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Accuracy of analytical standard solutions and the uncertainty in their nominal concentrations

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1. Summary

In the last step of the analysis, the accuracy of analytical standard solutions has a decisive influence on the measurement results of pesticide residues, as well as all other chemical contaminants, and, in the case of actual concentrations differing from nominal ones, can result in a systematic error during the determination of sample components. Therefore, most of the laboratories who feel responsible for their results pay special attention to the preparation and storage of standard solutions, and to replenishing solvent losses due to possible evaporation, based on the mass measurements of vessels storing standard solutions before and after use. However, in our experience, much less attention than necessary is paid in practical work to monitoring the possible decomposition of individual active ingredients, and not appropriate statistical methods are used for the evaluation of the monitoring results. In our article, we present the standard preparation methods of two laboratories employing "good analytical practices", analyze the uncertainties of the different steps, and make a recommendation for the preparation of the most accurate standard solutions.

2. Introduction

For authority and national reference laboratories of European Union member states it is mandatory to participate in the relevant proficiency tests. A ring test (EU-RT-FV17) was organized by the European Union Reference Laboratory for Pesticide Residues in Fruit & Vegetables to reveal the reasons for results that proved to be inadequate, based on the criteria used for the evaluation of the performance of participating laboratories. The test sample contained 11 pesticide active ingredients in controlled concentrations in a pure solvent (***Table 1***). Mainly laboratories reporting inadequate results were included in the ring test, but participation was open to all interested laboratories (Carmen Ferrer Amate, Universidad De Almería-Edificio de Química, personal communication). Test results were reported by 36 laboratories, but in certain cases not for all of the components of the standard mix. No official evaluation of the test results has been published yet, therefore, ***Table 1*** lists only the relevant partial results. Results show that 17 to 23 testing organizations of the authority and reference laboratories of the EU could determine the concentrations of the 11 active ingredients in the test sample with errors greater than the maximum acceptable difference (<10%) **[1]**. When looking at relative differences, the situation is even more critical, because pesticide residue concentrations at least twice as high as the actual concentrations in the test sample will be measured by laboratories with relative differences ≤- 50%, and the batch will be erroneously classified as unacceptable. However, in the case of a relative standard error of >+100%, a pesticide residue concentration of ≤50% of the actual one will be measured and reported, which makes marketing of batches containing pesticide residues in amounts exceeding the limit value possible. Thus, false "acceptable" results pose a food safety risk as well.

Taking into consideration the importance of the accuracy of standard solutions, the accuracies of the standard preparation methods, differing in certain details, of two Hungarian laboratories performing excellently and well in European proficiency tests **[2]** have been examined, as well as the uncertainties of the nominal concentrations of the standard mixes thus prepared. In this communication, methods for the preparation of standard solutions and their repro-

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ducibilities are presented, as well as the effects of the equipment used during the preparation of solutions and multi-component standard mixes on the accuracies of the concentrations of the components in the solutions prepared and on the uncertainty of the nominal concentration. The statistical method that can be used for the determination of the difference between the actual concentrations of the components of the standard solutions and the concentration of the reference standard solution is published in a separate communication **[3]**.

3. Methods used for the preparation of analytical standards

Application of the equipment used is performed in similar ways in the laboratories carrying out the tests. The procedure is only described in detail on the first occasion. A preliminary checking of the effectiveness of the method developed for cleaning the equipment is performed by the laboratories

Analytical standards are purchased from manufacturers with ISO 9001 certifications, who verify and certify the purity of the standards batch by batch.

3.1. Laboratory 'A'

Volumetric flask are of Class A, centrifuge tubes have been calibrated for each milliliter by the scientific glassblower with an accuracy of 0.1%.

A.1: *1 mg/ml stock solution*

The analytical standard, that is kept in a freezer at a temperature of <-20 °C is warmed to room temperature in a desiccator over activated silica gel and, taking into consideration its purity, 25 mg of it, with an accuracy of 0.01 mg, is measured into a 25 ml beaker, using a *Mettler AX 205-DR* type analytical balance, calibrated daily. The weighed standard is dissolved in 5ml of acetone, or another suitable solvent, and the solution is poured into a *25 ml Class A volumetric flask* (V_{25}). The beaker is rinsed with 3x5 ml of acetone, which is poured into the volumetric flask and, if necessary, complete dissolution is aided by placing the flask in an ultrasonic bath.

