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EURL Guidance Document on Stability Studies in the Field of the Analysis of Residues of Pharmacologically Active Substances

Contents

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Preamble

This document serves as a guidance to support official control laboratories in the harmonised implementation of stability studies. Laboratories operating under Commission Implementing Regulation (EU) 2021/808 are not obliged to follow this guidance minutely; different approaches are acceptable, if they provide the same level and quality of information.

Abbreviations

CCα	Decision Limit
CCβ	Detection Capability for Screening
CIR	Commission Implementing Regulation
CR	Commission Regulation
CV	Coefficient of Variation
LCL	Lowest Calibrated Level
ML	Maximum Level
MMPR	Minimum Method Performance Requirements
MRL	Maximum Residue Limit
RPA	Reference Point for Action
STC	Screening Target Concentration

1 Introduction

Being aware of analyte stability in matrix and in solution is essential for the provision of reliable analytical results. Stability studies are used to determine appropriate storage conditions and storage duration of matrix samples and solutions, before the degree of the degradation of the analytes of interest becomes unacceptable. For matrix samples, stability studies are therefore critical for informing on maximum turnaround times of analysis, and for informing on storage durations after which the result is deemed unreliable.

Commission Implementing Regulation 2021/808[1] lists stability studies as an essential element of validation studies for analytical methods in the field of veterinary medicinal products. This requirement is essential and holds for all method types (quantitative-qualitative, confirmation-screening[1]). The required data does not necessarily need to be determined within the validation study and within the laboratory itself. To reduce the workload, data from other sources such as previously conducted in-house studies, information provided by the EURLs or data from the literature, can be referenced if the applied conditions are identical.

In accordance with CIR 2021/808[1] stability information is required for solutions, as well as analyte or matrix constituents in the sample during storage or analysis. With regard to the analytes of interest in solutions it is further specified in the regulation that stability information is needed for:

- the calibration standard, matrix-matched standard and/or matrix-fortified standards
- solutions of the analyte, which are used for fortification,
- analyte solutions, used for final analysis (i.e. sample extracts),
- any other solution that is of interest.

The guidelines for matrix stability hereafter, relates to stability of samples as received at the laboratory, and assesses stability during storage in the laboratory before and after analysis. Examples of stability studies are given in Annex 1 Examples.

Not all required stability information needs to be obtained in dedicated stability studies. If elements of stability are included in the validation experiments – for example interruption of a preparation procedure, storage of an extract prior to injection into the measurement instrument – and the method performance parameters prescribed by CIR 2021/808[1] are still met, the stability can be assumed to be sufficient. Several stability study subjects for which this might be a viable approach are given in Table 1.

[1] Table 5 of CIR 2021/808[1] is lacking an ‚x' for stability for qualitative confirmation methods. Still, stability information is an integral part of ensuring method performance, regardless of specific method type and therefore stability information needs to be available also for methods validated as qualitative confirmation methods.

It is important to acknowledge that if stability information is obtained indirectly within the framework of a validation plan and without a dedicated stability study, then the analytical procedure implemented for routine analysis must be the same as the procedure used during the validation study. Accepting any interruption of the sample preparation procedure not included in the validation study would require weighing the risks and opportunities.

In order to potentially further reduce the number of required stability experiments, it should be evaluated which stability studies might be combined, where the study subject is deemed to be very similar. For example, a calibration standard might be equivalent to an analyte solution used for fortification; a sample extract ready for injection might be equivalent to a matrix-fortified standard.

Table 1: Solutions, extracts, and laboratory samples for which stability information might be required and how this information can be obtained (validation or dedicated stability study). In many cases referring to literature data would also be possible.

Stability study subject	**Strategy for obtaining stability information**
Calibration standards, matrix-matched standard, matrix-fortified standard	Could be included in the validation study
Analyte solutions used for fortification	Usually requires stand-alone stability studies
Extracts ready for injection	Could be included in the validation study
Analyte solutions (extracts) before interruption of the clean-up procedure	Could be included in the validation study
Matrix sample stored in the laboratory	Usually requires stand-alone stability studies

2 Definitions

Informed interpretation of stability studies is facilitated by a better understanding of all the terms involved. A commonly used term is “degradation”, which is often understood as the elimination of an analyte from a sample. For the purpose of this document “degradation” will be used to signify the reduction of an analyte’s concentration in a sample. It should be remembered that degradation is not equivalent to a mineralisation, i. e. the conversion of the analyte of interest into inorganic reaction products. Under the conditions applied in stability studies organic compounds rather undergo transformation processes yielding reaction products which are usually slightly modified derivatives of the parent compound. Metabolisation processes are transformation processes, which are mediated by living organisms. Metabolisation can yield the same products as other transformation processes. Also, transformation products of analytes of the same substance group may be similar or identical, which needs to be taken into account for the interpretation of the study results.

2.1 Degradation

Reduction of the analyte concentration in a sample.

2.2 Deterioration

Process of breakdown of matrix constituents (2.17).

2.3 Transformation

Process of conversion of analytes into reaction products. An analyte transformation coincides with analyte degradation.

2.4 Metabolisation

Transformation of a substance through biochemical processes mediated by living organisms (biotransformation).

2.5 Stability

Constancy of a property over time[2]. For the purpose of this document an analyte is considered to be stable, if the stability criteria (7.3) are fulfilled. This means that the analyte concentration does not change significantly.

2.6 Homogeneity

Uniformity of a certain property value within a defined portion of material.[3]

2.7 Solution

A liquid consisting of a solvent or solvent mixture and analytes of interest dissolved in the solvent or solvent mixture. For the purpose of this Guidance Document the term encompasses stock solutions (2.82.8), dilutions (2.9) and mixes (2.10).

2.8 Stock Solution

Solution prepared by weighing an appropriate portion of a solid or by measuring out an appropriate volume of a liquid and dissolving it in the weighed mass of solvent or by adding solvent to a given volume.[2]

2.9 Dilution

Solution prepared by adding a portion of an original solution to a defined amount of solvent to give a new solution with a concentration lower than that of the original solution. For the purpose of this document the term encompasses solutions of single analytes and analyte mixes (2.10).

2.10 Mix

Solution prepared by adding portions of several stock solutions (2.8) or dilutions (2.9) containing different analytes to a defined amount of solvent to give a solution which contains multiple analytes.

2.11 Laboratory Sample

Sample sent to or received by the laboratory with the intention of laboratory inspection or testing (Figure 1). A laboratory sample comprises material of one individual matrix from one individual animal. For example, seven laboratory samples of bovine muscle mean seven muscle samples from seven different bovines. In previous versions of the EURL Guidance Documents the term is also referred to as ‘batch’.

2.12 Test Sample

The sample, prepared from the laboratory sample (2.11), from which test portions (2.13) are removed for testing or for analysis (Figure 1). The laboratory sample (2.11) and the test sample can also be identical.

2.13 Test Portion

Quantity of material taken from the test sample (2.12) (or if both are the same, from the laboratory sample (2.11)) and on which the test or observation is actually carried out (Figure 1). In CIR 2021/808[1] this term is also referred to as 'aliquot'.

2.14 Extract

Resulting liquid following the process of transferring a substance from any matrix to an appropriate liquid phase.[2] For the purpose of this Guidance Document the term refers to the liquid which is obtained after following a sample preparation procedure performed on a test portion (2.13). Extracts may contain the analytes of interest or may be extracts of blank matrix.

2.15 Replicate Measurement

Multiple measurements performed on a single extract (2.14).

2.16 Influence Parameter

Any characteristic of the storage condition, which might influence the stability.

2.17 Matrix Constituents

Matrix components other than the analytes of interest.

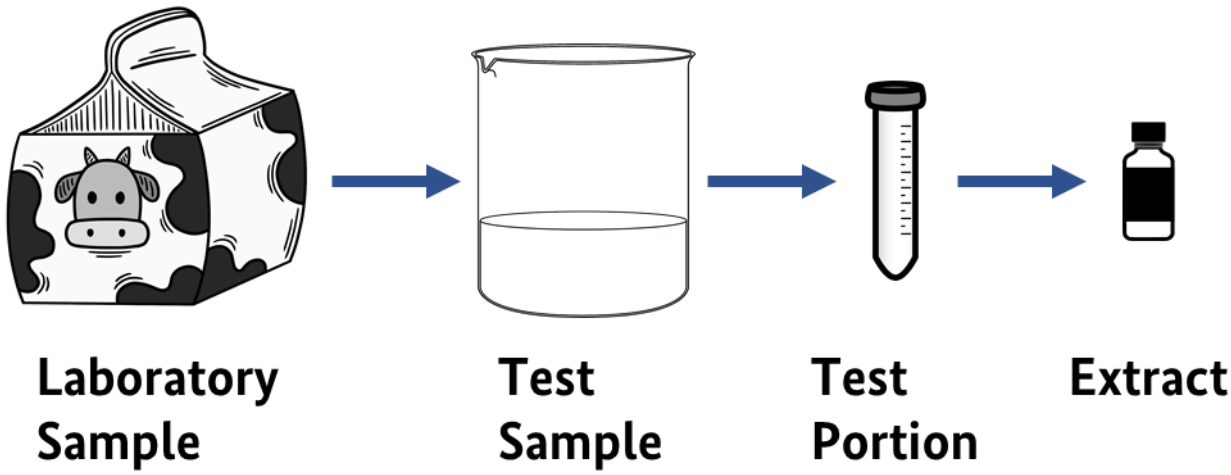

Figure 1: Relation of the terms 'Laboratory Sample', 'Test Sample', 'Test Portion', and 'Extract'. All images obtained via pixabay (users Idea Tivas TLM, OpenClipart-Vectors, Mohamed_hassan).

3 Types of Stability Studies

3.1 Chronological Stability Studies

In a chronological stability study all test portions are fortified at the same time and then subjected to sample preparation and measurement subsequently as time elapses, i. e. the test portions are analysed initially (t=0) and then per storage duration (t=x_1, x_2, ..., x_n) (Figure 2). The test portions for the different storage durations are therefore measured under intermediate precision conditions (within-laboratory reproducibility). The stability is assessed by comparing the concentrations to the analyte concentration in the beginning of the study (t=0). This type of study is suitable for incurred and fortified matrix material, the corresponding extracts, as well as solutions. The stability study schemes proposed in sections 2.5.1 and 2.5.2 of CIR 2021/808[1] describe chronological stability studies.

The advantage of chronological studies is that their concept is straight-forward and they are easy to plan. The main disadvantage is the need for subsequent measurements. The test portions need to be investigated as soon as possible after their assigned storage period has elapsed, resulting in a high workload over the duration of the stability study. Furthermore, the analytical results contain contributions from repeatability and within-laboratory reproducibility, which may hinder the identification of significant instabilities.

