383	6

The standard deviations observed for all-trans retinol were of a similar order of magnitude. This indicates that within the range of analyte concentrations studied, the standard deviation is independent of analyte concentration. In this case, the uncertainty associated with method precision, $u(P)$, for samples with all-trans retinol concentrations ranging from approximately 5 mg kg^{-1} to 10 mg kg^{-1}, was estimated as the pooled standard deviation. This was obtained using Eq. 4.1:

$$s_{pool} = \sqrt{\left(\frac{\left(3\times 0.146^2\right)+\left(3\times 0.247^2\right)+\left(3\times 0.217^2\right)+\left(5\times 0.285^2\right)}{3+3+3+5}\right)} = 0.238$$

$u(P)$ for all-trans retinol is therefore 0.238 mg kg^{-1}.

[§] Note: The examples given in Annex 4 were calculated using a spreadsheet. The values presented in the tables and equations have been rounded. Reproducing the calculations with the values given may therefore result in slightly differing answers.

For α-tocopherol, the relative standard deviations observed for the different samples were all of a similar order of magnitude. This indicates that the standard deviation is approximately proportional to analyte concentration, across the range studied. In such cases it is appropriate to pool the relative standard deviations to obtain the estimate of $u(P)$, using Eq. 4.2:

$$RSD_{pool} = \sqrt{\left(\frac{\left(3 \times 0.0376^2\right) + \left(3 \times 0.0312^2\right) + \left(3 \times 0.0463^2\right) + \left(5 \times 0.0383^2\right)}{3+3+3+5} \right)} = 0.0387$$

$u(P)/P$ for α-tocopherol is therefore 0.0387 as a relative standard deviation.

A4.1.3 Trueness study

Calculation of $\bar{R}_m$ and $u(\bar{R}_m)$

In this case a certified reference material, SRM 1846 produced by the National Institute of Standards and Technology (NIST), was available. The CRM is an infant formula certified for a number of vitamins, including all-trans retinol and α-tocopherol. Six portions of the CRM were analysed. The results, together with the certified values for the material, are summarised in Table A4.2.

Table A4.2: Results from the analysis of infant formula SRM 1846

Analyte	Certified values			Observed values	
	Concentration C_{CRM} (mg kg^{-1})	Quoted uncertainty (mg kg^{-1})[1]	Standard uncertainty $u(C_{CRM})$ (mg kg^{-1})[2]	Mean $\bar{C}_{obs}$ (mg kg^{-1})	standard deviation s_{obs} (mg kg^{-1})
all-trans retinol	5.84	0.68	0.35	5.32	0.285
α-tocopherol	271	25	12.8	278.51	10.663

[1] The quoted uncertainty is an expanded uncertainty given at the 95% confidence level.

[2] The standard uncertainty is obtained by dividing the expanded uncertainty by 1.96.

Eq. 4.3 and Eq. 4.4 were used to calculate $\bar{R}_m$ and $u(\bar{R}_m)$ respectively.

For all-trans retinol:

$$\bar{R}_m = 5.32 / 5.84 = 0.911$$

$$u(\overline{R}_m) = 0.911 \times \sqrt{\left(\frac{0.285^2}{6 \times 5.32^2}\right) + \left(\frac{0.35}{5.84}\right)^2} = 0.0581$$

Similar calculations for α-tocopherol gave values of $\overline{R}_m$ = 1.028 and $u(\overline{R}_m)$ = 0.0511.

To determine the contribution of $\overline{R}_m$ to the combined uncertainty, the value calculated above was compared with 1, using Eq. 4.18. For all-trans retinol:

$$t = \frac{1 - 0.911}{0.0581} = 1.53$$

In this case, t was compared with the coverage factor, k = 2. As t is less than two there is no evidence to suggest that $\overline{R}_m$ is significantly different from 1. $\overline{R}_m$ was therefore assumed to equal 1 with an uncertainty, $u(\overline{R}_m)$, of 0.0581 (see Section 4.2.5, case 1). A similar calculation for α-tocopherol also indicated that $\overline{R}_m$ was not significantly different from 1. $u(\overline{R}_m)$ was therefore estimated as 0.0511.

Calculation of $u(R_s)$

R_s was estimated from spiking studies on infant formula A used in the precision study. It was not possible to produce a homogeneous bulk spiked sample so individual portions of the infant formula were spiked at the required concentration. Samples were prepared at the concentrations indicated in Table A4.3. Four samples were analysed at each concentration, in separate extraction and HPLC batches. The samples were spiked by adding solutions of all-trans retinol and α-tocopherol to approximately 10 g of sample in a saponification flask, prior to the addition of any other reagents. The mean recovery for each sample was calculated using Eq. 4.12. C_{native} was taken as the mean of the results obtained from the precision study. The results are summarised in Table A4.3.

Table A4.3: Summary of results from the study of R_s for the determination of all-trans retinol and a-tocopherol

Spiking level	all-trans retinol			a-tocopherol		
	Approx target concentration (mg kg^{-1})	Mean recovery	***n***	Approx target concentration (mg kg^{-1})	Mean recovery	***n***
1	14.0	0.981	4	84.0	1.028	4
2	18.0	0.991	4	99.0	1.103	4
3	22.0	0.996	4	114.0	1.167	4
	$u(R_s)$	0.00767		$u(R_s)$	0.0696	

$u(R_s)$ is the standard deviation of the mean recoveries obtained at each concentration.

Calculation of ***R*** and ***u(R)***

In this case, as a representative CRM was available for the estimation of $\overline{R}_m$ and $u(\overline{R}_m)$ for both all-trans retinol and α-tocopherol, there was no need for the R_{rep} term. Both $\overline{R}_m$ and R_s are assumed to be equal to 1. R is therefore also equal to 1. $u(R)$ was calculated using Eq. 4.26. For all-trans retinol:

$$u(R) = \sqrt{0.0581^2 + 0.00767^2} = 0.0586$$

A similar calculation for α-tocopherol gives an estimate of $u(R) = 0.0863$. Note that since in both cases $R = 1$, the uncertainty is the same whether expressed as a standard deviation or a relative standard deviation.

A4.1.4 Evaluation of other sources of uncertainty

Ruggedness study

There were a number of method parameters which were not representatively varied during the precision and trueness studies. These were investigated using a ruggedness study. The extraction and HPLC stages of the method were examined in separate studies. Only the ruggedness study for all-trans retinol is presented. A similar study would be required for α-tocopherol to complete the uncertainty budget. The parameters investigated and the levels chosen are summarised in Table A4.4 and Table A4.5 below. The study was carried out on the Certified Reference Material.

Table A4.4: Parameters investigated in the ruggedness study of the extraction procedure for all-trans retinol

Parameter	Value			
Weight KOH used in ethanolic KOH	A	42 g	a	21 g
Saponification time	B	20 mins	b	40 mins
Number of 250 ml ether extractions	C	2	c	1
Shaking time for each ether extraction	D	2 mins	d	1 min
Number of water washes*	E	to neutral	e	3 extra
Rotary evaporation time**	F	to dryness	f	5 additional mins
Rotary evaporation temperature	G	30 °C	g	50 °C

* The method currently states that the ether extract should be washed with water until the wash extracts are neutral. For parameter (e) the ether extract was washed with a further 3 portions of water.

** The method currently states that the sample extract should be evaporated to dryness.

For parameter (f) the evaporation was continued for a further 5 minutes after dryness had been reached.

Table A4.5: Parameters investigated in the ruggedness study of the HPLC procedure for the determination of all-trans retinol

Parameter	Value			
Column age	A'	new	a'	old
Flow rate	B'	0.8 ml min^{-1}	b'	1.2 ml min^{-1}
Dummy variable	C'	+	c'	-
Mobile phase composition (methanol/water)	D'	90/10	d'	95/5
Injection volume	E'	15 µl	e'	25 µl
Detection wavelength	F'	320 nm	f'	330 nm
Column temperature	G'	30 °C	g'	oven off

Results

The results from the ruggedness studies were evaluated as described in Section 4.3.3.

Extraction and clean-up stage

The results obtained from the ruggedness testing of the extraction procedure for all-trans retinol are presented in Table A4.6. The differences for each parameter, Dx_A to Dx_G, were calculated as described in Section 4.3.3.1.