The volumetric flask is filled to the mark using a Pasteur pipette, it is labeled, weighed, and the following data are recorded in the standard log: code and mass of the stock solution prepared, identifier, purity and weighed mass of the solid standard used, concentration of the stock solution prepared, the solvent used, the final volume and mass of the stock solution prepared, the dates of preparation and expiration, and the short signature and initials of the employee preparing the solution. Until further use, the solution is kept in a refrigerator at <7 °C.

Usual storage times are summarized in **Table 2**, noting that, in our experience, components more prone to decomposition are only stored for shorter periods.

A.2. *Preparation of a 10 µg/ml standard mix in acetone with no more than 100 components*

The masses of the stock solutions, taken out of the refrigerator, are checked after their temperature reached room temperature. If there is a mass difference due to evaporation, then the mass of the stock solution is restored to the last value recorded in the log using acetone. Using a 500 µl Hamilton syringe ($\mathbf{H_{500}}$) 250 µl of the 1 mg/ml stock solutions are transferred into the V_{25} flask. The syringe is rinsed with acetone at least 3 to 5 times before use, and 5 to 7 times after the transfer. After transferring the stock solutions, the flask is filled to the mark using a Pasteur pipette, it is shaken, labeled, its mass is measured and recorded.

A.3. *10 µg/ml standard solutions with several components*

Standard mixes for ad hoc analyses are prepared according to procedure A.2, but 100 µl of the stock solution is transferred to a 10 ml screw cap calibrated centrifuge tube (T_{10}) using a H_{250} syringe.

A.4. *Preparation of a 20 µg/ml standard solution with ≤ 50 components*

From the individual stock solutions (A.1) taken out of the refrigerator, 1 ml is transferred to a 50 ml volumetric flask (V_{50}) using a 1000 µl Hamilton syringe (H_{1000}), after checking their masses at room temperature. After measuring the standards, the flask is filled to the mark using a Pasteur pipette, it is labeled, shaken its mass is measured and recorded. Standard solutions with several hundred components are prepared from the so-called sub-mixes with concentrations of 20 µg/ml.

A.5 Preparation of a *5 µg/ml standard solution with 350 components*

From the 7 sub-mixes prepared according to procedure A.4, 1000 µl of each is transferred to an 8 ml calibrated screw cap centrifuge tube (T_8) using a H_{1000} syringe. The volume is reduced to <1 ml using a gentle flow of air, and then the transfer of 7x1000 µl is repeated. The tube is filled to the mark using a Pasteur pipette. The nominal concentration of the standard mix thus prepared is 5 µg/ml. This standard mix can be used for three months, for example for recovery tests at the 0.1 mg/kg level.

A.6. 1 µg/ml: 1000 µl of the 10 µg/ml solution is filled to the mark in a 10 ml centrifuge tube using a H_{1000} syringe.

Preparation of calibration series from a 1 µg/ml acetone solution by solvent exchange

A.7. 0.1 µg/ml: 200 µl is transferred to a 10 ml centrifuge tube using a H_{250} syringe, concentrated by a weak flow of air or allowed to evaporate, and then filled to the 2 ml mark using a liquid chromatography eluent.

A.8. 0.05 µg/ml: 100 µl is taken using a H_{250}, and the solvent is exchanged according to A.7.

A.9. 0.01 µg/ml: 20 µl is taken using a 25 µl Hamilton syringe (H_{25}), and the solvent is exchanged to 2 ml of a liquid chromatography eluent.

A.10. 0.005 µg/ml: 20 µl is taken using a H_{25} syringe, and the solvent is exchanged to 4 ml of a liquid chromatography eluent.

Preparation of calibration series from a 10 µg/ml acetone solution without solvent exchange

A.11. 0.1 µg/ml: 20 µl of a 10 µg/ml standard solution is taken using a H_{25} syringe, and is filled to the 2 ml mark directly using a liquid chromatography eluent.

A.12. 0.05 µg/ml: 10 µl of a 10 µg/ml standard solution is taken using a H_{25} syringe, and is filled to the 2 ml mark directly using a liquid chromatography eluent.