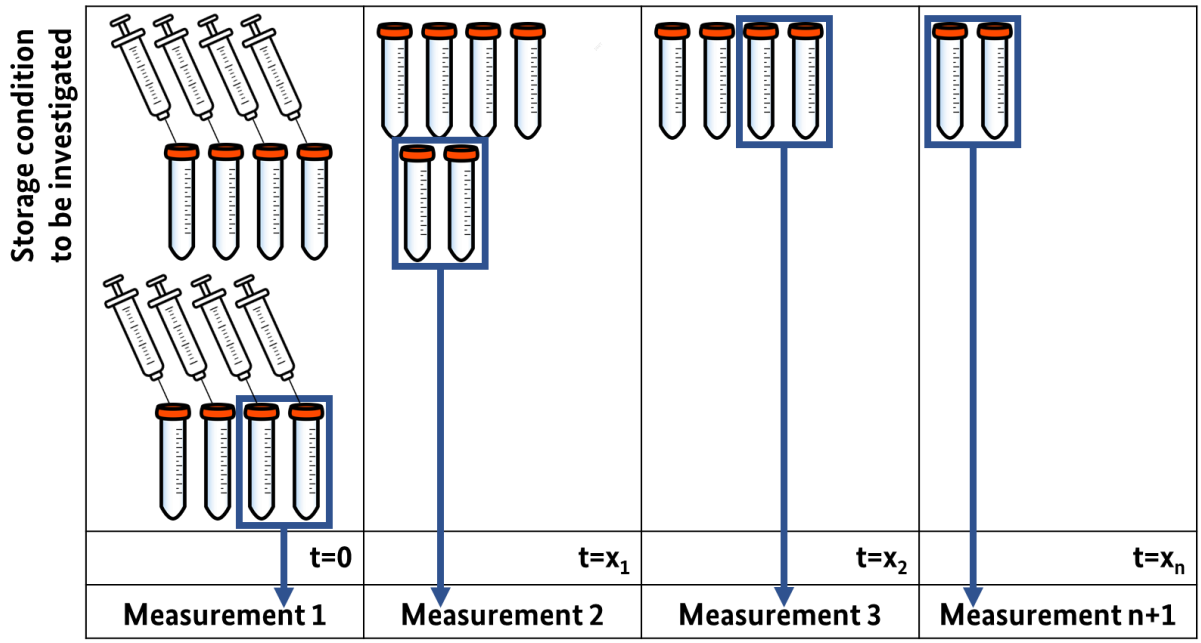

Figure 2: Illustration of a chronological stability study spanning x_n storage durations to be investigated. A number of n+1 measurements is required. Syringes illustrate when the test portions (represented by test tubes) are fortified. The number of test portions given in the illustration is arbitrary and should be adapted to serve the purpose of the specific stability study. Test samples can also be fortified as a batch before dividing into test portions.

3.2 Isochronous Stability Studies

An isochronous stability study is a stability study normalising to reference conditions, which are assumed not to induce any change in the sample composition, i. e. the test portions stored under the reference condition are treated as 'conserved'[4]. Commonly, the reference condition is storage at ultra-freezing temperatures (-80 °C). This type of study is suitable for incurred and fortified matrix material, the corresponding extracts, as well as solutions. The possibility of isochronous stability studies is also mentioned in CIR 2021/808[1].

In practice all test portions would be prepared in the beginning of the study, with a sufficient number of test portions immediately subjected to the reference conditions and the conditions to be investigated[2]. After the required storage period, the test portions stored under the conditions to be investigated are subsequently redistributed to the reference conditions (Figure 3). It is also possible to start the study with all test portions kept at the reference condition and then subsequently re-distributing to the conditions to be investigated (Figure 4). After all test portions have been stored for the intended time period under the conditions of interest, they can be analysed in a single[3] analytical series under repeatability conditions.

One main advantage of isochronous studies is the increased flexibility, as the test portions do not need to be analysed right away, but can be kept under the reference conditions. Additionally, the analytical results only contain contributions from the repeatability which makes significant changes in the analyte concentration easier to detect.

However, the isochronous approach also comes with some disadvantages. The most prominent disadvantage is that the test portions need to be stable under reference conditions, but this cannot always be easily proven. For the purpose of stability studies of analytes in matrix, one measure to increase confidence in the results of an isochronous study can be to include an additional quality control sample which serves to demonstrate the stability of the test portions stored at the reference conditions (section 6.3).

Moreover, disadvantages of isochronous studies include:

[2] Depending on the design of the isochronous study it can also be acceptable to fortify the test portions for the different combinations of storage condition and storage duration on different days. The prerequisite is that the solution used for fortification is the same and is stable over the course of the study.

[3] If the number of test portions is very large, it is usually not manageable to include them all in a single analytical series. In this case the test portions should be analysed in multiple analytical series in close temporal proximity. Ideally, the test portions are randomised across the different analytical series, or a block design (e.g., two test portions of the reference temperature of -80 °C can be used for both the 3-day and 7-day stability in one analytical series)is used so as to minimise the influence of inter-run effects on the results.

- results are only available after the longest storage period.
- the number of test portions to be subjected to sample preparation upon completion of the study can be quite large.

To improve these aspects, it can be beneficial to divide the study into multiple, overlapping isochronous studies covering broader phases like short-term, mid-term and long-term stability (Figure 5). For every phase a group of test portions subjected to the conditions to be investigated will be analysed together with one or more test portions stored under reference conditions for the same period. This means that for every phase the results are obtained under repeatability conditions whereas the results for the different phases are obtained under reproducibility conditions.

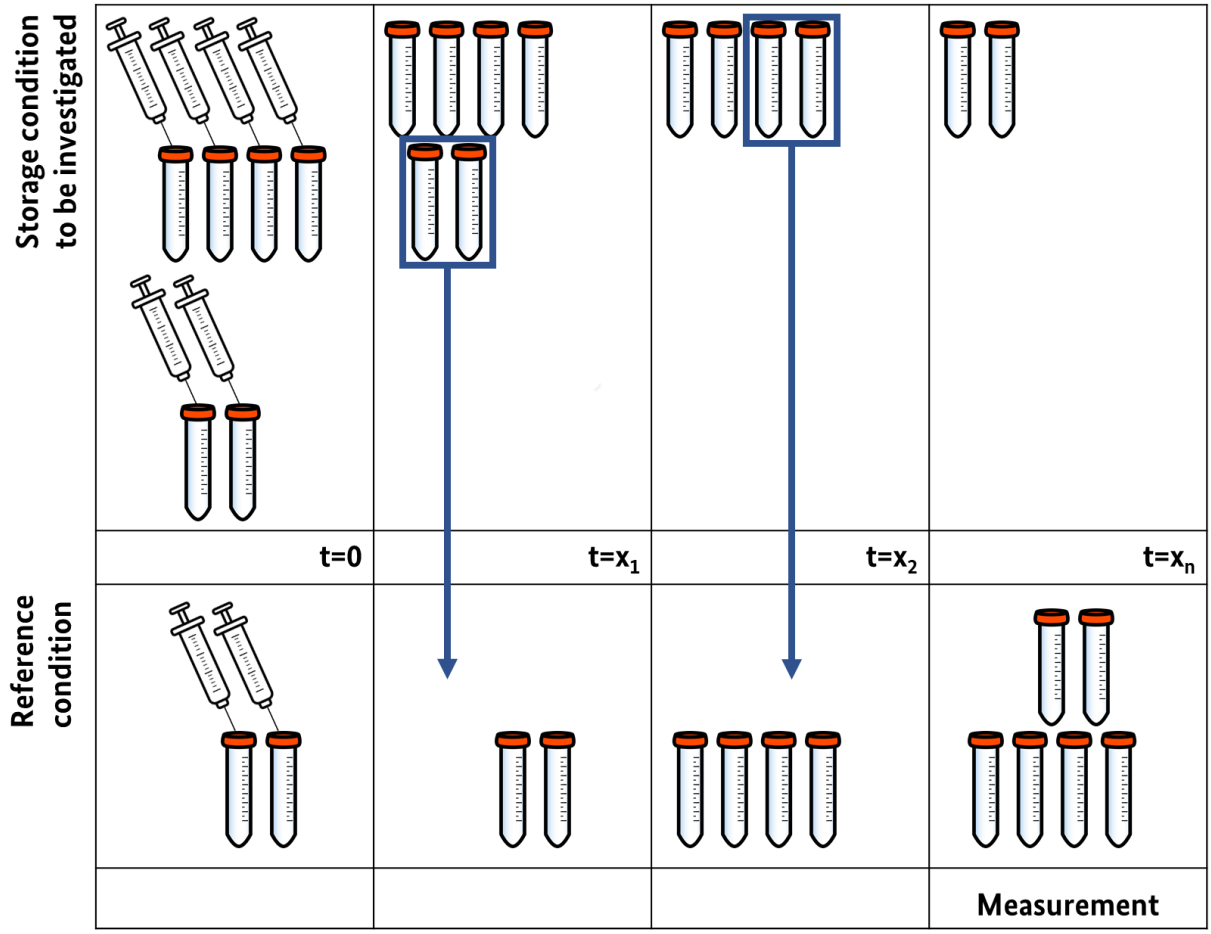

Figure 3: Illustration of an isochronous stability study spanning x_n storage durations to be investigated. All test portions are prepared and measured in a single analytical series at $t=x_n$. The test portions are initially subjected to the conditions to be investigated and then subsequently re-distributed to the reference conditions. Syringes illustrate when the test portions (represented by test tubes) are fortified. The number of test portions given in the illustration is arbitrary and should be adapted to serve the purpose of the specific stability study. Test samples can also be fortified as a batch before dividing into test portions.

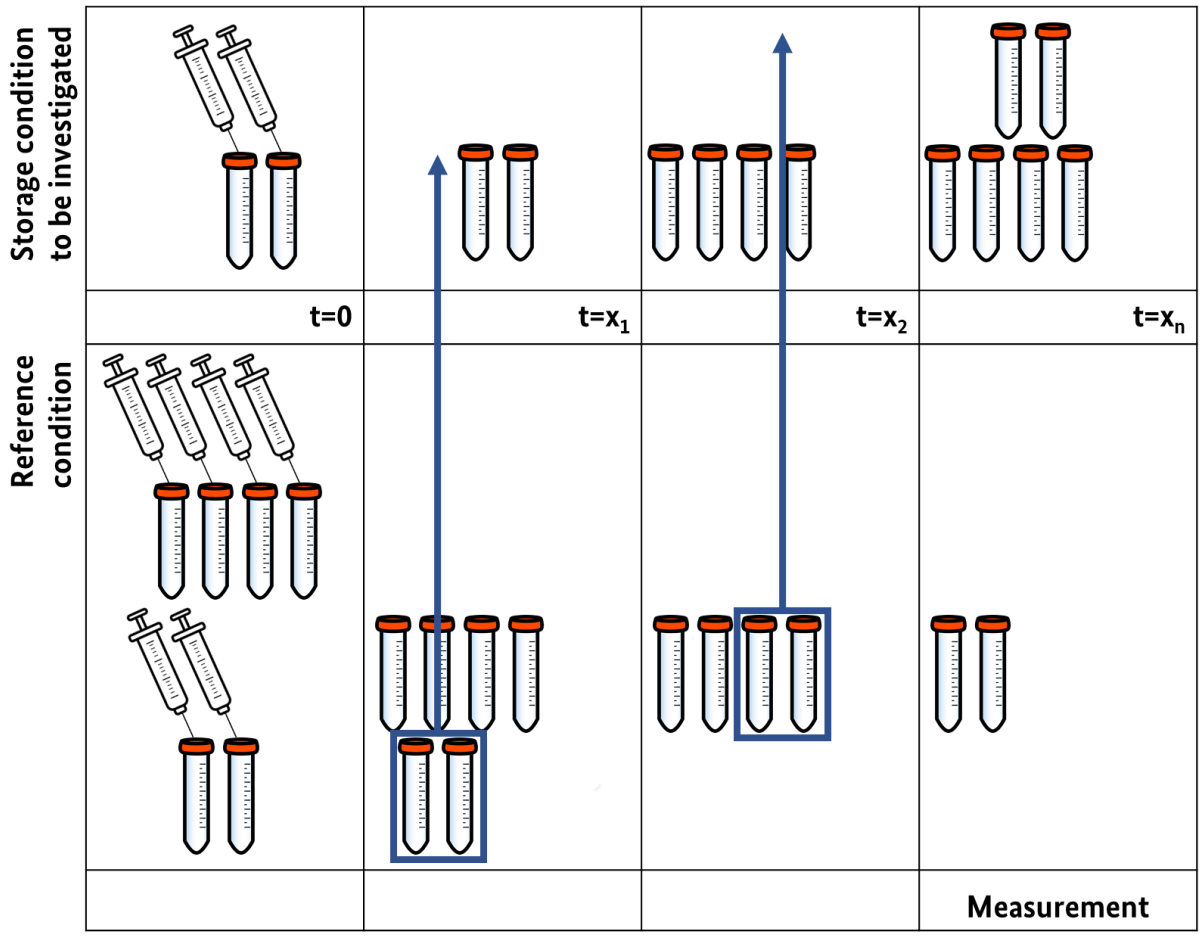

Figure 4: Illustration of an isochronous stability study spanning x_n storage durations to be investigated. All test portions are prepared and measured in a single analytical series at $t=x_n$. The test portions are initially stored at the reference conditions and then subsequently redistributed to the conditions to be investigated. Syringes illustrate when the test portions (represented by test tubes) are fortified. The number of test portions given in the illustration is arbitrary and should be adapted to serve the purpose of the specific stability study. Test samples can also be fortified as a batch before dividing into test portions.