Table A4.6: Results form the ruggedness testing of the extraction of all-trans retinol from infant formula

Observed result (mg kg^{-1})							
s	**t**	**u**	**v**	**w**	**x**	**y**	**z**
5.25	3.98	4.22	4.43	5.51	4.78	4.35	4.96
Calculated differences							
Dx_A	Dx_B	Dx_C	Dx_D	Dx_E	Dx_F	Dx_G	
-0.4329	0.3898	0.2948	-0.1009	0.2387	0.7036	0.03608	

The precision for the method had previously been estimated as 0.238 mg kg^{-1} with 14 degrees of freedom (see Section A4.1.2). Using Eq. 4.29, *t* values were calculated for

each parameter. These were then compared with the critical value t_{crit} = 2.145 (2-tailed, ν = 14, 95% confidence). The results are presented below:

Table A4.7: Identification of parameters having a significant effect on the extraction of all-trans retinol from infant formula

Parameter	*t* value	Significant effect at 95% confidence?
Weight KOH used in ethanolic KOH	2.573	Yes
Saponification time	2.316	Yes
Number of 250 ml ether extractions	1.752	No
Shaking time for each ether extraction	0.599	No
Number of water washes	1.419	No
Rotary evaporation time	4.181	Yes
Rotary evaporation temperature	0.214	No

In cases where the effect of a parameter was found not to be significant, Eq. 4.30 was used to calculate the uncertainty:

$$u(y(x_i)) = \frac{\sqrt{2} \times 2.145 \times 0.238}{\sqrt{4} \times 1.96} \times \frac{\delta_{real}}{\delta_{test}} = 0.184 \times \frac{\delta_{real}}{\delta_{test}}$$

Number of ether extractions

The method specifies two ether extractions. In the ruggedness study, the effect of using only one ether extraction was investigated. The results indicated that using only one extraction reduced the recovery of all-trans retinol, although the effect was not significant. If the method is applied correctly it is unlikely that δ_{real} would equal δ_{test}. However, there is likely to be some variation in the efficiency of the extractions from analysis to analysis, due to variations in the vigour with which the mixture is shaken. This effect will be covered by the estimate of uncertainty associated with precision. For these reasons, an additional uncertainty has not been included for this parameter.

Shaking time for ether extractions

The method was developed using a shaking time of 2 minutes per extraction. In the ruggedness test this was reduced to 1 minute. δ_{test} is therefore equal to 1 minute. It is estimated that during normal operation of the method, the shaking time might vary between 1.5 minutes and 2.5 minutes. δ_{real} is therefore also 1 minute. $u(y(x_D))$ is therefore 0.184 mg kg^{-1}.

Number of water washes

The method currently specifies that the ether extract should be washed with 150 ml portions of water until the wash solution is neutral to phenolphthalein. The results of the ruggedness test indicate that additional washes of the extract beyond this leads to lower results. In the ruggedness study the effect of three extra washes was investigated (δ_{test} = 3). During routine use of the method it was considered unlikely that as many as three extra washes would be carried out. However, one additional wash may be likely, particularly if neutrality has only just been reached on the previous wash. δ_{real} was therefore estimated as being equal to 1. The uncertainty, $u(y(x_E))$, is therefore 0.0613.

Rotary evaporation temperature

The method has been developed using a rotary evaporation temperature of 40°C. It is estimated that this is controlled to ±2°C in normal use (*i.e.*, δ_{real} = 4°C). In the ruggedness test the temperature was set at 30°C and 50°C, giving a δ_{test} value of 20°C. $u(y(x_G))$ is therefore 0.0368 mg kg^{-1}.

For parameters identified as having a significant effect on the extraction, the procedure described in Section 4.3.3.3 was followed.

Weight of potassium hydroxide used to prepare ethanolic potassium hydroxide solution

Under normal operating conditions, 42±0.1 g potassium hydroxide is used in the preparation of the ethanolic potassium hydroxide solution required for the saponification reaction. In the ruggedness study the amount of potassium hydroxide used was reduced by 21 g. This resulted in a difference, Dx_A, of -0.4329 mg kg^{-1}. Using Eq. 4.31, the sensitivity coefficient, c_A, was calculated as:

$$c_A = \frac{0.4329}{21} = 0.0206 \text{ mg kg}^{-1} \text{ g}^{-1}$$

The uncertainty in the parameter, $u(x_A)$, was obtained from the control limit on the weight of potassium hydroxide specified in the method, assuming a rectangular distribution. Therefore, $u(x_A) = 0.1/\sqrt{3} = 0.0577$ g. The uncertainty in the final result due to variations in parameter *A*, $u(y(x_A))$ was calculated using Eq. 4.32:

$$u(y(x_A)) = 0.0577 \times 0.0206 = 0.00119 \text{ mg kg}^{-1}$$

Saponification time

The method was developed using a saponification time of 30 minutes. It is estimated that during routine use of the method this will vary by ±5 minutes, giving an uncertainty, u_B, of 2.89 minutes (assuming a rectangular distribution). In the ruggedness study the parameter was varied from 20 minutes to 40 minutes. This resulted in a difference, Dx_B, of 0.3898 mg kg^{-1}. The sensitivity coefficient, c_B, is therefore 0.0195 mg kg^{-1} min^{-1}. The uncertainty in the final result due to variation in this parameter, $u(y(x_B))$, is therefore 0.0564 mg kg^{-1}.

Rotary evaporation time

The results of the ruggedness study showed that allowing the rotary evaporation to continue after the sample had reached dryness caused a reduction in the amount of all-trans retinol observed. This is likely to be due to decomposition of the analyte. In the ruggedness study, rotary evaporation was allowed to continue for an extra 5 minutes after dryness had been reached. This resulted in a difference, Dx_F, of 0.7036 mg kg^{-1}. The sensitivity coefficient is therefore 0.141 mg kg^{-1} min^{-1}. In the routine application of the method, it was estimated that the rotary evaporation may continue for between 0 and 5 minutes after dryness has been reached. This was taken as a rectangular distribution of 2.5±2.5 minutes. u_F is therefore equal to 1.443 minutes. The uncertainty, $u(y(x_F))$, is therefore 0.203 mg kg^{-1}.

The effect of all the above parameters was considered to be proportional to the analyte concentration. The uncertainties were therefore converted to relative standard deviations by dividing by 5.32 mg kg^{-1}, the mean of the results obtained from previous analyses of SRM 1846 under normal method conditions.

HPLC stage

The results from the ruggedness testing of the HPLC procedure for the determination of all-trans retinol are presented in Table A4.8.

Table A4.8: Results form the ruggedness testing of the HPLC procedure for the determination of all-trans retinol

Observed result (mg kg^{-1})							
s	**t**	**u**	**v**	**w**	**x**	**y**	**z**
5.47	5.33	5.35	5.07	4.59	4.75	4.79	5.09
Calculated differences							
Dx_A'	Dx_B'	Dx_C'	Dx_D'	Dx_E'	Dx_F'	Dx_G'	
0.4997	-0.04021	-0.01202	0.2291	0.2192	-0.000975	-0.06682	

The calculated differences were tested for significant variation from zero. The standard deviation used in the significance test was based on pooled within batch precision data from three standard solutions of all-trans retinol, each analysed four times. This gave a pooled standard deviation of 0.167 mg kg^{-1} ($\nu = 9$). The appropriate t_{crit} value, at the 95% confidence level, is 2.262.

For parameters identified as having no significant effect, the uncertainty was estimated as $0.136 \times (\delta_{real}/\delta_{test})$ using Eq. 4.30.

Flow rate

The method has been developed using a flow rate of 1 ml min^{-1}. Information in the literature suggests that a typical variation in flow rate is ±0.3 %. This corresponds to 0.003 ml min^{-1} in this case. δ_{real} is therefore equal to 0.006 ml min^{-1}. In the ruggedness study the flow rate was changed from 0.8 ml min^{-1} to 1.2 ml min^{-1}. δ_{test} is therefore 0.4 ml min^{-1}. The uncertainty in the final result due to variations in the flow rate, $u(y(x_B'))$ is therefore 0.00204 mg kg^{-1}.

Mobile phase composition

The method specifies a mobile phase composition of methanol:water (90:10 v/v). From the literature it is estimated that the variation in mixing the mobile phase is unlikely to exceed more than ±1 % in either of the components. In the ruggedness study the composition was changed to 95:5 (v/v) methanol:water, a much larger change than would be expected in the normal application of the method. As a first estimate, it was assumed that, during normal use of the method, the effect on the final result due to variations in the mobile phase composition would be one tenth of that observed in the ruggedness test. The uncertainty, $u(y(x_D'))$, is therefore 0.0136 mg kg^{-1}.

Injection volume

Information in the literature indicates 1.5% to be a typical coefficient of variation for the volume delivered by an auto sampler. The method specifies an injection volume of 20 µl. δ_{real} is therefore 0.6 µl. In the ruggedness study the injection volume was varied by 10 µl (δ_{test}). The uncertainty, $u(y(x_E'))$, is therefore 0.00816 mg kg^{-1}.

Detection wavelength

In the ruggedness study the detection wavelength was varied by 10 nm. Based on information in the literature, a wavelength accuracy of ±2 nm is assumed. The uncertainty, $u(y(x_F'))$, is 0.0544 mg kg^{-1}.

Column temperature

As part of the ruggedness study, the effect of using an un-thermostatted column was investigated. During the study the laboratory temperature averaged at 22.2°C with a %CV of 1%. The column is normally maintained at 30°C. It is estimated that this is controlled to within ±1°C. δ_{real} and δ_{test} are therefore equal to 2°C and 7.8°C respectively. The uncertainty, $u(y(x_G'))$, is therefore 0.0349 mg kg^{-1}.

The effect of all the above parameters was considered to be proportional to the analyte concentration. The uncertainties were therefore converted to relative standard deviations by dividing by 5.32 mg kg^{-1}

Only one parameter was found to be having a significant effect on the method performance - the age of the column. A lower result was obtained when an old column was used compared to a brand new one. Further investigation of the data revealed that this was due to the peak area for the standard increasing on the old column, whilst the peak area for the sample decreased, leading to a decrease in the calculated concentration.