A.13. 0.01 µg/ml: 20 µl of a 1 µg/ml standard solution is taken using a H_{25} syringe, and is filled to the 2 ml mark directly using a liquid chromatography eluent.

A.14. 0.005 µg/ml: 10 µl of a 1 µg/ml standard solution is taken using a H_{25} syringe, and is filled to the 2 ml mark directly using a liquid chromatography eluent.

Notes:

- It is not recommended to take solutions with volumes less than 10 µl. If you want to prepare solutions with other concentrations, the Hamilton syringe to be used should be selected in accordance with the volume to be measured.
- If a different concentration without solvent exchange is required, then care should be taken that, without evaporation, no more than 50 µl of acetone remains in the liquid chromatography eluent with a volume of 2 ml (i.e., one volume unit of the liquid chromatography eluent contains no more than 2.5% of acetone).
- Number of calibration points: should be ≥3 in the case of screening tests; ≥5 in the case of quantitative confirmation, validation.

3.2. Laboratory 'B'

General practice:

The analytical standard, allowed to warm to room temperature in a desiccator over activated silica gel, is measured into a 25 ml volumetric flask with an accuracy of ± *0.04 ml*, previously washed, rinsed with acetone, dried in a drying cabinet and stored in a desiccator for two days. The mass to be measured accurately (±0.01 mg) is determined taking into account the purity of the substance, and it is measured using an analytical balance calibrated daily. In the case of solid standards, disposable plastic spatulas, in the case of liquid substances, automatic pipettes are used for the measurements.

The standard is dissolved in a volumetric flask in the appropriate solvent (acetone, methanol, hexane, etc.,), complete dissolution is ensured by placing the flask in an ultrasonic bath, and then the flask is filled to the mark using a pipette. For the identification of the standard solution prepared, a form is prepared, containing the name of the substance, its laboratory ID code, the mass measured, code number of the solution, the concentrations of the components, the solvent used, the dates of preparation and expiration, and the name of the person preparing the solution. The solution is stored in a screw cap brown glass bottle with a Teflon liner. A label is placed on the bottle containing the information regarding the standard solution, and the total mass is measured, which is then entered into the standard registration. Reference standards are stored in a freezer at a temperature of <-20 °C, while standard solutions are stored in a refrigerator at a temperature of ≤7 °C.

In most cases, 2 mg/ml stock solutions are prepared using acetone (taking into account the solubilities of the known standards), thus avoiding the need for solvent exchange. Occasionally, the appropriate amount of internal standard is mixed in or, in the case of matrix-matched calibration, a previously prepared blank matrix solution is used instead of acetonitrile with formic acid.

After use, syringes are washed with acetone 10 to 15 times, then dried before using them again.

B.1 2 mg/ml stock solution

As an example, take a solid analytical standard of 99.8% purity, of which 50.10 mg is measured into a 25 ml volumetric flask (V_{25B}), acetone is added, and

dissolution is completed in an ultrasonic bath. Then the flask is filled to the mark.

B.2 20 µg/ml standard solution with ≤50 components

The stock solutions prepared according to B.1 are taken out of the refrigerator, and allowed to warm to room temperature over 2 hours in a desiccator over activated silica gel. 100 µl is taken using a H_{100} syringe, and it is injected into a 10 ml volumetric flask *(±0.04* accuracy, V_{10B}) washed for this purpose, into which 1 ml of acetone was placed in advance. From each stock solution, the appropriate amount is measured into the flask, corresponding to its concentration, then the contents of the flask are homogenized by vortexing, and filled to the mark using a pipette. The standard mix thus prepared is stored at ≤-20 °C in a dark bottle in a freezer, and can be used for half a year.

B.3 1 µg/ml standard solution with several hundred components

20 µg/ml solutions prepared according to procedure **B.2** are allowed to warm to room temperature in a desiccator. 500 µl of each is measured into a V_{10B} volumetric flask using a H_{500} syringe, the solution is homogenized, filled to the mark with acetone, stored in a labeled brown bottle in a freezer. It can be used for no more than 3 months.