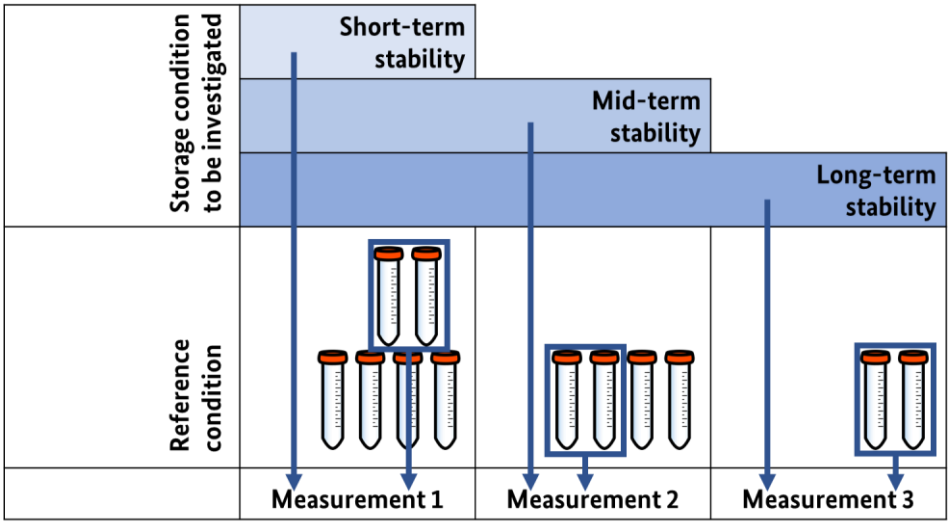

Figure 5: Illustration of multiple, overlapping isochronous stability studies spanning several storage durations to be investigated. The test portions are prepared, measured and compared to the test portions stored under reference conditions at three different time points. This is an example and the numbers of test portions, and the study durations are chosen arbitrarily.

3.3 Other Approaches for Stability Studies

The stability study types described in sections 3.1 and 3.2 are only two suggestions for an efficient study design and it is possible to modify these layouts. An approach which allows for the analysis of the stored, fortified test portions under repeatability conditions while also foregoing the need for defining a reference condition is displayed in Figure 6. After designing the study, a sufficient amount of solution to be used for fortification of the blank test portions needed for the study is prepared. Blank test portions are then subsequently removed from general storage, fortified and subjected to the storage conditions to be investigated. After preparing and storing all test portions for the intended duration, all stored test portions are analysed in a single analytical series together with blank test portions fortified that day, which will represent the concentration at t=0. This type of study is suitable for fortified matrix material, the corresponding extracts, as well as solutions.

The advantage of this approach is that all test portions can be analysed under repeatability conditions. The disadvantages are that this approach is not suitable for incurred material and that the solution used for fortification needs to remain stable over the course of the study. In order to evaluate this, it is recommended to verify the solution's concentration at the beginning and at the end of the study or to reference data from other sources proving solution stability over the course of the study.

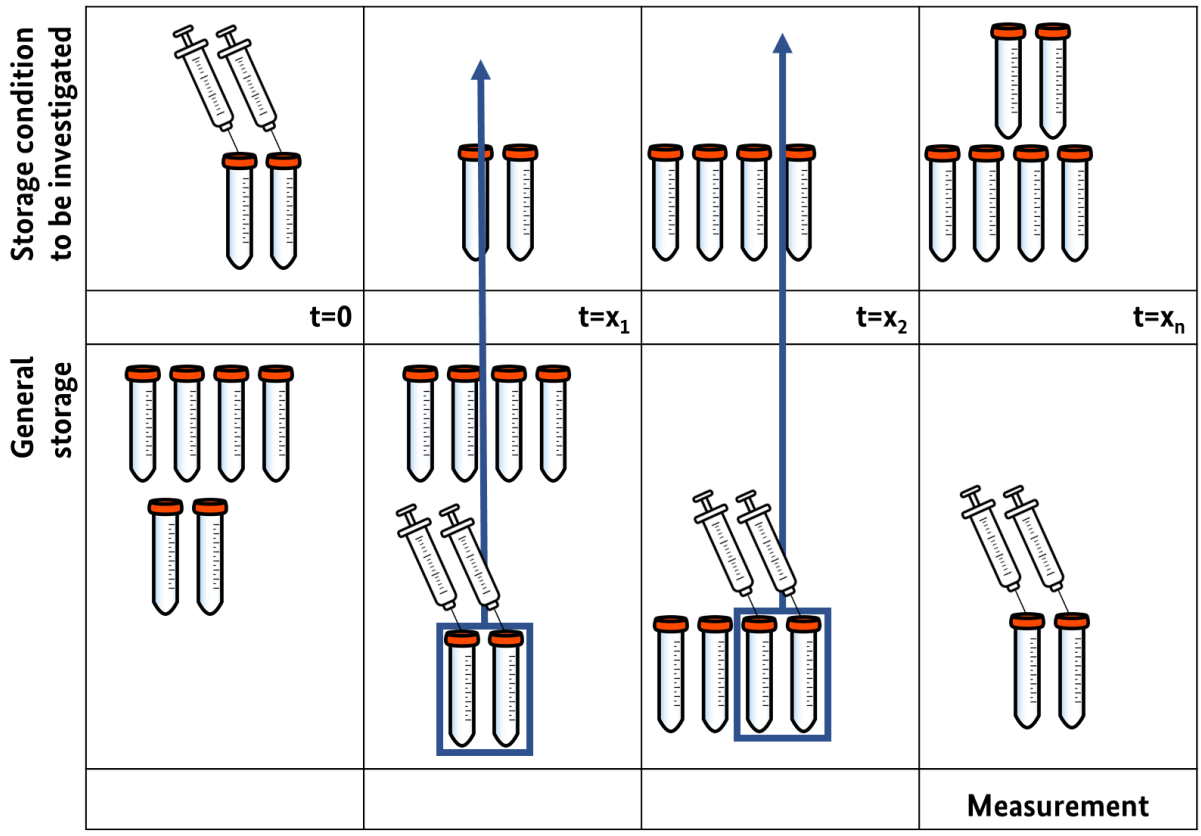

Figure 6: Illustration of a modified stability study spanning xn storage durations to be investigated. Test portions are fortified subsequently before storing under the conditions to be investigated. All test portions are finally prepared and measured in a single analytical series. Syringes illustrate when the test portions (represented by test tubes) are fortified. The number of test portions given in the illustration is arbitrary and should be adapted to serve the purpose of the specific stability study.

3.4 Stability Studies for Other Purposes

Depending on the specific circumstances of the storage and treatment of the solutions, extracts, and laboratory samples, other types of stability studies can be relevant. For example, in the case of known stability issues, it can be helpful to determine the stability after several freeze-thaw-cycles.

It is also a requirement of CIR 2021/808[1] that matrix constituents in the sample are checked for stability. Since there are no universal and quantifiable markers for matrix stability commonly applied for food samples within the realm of veterinary drug residue analysis, it is recommended that the matrix integrity is evaluated in a qualitative manner. This is necessary as matrix degradation may influence the extractability of analytes or promote the formation of metabolites, which might be considered the marker residue in accordance with CR 37/2010[5]. Qualitative markers of matrix instability can be changes in colour, smell, consistency (also phase separation) and formation of water crystals. If it is noted that changes in the matrix constituents impair the analysis, this should be adequately considered, for example by limiting the storage time of affected samples.

4 Stability Study Design

4.1 Objective

The designs and interpretations of the stability studies given in CIR 2021/808[1] are to be understood as recommendations rather than prescriptions. This is signified by the phrasing utilising "should" (signifying a recommendation) rather than "shall" (signifying a requirement), the reference to other possible approaches like the isochronous studies, and the possibility to use data from other sources. For any deviation from the designs laid out in CIR 2021/808[1] the responsible analysts should make sure to use plausible statistical models.

Clear defining the objective of the intended stability study greatly helps with deciding on a suitable study design. The responsible analysts should ask themselves what they would like to use the obtained data for, which prescriptions are already in place in their laboratory (standard operation procedures), which conditions are to be encountered during routine operations, what safety margin should be included, as well as what is already known about the behaviour of the analytes of interest. Especially the aspects laid out in sections 4.2-4.6 should be considered.

4.2 Storage Conditions

With regard to the storage temperatures, CIR 2021/808[1] recommends studying analyte behaviour at -20 °C, +4 °C and +20 °C. In the EURLs' interpretation, deviation from these temperatures is acceptable as they are to be understood as the individual laboratory's conditions for storage under freezing, cooled, and ambient conditions. At a minimum, laboratories should have access to stability data for the conditions under which solutions, extracts, and laboratory samples are usually stored in their facility. Information obtained for storage at elevated temperatures has the advantage that changes in the analyte concentration usually become apparent after shorter storage times, allowing for an improved understanding of the analyte's general susceptibility to degradation processes.

With regard to the presence/absence of light, CIR 2021/808[1] proposes to study both. This is to ensure stability during handling. However, it should be noted that the degradation pathway and the degradation kinetics under irradiation can differ significantly from those of temperature-driven degradation. Study results obtained for storage in the dark and in the light are therefore usually not comparable. If the influence of light on the stability is deemed irrelevant due to practical reasons (e. g. exclusive use of amber glassware), this factor would not need to be studied.

What also requires consideration is a possible change of vessel material (e. g. untreated glassware, silanised glassware, different polymers) used for the storage of solutions, extracts, and laboratory samples[4]. For example, when analytes are known to be prone to adsorption to untreated glassware, or

[4] also test samples or test portions

are assumed to be stable in amber glassware, it would be advisable to conduct a study comparing the stability in different vessels in order to ensure that previously obtained data using different vessels or data from sources other than your own laboratory can be considered sufficiently similar. This is a direct consequence of the prescription of CIR 2021/808[1] that existing stability data can only be referenced if identical conditions are applied (see also section 8.1). When deciding to reference stability data from sources other than your own laboratory, the risks and benefits should be thoroughly considered, and where necessary, additional stability studies should be planned.

Generally, if laboratories study only a minimum number of stability conditions, they should establish a laboratory policy for those cases, in which these routine conditions are not met. Such a policy could for example encompass those solutions kept under elevated temperatures for prolonged periods need to be disposed of. Alternatively, laboratories could evaluate the risks and opportunities in such cases. Regardless of how these issues are approached, their treatment should be documented.

4.3 Storage Periods

CIR 2021/808[1] prescribes to conduct stability studies until first signs of degradation are noticed. For very stable analytes this is not always practical, as the stability of these analytes might far surpass the duration that solutions, extracts, and laboratory samples are usually retained for in the laboratory. It is therefore recommended that the storage durations to be included in a stability study should at the very least represent the expected treatment of the solutions, extracts, and laboratory samples in the laboratory.