The results of the ruggedness test indicate that as the column ages the method performance will change. During routine application of the method this should be monitored using an HPLC QC standard. Only when this is performing adequately (after adjustment of the HPLC conditions if necessary) will sample extracts be presented for analysis. Due to the relatively short timescale it is unlikely that the precision study undertaken as part of the validation study would include variability in method performance due to column ageing. However, when the method is in routine use a check sample will be analysed and data collected over a period of time. The variation observed in these results will include the variability due to changes in the column performance, within the bounds permitted by the HPLC QC standard. If the long term precision estimate obtained from the QC sample is significantly different from that obtained from the precision study described earlier, then it can be used to generate a revised uncertainty estimate at a later data. For the above reasons, an estimate of the uncertainty due to ageing of the HPLC column has not been included in the present uncertainty budget.

Remaining sources of uncertainty

The precision and trueness studies were designed to cover as many of the sources of uncertainty as possible (see cause and effect diagram, Figures A1.1 and A1.2), for example, by analysing a range of concentration levels, and by preparing new standards and HPLC mobile phase for each batch of analyses. Parameters which were not adequately varied during these experiments, such as the extraction and HPLC conditions, were investigated in the ruggedness tests. There are however, a small number of parameters which were not covered by the above experiments. These generally related to the calibration of volumetric glassware and balances used in the preparation of the standards and samples. For example, during this study the same balance was used to weigh out all the samples. Although the precision associated with this operation is included in the overall precision estimate, the effect of the accuracy of the balance has not been included in the uncertainty budget so far. The balance used in the study may typically have a positive bias of 0.0001 g. In the future a different balance, or the same balance after re-calibration, may have a negative bias of 0.0001 g. Since this possible variation is not already included in the uncertainty budget it should be considered separately. However, previous experience[18] has shown us that uncertainties associated with the calibration of analytical balances and volumetric glassware are generally small compared to other sources of uncertainty such as overall precision and recovery. Additional uncertainty estimates for these parameters have not therefore been included in the uncertainty budget.

A4.1.5 Calculation of combined and expanded uncertainties

Table A4.9 presents the magnitudes of the individual uncertainty components for the determination of all-trans retinol in infant formula.

Table A4.9: Uncertainty budget for the determination of all-trans retinol in infant formula

Parameter		Standard uncertainty
Precision	$u(P)$	0.238 mg kg^{-1}
Recovery	$u(R)$	0.0586
Weight KOH used in ethanolic KOH	$u(y(x_A))$	0.000224
Saponification time	$u(y(x_B))$	0.0106
Shaking time for each ether extraction	$u(y(x_D))$	0.0346
Number of water washes	$u(y(x_E))$	0.0115
Rotary evaporation time	$u(y(x_F))$	0.0382
Rotary evaporation temperature	$u(y(x_G))$	0.00692
Flow rate	$u(y(x_B'))$	0.000383
Mobile phase composition	$u(y(x_D'))$	0.00256
Injection volume	$u(y(x_E'))$	0.00153
Detection wavelength	$u(y(x_F'))$	0.0103
Column temperature	$u(y(x_G'))$	0.00656
Note that standard uncertainties given without units are expressed as relative standard deviations.		

The sources of uncertainty that were identified as being proportional to analyte concentration were combined by calculating the root sum of squares as in Eq. 5.1:

$$\frac{u(y)}{y} = 0.0809$$

This was combined with the uncertainty associated with method precision, which was found to be independent of analyte concentration, using Eq. 5.3. For example, for a sample containing 5 mg kg^{-1} all-trans retinol:

$$u(y') = \sqrt{0.238^2 + \left(5 \times 0.0809\right)^2} = 0.47 \text{ mg kg}^{-1}$$

Similar calculations lead to the following estimates of uncertainty for a range of typical all-trans retinol concentrations:

Table A4.10: Standard uncertainty estimates for the determination of all-trans retinol in infant formula		
Concentration (mg kg^{-1})	Standard uncertainty (mg kg^{-1})	Relative standard uncertainty
5	0.47	0.094
7.5	0.65	0.087
10	0.84	0.084

Expanded uncertainties were obtained by multiplying the standard uncertainties by a coverage factor, $k = 2$, which gives a level of confidence of approximately 95%:

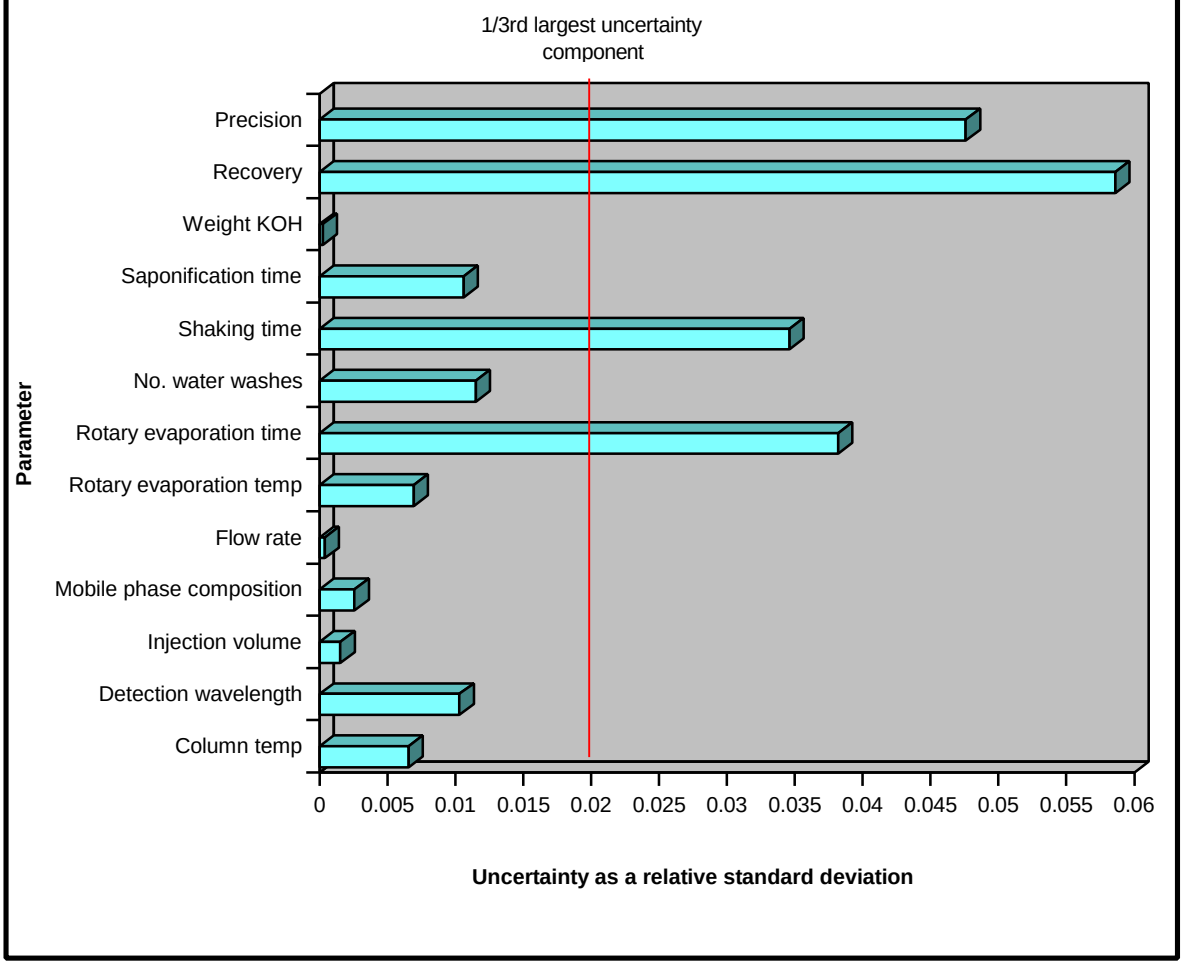
Table A4.11: Expanded uncertainty estimates for the determination of all-trans retinol in infant formula		
Concentration (mg kg^{-1})	Expanded uncertainty (mg kg^{-1})	Relative expanded uncertainty
5	0.94	0.19
7.5	1.30	0.17
10	1.69	0.17

Comments on uncertainty budget

Figure A4.2 illustrates the contributions to the measurement uncertainty for the determination of all-trans retinol in infant formula at a concentration of 5 mg kg^{-1}. The vertical line indicates one third of the magnitude of the largest contribution to the uncertainty budget (in this case recovery). Only parameters with uncertainties greater than this value need to be considered as significant sources of uncertainty for the method. In this case the four major contributions were identified as precision and recovery for the entire method, and the effects of the length of time each ether extract is shaken with water and of evaporating the ether extract to dryness. Both the latter uncertainties were evaluated using data from the ruggedness test. The test is designed to highlight the parameters which have a significant effect on method performance and also to give an initial uncertainty estimate for each parameter. If the uncertainty estimate so obtained is a significant contribution to the uncertainty estimate, ideally the effects of changes in that parameter should be investigated further. In this case, for example, that could involve separate studies to investigate the effect of shaking time and rotary evaporation conditions on the result of the analysis. It will then be possible to include a refined uncertainty estimate in the uncertainty budget. If the uncertainties associated with these

parameters are unacceptably large, the parameters will have to be more strictly controlled in the method protocol, for example by specifying a shaking time of 2 minutes±10 seconds. If it is not possible to control the parameter sufficiently, *i.e.*, if the control limits required would be so tight as to be impossible to achieve during routine application of the method, then further method development will be required.