Preparation of calibration series using standard mixes

Start with the 1 µg/ml acetone mix:

B.4 0.001 µg/ml: take 5 µl using a H_{10} syringe, and fill to the 5 ml mark in a screw cap calibrated test tube (T_{5B}) using acetonitrile with formic acid.

B.5 0.002 µg/ml: take 10 µl using a H_{10} syringe, and fill to the 5 ml mark in a T_{5B} test tube using acetonitrile with formic acid.

B.6 0.005 µg/ml: take 10 µl using a H_{10} syringe, and fill to the mark in T_{2B} using acetonitrile with formic acid.

B.7 0.01 µg/ml: take 10 µl using a H_{10} syringe, and fill to the 1 ml mark in T_{2B} using acetonitrile with formic acid.

B.8 0.1 µg/ml: take 100 µl using a H_{100} syringe, and fill to the 1 ml mark in T_{2B} using acetonitrile with formic acid.

3. Nominal accuracy of the calibrated equipment used for the preparation of standard solutions

Calibrated glassware and analytical balances with an accuracy of 0.01 mg are used by the laboratories for the preparation of standard solutions. Characteristic parameters of the equipment used are listed in ***Tables 3, 4, and 5***.

In the case of analytical standards, the actual active ingredient content can take any value within the given range. Accordingly, a rectangular distribution was assumed, and the standard deviation is calculated from the given purity range by division by the square root of 3 **[4]**. If, according to the specification, the purity of the analytical standard is 99.8±0.5%, then the standard deviation (s) for the calculation of the uncertainty of the measurement result (s), for a weighing of 1 mg standard:

s=1*0.98*0.005/√3=2.829E-3 mg, corresponding to a coefficient of variation (CV) of 2.829E-3.

In the tables and in the text 'E' refers to the power of base 10: e.g., 2.829E-3 = 2.829x10^{-3}

Based on previous experience, assuming a triangular distribution, the standard deviation is obtained by division of the specified tolerance limits of calibrated glassware by the square root of 6. The CV is then calculated taking into account the nominal volume **[4]**.

Reproducibilities of the balances, resulting from the combination of the resolution and the linearity, were calculated from data provided by the manufacturer, the standard deviation, assuming a normal distribution, was calculated by a division by 1.96, while the coefficient of variation was calculated taking into account the amount weighed. For example, if the reproducibility is ±0.04 mg, then the coefficient of variation (CV) of the weighing of 25 mg of a standard is 8.16E-4. In the case of measurements carried out under repeatability conditions and under reproducibility conditions within the laboratory, the coefficients of variation are indicated by CV_r and CV_R, respectively, in accordance with international practice.

Table 4 shows the coefficients of variation calculated from the accuracy specifications of the manufacturer relative to the volume measured (CV_{RT}), and the coefficient of variation calculated from the results of reproducibility measurements (CV_{Exp}), containing the coefficient of variation of filling to the mark (CV_{fill}). CV_{fill} was calculated from the values of CV_{RT} and CV_{Exp}:

$$CV_{Rfill} = \sqrt{CV^2_{Rexp} - CV^2_{RT}}$$

4. Reproducibility of the preparation of analytical standard solutions

The accuracies of the weighings are determined by the manufacturing accuracy of the equipment used, defined by the specified tolerance (***Table 4***), and by the experience of the analyst performing the weighing, and his or her personal abilities (vision, dexterity, ability to concentrate).

Repeatability and reproducibility studies were carried out by the same persons, who prepares standards regularly. Measurements were carried out with an accuracy of 0.01 mg using the Mettler balances listed in ***Table 2***, calibrated daily. For the determination of repeatability, the same equipment was used 10 times. Measurements were carried out at a temperature of 24 to 25 °C.

5.1. Determination of repeatability

Mass measurement:

Before measurement, the balance was zeroed, the clean and dry container to be measured was placed on the balance, and its mass was recorded to five decimal places. After each measurement, the container was removed from the balance, the balance was zeroed, and the container was placed on the pan of the balance again using tongs, and its mass was measured again. The procedure was repeated 10 times in succession, and the temperature of the balance room was recorded.

Volume measurement:

The net mass of the clean equipment to be tested, dried in a desiccator, was measured, and then it was filled accurately to the mark using distilled water and a Pasteur pipette, the mass of the vessel was measured again, and the measurement results were recorded in an Excel worksheet. The mass of the water weighed was calculated from the mass differences.