The storage durations to be included in the study should be selected based on the study's objective. In practice that might mean that stability studies for extracts can be kept rather short, as extracts are usually not routinely stored for longer periods of time. Laboratories usually also have rules in place for maximum storage of stock solutions, dilutions, and mixes. These can serve as an upper limit for the storage durations to be investigated in the study. Other sensible storage periods can be derived by considering the lifecycle of the study object, by setting a number of intervals leading up to the maximum storage duration, or by including a storage duration which is expected to result in analyte/matrix degradation.

Studies for laboratory samples should at least be as long as the maximum retention period as prescribed by the laboratory's policy. Studying analyte behaviour under storage durations longer than prescribed by laboratory policy or by the relevant method description offers the advantage that stability information is available in exceptional cases such as follow-up analyses or the need for postponed measurements in the case of unexpected malfunctions of the measuring instrument.

For selecting appropriate storage periods, it is also recommended to consider all available information, such as data from previous studies, other laboratories, or literature. If analytes are known to be sensitive, then the storage periods to be studied should be adjusted accordingly.

In order to allow for the performance of a linear regression and to be able to test the study results for trends, it is preferable to include additional timepoints for any given storage condition, ideally at least three timepoints per storage condition. It is noted that additional timepoints above three, can be more beneficial to allow for better assessment of stability, and to verify apparent changes in the analyte concentration.

4.4 Concentrations

When it comes to the selection of suitable analyte concentrations for a stability study, the most important criterion is the study object. For the stability of stock solutions, the analysts should use those stock solutions, which are usually prepared in their laboratory. For dilutions and mixes, which might also be used for the preparation of calibration curves, at least the most relevant concentration should be assessed.

For laboratory samples and extracts, it is suitable to differentiate based on the authorisation status of the relevant analytes. In the case of authorised compounds, the main objective is to ensure that the decision on the sample conformity remains identical over the course of the allowed storage period. Therefore, for assessment of stability the analytes should be present in the matrix around the maximum residue limit (MRL) or maximum level (ML). Extracts to be studied for their stability should be prepared from test samples/test portions with analyte concentrations around the MRL or ML. If a stability study is designed only for the purpose of testing a screening method, then it is recommended to fortify test samples/test portions and extracts around the Screening Target Concentration (STC).

In the case of non-authorised or prohibited compounds, any confirmed presence of the analyte would equate a non-conforming sample. However, for very low concentrations – i. e. usually around the lowest calibrated level (LCL) – achieving analyte confirmation might be difficult. Selecting a slightly higher concentration, for example around the minimum method performance requirement (MMPR) or reference point for action (RPA), balances the requirement for low concentrations while still maintaining analyte confirmation.

It is necessary to note that degradation kinetics of many analytes are not studied in detail and hence inferring the stability for concentrations not previously investigated is not recommended. For further considerations refer to section 8.1.

4.5 Selection of Samples

CIR 2021/808[1] prescribes using five test portions subjected to the same storage condition and storage duration for the evaluation of the study. Since this prescription aims at obtaining statistically-sound information on the analyte behaviour, it is reasonable to reduce the number of test portions, if the trust in the overall conclusion on the analyte's stability can be increased by other means. In practice this could be achieved by studying more than one storage duration. In this case the number of test portions per temperature-time combination can be decreased. Ideally, the minimum number of test portions

per condition is at least three to allow for the calculation of a standard deviation and subsequent comparison to the respective analytical method's precision parameters. The number of test portions and replicate measurements per corresponding extract should be selected in accordance with the method description of the applied analytical method and the laboratory policy.

For the design of stability studies in matrix, it is also necessary to consider how many different laboratory samples to use. When using multiple laboratory samples in a single study there is a risk that the intra-species variability hinders the detection of significant changes in the analyte concentration. It is therefore recommended to use only a single laboratory sample for a single stability study. Whether or not supplementary studies using more laboratory samples are necessary depends on the method performance and all information available. Generally, if the criteria for the precision and the matrix effect outlined in CIR 2021/808[1] are fulfilled, a single study using only one laboratory sample is sufficient in order to reduce the overall workload.

For analytical methods which cover a variety of matrix-species combinations, in principle stability data for all combinations is required. This would often be associated with a high workload. The EURLs therefore concede that stability information for certain matrix-species combinations, for which the responsible laboratory only analyses a very low number of samples per year, is not required. The laboratories should also take into account the frequency of positive findings and the authorisation status of compounds for the prioritisation of their stability studies. This is not to be misunderstood as *carte blanche* for studying only the stability of a single matrix-species combination – stability information for the most relevant combinations needs to be available. This requirement also applies to the sample condition: Laboratories are required to have access to stability information for the condition the laboratory samples are most often received in (e. g. fresh raw milk, dehydrated milk).

4.6 Analytes to be Monitored

In general, all relevant analytes should be monitored in a stability study. In cases where multiple marker residues are expected, at least those given in the residue definition should be monitored.

If the study object includes both a parent compound and metabolites (transformation products), the analyst should be aware that the metabolite concentration might at first increase or remain stable instead of decreasing, due to the ongoing formation of the metabolite from the parent compound. It can be preferable to monitor the sum of parent compound and metabolites, expressed as equivalents of one or the other (ideally as the marker residue given in CR 37/2010[5], if available[5]) so as to be able to gain an understanding on the overall change in concentration. In order to improve the confidence

[5] If both the parent compound and the transformation products or also the isomers of a pharmacologically active substance are listed as separate marker residues in CR 37/2010, then the stability also needs to be determined separately for each of the relevant marker residues.

in the results, additional studies in which only the parent compound or only individual metabolites are investigated, can be conducted.

Conjugates are a special case, as there are usually no standards available. In consequence, solutions are not prepared and stability studies for these are not required. For any method that includes a deconjugation step and provides a result for the deconjugated entity, only, a stability study for the conjugate in extract is also not required.

In incurred matrix, many compounds are present in a conjugated form. This means that a stability study on a blank matrix fortified with a solution of the unconjugated analyte is not an adequate representation of an actual laboratory sample and the stability of the marker residue in this laboratory sample. Still, such a study may provide indications as to the analyte's general stability. It is therefore recommended to gather this kind of information regardless and assume similar stability for the analyte and its conjugate, unless otherwise known. If repeated analyses of an incurred material indicate instability of the analyte of interest[6], then this should be accounted for, for example by limiting the storage period for any laboratory samples of the same combination species-matrix-analyte. Should standards of relevant conjugates be available, then stability studies should also be conducted for the conjugates.

4.6.1 Studies for Multiple Analytes

Usually, it is inefficient to conduct stability studies for only a single analyte at a time. For the majority of laboratories this would also not adequately represent their routine workflow. It is therefore preferable to use the mix solutions used for routine analyses also for the stability studies. Ideally, the stability studies are designed at the validation stage of an analytical method and the solutions used for the validation are also employed for the stability study.

If stability issues are identified for mix solutions, it should be remembered that these could also stem from an interaction of the analytes among themselves instead of a degradation. Furthermore, whenever parent compounds and marker residues are present in the same solution, it might be better to monitor the sums, so as not to misinterpret changes in the individual analyte's concentrations. The same approach can be used when two analytes are transformed to the same product (e. g. metronidazole and ronidazole are both transformed to HMMNI).

4.6.2 Stability Studies for Internal Standards

Internal standards require nuanced consideration. For any internal standard it is of prime importance that it behaves just as the native standard it is used as reference for, i. e. remains stable if the native standard remains stable or degrades in the same fashion as the native standard, they are mimicking

[6] in this case it would be the conjugate of interest

under all conditions of the sample preparation procedure. If that is the case, the internal standard stability does not influence the analytical results.

However, especially in the case of deuterated internal standards a deuterium-hydrogen exchange may take place in protic solvents, resulting in the conversion of the deuterated compound to the native analyte of interest. In extracts this would lead to a perceived increase in the concentration of the analyte of interest for repeated measurements, which should be taken as an alarm signal. Note that when calibration standards are stored alongside the extract and investigated together this cannot be detected and the measurement result remains identical. It should also be noted that referring to the ratio of the analyte of interest and the internal standard for assessing the stability in extracts can be misleading. If both degrade with a very similar rate, then the concentration is overall perceived as stable, despite only the ratio being stable. The role of an IS in this context is explained in more detail in section 7.2.

If the design of the stability study reflects routine workflow, the effect of an increase in the analyte of interest due to IS does not need to be further considered. But if the design of the stability study is different from the routine approach (e. g. use of a freshly prepared calibration curve for the quantification of stored extracts), the analysts should be aware of the possibility of an influence of the internal standard stability on the overall result. The stability of the internal standard should then be assessed, or rules should be set to always re-extract test samples to be investigated and calibration samples for a repeated analysis.

Furthermore, laboratories should regularly investigate solutions of labelled internal standard for the presence of the native compound. This can also be incorporated into the analytical routine by analysing an additional quality control sample such as a blank sample fortified with a solution of the internal standard.

5 Sample Preparation

In order to study the stability in solutions and extracts, a sufficient number of aliquots needs to be prepared. Since the design of the stability study determines the required amount, it is recommended to prepare the solutions, extracts, or fortified test sample/test portions to be studied only after the study layout is finalised, so as to make sure that the same solutions are used for all stability study samples. If extracts are studied, a sufficient amount should be prepared from incurred matrix or blank matrix material fortified with the analytes of interest prior to extraction. In the case of incurred matrix, it should be decided how many test portions can be used for the stability study.

For studying stability in matrix, incurred laboratory samples are the preferred option as they represent the actual interactions of analyte (conjugate) of interest with matrix components. Fortified blank matrix is to be used when incurred matrix material is not available. In order to ensure an adequate interaction

of the analytes fortified into blank matrix, the test sample/test portions should be adequately mixed. In the case of solid matrix sufficient interaction cannot be guaranteed, especially when there is no effective homogenisation step. This risk needs to be acknowledged and can explain differences in the results of stability studies for incurred matrix material and fortified matrix material. It is recommended that when preparing individually fortified test portions of solid matrix material the solvent introduced through the solution used for fortification is left to evaporate and the test portions are vortexed.

For preparing fortified matrix, the same solution should be used for all samples. Ideally, a portion of the solution is retained for ensuring stability of the solution over the course of the stability study.

5.1 Homogeneity

In order to obtain credible results for the stability studies all test portions need to be sufficiently homogeneous. However, this Guidance Document aspires to provide recommendations for stability with regard to fitness for purpose of analytical methods and therefore the effort taken for homogeneity assessment should be limited. In many cases it is reasonable to not assess the homogeneity at all, but to rather follow best practices.

Achieving homogeneity is usually much easier for liquid matrix material. All solutions, extracts, and liquid test samples should be fully defrosted (if applicable) and agitated before sub-sampling. If feasible, all liquid laboratory samples which might contain precipitate or multiple phases (e. g. extracts, unfiltered urine, raw milk) should be mixed thoroughly before pipetting test samples or test portions. In these cases, it is also recommended to subject test portions of the required volume to the storage conditions, that they can directly be used for the analyses without the need for further sub-sampling.

In the case of solid matrix material (e.g., tissue) the homogeneity can be assumed sufficient if test portions are fortified individually and the test portions are subsequently subjected to sample preparation without further sub-sampling.

For batch-fortified test samples or incurred solid laboratory samples/test samples sufficient homogenisation is harder to achieve. For both types a prerequisite for achieving a uniform and homogeneous material is sufficient grinding, before dividing into test portions. Ideally, the homogeneity is assessed prior to the start of the stability study, so that additional homogenisation steps can be conducted in cases where significant heterogeneity is detected. But if the particle size is relatively small and the analyst is experienced with preparing fortified material, it may also be possible to accept the risk of heterogeneity and assess the homogeneity only post-study. Information on homogeneity tests can i. a. be gathered from ISO 33405[6].