Figure A4.2: Contributions to the measuremrnt uncertainty for the determination of all-trans retinol in infant formula (analyte concentration 5 mg kg^{-1})



A4.2 The determination of markers in oils

A4.2.1 Outline of method

The quantification of three markers - solvent red 24, quinizarin and solvent yellow 124 - in oils is required. The samples are analysed by reversed phase high performance liquid chromatography with UV-visible diode array detection, after a clean up stage. The sample (10 ml) is passed through a 500 mg silica cartridge. The cartridge is washed under vacuum with 10 ml hexane to remove residual oil. The solvent red 24, quinizarin and solvent yellow 124 are then eluted under gravity with 10 ml butan-1-ol in hexane (butan-1-ol 10% v/v). The eluant is evaporated to dryness under a stream of air, heating if necessary, and the residue dissolved in 2.5 ml acetonitrile. The solution is placed in an ultrasonic bath for 5 minutes and then filtered through a 0.45µm filter. 50 µl of the resulting solution is analysed by HPLC using a 5 µm phenyl-hexyl column (250 mm x 4.6 mm) with a gradient elution of water and acetonitrile at a flow rate of 1 ml min^{-1}. The column is maintained at a temperature of 30 °C. Detection is by means of a diode array detector set at 475 nm for the determination of solvent yellow 124 and 500 nm for the determination of solvent red 24 and quinizarin. Calibration is by means of a single standard in acetonitrile containing solvent red 24 and solvent yellow 124 at a concentration of 20 mg l^{-1}, and quinizarin at a concentration of 10 mg l^{-1}. The concentration of the markers, C_{marker} in mg l^{-1}, is given by:

$$C_{\text{marker}} = \frac{A_S \times V_F \times C_{STD}}{A_{STD} \times V_S}$$

where:

A_S is the peak area recorded for the sample solution;

A_{STD} is the peak area recorded for the standard solution;

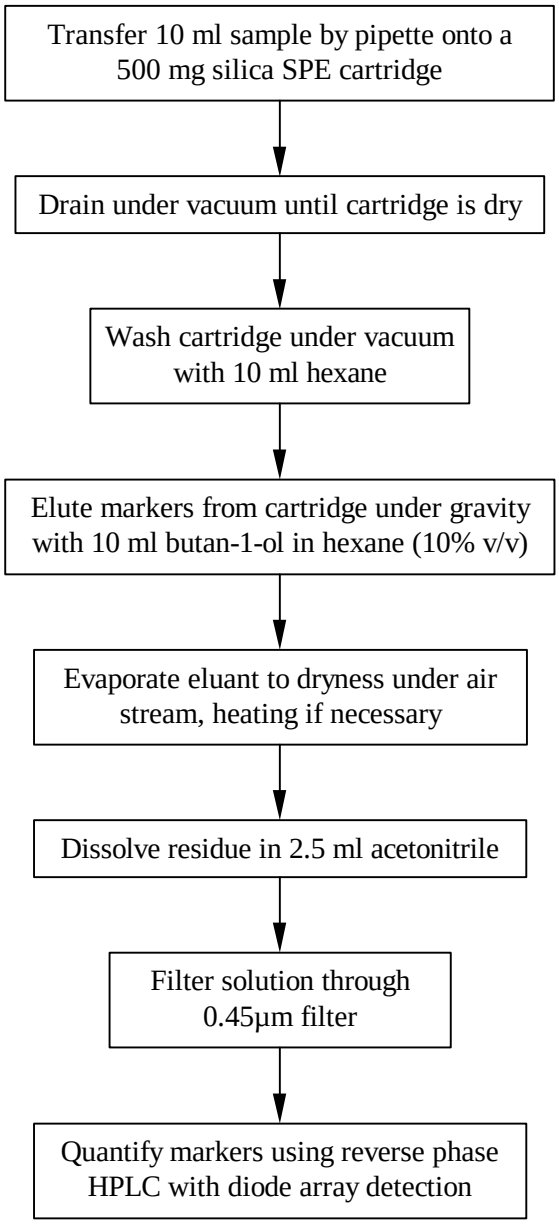
V_F is the final volume of the sample solution (ml);

V_S is the volume of the sample taken for analysis (ml);

C_{STD} is the concentration of the standard solution (mg l^{-1}).

A flow diagram illustrating the main stages in the method is presented in Figure A4.3.

Figure A4.3: Flow diagram illustrating the stages in the method for the determination of markers in diesel oil



A4.2.2 Precision study

Samples of three types of unmarked oils (A to C) were fortified with solvent red 24, quinizarin and solvent yellow 124 according to Table A4.12, giving a total of 15 samples.

Table A4.12: Samples prepared for precision study on the determination of markers in oil

Analyte	Concentration of compound in sample (mg l^{-1})				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Solvent red 24	0.041	1.02	2.03	3.05	4.06
Quinizarin	0.040	0.498	0.996	1.49	1.99
Solvent yellow 124	0.040	1.20	2.40	3.99	4.99

Oil B was a diesel sample, and this represented a sample of typical viscosity. Oil A was a kerosene and oil C was a lubricating oil. These oils are respectively less viscous and more viscous than oil B. Initially, 12 sub-samples of the diesel (B) sample 3 were analysed. The extraction of the samples was carried out in two batches of six on consecutive days. The markers in all 12 samples were quantified in a single HPLC run with the order of analysis randomised. The results are summarised in Table A4.13 (note that analysis of one of the samples for quinizarin was unsuccessful, giving n = 11). This initial study was followed by the analysis, in duplicate, of all 15 samples. The results are summarised in Table A4.14, Table A4.15 and Table A4.16.

Table A4.13: Summary of results from the replicate analysis of matrix B fortified at concentrations of 2.03 mg l^{-1} solvent red 24, 0.996 mg l^{-1} quinizarin and 2.40 mg l^{-1} solvent yellow

Analyte	mean (mg l^{-1})	standard deviation (mg l^{-1})	relative standard deviation	n
Solvent red 24	1.92	0.0621	0.0323	12
Quinizarin	0.913	0.0216	0.0236	11
Solvent yellow 124	2.35	0.0251	0.0107	12

Table A4.14: Summary of results from the duplicate analysis of oil samples spiked with solvent red 24 at a range of concentrations

Matrix	Target conc (mg l^{-1})	Observed conc (mg l^{-1})		mean	Difference	Normalised difference
A	0.041	0.033	0.036	0.035	-0.003	-0.0883
A	1.02	0.989	0.969	0.979	0.019	0.0199
A	2.03	1.96	1.98	1.97	-0.016	-0.00837
A	3.05	2.98	2.88	2.93	0.098	0.0334
A	4.06	3.99	4.01	4.00	-0.019	-0.00474
B	0.041	0.044	0.036	0.040	0.007	0.181
B	1.02	0.945	0.927	0.936	0.018	0.0191
B	2.03	1.98	1.96	1.97	0.013	0.00669
B	3.05	2.83	2.90	2.87	-0.065	-0.0226
B	4.06	3.90	3.88	3.89	0.025	0.00650
C	0.041	0.039	0.040	0.040	-0.001	-0.0195
C	1.02	0.921	0.963	0.942	-0.043	-0.0455
C	2.03	1.93	1.87	1.90	0.064	0.0339
C	3.05	2.96	3.02	2.99	-0.061	-0.0205
C	4.03	3.92	4.04	3.98	-0.120	-0.0302
				s	0.0532	0.0585
				$s/\sqrt{2}$	0.0376	0.0414

Table A4.15: Summary of results from the duplicate analysis of oil samples spiked with quinizarin at a range of concentrations						
Matrix	Target conc (mg l^{-1})	Observed conc (mg l^{-1})		mean	Differences	Normalised difference
A	0.040	0.030	0.029	0.030	0.001	0.0398
A	0.498	0.359	0.360	0.360	-0.002	-0.00422
A	0.996	0.743	0.700	0.722	0.042	0.0587
A	1.49	0.999	1.13	1.07	-0.135	-0.127
A	1.99	1.58	1.59	1.58	-0.013	-0.00815
B	0.040	0.030	0.027	0.029	0.003	0.100
B	0.498	0.421	0.443	0.432	-0.023	-0.0529
B	0.996	0.909	0.892	0.900	0.018	0.0196
B	1.49	1.37	1.35	1.36	0.022	0.0160
B	1.99	1.88	1.87	1.88	0.010	0.00533
C	0.040	0.034	0.031	0.032	0.003	0.0954
C	0.498	0.355	0.248	0.302	0.107	0.354
C	0.996	0.779	0.665	0.722	0.114	0.158
C	1.49	1.20	1.11	1.16	0.089	0.0769
C	1.99	1.53	1.63	1.58	-0.095	-0.0602
				s	0.0664	0.111
				$s/\sqrt{2}$	0.0470	0.0788