A portion of the water weighed was removed using a pipette, taking care to keep the wall of the volumetric flask of the test tube dry, then the vessel was filled to the mark again and its mass was measured.

5.2. Determination of reproducibility (CV_R)

To determine the reproducibility of volume measurement, 10 pcs from each of either the calibrated volumetric flasks, graduated centrifuge tubes or test tubes of the laboratory were filled to the mark using distilled water of a temperature of 24 to 25 °C, and the amount of water was weighed to the nearest 0.01 mg. The actual average volume of the volume measurement equipment used was calculated from the average mass and density of the distilled water used to fill to the mark in 10 repetitions. A value of 0.9971749 was used **[5]** in the calculations as the density of water of 24.5 °C, was considered to be the average temperature. The error thus introduced is practically negligible, because the densities of distilled water at 24 °C and 25 °C are 0.9972994 g/ml and 0.9970480 g/ml, respectively. The volumes calculated from the average mass obtained when filling the 25 ml volumetric flask to the mark are 24.89410 ml and 24.88844 ml, meaning a difference of 0.025%.

Results are summarized in ***Table 6***. In the table, the individual equipment are listed using the code used at the preparation of standard solutions. Considering that the combined uncertainties of the nominal concentrations of the components of the standard solutions prepared and concentrations based on mass measurements are calculated as the result of our work, the data necessary for performing the calculations are summarized in the table. The measurement sequences used for the calculations are indicated with (*) in Table 6.

6. Accuracy of the analytical standard solutions prepared and the uncertainty of their nominal concentrations

The weighed amounts of standards of known purity are given below in milligrams and the volumes in milliliters. For simplicity, a purity of 100% is assumed for the calculations, because the percentage purity of the standard can be taken into consideration easily during the weighing. For example, if the analytical standard to be weighed is of 98% purity then, instead of the nominal mass of 25 mg, ca. 25.51 g is weighed.

It should be noted that it is not necessary to weigh the target mass accurately (0.00001 g), because this could lead to a great loss of time, a loss of material and possible contamination of the pure standard. The nominal concentrations of the standard solutions prepared can be calculated easily by taking into account the actual accurately weighed mass (±0.00001 g).

Minimum, average and maximum values of the concentrations of the standard solutions prepared, based on mass measurement, were calculated from the appropriate data of 10 repeat measurements, resulting in maximum uncertainty. Results are summarized in ***Table 7***. The calculation procedure is demonstrated on the method used in laboratory 'A'.

6.1. Standard preparation method of laboratory 'A'

The procedures used are described in Section 3.1., where the symbols representing the equipment used for weighing were explained.

A1: *1 mg/ml stock solution*: $C_{A1} = \frac{m*p}{V_{25}} = \frac{25*1}{25} = \frac{mg}{ml}$

The coefficient of variation of the stock solution CV_A:

$$C_{A1} = \sqrt{CV^2_{w25} + CV^2_P + CV^2_{v25}}$$

Where, m= the mass of the weighed standard of known purity in milligrams; p= the purity of the standard in mass fraction; V_{25} denotes the 25 ml volumetric

flask. The coefficient of variation of weighing 25 mg is CV_{w25}= 8.163E-4; the coefficient of variation of the purity of the standard in the case of weighing 25 mg is 2.829E-3; for filling the 25 ml volumetric flask to the mark it is CV_{V25}=7.32E-3.

$CV_{A1}=(0.0008163^2 + 0.002829^2 + 0.00732^2)^{½}$
=0.00789=0.789%

Actual (min.-max.) concentration: 1.004372 mg/ml (0.9840-1.00842 mg/ml).