6 Analytical Methods for Stability Studies

If stability studies are conducted as part of fulfilling the requirements of CIR 2021/808[1] , then the analytical results should be obtained by applying analytical methods also validated in accordance with

this regulation.[7] Use of a validation method for assessing solvent stability may not always be required, or necessary, however, it is recommended that a well-established method/technique should be used. Preference should be given to quantitative or semi-quantitative methods of adequate precision. If the precision is insufficient, the interpretation of the results is hindered and instabilities may become unnoticeable. Depending on the study type, sufficient repeatability, within-laboratory reproducibility or both are of the essence. If an isochronous study is planned, analytical methods with a good repeatability for the relevant analytes are to be preferred. In the case of chronological stability studies, it is necessary that the measurements carried out at different points in time can be compared with each other well. Therefore, a method with good within-laboratory reproducibility should be selected. For multiple, parallel isochronous studies the analytical methods to be used should ideally exhibit both sufficiently good repeatability and intermediate precision. In case of doubt, however, analytical methods with optimised repeatability should be preferred.

If it is already known that the inadequate precision of the analytical methods to be used for the purpose could make it difficult to evaluate the results of the stability study, it may be useful to increase the number of replicate measurements performed on each test portion (e. g. three measurements instead of only one). Furthermore, it may be advisable to increase the number of test portions stored per combination of storage period and storage condition or to adjust the number of combinations of storage period and storage condition examined in order to obtain a better data basis for the stability assessment. In cases where the number of test portions or replicate measurements is modified, the impact on the measurement uncertainty should be taken into account for the evaluation of the results.

6.1 Qualitative Methods

Stability information is an integral part of ensuring the reliability of an analytical method, regardless of the specific method type. Therefore, information on analyte stability needs to be available also for methods validated as qualitative confirmation methods or qualitative screening methods. When planning a stability study using a qualitative method for the determination of the analytes of interest, it is advisable to assess whether it would also be possible to obtain at least semi-quantitative data by applying the analytical method in question. Semi-quantitative data have the advantage that the analyte behaviour over the storage duration can be characterised to some extent and is not limited to the outcomes ‘analyte is present’ and ‘analyte is not present’. This approach would usually be feasible for all those analytical methods validated in accordance with CIR 2021/808[1] which are characterised as qualitative methods merely because they do not fulfil all requirements imposed for precision and accuracy. If laboratories require stability information for a truly qualitative method, these analytical

[7] Data from external sources using analytical methods not validated in accordance with CIR 2021/808[1] can be acceptable. The provided method information should be carefully studied by the responsible expert with regards to fitness for purpose.

methods can also be used for the stability study. Care should be taken to use an appropriate number[8] of test portions per storage condition, so that the confidence in the qualitative result suffices. Alternatively, the responsible analyst could consider referencing appropriate stability data from external sources (if available). If qualitative screening methods utilising a biological or biochemical detection principle are employed, it should be noted that the change in matrix composition over the course of the storage may result in changes in cross-reactivity, possibly leading to an increased false positive and false negative rate. It is therefore recommended to verify the outcome of the external stability study by reproducing the results for t=0 and the intended maximum storage duration t=max.

6.2 Sample Preparation and Analysis

Generally, the number of test portions and the number of replicate measurements for each test portion should follow the instructions laid down in the respective method description. In the case of solutions, stability testing can either be performed directly on portions of the stored solutions or - if the concentrations are too high, e. g. in the case of stock solutions - they should be diluted immediately before analysis. The procedure for analysing the test portions should adhere to the respective method description and standard operation procedures. The order in which the different solutions or extracts are injected should be randomised so as to distinguish the concentration change due to storage during the stability study from an analytical trend.

6.3 Quality Control Samples

For the analyses required for the stability study, the usual set of quality control samples should be used (refer to the EURL Guidance on the Quality Control During Routine Analysis[7] for details). In cases where analyte stability in fortified matrix is being assessed, the solution used for the fortification should also be employed for the purpose of quality control. Additionally, it should be demonstrated that the employed solution is stable over the course of the stability study. For chronological stability studies (section 3.1) ideally the same set of quality control samples is prepared for every analytical series. Their results should not deviate by more than the analytical method's within-laboratory reproducibility as derived from the validation experiments.

For isochronous stability studies (section 3.2) available information suitable to demonstrate the stability under reference conditions should be gathered. In order to further increase confidence in the appropriateness of the reference condition for fortified matrix samples, a specific quality control sample is recommended: A recovery control sample freshly prepared by fortification with the same

[8] Appropriate numbers of test portions per storage condition would usually be higher if purely qualitative methods are to be applied instead of methods yielding at least semi-quantitative data.

solution as used for the fortification of the test portions should be prepared. The deviation between this recovery sample and the test portions stored under reference conditions should be in agreement, taking into account the repeatability as derived from the validation experiments.

For the purpose of monitoring the stability of isotopically labelled internal standards a blank sample fortified with a solution of the internal standard can be used. This allows for changes in the composition of the employed internal standard, especially the formation of the native compound from deuterium-labelled standards.

6.4 Confirmation Criteria

Since information on analyte stability is required for analytical methods validated in accordance with CIR 2021/808[1], it is reasonable that the stability information at least matches the data quality obtained by the analytical method. This means that for a semi-quantitative screening method the stability information should also be obtained at least using a semi-quantitative screening method. Using information of higher information value for example,from a quantitative confirmation method, is possible. This implies that for any quantitative confirmation method the stability information should also consider confirmation criteria. When more than one test portion is analysed per temperature-time combination the confirmation status can be derived from averaging the ion ratios for these test portions. In case there are individual results which do not seem to correspond to the remainder of the group (i. e. outliers), these should be carefully evaluated with regard to possible errors in the sample preparation, measurement, etc. If there is evidence for errors, it can be adequate to remove the outliers. If there are no such indications, then outlying values might suggest stability issues or insufficient homogeneity. It is advised to decide how to treat the anomalous data point only after reviewing the complete set of stability data.

It is important that the requirement toconfirmthe analytes of interest, should also be taken into account for the design of the study. In addition, it is particularly important to ensure that the selected concentration for marker residues of unauthorised pharmacologically active substances, is not set too low.Once the analyte of interest is degraded to a substantial degree, the confirmation might no longer be possible due to significant changes in the ion ratio of quantifier and qualifier. This should be considered in drawing conclusion from the stability study. It is noted however, if a signal for the quantifier is still detected and its retention time fulfils the confirmation criteria, the analyte could possibly still be considered sufficiently stable for screening purposes.

6.5 Further Considerations

When storing solutions, extracts, and laboratory samples there are other factors which should be minded, especially in cases where stability is unexpectedly found to be insufficient. With solutions and extracts, specifically where more volatile solvents are involved, it can be helpful to check for possible

solvent evaporation. This can be done by regularly recording the weight of the vessels used for storing the solutions and the extracts.

Similarly, solutions, extracts, and liquid test portions should be checked for precipitation. Where precipitation is expected, accepted for routine analyses, and the respective solutions, extracts or test portions are usually ultrasonicated, the same approach should be used also for stability studies. When problems with the stability of solutions arise and precipitation is observed, it is recommended to store and handle the solutions in lower concentrations. Usually there is no need to study the influence of ultrasonication separately.

Another factor which might be relevant, is the duration between removal of solutions, extracts, and test portions from freezing or refrigerated conditions before further processing or measurement. The general recommendation is to keep these transition times as short as possible, while still guaranteeing complete phase transition and adjustment to room temperature. In case of unexpected results in the stability study, additional investigations using, for example, prolonged storage at room temperature, are advised.

7 Data Evaluation and Interpretation

7.1 Data Evaluation

To evaluate the stability the solutions, extracts, and test portions are processed and assessed for changes in the analyte concentration under the different storage conditions. In principle, two evaluation approaches are possible:

(1) Concentration-based: Evaluation using a calibration curve and comparison of the concentrations

(2) Peak area-based: direct comparison of the measurement results obtained. If an internal standard is used, the area ratio of analyte to internal standard should be used, as this helps mitigate intra-run and inter-day variability.

Particularly for stability studies in matrix and if, for example, it is known that the response of the measuring device fluctuates greatly between measurement days, it is recommended to evaluate by referencing a calibration curve (Approach 1). This makes it easier to compare the results between the different analytical series because the influence of the measuring device is somewhat compensated for. Another advantage of a concentration-based approach is that the degradation processes can be quantified and not just be expressed in relative terms. Approach 2 offers the advantage that fewer test portions have to be prepared and measured, as no calibration curve is required. However, a comparison with results from tests carried out previously is made more difficult. A combination of both approaches is also possible.

7.2 Role of Internal Standards

Using internal standards in stability studies is highly recommended (see above). Still, the analyst needs to be aware of stability issues which might be overlooked in case of application of an internal standard. Whenever the stability of extracts[9] is studied, the situation may arise where the analyte is perceived as stable, because the ratio of the analyte of interest and the internal standard remains more or less identical (i. e. fulfils the criteria given in section 7.3). Yet, at the same time the area of the analyte of interest may really have decreased significantly along with the area of the internal standard due to undetected degradation processes. If quantified against a matrix(-matched) calibration subjected to the same storage conditions, the resulting analyte concentration in the stored extract would be assumed to be identical to that obtained for sample extract and calibration measured immediately after preparation, despite degradation having taken place. In order to differentiate this occurrence from actual analyte stability (i. e. no changes in the area whatsoever), the term extract equivalency is used. The purpose of such assessment is to demonstrate that the concentration measured after storage for a given time, is equivalent to the concentration measured initially, despite their potentially being a change in analyte response in the extract. Assessing extract equivalency rather than stability is only acceptable for extracts, not for solutions or matrix.

The implications of assessing extract equivalency as opposed to stability pertain to the analyte confirmation (a) and also the handling of repeated analysis (b).

(a) A decline in the number of analyte molecules present in the extract to be quantified can imply a shift in the ion ratios of quantifier and qualifier, possibly leading to failure of the confirmation criteria.
(b) If a sample needs to be re-analysed, it is recommended that the calibration to be used for quantification is also freshly prepared.

Overall, the assessment of extract equivalency will likely yield more practical information relating to how long extracts may be stored without impacting the analytical result. It is also noted that the use of extract equivalency is reliant on the use of internal standards that effectively mimic the behaviour of the analyte of interest. If the internal standard employed is shown to be less effective for correcting for deterioration of the analyte response, for example due to matrix effect, then the use of extract equivalency for assessing extract storage may be less appropriate.

[9] Bear in mind that matrix calibrations and matrix-matched calibrations are also extracts.

7.3 Assessment Criteria

7.3.1 Criteria Defined in CIR 2021/808

For the assessment of solution stability, CIR 2021/808[1] gives 15 % as a quality criterion: If the mean analyte concentrations in fresh and stored solutions do not differ by more than 15 %, the analyte is considered to be stable under the investigated conditions.

Analyte stability in matrix and extracts is deemed sufficient, if the mean analyte concentrations in fresh and stored test portions do not differ by more than the within-laboratory reproducibility. In some cases, the within-laboratory reproducibility can be rather small, if some contributions to the overall measurement uncertainty are not included. It could therefore be acceptable to use either the within-laboratory reproducibility or 15 % as a quality criterion, whichever is larger.

For strictly qualitative screening methods the solutions, extracts, and laboratory samples are deemed stable, if the result of the screening test remains 'screen positive' over the course of the stability study.

In any case where the difference between fresh and stored test portions does not meet the criteria described above, the laboratories are advised to document how these cases will be handled in routine[10]. This decision can also be supported by the results of the additional assessment approaches described in section 7.3.2.