Table A4.16: Summary of results from the duplicate analysis of oil samples spiked with solvent yellow 124 at a range of concentrations						
Matrix	Target conc (mg l^{-1})	Observed conc (mg l^{-1})		mean	Differences	Normalised difference
A	0.040	0.038	0.031	0.034	0.007	0.206
A	1.20	1.16	1.15	1.16	0.009	0.00796
A	2.40	2.32	2.27	2.30	0.054	0.0236
A	3.99	3.78	3.80	3.79	-0.022	-0.00574
A	4.99	4.86	4.82	4.84	0.041	0.00857
B	0.040	0.032	0.036	0.034	-0.004	-0.121
B	1.20	1.16	1.14	1.15	0.020	0.0173
B	2.40	2.37	2.32	2.35	0.052	0.0220
B	3.99	3.90	3.85	3.88	0.054	0.0139
B	4.99	4.97	4.93	4.95	0.039	0.00781
C	0.040	0.043	0.040	0.041	0.003	0.0780
C	1.20	1.17	1.15	1.16	0.024	0.0211
C	2.40	2.38	2.30	2.34	0.081	0.0348
C	3.99	3.85	3.81	3.83	0.041	0.0107
C	4.99	4.79	4.85	4.82	-0.057	-0.0118
				s	0.0349	0.0656
				$s/\sqrt{2}$	0.0247	0.0464

In addition, a sample of diesel spiked with the three markers was analysed 48 times in 3 batches of 16 analyses as part of the trueness study (see Section A4.2.3). The results are summarised in Table A4.17. The estimates of s_{total} were obtained from analysis of variance (ANOVA).[4]

Solvent Red 24

In the case of solvent red 24, there was no statistically significant difference between the three estimates of the relative standard deviation obtained (see Table A4.13, Table A4.14 and Table A4.17), although the test was borderline. This indicates that across the range studied (0.04 mg l^{-1} to 4 mg l^{-1}) the precision is approximately proportional to analyte concentration. However, to avoid producing an uncertainty estimate which would be an under estimate for any particular matrix or concentration, it was decided to use the estimate of 0.0414 as a "worst case" uncertainty associated with precision, $u(P)$. This estimate was obtained from the analysis of different matrices and concentrations and is therefore likely to be a more representative of the precision across the method scope.

Quinizarin

The various estimates of the standard deviation and relative standard deviation were not comparable. In particular, the estimates obtained from the duplicate results were significantly different from the other estimates. However, there were no obvious patterns in the data so no particular matrix and/or concentration could be identified as being the cause of the variability. There was therefore no justification for removing any data and restricting the coverage of the uncertainty estimate, as in the case of solvent yellow 124 (see below). The results of the precision studies indicate that the method is more variable across different matrices and analyte concentrations for quinizarin than for the other markers. The uncertainty associated with the precision was therefore taken as the estimate of the relative standard deviation obtained from the duplicate results, 0.0788. This represents a worst case estimate but it should ensure that the uncertainty is not underestimated for any given matrix or concentration (although it may result in an overestimate in some cases).

Solvent yellow 124

There was no significant difference between the estimates of the relative standard deviation obtained for samples at concentrations of 2.4 mg l^{-1} and 4.99 mg l^{-1}. However, the estimate obtained from the duplicate analyses was significantly greater than the other estimates. Inspection of that data revealed that the normalised differences observed for the samples at a concentration of 0.04 mg l^{-1} were substantially larger than those observed at the other concentrations. Removing these data points gave a revised estimate of the relative standard deviation of 0.00903. This was in agreement with the other estimates obtained (F-tests, 95% confidence). The three estimates were therefore pooled using Eq. 4.2 to give a single estimate of the relative standard deviation of 0.0114. As it stands, the uncertainty estimate cannot be applied to samples with concentrations below 1.2 mg l^{-1}. Further study would be required to investigate in more detail the precision at these low levels.

A4.2.3 Trueness study

Calculation of $\bar{R}_m$ and $u(\bar{R}_m)$

In this case there was no suitable CRM available for the evaluation of $\bar{R}_m$. The best option available was to use spiked samples. In this example, the behaviour of spiked samples is likely to be a good representation of that of test samples, as oils are marked simply by adding and mixing in the required amount of the markers. A 2000 ml sample of unmarked BP diesel was spiked with standards in toluene containing solvent red 24, quinizarin and solvent yellow 124 at concentrations of 0.996 mg ml^{-1}, 1.02 mg ml^{-1} and 1.97 mg ml^{-1} respectively to give concentrations in the diesel of 4.06 mg l^{-1}, 1.99 mg l^{-1} and 4.99 mg l^{-1} respectively. Forty-eight aliquots of this sample were analysed in three batches of 16 analyses. The results are summarised in Table A4.17.

Table A4.17: Results from the replicate analysis of a diesel oil spiked with solvent red 24, quinizarin and solvent yellow 124

Analyte	Target concentration C_{spike} (mg l^{-1})	Mean $\overline{C}_{obs}$ (mg l^{-1})	Standard deviation of the mean, s_{mean} (mg l^{-1})[a]	Total standard deviation, s_{total} (mg l^{-1})[b]	Relative standard deviation
Solvent red 24	4.06	3.88	0.0360	0.112	0.0289
Quinizarin	1.99	1.89	0.00370	0.0256	0.0136
Solvent yellow 124	4.99	4.99	0.0167	0.0618	0.0124

[a]Estimated from ANOVA of 3 groups of 16 replicates according to ISO 5725:1994[19]
[b]Estimated from ANOVA[4]

$\overline{R}_m$ was calculated as described in Section 4.2.2.1. The mean recovery and its uncertainty were calculated using Eq. 4.5 and Eq. 4.6 respectively, where s_{mean} given in Table A4.17 is equivalent to $s_{obs}/\sqrt{n}$.

For **solvent red 24**:

$$\overline{R}_m = 3.88 / 4.06 = 0.956$$

Based on information on the purity of the material used to prepare the spiked sample, and the accuracy and precision of volumetric glassware and analytical balances used, the uncertainty in the concentration of solvent red 24 in the sample, $u(C_{spike})$, was estimated as 0.05 mg l^{-1}.** The uncertainty in $\overline{R}_m$ is therefore given by:

$$u(\overline{R}_m) = 0.956 \times \sqrt{\left(\frac{0.0360}{3.88}\right)^2 + \left(\frac{0.05}{4.06}\right)^2} = 0.0148$$

The uncertainties associated with the concentration of **quinizarin** and **solvent yellow 124** in the spiked sample were estimated as 0.025 mg l^{-1} and 0.062 mg l^{-1} respectively. Similar calculations as above for quinizarin and solvent yellow 124 produced the following estimates:

quinizarin: $\overline{R}_m$ = 0.949, $u(\overline{R}_m)$ = 0.0121

solvent yellow 124, $\overline{R}_m$ = 1.00, $u(\overline{R}_m)$ = 0.0129

To determine the contribution of $\overline{R}_m$ to the combined uncertainty, the value calculated above was compared with 1, using Eq. 4.18. For **solvent red 24**:

** Detailed information on the estimation of uncertainties of this type is presented in reference 2

$$t = \frac{1 - 0.956}{0.0148} = 2.97$$

In this case, t was compared with coverage factor, $k = 2$. A t value greater than two suggests that $\overline{R}_m$ is significantly different from 1. However, in the normal application of the method, no correction will be made to take account of the fact that the method recovery is significantly different from 1. This is an example of case 3 discussed in Section 4.2.5. Accordingly, Eq. 4.20 was used to calculate the uncertainty $u(\overline{R}_m)''$:

$$u(\overline{R}_m)'' = \sqrt{\left(\frac{1 - 0.956}{2}\right)^2 + 0.0148^2} = 0.0262$$

The significance test also indicated that the recovery of **quinizarin** was significantly different from 1. Applying Eq. 4.20 gives a value of $u(\overline{R}_m)''$ of 0.0283. In the case of **solvent yellow 124**, the significance test indicated that $\overline{R}_m$ was not significantly different from 1. This is an example of case 1 discussed in Section 4.2.5. In such cases the uncertainty associated with $\overline{R}_m$ is the value of $u(\overline{R}_m)$ calculated above (*i.e.*, 0.0129).

Calculation of R_s

R_s was calculated using the results from the samples analysed in the precision study and the data from the determination of $\overline{R}_m$. $u(R_s)$ is the standard deviation of the mean recoveries obtained for the different matrices and analyte concentrations. The data are summarised in Table A4.18 to Table A4.20.