A2: 10 µg/ml dilution:

$$C_{A2}= C_{A1} * \frac{^{0.25}H_{500}}{^{25}V_{25}} = 0.01C_{A1}$$

$$C_{A1}= \sqrt{CV^2_{w25} + CV^2_{p} + CV^2_{V25} + CV^2_{H0.25} + CV^2_{V25}}$$

$$C_{A2}= \sqrt{CV^2_{A1} + CV^2_{H0.25} + CV^2_{V25}}$$

A3: 10 µg/ml dilution:

$$C_{A3}= C_{A1} \frac{^{0.1}H_{250}}{^{10}T_{10}} = 0.01C_{A1}$$

$$C_{A2}= \sqrt{CV^2_{A1} + CV^2_{H00} + CV^2_{T10}}$$

A4: 20 µg/ml sub-mix:

$$C_{A4}= C_{A1} * \frac{^{1}H_{1000}}{^{50}V_{50}} = C_{A1} * 0.02$$

$$CV_{A4}= \sqrt{CV^2_{A1} + CV^2_{H1000} + CV^2_{V50}}$$

Calculated concentrations are for a given component, and they depend on the weighed amount. CV_{A4} is for each component.

A5: 5 µg/ml combined mix with 350 components:

$$C_{A5}= C_{A4} * \frac{^{1}H_{1000}+^{1}H_{1000}}{V_{Tg}} = C_{A4} * 0.25$$

$$C_{2x1ml} = \frac{\sqrt{2xSD^2_{H1000}}}{2}$$

The standard deviation of the 1000 µl weighing was calculated from the square root of the average variance of the measurement repeated 7 x 10 times: SD_{ave}=2.0021E-3. Considering that, from an A4 submix, 2 x 1 ml were weighed, the value of CV for 1 ml of standard solution is obtained by dividing the calculated standard deviation by 2: CV_{2*1ml}=1.4157E-3

$$CV_{A5}= \sqrt{CV^2_{A4} + CV^2_{2xH1000} + CV^2_{T8}}$$

Preparation of calibration series with solvent exchange

The uncertainty due to solvent evaporation was not taken into consideration because of the disproportionately large measurement uncertainty of the small masses weighed. In order to obtain accurate results, the evaporation has to be performed using a very gentle flow of air.

A6: 1 µg/ml dilution:

$$C_{A6}= C_{A3} \frac{^{1}H_{1000}}{^{10}T_{10}} = 0.1C_{A3}$$

$$CV_{A6}= \sqrt{CV^2_{A3} + CV^2_{H1000} + CV^2_{T10}}$$

A7: 0.1 µg/ml:

$$C_{A7}= C_{A6} * \frac{^{0.2}H_{250}}{^{2}T_{10}} = 0.1C_{A6}$$

$$CV_{A7}= \sqrt{CV^2_{A6} + CV^2_{H200} + CV^2_{T2}}$$

A8: 0.05 µg/ml:

$$C_{A8}= C_{A6} * \frac{^{0.1}H_{250}}{^{2}T_{10}} = 0.05C_{A6}$$

$$CV_{A8}= \sqrt{CV^2_{A6} + CV^2_{H100} + CV^2_{T10.2}}$$

A9: 0.01 µg/ml:

$$C_{A9}= C_{A6} * \frac{^{0.02}H_{25}}{^{2}T_{10}} = 0.01C_{A6}$$

$$CV_{H}= \sqrt{CV^2_{A6} + CV^2_{H20} + CV^2_{T10\,2}}$$

A10: 0.005 µg/ml:

$$C_{A10}= C_{A6} * \frac{^{0.02}H_{25}}{^{4}T_{10}} = 0.005C_{A6}$$

$$CV_{1}= \sqrt{CV^2_{A6} + CV^2_{H20} + CV^2_{T10.4}}$$

Preparation of calibration series without solvent exchange

A11: 0.1 µg/ml:

$$C_{11}= C_{A2} * \frac{^{0.02}H_{25}}{^{2}T_{10}} = 0.01C_{A2}$$

$$CV_{A11}= \sqrt{CV^2_{A2} + CV^2_{H20} + CV^2_{T10\,2}}$$

A12: 0.05 µg/ml:

$$C_{A12}= C_{A2} * \frac{^{0.01}H_{25}}{^{2}T_{10}} = C_{A2}0.005$$

$$CV_{A12}= \sqrt{CV^2_{A2} + CV^2_{H20} + CV^2_{T10\,2}}$$

A13: 0.01 µg/ml:

$$C_{13}= C_{A6} * \frac{^{0.02}H_{25}}{^{2}T_{10}} = 0.01C_{A6}$$

$$CV_{A13}= CV_{A9} = \sqrt{CV^2_{A6} + CV^2_{H20} + CV^2_{T10\,2}}$$

A14: 0.005 µg/ml:

$$C_{A14}= C_{A6} * \frac{^{0.01}H_{25}}{^{2}T_{10}} = 0.005C_{A6}$$

$$CV_{A14} = \sqrt{CV_{A6}^2 + CV_{H100}^2 + CV_{T10.2}^2}$$

Detailed concentration calculation for standard solution **A14**

$$C_{A14} = \frac{25_w * 1_p * 0.1_{H250} * 1_{H1000} * 0.01_{H25}}{25_{v25} * 10_{T10} * 10_{T10} * 2_{T10}}$$

6.2. Analytical standard preparation methods of laboratory 'B'

The procedures used were described in ***Section 3.2***. Actual concentrations of the standard solutions and their coefficients of variations were calculated using the procedure described in ***Section 5.1***.

The coefficient of variation of the purity of the analytical standard is the same as in the case of laboratory 'A' CV_p = 2.829E-3. The coefficient of variation of weighing 50 mg (Table 5) is 5.10E-4.

B1: 2 mg/ml stock solution $C_{B1} = \frac{m*p}{V_{25}} = \frac{50*1}{25} = \frac{2mg}{ml}$

CV_{B1}: $CV_{B1} = \sqrt{CV_{w50}^2 + CV_P^2 + CV_{V25}^2}$

B2: 20 µg/ml $C_{B2} = C_{B1}\frac{0.1_{H100}}{10_{V10B}}$

B3: 1 µg/ml $C_{B3} = C_{B2}\frac{0.5_{H500}}{10_{V10B}}$

B4: 0.001 µg/ml $C_{B5} = C_{B3}\frac{0.005_{H10}}{5_{T5B}}$

B5: 0.002 µg/ml $C_{B6} = C_{B3}\frac{0.01_{H10}}{5_{T5B}}$

B6: 0.005 µg/ml $C_{B7} = C_{B3}\frac{0.01_{H10}}{2_{T5B}}$

B7: 0.01 µg/ml $C_{B8} = C_{B3}\frac{0.01_{H10}}{1_{T2B}}$

B8: 0.1 µg/ml $C_{B10} = C_{B3}\frac{0.1_{H100}}{1_{T2B}}$

B6 0.005 µg/ml: $C_{B6} = \frac{50*1_P*0.1_{H10}*0.5_{H500}*0.01_{H10}}{25_{V25B}*10_{V10}*10_{V10}*5_{TB}}$

6. Evaluation of the results

When applying the equipment used for measurements, coefficients of variation calculated from their specifications are listed in ***Tables 3 and 4***.

Results of the reproducibility analyses based on mass measurements in 10 repetitions are summarized in ***Table 7***. Calculations of the minimum and maximum concentrations were performed using the formulas below:

$$C_{min} = \frac{m_{min}}{v_{max}} \text{ and } C_{max} = \frac{m_{max}}{v_{min}}$$

Where m_{min} and m_{max}, and V_{min} and V_{max} denote the minimum and maximum masses measured during the 10 repetitions, and the corresponding volumes calculated with the density of distilled water [mxρ].

The uncertainty of the target concentration was estimated with the CV_R value calculated from the reproducibility measurement results of the equipment used, according to the formulas given in ***Section 5.1***.

Relative differences [D_i] between C_{max} and C_{min}, and the target nominal concentration, C_0, were calculated according to the following formulas:

$$\Delta_{Cimax} = \frac{C_{imax} - C_{io}}{C_{io}} \text{ and } \Delta_{Cimin} = \frac{C_{imin} - C_{io}}{C_{io}}$$

while the relative difference between the average concentration and the target concentration was calculated as follows:

$$\bar{\Delta}_{Ci} = \frac{C_{io} - \bar{C}_i}{\bar{C}_{io}}$$

where 'i' denotes the different nominal concentrations.

The relative difference of the average measured concentrations and target concentrations ($\bar{\Delta}_{Ci}$%) showed a negative deviation of ≈<10% in laboratory 'A'. In laboratory 'B', the deviation was positive (<4.1%). More relevant information regarding the reliability of the results can be gained by considering the relative difference between the minimum and maximum concentrations obtained from 10 repeat measurements (Δ_{Ci}), which slightly exceeded 10% in both laboratories in the case of solutions used for low concentration calibration. Such a difference in the accuracies of standard solutions cannot be accepted, and justifies mass measurement based preparation of standard solutions, because in this case the concentration of the solution prepared is known much more accurately.