7.3.2 Supplementary Assessment Approaches

Despite careful planning of a stability study, it is possible that the stability criteria in accordance with CIR 2021/808[1] are not met. Nevertheless, the data obtained can be useful for risk-based decisions[Fehler! Unbekanntes Schalterargument.] regarding stability in your laboratory (the data can be useful even if it does not meet all criteria). The following sub-sections provide two examples on supplementary data analysis approaches.

7.3.2.1 Bias Assessment

The results of the stability study for the different storage durations are assessed against the results obtained for the reference. The reference can be the initial measurements performed at t=0, results for test portions kept at reference conditions for the duration of the study and also results for test portions prepared the same day that the stored test portions are subjected to analysis. Whether a statistically significant difference between two values is apparent, can be checked by a bias assessment. For this it is necessary to calculate the difference between the results of the stored test portions and the

[10] For example, the decision might take into account the potential risk of falsely classifying an official sample as compliant. Laboratories could for example evaluate the difference in fresh and stored test portions as 'not meeting the criteria, but not relevant' or 'acceptable with respect to additional influencing factors (contribution of uncertainty of the standard solution not included in within-laboratory reproducibility)'.

reference Δc (equation (1)), as well as their combined uncertainty $u_{\Delta c}$ (equation (2)). The difference in concentration is then compared with the combined uncertainty. If Δc is smaller than or equal to twice the combined uncertainty (equation (3)), the results are not significantly different and the analyte in question is considered stable for the applied storage conditions. If Δc is larger than twice the combined uncertainty (equation (4)), the results are significantly different and the analyte in question is considered unstable for the applied storage conditions.

$$\Delta c = |c_{stored} - c_{reference}| \quad (1)$$

$$u_{\Delta c} = \sqrt{u_{stored}^2 + u_{reference}^2} \quad (2)$$

where
$c_{reference}$ - (mean) concentration of the reference sample(s)
c_{stored} – (mean) concentration of the stored sample(s)
$u_{reference}$ – uncertainty (of the mean) of the reference sample(s)
u_{stored} – uncertainty (of the mean) of the stored sample(s)

$$\Delta c \leq 2 \cdot u_{\Delta c} \quad (3) \quad \text{No statistically significant difference}$$

$$\Delta c > 2 \cdot u_{\Delta c} \quad (4) \quad \text{Statistically significant difference}$$

The input for the concentrations in equation (1) should be the mean of all test portions used as reference ($c_{reference}$) and all test portions stored under the condition to be evaluated (c_{stored}) (only one temperature-time combination), respectively. The uncertainties to be used for this assessment should be selected in accordance with the stability study scheme and are obtained by correcting the respective standard deviation for the number of replicates N (equation (5)). If both the reference and the stored test portions are analysed within the same analytical series, the repeatability should be used. If reference samples and stored samples are analysed within different analytical series, the within-laboratory reproducibility should be used.

$$u_{stored,\,reference} = \frac{s}{\sqrt{N}} \quad (5)$$

where
s – repeatability; within-laboratory reproducibility; estimate
N – number of replicate analyses

While the assessment described above yields helpful results for the comparison of two data sets, it does not consider different storage durations for the same storage condition.

7.3.2.2 t-test

When studying multiple timepoints for the same storage condition a convenient approach for assessing the analyte stability is to perform a *t*-test. To obtain the required parameters in principle a linear regression on the data is performed and it is checked whether the slope significantly differs from zero.

Note that this approach can only be applied to (pseudo-)linear functions of analyte degradation. In more detail:

Calculate the slope *b* of a linear regression over the data obtained for a single storage condition (equation (6)).

$$b=\frac{\sum(x_i-\overline{x})\,(y_i-\overline{y})}{\sum(x_i-\overline{x})^2} \quad (6)$$

where
b – slope
x_i – actual ith duration
$\overline{x}$ – mean duration
y_i – actual ith concentration
$\overline{y}$- mean concentration

Calculate the standard error of the slope s_b (equation (7)) and find the *t*-value by dividing the slope *b* by its standard error s_b (equation (8)).

$$s_b=\sqrt{\frac{1}{n-2}\cdot\frac{\sum(y_i-\hat{y}_i)^2}{\sum(x_i-\overline{x})^2}} \quad (7)$$

where
n- total sample size (number of studied time points for temperature to be assessed)
$\hat{y}_i$ – ith concentration predicted from linear regression curve

$$t=\frac{b}{s_b} \quad (8)$$

t – *t*-value of the linear regression slope

Finally, the *t*-value is compared to the critical *t*-value at the 0.05 significance level for n-2 degrees of freedom (equations (9), (10)). If the critical *t*-value is larger than the *t*-value derived from the experimental data, no statistically significant difference of the slope from zero is detected and the analyte concentration can be assumed to be stable for the investigated duration.

$t_{critical,\,0.05,\,n-2}>t$ No statistically significant difference of the slope from zero (9)
$t_{critical,\,0.05,\,n-2}\leq t$ Statistically significant difference of the slope from zero (10)

where
$t_{critical,\,0.05,\,n-2}$ – critical *t*-value at the 0.05 significance level for n-2 degrees of freedom

7.4 Validity of Results

Due to the unavoidable inter-sample variability, a situation can sometimes arise where the analyte concentration does not gradually decrease with a prolonged storage duration but rather seems to fluctuate. These unexpected results are however acceptable, if the criteria given in section 7.3.1 are met at every time point.

If the stability criteria are not met for a time point, a differentiated assessment is required. If the results obtained for a single storage temperature,duration A, do not correspond to the remaining results obtained for this storage condition, but the results of at least two of the following (longer) storage durations B and C are consistent, then the results for A can be considered an outlier. It is recommended to exert expert judgement and refer to outlier tests (e.g., Grubbs' test)) in order to confirm and further support this decision.

7.5 Trends and Extrapolation

In some cases, the stability criteria outlined in section 7.3.1 might be fulfilled, but a trend in the analyte concentration is noticeable. Such an occurrence does not need to be investigated any further, if the laboratory's maximum storage period is covered by the stability study. Still, it can be helpful to document the observed trend for future reference.

Since the stability studies designed in accordance with CIR 2021/808[1] are usually unfit to obtain a detailed understanding of the degradation kinetics, extrapolation of analyte stability is discouraged.

8 Stability Data from External Sources

It is perfectly acceptable that laboratories reduce their workload for stability studies by referring to data from external sources. Stability data can be obtained from literature, exchange with other laboratories or databases. The EURLs active in the field of the analysis of veterinary medicinal products provide a platform for the exchange of stability data for their laboratory network[11]. Any network laboratory may submit data for consideration using the data submission form (Annex 3 Stability Data Submission Form). The submission then undergoes the process described in the order of business (Annex 2 Stability Database Order of Business).

In case external data is used, it is necessary to consider the following:

(i) Expert opinion
To which extent data from external sources can be used as a reference is always a case-by-case decision and requires evaluation by an expert.

(ii) Applied methods
For studies conducted by other entities the applied analytical methods are usually not disclosed entirely. If the applied analytical method has been validated in accordance with CIR 2021/808[1] this usually means that quality control is emphasised and the data is probably reliable. For literature references it can be hard to assess the confidence in stability data. Careful study of the experimental section is advised.

[11] The database is currently in preparation.

For extract stability, results from external sources can be used for comparison, if they use an identical or nearly identical extraction approach (see section 8.1.1); otherwise, it is recommended that the laboratories should do their own extract stability.

(iii) Comparable storage conditions

CIR 2021/808[1] prescribes that data from external sources may only be assumed to hold in another laboratory, 'if identical conditions are applied'. As many influencing factors as possible should be consistent with the conditions in the laboratory interested in referencing data from external sources. For detailed considerations see section 8.1.

(iv) Additional studies in case of data gaps

If the data gathered from external sources does not adequately cover the analyte scope, concentrations, matrix etc. additional stability studies should be conducted.

These principles can be summarised by the acronym 'STABLE':

***S**tability studies = routine*

The preparation and measurement of samples for stability studies should be identical to what is required by the respective method description.

***T**ake a risk (-based decision)*

All decisions with regard to the applicability of data from external sources or the handling of insufficient stabilities should be risk-based and documented.

***A**vailable information*

Existing information can provide indications as to an analyte's stability and should be used as a reasonable starting point for planning additional studies more suited to a laboratory's requirements.

***B**are minimum = routine conditions*

The laboratories should analyse which aspects of stability are already covered by their validation experiments and supplement with additional studies where required. If for example an interruption of the sample clean-up procedure was included in the ruggedness study and was evaluated not to have a significant influence on the measurement results, the analytes can be assumed to be sufficiently stable during the interruption.

Also, laboratories should have stability information available at least for the routine conditions in accordance with their laboratory policy. For

example, this would mean that laboratories storing their solutions at-20 °C should at least have stability data for this condition available. Data for additional conditions improves the understanding of analyte susceptibility to degradation but is not mandatory.

***L**egitimate doubt calls for supplementary studies*

Whenever available information contradicts itself or the conditions which are relevant for the laboratory are not represented by available data, a new in-house study should be conducted.

***E**xert expert judgement*

For all considerations with regard to the transferability of existing data to the conditions present in their own laboratory, the analysts should use expert judgment.

If there is no prior knowledge of the stability of an analyte of interest in a specific solvent or matrix, all available information on the stability of closely related analytes and similar solvents and matrices should be considered so as to define suitable storage conditions and a maximum acceptable storage period. If there is no such information available, laboratory samples should usually be stored under freezing conditions, and they should be analysed as soon as the laboratory operations allow. These decisions should be taken by weighing risks and opportunities and should be adequately documented.

8.1 Comparable Conditions

In order to reduce the additional workload associated with stability studies, CIR 2021/808[1] allows to reference stability data published in literature or obtained from other laboratories. However, for the stability of analytes in solutions and extracts, this data may only be accepted if 'identical conditions' are applied. Different relevant aspects are discussed below regarding the degree of similarity which is deemed acceptable (see Flowchart on Figure 7 and the section below). In any case accepting external data as a valid reference for their own laboratory should always be a case-by-case decision handled by an expert. For choosing acceptable stability data from external sources, the responsible expert's priority should be to keep as many influencing factors as possible consistent with the conditions in their laboratory.