Table A4.18: Summary of recovery data for solvent red 24			
Matrix	Target concentration (mg l^{-1})	Mean recovery	***n***
diesel oil	4.06	0.957	48
A	0.041	0.857	2
A	1.02	0.964	2
A	2.03	0.971	2
A	3.05	0.962	2
A	4.06	0.985	2
B	0.041	0.986	2
B	1.02	0.922	2
B	2.03	0.970	2
B	2.03	0.947	12
B	3.05	0.941	2
B	4.06	0.958	2
C	0.041	0.975	2
C	1.02	0.928	2
C	2.03	0.935	2
C	3.05	0.983	2
C	4.06	0.986	2
standard deviation (*s*)		0.0322	

Table A4.19: Summary of recovery data for quinizarin			
Matrix	Target concentration (mg l^{-1})	Mean recovery	***n***
diesel oil	1.99	0.949	48
A	0.040	0.745	2
A	0.498	0.722	2
A	0.996	0.725	2
A	1.49	0.714	2
A	1.99	0.794	2
B	0.040	0.725	2
B	0.498	0.868	2
B	0.996	0.904	2
B	0.996	0.945	11
B	1.49	0.910	2
B	1.99	0.941	2
C	0.040	0.806	2
C	0.498	0.606	2
C	0.996	0.725	2
C	1.49	0.775	2
C	1.99	0.792	2
standard deviation (*s*)		0.0932	

Table A4.20: Summary of recovery data for solvent yellow 124			
Matrix	Target concentration (mg l^{-1})	Mean recovery	***n***
diesel oil	4.99	1.00	48
A	0.040	0.857	2
A	1.20	0.966	2
A	2.40	0.959	2
A	3.99	0.949	2
A	4.99	0.970	2
B	0.040	0.841	2
B	1.20	0.957	2
B	2.40	0.979	2
B	2.40	0.978	12
B	3.99	0.971	2
B	4.99	0.992	2
C	0.040	1.04	2
C	1.20	0.969	2
C	2.40	0.977	2
C	3.99	0.958	2
C	4.99	0.966	2
standard deviation (*s*)		0.0426	
s excluding recoveries at 0.04 mg l^{-1}		0.0138	

For **solvent red 24**, the standard deviation of the mean recoveries obtained at various concentrations and from various matrices was calculated as 0.0322. As discussed in Section 4.2.6, this was used as the estimate of $u(R_s)$.

In the case of **quinizarin**, the standard deviation of the mean recoveries was 0.0932. The large standard deviation is due to the fact that the recoveries obtained for matrix B were generally higher than those obtained for matrices A or C. In this example, a single uncertainty estimate will be reported for all the matrices studied. However, if this estimate was found to be unsatisfactory for future applications of the method, separate budgets could be calculated for individual matrices.

Due to the problems associated with the precision of the determination of **solvent yellow 124** at low concentrations discussed in Section A4.2.2, the present uncertainty budget will not cover samples with a concentration below 1.2 mg l^{-1}. When calculating $u(R_s)$ the results obtained for samples containing 0.04 mg l^{-1} were therefore discounted. This resulted in a standard deviation of mean recoveries of 0.0138. This was taken as the estimate of $u(R_s)$.

Calculation of *R* and *u(R)*

As mentioned previously, in this example a spiked sample can be considered a reasonable representation of test samples of marked diesel oils, as fuel is marked simply by adding the required quantity of the marker. There is therefore no need for R_{rep} and $u(R_{rep})$ terms. Both $\overline{R}_m$ and R_s are assumed to be equal to 1. R is therefore also equal to 1. $u(R)$ is calculated using Eq. 4.26. For **solvent red 24**:

$$u(R) = \sqrt{0.0262^2 + 0.0322^2} = 0.0415$$

Similar calculations for **quinizarin** and **solvent yellow 124** give values of 0.0974 and 0.0187 respectively.

A4.2.4 Evaluation of other sources of uncertainty

Ruggedness study

There were a number of method parameters which were not adequately varied during the precision and recovery studies. These were investigated using a ruggedness test as described in Section 4.3.3. The extraction and HPLC stages of the method were examined separately. The parameters studied and the levels chosen are presented in Table A4.21 and Table A4.22. The ruggedness test was applied to matrix B sample 3 used in the precision study (see Table A4.12).

Table A4.21: Parameters investigated in the ruggedness study of the extraction procedure for the determination of markers in oil

Parameter	Value			
Brand of silica cartridges	A	Brand A	a	Brand B
Sample volume	B	10 ml	b	12 ml
Rate of elution of oil with hexane	C	vacuum	c	gravity
Volume of hexane wash	D	12 ml	d	8 ml
Conc butan-1-ol/hexane	E	12%	e	8%
Vol 10% butan-1-ol/hexane	F	12 ml	f	8 ml
Evaporation temperature	G	50 °C	g	80 °C

Table A4.22: Parameters investigated in the ruggedness study of the HPLC procedure for the determination of markers in oil				
Parameter	Value			
Grade of acetonitrile in mobile phase	A'	Far UV	a'	HPLC
Flow rate	B'	0.8 ml min^{-1}	b'	1.2 ml min^{-1}
Injection volume	C'	40 µl	c'	60 µl
Column temperature	D'	25 °C	d'	35 °C
Detector wavelength (A)	E'	465 nm	e'	485 nm
Mobile phase degassing	F'	degassed	f'	not degassed
Detector wavelength (B)	G'	490 nm	g'	510 nm

Results

The results from the ruggedness studies were evaluated as described in Section 4.3.3.

Extraction stage

The results obtained from the ruggedness testing of the extraction procedure for solvent red 24, quinizarin and solvent yellow 124 are presented in Table A4.23. The differences for each parameter, D_{X_A} to D_{X_G} were calculated as described in Section 4.3.3.1.

Table A4.23: Results form the ruggedness testing of the extraction of solvent red 24, quinizarin and solvent yellow 124 from fuel

Solvent red 24 Observed result (mg l^{-1})							
s	**t**	**u**	**v**	**w**	**x**	**y**	**z**
2.02	2.00	2.12	2.17	1.87	1.71	2.34	2.36
Calculated differences							
Dx_A	Dx_B	Dx_C	Dx_D	Dx_E	Dx_F	Dx_G	
0.0075	-0.3525	0.0275	0.2125	-0.0425	0.0625	-0.0275	
Quinizarin Observed result (mg l^{-1})							
s	**t**	**u**	**v**	**w**	**x**	**y**	**z**
1.10	1.06	1.15	1.01	0.96	0.86	1.28	1.26
Calculated differences							
Dx_A	Dx_B	Dx_C	Dx_D	Dx_E	Dx_F	Dx_G	
-0.0075	-0.18	0.07	0.1775	0.0175	-0.005	-0.0425	
Solvent yellow 124 (mg l^{-1}) Observed result							
s	**t**	**u**	**v**	**w**	**x**	**y**	**z**
2.41	2.38	2.52	2.66	2.19	2.15	2.76	2.87
Calculated differences							
Dx_A	Dx_B	Dx_C	Dx_D	Dx_E	Dx_F	Dx_G	
-0.0025	-0.4225	-0.02	0.225	-0.01	0.08	0.0075	

The precision of the method for the analysis of the sample used in the ruggedness study had previously been estimated as:

solvent red 24: 0.0621 mg l^{-1} (ν = 11)

quinizarin: 0.0216 mg l^{-1} (ν = 10)

solvent yellow 124: 0.0251 mg l^{-1} (ν = 11)

Using Eq. 4.29, *t* values were calculated for each parameter and each analyte. These were compared with the appropriate critical values of *t* at 95% confidence (t_{crit} = 2.201 for solvent red 24 and solvent yellow 124, and 2.228 for quinizarin).

Solvent red 24

In cases were the effect of a parameter was found not to be significant, Eq. 4.30 was used to calculate the uncertainty:

$$u(y(x_i)) = \frac{\sqrt{2} \times 2.201 \times 0.0621}{\sqrt{4} \times 1.96} \times \frac{\delta_{real}}{\delta_{test}} = 0.0493 \times \frac{\delta_{real}}{\delta_{test}}$$

Brand of silica cartridge

The ruggedness test investigated the effect of changing between brands of silica cartridge. Since this is likely to happen during the routine application of the method, δ_{real} can be considered as being equal to δ_{test}. The uncertainty, $u(y(x_A))$, is therefore 0.0493 mg l^{-1}.

Rate of elution of oil with hexane

The effect of the rate of elution of oil by hexane from the cartridge was investigated by comparing the difference between elution under a vacuum and elution under gravity. When the method is used routinely, the oil will be eluted under vacuum. Variations in the vacuum applied from one extraction to another will affect the rate of elution of the oil and the amount of oil eluted. However, the effect of variations in the vacuum will be small compared to the effect of having no vacuum present. It can therefore be assumed that variations in the observed concentration of the markers, due to variability in the vacuum, will be small compared to the differences observed in the ruggedness test. As a first estimate, the effect of variation in the vacuum during routine application of the method was estimated as one tenth of that observed during the ruggedness study.

Concentration of butan-1-ol in hexane

The method was developed using a concentration of 10% (v/v) butan-1-ol in hexane. Based on the manufacturers' specification and typical precision data for the volumetric glassware used to prepare the solution, it was estimated that the concentration may vary by ±0.1% (v/v), *i.e.*, δ_{real} is 0.2% (v/v). From the ruggedness test, δ_{test} is equal to 4% (v/v). The uncertainty, $u(y(x_E))$, is therefore 0.00247 mg l^{-1}.

Volume of 10% (v/v) butan-1-ol in hexane

The method was developed using 10 ml of 10% (v/v) butan-1-ol in hexane for elution of the markers. The likely variation in this volume was estimated as ±0.04 ml (based on the manufacturer's specification for the pipette and typical precision data for pipettes of this type). In the ruggedness test, the volume was changed by 4 ml. δ_{real} and δ_{test} are therefore equal to 0.08 ml and 4 ml respectively. The uncertainty, $u(y(x_F))$, is therefore 0.000986 mg l^{-1}.