The coefficients of variation of standard solutions prepared by weighing was typically around 2 to 3% for low concentration standard solutions, resulting primarily from the coefficient of variation of filling to the mark (CV_{Rfill}) (***Table 4***). A 2 to 3% coefficient of variation is considered a borderline case, because correct results are obtained by linear regression, which is applied by software calculations of the calibration curves, if the uncertainty of the calibration solutions is negligible compared to the relative repeatabilities of the signals obtained. For comparison, under routine operating conditions, the average signal reproducibility of the LC/MS-MS measurements of laboratory 'A', calculated from the signals measured at the beginning and at the end of the batch over a 10-day period was 1.8%.

The percentage contributions of the variances of the individual steps to the uncertainty of the solution prepared are illustrated in ***Figures 1 and*** 2, using as examples calibration solutions A.14 (0.005 µg/ml) and B.6 (0.005 µg/ml).

The total variances of the preparation procedures of standard solutions A.14 and B.6 are 4.34E-4 and 6.96E-5, respectively.

Figure 1 shows that, in the last step of the preparation of standard solution A.14, filling the 10 ml graduated centrifuge tube to the 2 ml mark is the step with the greatest uncertainty, and it contributes to the total uncertainty of the standard solution with nearly 64%. The combined uncertainty can be improved by replacing this step by the application of a more reproducible equipment.

Contributions of the steps of calibration solution B6 to the combined uncertainty are more even. Somewhat better results can be obtained by improving the repeatability of the weighing of the 10 µl solution.

Approximately one half of the uncertainty values resulting from the volume specifications of the measuring equipment were lower than the uncertainty values of filling to the mark and, accordingly, only contributed to the uncertainty of the concentration of the standard prepared to a small extent. However, in certain cases, CV_{RT} was greater than CV_{Exp}, and, consequently, it was the main source of the inaccuracy of the calibration of the equipment. If the preparation of standard solutions is based on mass measurement, then this source of uncertainty can be eliminated.

The uncertainty of the purity of the reference materials only contributed to the uncertainty of the concentration of the stock solution. The uncertainty of a measurement with an accuracy of 0.01 mg is orders of magnitude lower than that of the volumetric flasks used for weighing, therefore, the accuracies and uncertainties of dilutions carried out in several steps are practically not affected by it.

It should be noted that balances with an accuracy of 0.1 mg cannot be used for the preparation of standard solutions as described in Chapter 2.

8. Recommendations

Our results clearly show that standard solutions can be prepared with much greater accuracy by using mass measurement of 0.01 mg accuracy up until the last step, where concentrations expressed in g/g before have to be expressed in g/ml units, since µl quantities of standard solutions and samples are injected into the chromatographic systems.

The use of more accurate balances is not justified, because measurements with an accuracy of 0.01 mg practically do not influence the accuracy of the standard solutions prepared and the uncertainty of the nominal concentration.

Standard solutions should be prepared in as few steps as possible, and the final volume should be adjusted in class A or individually calibrated volumetric flasks.

Even with the most careful work, measurement errors can still occur, modifying the actual concentration of the standard solution to an unknown extent. To eliminate the effect of a possible measurement error, it is advisable to prepare the stock solution and the intermediate standards used for further dilutions (e.g., A6, B3) in duplicate, using independent weighings. If the difference in the detected signals of 0.002 µg/ml or 0.005 µg/ml solutions prepared from these is smaller than the value determined by the quality manual of the laboratory (e.g., 5%), then the two solutions are combined and used for further dilutions, calculating with the nominal concentration. If the difference is greater, then preparation of a third solution is necessary. After examination of the three results, the two solutions showing the smaller difference are combined and the solution obtained is used for further measurements.

Standard solutions prepared according to the above can be used to monitor the stability of the components of solutions prepared earlier. According to the instructions of DG SANTE, the old standard can be used if the difference between the concentrations of the old and new solutions is <10%.

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