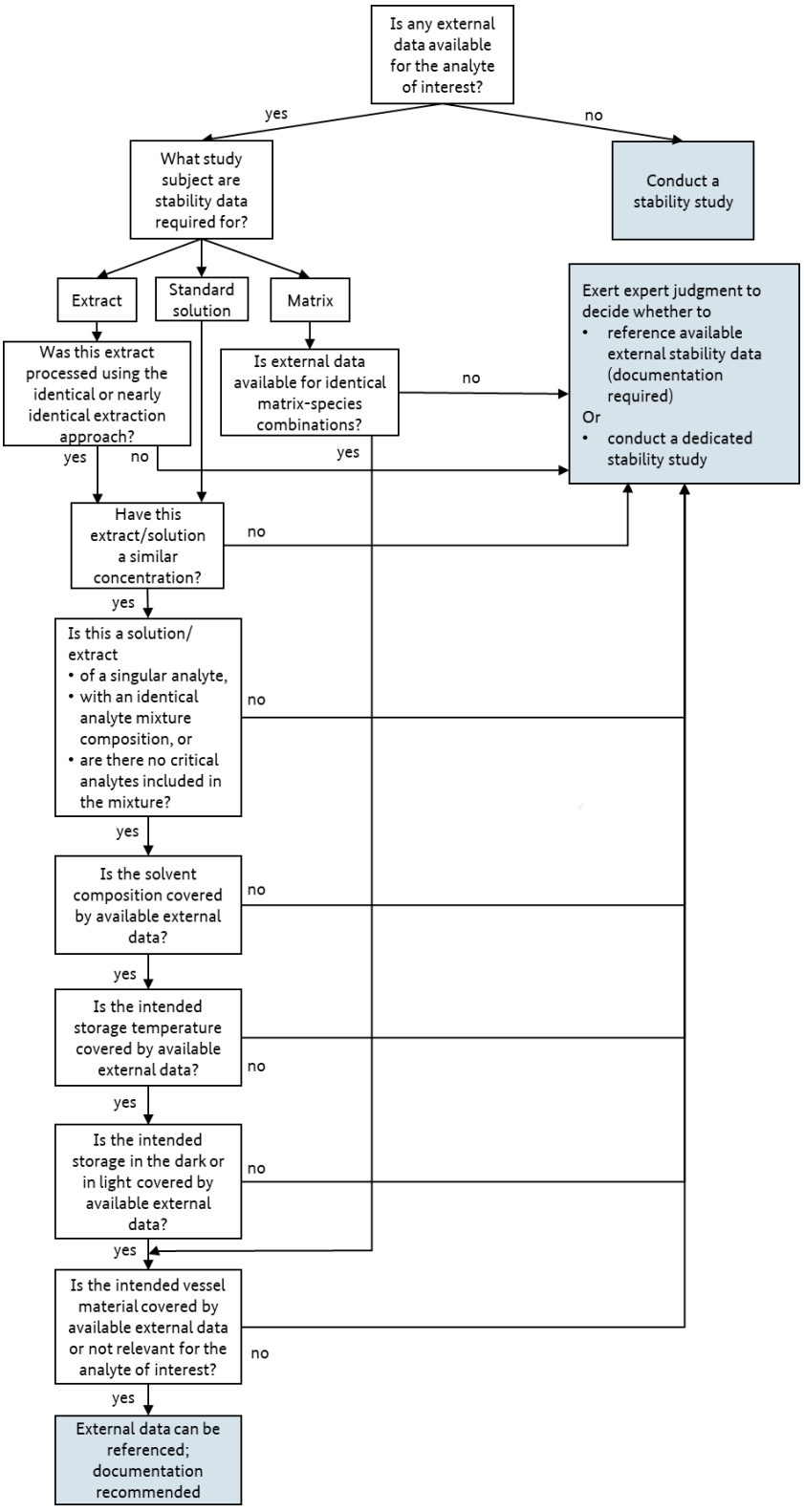

Figure 7: Flow chart highlighting aspects to be considered when comparing stability data from external sources with the conditions in another laboratory.

8.1.1 Extraction approach (for extracts)

A nearly identical extraction approach means that the extraction leads to an extract with essentially the same composition and stress factors for the analyte.

In practice, the following parameters are considered critical as the can change the composition during the extraction and of the final extract and therefore, should be identical (or extremely close):

- Composition of extraction and reconstitution solvents (see requirements for solvent composition below)
- Major clean-up steps (for instance QuEChERs vs SPE vs IAC) as it impacts the co-extractives and then the extract composition.
- Temperature during extraction steps (note: stability can be assumed for lower temperatures, see requirements for temperature below)

Note: the above list is not exhaustive. Other factors not listed here, should be considered if is known based on expert knowledge, that such factors may impact on the final composition of the extract.

The following parameters can be seen as non-critical if justified, e.g.:

- Sample mass
- Extraction Solvent(s) volume
- Filtration vs centrifugation
- Centrifugation speed
- Shaking conditions
- Durations of the extraction steps (within reasonable limits)

8.1.2 Temperature

Certainly, one of the most important conditions to be controlled with regard to analyte stability is temperature. When contemplating whether data obtained under slightly different temperature conditions might also hold for the conditions applied in their own laboratory, the analysts should remember that (i) analyte degradation is often a function of temperature, (ii) lower temperatures usually mean lower degradation rates, and (iii) phase changes are critical. This means that the melting points of the relevant solvents or matrix need to be considered. The temperature ranges given in Table 2 are usually considered to be equivalent. If stability is demonstrated for higher temperatures, then stability can also be assumed for lower temperatures. However, these considerations only hold in case the solvent or matrix does not undergo a phase change.

Table 2: Usually acceptable storage temperatures for different conditions.

Condition	Acceptable temperature range
Ultra-Freezing	-80 °C to -60 °C
Freezing	-25 °C to -10 °C
Refrigerated	2 °C to 8 °C
Room temperature	18 °C to 25 °C

8.1.3 Solvent Composition

Since solvents or additives could potentially engage in or catalyse reactions with the analytes of interest, the solvent composition of solutions, extraction solvents or final extracts is another critical aspect. When comparing stability in pure, non-polar, aprotic solvents the behaviour of the analyte of interest is assumed to be similar – the probability of these solvents engaging in a reaction with the analytes of interest without the presence of additives or catalysts is assumed to be limited.

For protic and polar solvents, it is the authors' experience that the analyte stability is very much dependent on the exact solvent composition. Therefore, a dedicated stability study is usually recommended, if no data is available. For solvent mixtures with the same components, but different ratios the following approach is proposed: When two or more sets of data for the analyte of interest in solvent mixtures with the same components, but different ratios are available and the outcome of the stability studies is comparable, it may be assumed that the analyte of interest is also stable for any of the ratios covered by these two or more data sets.

> Consider this hypothetical example: Metamizole is stable in a mixture of acetonitrile/water 1/1 v/v over the course of one month (study A). Metamizole is also found to be stable in a mixture of acetonitrile/water 1/8 v/v over the course of one month (study B). Metamizole can be assumed to be stable for at least one month in any mixture of acetonitrile/water with ratios between 1/1 and 1/8 v/v.

The presence or absence of additives can also be decisive for an analyte's stability in solution. Hence, it is usually not advisable to assume that the analyte stability for the same solvent composition but with different or no additives is identical. Similarly, a different pH for aqueous solution can be considered critical.

The composition of extracts is usually very specific to the applied analytical methods. The comparability of stability data from external sources is therefore very limited and should only be taken as an indication as to the stability for similar extract compositions. As a result, it is advised that laboratories validating their analytical methods in accordance with CIR 2021/808[1] re-inject several extracts obtained during the validation experiments and compare the results to those initially obtained.

8.1.4 Mix Composition

With regard to stability data from external sources it can be hard to obtain information on all analytes present in a mix solution. Mixes can be problematic as analytes may undergo reactions with one another (section 4.6.1). If stability information for a different mix composition but otherwise comparable conditions is available, one option is to assume stability also for the modified mix solution. If over time stability issues become apparent, the mix composition should be considered as a possible influence.

8.1.5 Matrix

If multiple data sets for the analyte of interest are available for the matrix-species combination of interest, the responsible expert should carefully evaluate the design of all studies. It is to be expected, that details of the studies differ, possibly leading to preference of one data set. If several data sets are comparable with regard to their design but still yield different results in terms of analyte stability, this information can be taken as an indication. In such a case it is advisable to plan an in-house stability study for the analyte of interest.

8.1.6 Analyte Concentration

The rate of an analyte's degradation can depend on its concentration. When transferring data from external sources the concentration of the analyte of interest is therefore a factor that begs consideration. First of all, it should be noted that there is no general rule that lower concentrations degrade with a faster rate than higher concentrations. However, when two data sets with sufficiently similar conditions but different concentrations are available and their result with regard to stability is the same, it can be assumed that the analyte also behaves in the same fashion for all concentrations covered by these two data sets.

> Hypothetical example: A dimetridazole solution of 1 ng/mL is stable for three months (data set A). Another study shows that a comparable dimetridazole solution of 100 ng/mL is also stable for at least three months. Hence, all similar solutions of dimetridazole in the range 1-100 ng/mL are considered stable for at least three months. The influence of transformation products on the degradation rate is assumed to be negligible.

A general recommendation for pharmacologically active substances and marker residues with frequent MRL/ML changes is to include more than a single concentration in a dedicated stability study, in order to be prepared for changes in the legislation. If data for the concentration of interest is not available from external sources, it is recommended to conduct a dedicated in-house stability study, rather than infer stability from data at a different concentration.

8.1.7 Light

When it comes to the presence and absence of light, it is to be noted that results obtained for samples stored in the dark cannot usually be transferred to samples stored under the influence of light. The

reason is that irradiation can favour other pathways than temperature-driven analyte degradation. The degree of light absorbance of the storage vessels is often unknown, and it is therefore not recommended to assume that the analyte behaviour when stored in the dark and in the light in amber vessels is equivalent. A risk-based approach should be adopted for deciding on appropriate light conditions, based on any prior knowledge of the analytes of interest, and with consideration to the practicalities of the laboratory environment.

8.1.8 Vessels

Another aspect that needs consideration is the vessel material. This is of heightened importance for any solution, extract, or liquid matrix. If analytes are known to be prone to surface adsorption, it might be necessary to use de-activated glassware/special polymers or to reverse adsorption by ultrasonication (if applicable). Storage conditions would only be considered equivalent, if the specific requirements with regard to the vessel material are fulfilled.

Annex 1 Examples – Real life examples

Example for a Chronological Stability Study

Study Design

Study Object	Intermediate working solution of avermectins
Analytes to be Monitored	Abamectin, doramectin, emamectin, eprinomectin, ivermectin, moxidectin
Study Type	Chronological study
Storage Conditions	-20 °C Note: only one storage temperature is selected for practical reasons, as it best represents the laboratory procedure. As noted in Section 4.2, this is deemed acceptable, as laboratories must, at a minimum, have access to stability data for the conditions under which the solutions are usually stored
Storage Periods	**Condition / Duration** | **-20 °C** **T0 = 0 d** | x **T1 = 71 d** | x **T2 = 131 d** | x
Concentrations	1.5MRL
Selection of Samples	Intermediate working solution of the mix of analytes of interest in methanol
Number of test portions	1 flask containing the solution, 6 replicate injections per storage condition
Vessels	Polypropylene flask with polypropylene caps
Analytical Method	Method for detecting and quantifying avermectin and moxidectin residues in fish, milk and liver by HPLC-FLD

Implementation

- Prepare the working solution to be studied at the target concentration(s).
- Store it under the storage condition to be investigated.
- At each time point, analyse of six replicate injections of this stored solution, as well as six (min 5) replicate injections of a freshly prepared solution.

Data Evaluation

- Evaluate the results by
  - calculating the means of the responses obtained for the stored and fresh solutions.
  - calculating the "analyte remaining" of the sample stored under the conditions to be studied by comparing its responses with the responses of the analyte in the freshly prepared solution.

$$Analyte\ Remaining\ (\%) = \frac{Ri}{Rfresh}\ x\ 100$$

With: Ri: Mean response at time point i
Rfresh: Mean response for the fresh solution

  - assessing whether the criterion for the maximum acceptable difference of 15% according to CIR 2021/808 is met for the investigated storage condition [1]

Table 3: Observed difference of the stored solution relative to the results obtained for the freshly prepared solutions. All results fulfilled the confirmation criteria.

Analyte / Duration	Abamectin	Doramectin	Emamectin	Eprinomectin	Ivermec-tin	Moxidectin	Difference (-20 °C) ≤15%?
71 d	+2.6 %	+3.1 %	+3.3 %	+1.6 %	+2.4 %	+5.5 %	Yes for all
131 d	-0.7 %	-1.1 %	-3.12 %	-0.67 %	-6.46 %	-2.36 %	Yes for all

Conclusion

All six avermectins are stable in methanol for at least 131 days at -20 °C.