Evaporation temperature

The method currently specifies that samples are evaporated to dryness at 50°C. The uncertainty in this value during routine use of the method was estimated as ±5°C. δ_{real} is therefore 10°C. δ_{test} was 30°C. The uncertainty $u(y(x_G))$ is therefore 0.0164 mg l^{-1}.

For parameters identified as having a significant effect on the extraction, the procedure described in Section 4.3.3.3 was followed.

Sample volume

In the routine application of the method, 10 ml of sample is taken for analysis using an automatic pipette. The uncertainty associated with this volume was estimated as 0.04 ml. In the ruggedness test, the volume was increased to 12 ml. This resulted in a difference, D_B, of -0.3525 mg l^{-1}. The sensitivity coefficient, c_B, is therefore 0.1763 mg l^{-1} ml^{-1}. The uncertainty, $u(y(x_B))$, was therefore calculated as 0.00705 mg l^{-1}.

Volume of hexane wash

The method was developed using 10 ml hexane (added by pipette) to wash the silica cartridge. The uncertainty associated with the volume delivered was estimated as 0.04 ml. This is $u(x_D)$. Eq. 4.31 was used to calculate the sensitivity coefficient, c_D:

$$c_D = \frac{0.2125}{4} = 0.05313 \text{ mg } l^{-1} \text{ ml}^{-1}$$

The uncertainty in the final result due to variation in parameter D, $u(y(x_D))$ was calculated using Eq. 4.32:

$$u(y(x_D)) = 0.04 \times 0.05313 = 0.00213 \text{ mg } l^{-1}$$

The effect of all these parameters was considered to be proportional to the analyte concentration. The uncertainty was therefore converted to a relative standard deviation by dividing by, 1.92 mg l^{-1}, the mean of the results obtained from previous analyses of the sample under normal method conditions.

Quinizarin

For the parameters identified as not having a significant effect on the method performance, $u(y(x_i))$ was calculated as $0.0174 \times (\delta_{real}/\delta_{test})$ using Eq. 4.30. The uncertainties were converted to relative standard deviations by dividing by 0.909 mg l^{-1}.

Brand of silica cartridge

Using the same arguments applied in the case of solvent red 24, the uncertainty was calculated as 0.0174 mg l^{-1}.

Concentration of butan-1-ol in hexane

Again applying the same reasoning as in the case of solvent red 24, the uncertainty was estimated as 0.000868 mg l^{-1}.

Volume of 10% (v/v) butan-1-ol in hexane

The uncertainty was calculated as 0.000347 mg l^{-1}, as discussed previously.

For the parameters identified as being significant, the following uncertainty estimates were calculated:

Sample volume

The uncertainty was calculated as 0.00360 mg l^{-1}, using the same reasoning as applied to solvent red 24.

Rate of elution of oil with hexane

As discussed above, the effect on the final result of variations in the vacuum when eluting the oil from the cartridge with hexane was estimated as one tenth that observed in the ruggedness test. The uncertainty, $u(y(x_C))$ was therefore estimated as 0.0070 mg l^{-1}.

Volume of hexane wash

The uncertainty was evaluated as described in the case of solvent red 24. This resulted in an uncertainty of 0.00177 mg l^{-1}.

Evaporation temperature

The uncertainty in the evaporation temperature was based on the assumption that the temperature could be controlled to ±5 °C. This was taken as a rectangular distribution and converted to a standard uncertainty by dividing by $\sqrt{3}$. The uncertainty $u(y(x_G))$ is therefore 0.00409 mg l^{-1}.

Solvent yellow 124

For the parameters identified as not having a significant effect on the method performance, $u(y(x_i))$ was calculated as $0.0199 \times (\delta_{real}/\delta_{test})$ using Eq. 4.30. The uncertainties were converted to relative standard deviations by dividing by 2.35 mg l^{-1}.

Brand of silica cartridge

Using the same arguments applied in the case of solvent red 24, the uncertainty was calculated as 0.0199 mg l^{-1}.

Rate of elution of oil with hexane

This was treated as for solvent red 24, giving an uncertainty of 0.00199 mg l^{-1}.

Concentration of butan-1-ol in hexane

Again applying the same reasoning as in the case of solvent red 24, the uncertainty was estimated as 0.0010 mg l^{-1}.

Evaporation temperature

The uncertainty was calculated as 0.00663 mg l^{-1}, following the reasoning given for solvent red 24.

For the parameters identified as being significant, the following uncertainty estimates were calculated:

Sample volume

The uncertainty was calculated as 0.00845 mg l^{-1}, using the same reasoning as applied to solvent red 24.

Volume of hexane wash

As for the other two markers, the volume of the hexane wash clearly influences the recovery of solvent yellow 124. Using the procedure described for solvent red 24, the uncertainty $u(y(x_D))$ was calculated as 0.00225 mg l^{-1}.

Volume of 10% (v/v) butan-1-ol in hexane

The results indicate that the volume of butan-1-ol/hexane used to elute the marker has a significant effect on recovery. As in the case of the hexane wash, the solution was added by automatic pipette. The uncertainty $u(y(x_F))$ was therefore calculated in the same way. $u(y(x_F))$ is therefore equal to 0.00080 mg l^{-1}.

HPLC stage

The results obtained from the ruggedness testing of the HPLC procedure for solvent red 24, quinizarin and solvent yellow 124 are presented in Table A4.24. The differences for each parameter, Dx_A' to Dx_G' were calculated as described in Section 4.3.3.1.

Table A4.24: Results form the ruggedness testing of the HPLC procedure for the determination of solvent red 24, quinizarin and solvent yellow 124 in fuel

Solvent red 24 Observed result (mg l^{-1})							
s	**t**	**u**	**v**	**w**	**x**	**y**	**z**
1.82	1.39	1.87	1.86	1.82	1.81	1.81	1.80
Calculated differences							
Dx_A'	Dx_B'	Dx_C'	Dx_D'	Dx_E'	Dx_F'	Dx_G'	
-0.0748	-0.1236	0.1152	-0.1303	0.1036	0.1082	0.1047	
Quinizarin Observed result (mg l^{-1})							
s	**t**	**u**	**v**	**w**	**x**	**y**	**z**
0.947	0.911	0.846	0.915	1.02	1.00	0.932	1.07
Calculated differences							
Dx_A'	Dx_B'	Dx_C'	Dx_D'	Dx_E'	Dx_F'	Dx_G'	
-0.1014	0.0283	-0.0406	0.0201	0.0239	0.0641	-0.0112	
Solvent yellow 124 Observed result (mg l^{-1})							
s	**t**	**u**	**v**	**w**	**x**	**y**	**z**
1.87	1.83	1.89	1.91	1.89	1.86	1.95	1.89
Calculated differences							
Dx_A'	Dx_B'	Dx_C'	Dx_D'	Dx_E'	Dx_F'	Dx_G'	
-0.0228	-0.0465	0.0284	-0.0023	-0.0161	0.0091	0.0198	

Previous replicate analyses of a standard solution of the three markers gave the following estimates of the precision of the HPLC system:

solvent red 24: 0.0363 mg l^{-1} (n = 69)

quinizarin: 0.0107 mg l^{-1} (n = 69)

solvent yellow 124: 0.0196 mg l^{-1} (n = 69)

Using Eq. 4.29, t values were calculated for each parameter and each analyte. These were compared with the appropriate critical value of t at 95% confidence (t_{crit} = 1.995, ν = 68).

Solvent red 24

All the parameters studied had a significant effect on the concentration of solvent red 24 observed. The relevant uncertainties were calculated using the procedure outlined in Section 4.3.3.3.

Flow rate

In the ruggedness test the flow rate was varied by 0.4 ml min^{-1}. This resulted in a difference of -0.1236 mg l^{-1}. The sensitivity coefficient, c_B', is therefore 0.309. The uncertainty in the flow rate was estimated as 0.00173 ml min^{-1} (see section on all-trans retinol ruggedness test). The uncertainty, $u(y(x_B'))$, is therefore 0.000535 mg l^{-1}.

Injection volume

The method specifies an injection volume of 50 µl. The uncertainty associated with this was estimated as 0.75 µl (see all-trans retinol ruggedness test). The sensitivity coefficient, c_C', was calculated as 0.00576. The uncertainty, $u(y(x_C'))$, is therefore 0.00432 mg l^{-1}.

Column temperature

During routine use of the method, the column is thermostatted at 30°C. The uncertainty associated with this was estimated as ±1°C (see section on all-trans retinol ruggedness test). In the ruggedness test the temperature was varied from 25°C to 35°C which resulted in a difference of 0.1303 mg l^{-1}. The sensitivity coefficient is therefore 0.01303 and the uncertainty, $u(y(x_D'))$, is 0.00752 mg l^{-1}.

Channel A wavelength

The wavelength for detector channel A is specified in the method as 475 nm. The uncertainty associated with this was estimated as ±2 nm (assuming a rectangular distribution). In the ruggedness study, a change in wavelength of 20 nm resulted in a difference of 0.1036 mg l^{-1}. The uncertainty, $u(y(x_E'))$, is therefore 0.00598 mg l^{-1}.

Channel B wavelength

The wavelength for detector channel B is specified in the method as 500 nm. In the ruggedness study a change of 20 nm resulted in a difference of 0.1047 mg l^{-1}. The uncertainty is therefore 0.00604 mg l^{-1}.

All the above uncertainties were converted to relative standard deviations by dividing by 1.92 mg l^{-1}.

Two other parameters also had a significant effect on the method performance: the type of acetonitrile used in the mobile phase and whether or not the mobile phase was degassed. The method was developed using HPLC grade acetonitrile. The ruggedness test indicated that changing to far-UV grade results in a lower recovery. The method

protocol will therefore specify that for routine use, HPLC grade must be used. The ruggedness test also indicated that not degassing the mobile phase causes a reduction in recovery. The method was developed using degassed mobile phase, and the method protocol will specify that this must be the case during future use of the method. As these two parameters are being controlled in the method protocol, uncertainty terms have not been included.

Quinizarin

The results of the ruggedness test indicated that the following parameters have a significant effect on the method performance:

Flow rate

Using the procedure discussed in the case of solvent red 24, the uncertainty was calculated as 0.000122 mg l^{-1}.

Injection volume

The uncertainty was calculated as 0.00152 mg l^{-1} (see solvent red 24 example).

Column temperature

The uncertainty was calculated as 0.00116 mg l^{-1} (see solvent red 24 example).

Channel A wavelength

Following the solvent red 24 example, the uncertainty was calculated as 0.00138 mg l^{-1}.

As in the case of solvent red 24, no additional uncertainties associated with the grade of acetonitrile used and the degassing of the mobile phase were included.

Channel B wavelength

The ruggedness test indicated that this parameter does not have a significant effect on the result of the analysis. The uncertainty was therefore evaluated using Eq. 4.30. δ_{real} and δ_{test} are equal to 4 nm and 20 nm respectively. The uncertainty was therefore calculated as 0.00154 mg l^{-1}.

All the uncertainties were converted to relative standard deviations by dividing by 0.909 mg l^{-1}.

Solvent yellow 124

The results of the ruggedness test indicated that the following parameters have a significant effect on the method performance:

Flow rate

The uncertainty was calculated as 0.00020 mg l^{-1} (see solvent red 24 example).

Injection volume

The uncertainty was calculated as 0.00107 mg l^{-1} (see solvent red 24 example).

As in the case of solvent red 24 and quinizarin, no additional uncertainties associated with the grade of acetonitrile used and the degassing of the mobile phase were included.

The results of the study indicated that remaining parameters do not have a significant effect on the method performance. The uncertainty was therefore calculated using Eq. 4.30 which gives $0.0141 \times (\delta_{true}/\delta_{test})$.

Column temperature

δ_{true} and δ_{test} are equal to 2°C and 10°C respectively. The uncertainty is therefore 0.00282 mg l^{-1}.

Channel A wavelength

δ_{true} and δ_{test} are equal to 4 nm and 20 nm respectively. The uncertainty is therefore 0.00282 mg l^{-1}.

Channel B wavelength

As in the case of the channel A wavelength, the uncertainty was calculated as 0.00282 mg l^{-1}.

The above uncertainties were converted to relative standard deviations by dividing by 2.35 mg l^{-1}.

Remaining sources of uncertainty

As discussed in Section A4.1.4 the uncertainties associated with a number of parameters, such as the calibration of the pipette used to dispense the samples, have not yet been considered. However, as these uncertainties are generally small, no additional contributions to the uncertainty budget have been included.

A4.2.5 Calculation of combined and expanded uncertainties

Table A4.25 summarises the magnitudes of the individual uncertainty components for the three makers.

Table A4.25: Uncertainty budget for the determination of markers in oil

Parameter		Standard uncertainties as relative standard deviations		
		Solvent red 24 (0.04 - 4 mg l^{-1})	Quinizarin (0.04 - 2 mg l^{-1})	Solvent yellow (1.2 - 5 mg l^{-1})
Precision	$u(P)$	0.0414	0.0788	0.0114
Recovery	$u(R)$	0.0415	0.0974	0.0187
Brand of cartridge	$u(y(x_A))$	0.0257	0.0190	0.00848
Sample volume	$u(y(x_B))$	0.00367	0.00394	0.00360
Rate of elution of oil with hexane	$u(y(x_C))$	0.00257	0.00767	0.000848
Volume of hexane wash	$u(y(x_D))$	0.00111	0.00195	0.000957
Concentration of butan-1-ol/hexane	$u(y(x_E))$	0.00128	0.000951	0.000424
Volume of butan-1-ol/hexane	$u(y(x_F))$	0.000514	0.000380	0.000340
Evaporation temperature	$u(y(x_G))$	0.00856	0.00480	0.00283
Flow rate	$u(y(x_B'))$	0.000279	0.000134	0.000855
Injection volume	$u(y(x_C'))$	0.00225	0.00167	0.000455
Column temperature	$u(y(x_D'))$	0.00392	0.00128	0.00120
Detector channel A wavelength	$u(y(x_E'))$	0.00311	0.00152	0.00120
Detector channel B wavelength	$u(y(x_G'))$	0.00315	0.00169	0.00120

For all three analytes, the sources of uncertainty were considered to be proportional to analyte concentration. The combined uncertainty was therefore calculated using Eq. 5.1:

solvent red 24: $u(y)/y = 0.065$

quinizarin: $u(y)/y = 0.13$

solvent yellow 124: $u(y)/y = 0.024$

Table A4.26 to Table A4.28 gives standard and expanded uncertainties for typical concentrations of the three markers. The expanded uncertainties were calculated using a coverage factor of $k = 2$ which gives a confidence level of approximately 95%.

Table A4.26: Standard and expanded uncertainty estimates for the determination of solvent red 24 in oil

Concentration (mg l^{-1})	Standard uncertainty (mg l^{-1})	Relative standard uncertainty	Expanded uncertainty (mg l^{-1})
0.04	0.0026	0.065	0.0052
1.5	0.098	0.065	0.20
2	0.13	0.065	0.26
4	0.26	0.065	0.52

Table A4.27: Standard and expanded uncertainty estimates for the determination of quinizarin in oil

Concentration (mg l^{-1})	Standard uncertainty (mg l^{-1})	Relative standard uncertainty	Expanded uncertainty (mg l^{-1})
0.04	0.0052	0.13	0.010
1.0	0.13	0.13	0.26
1.5	0.19	0.13	0.39
2	0.26	0.13	0.52

Table A4.28: Standard and expanded uncertainty estimates for the determination of solvent yellow 124 in oil

Concentration (mg l^{-1})	Standard uncertainty (mg l^{-1})	Relative standard uncertainty	Expanded uncertainty (mg l^{-1})
1.5	0.036	0.024	0.072
2	0.048	0.024	0.096
4	0.096	0.024	0.19
5	0.12	0.024	0.24

Comments on uncertainty budget

Solvent red 24 and solvent yellow 124

In the case of solvent red 24 and solvent yellow 124, the significant contributions to the uncertainty budget arose from overall precision and recovery, and the brand of the solid phase extraction cartridge used. If a reduction in the overall uncertainty of the method was required, useful approaches would be to specify a particular brand of cartridge in the method protocol, or to adopt matrix specific recovery corrections for test samples.

Quinizarin

The combined uncertainty for quinizarin, which is significantly greater than that calculated for the other markers, is dominated by the precision and recovery terms. The results of the precision study indicated variable method performance across different matrices and analyte concentrations. The uncertainty, $u(R_s)$, associated with the variation in recovery from sample to sample was the major contribution to the recovery uncertainty, $u(R)$. This was due to the fact that the recoveries obtained for matrix B were generally higher than those obtained for matrices A and C. However, in this study, a single uncertainty estimate for all the matrices and analyte concentrations studied was required. It was therefore necessary to use "worst case" estimates of the uncertainties for precision and recovery to adequately cover all sample types. If this estimate was found to be unsatisfactory for future applications of the method, separate budgets could be calculated for individual matrices and concentration ranges.

Annex 5: Proforma for documenting uncertainty

Uncertainty Budget		
Method Title:		Ref:
Scope:		

Sources of Uncertainty				
1. Precision				
Samples analysed				
Matrix	Concentration	No. replicates	Standard deviation	Analyst/ date
$u(P)$				

Uncertainty Budget							
Method Title:						Ref:	
2. Trueness							
Samples analysed							
Matrix (CRM/spike?)				Concentration	No. replicates	Result	Recovery	Analyst/ date
$\overline{R}_m$		$u(\overline{R}_m)$		$\overline{R}_m$ different from 1?		Correction applied?		
$u(\overline{R}_m)'$		$u(R_s)$		$u(R_s)$		R_{rep}		
$u(R_{rep})$		$\boldsymbol{R}$				$\boldsymbol{u(R)}$		

Uncertainty Budget		
Method Title:		Ref:

3. Other			
Source of Uncertainty	Standard Uncertainty	Method of evaluation	Analyst/ date

Combined standard uncertainty	
Expanded uncertainty	
Coverage factor applied and level of confidence	

Prepared by:		Date:	

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