Example for an Isochronous Stability Study

Study Design

<table>
<tr><td>Study Object</td><td>NSAIDs in lyophilised raw milk</td></tr>
<tr><td>Analytes to be Monitored</td><td>Phenylbutazone</td></tr>
<tr><td>Study Type</td><td>Isochronous study</td></tr>
<tr><td>Storage Conditions</td><td>-20 °C, +4 °C, room temperature (dark); reference: -80 °C</td></tr>
<tr><td>Storage Periods</td><td>
<table>
<tr><th>Condition / Duration</th><th>-80 °C</th><th>-20 °C</th><th>+4 °C</th><th>RT</th></tr>
<tr><td>T0 = 3 d</td><td></td><td>x</td><td>x</td><td>x</td></tr>
<tr><td>T1 = 7 d</td><td></td><td>x</td><td>x</td><td>x</td></tr>
<tr><td>T2 = 28 d</td><td></td><td>x</td><td>x</td><td>x</td></tr>
<tr><td>T3 = 3 m</td><td>x</td><td>x</td><td></td><td></td></tr>
</table>
</td></tr>
<tr><td>Concentrations</td><td>Phenylbutazone is a prohibited compound; MMPR=5 µg/kg</td></tr>
<tr><td>Selection of Samples</td><td>Lyophilised raw milk with incurred residues of phenylbutazone. The samples are prepared as a batch prior to the start of the stability study. Sample homogeneity is assessed in a separate study.</td></tr>
<tr><td>Number of test portions</td><td>three aliquot per combination of storage duration and storage condition; total=3x11=33 aliquots</td></tr>
<tr><td>Vessels</td><td>Polypropylene tubes with polypropylene caps</td></tr>
<tr><td>Analytical Method</td><td>Dedicated quantitative confirmatory method for the determination of phenylbutazone; within-laboratory reproducibility at MMPR s_{wR}(MMPR)=6.8 %</td></tr>
</table>

Implementation

- Prepare the sample material as a batch. Weigh a number of aliquots sufficient for the study design (here: 33 aliquots).
- Subject the samples to the storage conditions to be investigated. After the intended storage duration, re-distribute the stored samples to the reference condition (-80 °C) (compare Figure 3). Alternatively, initially store under the reference condition and subsequently distribute the samples to the conditions to be investigated (compare Figure 4).

- Analyse all samples and the usual set of quality control samples together in a single analytical series. Since an incurred sample is used, it is not possible to analyse an additional quality control sample for increasing the confidence in the results of the samples stored under reference conditions (section 6.3).

Data Evaluation

- Evaluate the results by
    - calculating the mean for every temperature-time combination
    - calculating the deviation of the results obtained for the samples stored under the conditions to be investigated from the results obtained for the samples stored under reference conditions
    - assessing whether the criterion for the maximum acceptable deviation is met for the investigated storage conditions

    and

    - assessing whether the analyte of interest can be confirmed at the investigated temperature-time combinations (Table)

Table 4: Deviation of the mean results relative to the results obtained for the samples stored under the reference conditions. All results fulfilled the confirmation criteria.

Condition / Duration	-20 °C	Deviation (-20 °C) ≤swR?	+4 °C	Deviation (+4 °C) ≤swR?	RT	Deviation (RT) ≤swR?
3 d	-2.5 %	Yes	-0.8 %	Yes	-12.4 %	No
7 d	-6.5 %	Yes	-5.4 %	Yes	-12.5 %	No
28 d	-4.8 %	Yes	-11.5 %	No	-13.4 %	No
3 m	-7.5 %	No	n. a.	n. a.	n.a.	n.a.

Conclusion

Phenylbutazone is stable in lyophilised bovine milk for 28 days at -20 °C and 7 days at +4 °C. The storage of lyophilised bovine milk containing phenylbutazone at room temperature is not suitable.

Example for a Different Stability Study Approach – Extract Equivalency

The purpose of this Extract Equivalency Study is not to determine the stability of analyte in extract, rather to determine the duration for which extracts can be stored under specified conditions, without impacting on the analytical result measured. This study aims to demonstrate that the concentration measured in a sample at timepoint x, is equivalent to the concentration measured a timepoint 0. This approach assumes use of internal standards as part of quantification, and also storage of calibration extracts under the same conditions as the test samples being measured. The approach outlined below, also captures any potential freeze-thaw effects of removing and returning samples to the storage condition.

Study Design

<table>
<tr><td>Study Object</td><td>A1c Steroids in milk (pasteurised, raw, lyophilized bovine milk)</td></tr>
<tr><td>Analytes to be Monitored</td><td>Ethinylestradiol</td></tr>
<tr><td>Study Type</td><td>Extract Stability – Equivalency Study</td></tr>
<tr><td>Storage Conditions</td><td>-80 °C</td></tr>
<tr><td>Storage Periods</td><td>
<table>
<tr><th>Condition / Duration</th><th>-80 °C</th><th>RT</th></tr>
<tr><td>T0 = 0 d</td><td></td><td>x</td></tr>
<tr><td>T1 = 3 d</td><td>x</td><td></td></tr>
<tr><td>T2 = 7 d</td><td>x</td><td></td></tr>
<tr><td>T3 = 14 d</td><td>x</td><td></td></tr>
<tr><td>T4 = 21 d</td><td>x</td><td></td></tr>
</table>
</td></tr>
<tr><td>Concentrations</td><td>Ethinylestradiol is a prohibited compound; No MMPR established for Milk. LCL of 0.5 ng/mL selected based on method sensitivity</td></tr>
<tr><td>Selection of Samples</td><td>Fortified Bovine milk, comprising of raw milk, pasteurised milk and lyophilized milk powder (reconstituted with water). Samples are those as extracted for within-laboratory reproducibility run 1</td></tr>
<tr><td>Number of test portions</td><td>6 different bovine milk samples fortified at 3 different concentrations (1.0, 2.0 and 3.0 LCL). Samples at each concentration level comprising of n = 2 raw bovine milks, n = 2 pasteurised bovine milk and n=2 bovine milk powder. Provides data for n=6 test portions at each time point, at three concentration levels</td></tr>
</table>

Vessels	Polypropylene HPLC vials with polypropylene caps
Analytical Method	Dedicated quantitative confirmatory method for the determination of ethinylestradiol

Implementation

- Samples are prepared in accordance with your design for assessing within-laboratory reproducibility, which requires a minimum of n=6 samples at three concentration levels, quantified against a calibration cure with a minimum of 5 points.
- Select one of the three within-laboratory reproducibility experiments and analyse as normal. The results obtained from this experiment are considered to be the reference timepoint, T0.
- Immediately after analysis is complete, store your extracts (for validation test portions and calibration curve) at the condition to be assessed (-80 °C, in the case of this example).
- After 3 days have passed, remove the extracts from the freezer storage, and repeat the analysis of the test portions and calibration. The results obtain correspond to T1 = 3days. Return the extracts to the -80 °C freezer immediately after analysis.
- After a further 4 days, remove the extracts from the freezer storage, and repeat the analysis of the test portions and calibration. The results obtain correspond to T2 = 7days. Return the extracts to the -80 °C freezer immediately after analysis.
- After a further 7 days, remove the extracts from the freezer storage, and repeat the analysis of the test portions and calibration. The results obtain correspond to T3 = 14days. Return the extracts to the -80 °C freezer immediately after analysis.
- After a further 7 days, remove the extracts from the freezer storage, and repeat the analysis of the test portions and calibration. The results obtain correspond to T4 = 21days. Return the extracts to the -80 °C freezer immediately after analysis).

Data Evaluation

- Evaluate the results at each concentration level by
    - calculating the mean concentration of the n=6 test portion for each time point
    - calculating the deviation of the results obtained for the samples stored under the conditions to be investigated relative to the results obtained for the samples stored under reference conditions (i.e., T0)
    - assessing whether the criterion for the maximum acceptable deviation is met for the investigated storage conditions

  and

- assessing whether the analyte of interest can be confirmed at the investigated temperature-time combinations (2)

Table 5: Deviation of the mean results (based on n=6 test portions) at each time point, relative to the results obtained for the samples stored under the reference conditions i.e. T0. All results fulfilled the confirmation criteria. Results presented for three concentration levels equivalent to 1.0, 2.0 and 3.0 LCL

Dura-tion	**Extracts fortified @ 1.0 LCL stored at -80 °C**		**Extracts fortified @ 2.0 LCL stored at -80 °C**		**Extracts fortified @ 3.0 LCL, stored at -80 °C**	
	% Diffe-rence (relative to T0)	**Deviation ≤15%?**	**% Difference (relative to T0)**	**Deviation ≤15%?**	**% Difference (relative to T0)**	**Deviation ≤15%?**
3 d	-1.2 %	Yes	-0.1 %	Yes	0.2%	Yes
7 d	-0.7 %	Yes	0.6 %	Yes	0.7 %	Yes
14 d	-0.6 %	Yes	1.0 %	Yes	0.0 %	Yes
21 d	-0.6 %	Yes	1.6 %	Yes	-0.8 %	Yes

Conclusion

At all three concentration levels, the results show that the measured concentration of ethinylestradiol in the final extract after storage for 21 days at -80 °C, are equivalent to the results obtained when analysed on day 0. Based on this, the study shows that sample extracts can be stored for at least 21 days at -80 °C before determination, without impacting on the analytical result.

Example for an Alternative Isochronous Matrix Stability Study

In this example, an alternative approach to an isochronous matrix stability study is presented. An alternative analysis approach for the stability study will be adopted, compared to that suggested in 2021/808. A large quantity of blank matrix is prepared, aliquoted and stored under the required conditions. At each time point, n=6 aliquots will be removed from storage and fortified with analyte at a suitable concentration, and the samples are returned to the freezer/fridge for the required duration. At the end of the storage period, the final n=6 aliquots will be fortified with analyte (representing time point T=0) and all time points will be retrospectively analysed on day T=0in the same analytical batch. This approach avoids the uncertainty associated with instrumental variation, which can be problematic when samples are analysed on different days with different instrumental conditions. The sample stability will be determined based on the percentage difference between the response of the n=6 replicates of T0 and each of the stored time points.

Note: the below approach does not require the use of a calibration curve for determination of concentration, rather incorporates the use of internal standard, which allows the use of response ratio for assessing the stability between time points. This relies on adding the internal standards at T0 just prior to sample analysis.

Study Design

Study Object	A1c Steroids in milk (pasteurised, raw, lyophilized bovine milk)
Analytes to be Monitored	17-β-Estradiol, 17-α-Estradiol, Ethinylestradiol
Study Type	Matrix Stability (short term) The study duration was selected with consideration to the agreed turn-around time for reporting of results to the client.
Storage Conditions	-20 °C Note: only one storage temperature is selected for practical reasons, as it best represents the laboratory procedure. As noted in Section 4.2, this is deemed acceptable, as laboratories must, at a minimum, have access to stability data for the conditions under which the solutions are usually stored”

<table>
<tr><td>Storage Periods</td><td>
<table>
<tr><th>Condition / Duration</th><th>-20 °C</th><th>RT</th></tr>
<tr><td>T0 = 0 d</td><td></td><td>x</td></tr>
<tr><td>T1 = 7 d</td><td>x</td><td></td></tr>
<tr><td>T2 = 28 d</td><td>x</td><td></td></tr>
<tr><td>T3 = 42 d</td><td>x</td><td></td></tr>
<tr><td>T4 = 56 d</td><td>x</td><td></td></tr>
</table>
</td></tr>
<tr><td>Concentrations</td><td>17-β-Estradiol, 17-α-Estradiol and Ethinylestradiol are all considered as prohibited substance in milk; No MMPR established for Milk. LCL of 0.5 ng/mL selected based on method sensitivity. Matrix stability carried out at a concentration equivalent to 3.0 LCL i.e., 1.5 ng/ml.</td></tr>
<tr><td>Selection of Samples</td><td>Fortified Bovine milk, comprising of raw milk, pasteurised milk and lyophilized milk powder (reconstituted with water).

No incurred material available</td></tr>
<tr><td>Number of test portions</td><td>6 aliquots of bovine milk samples, at each time point.

At each of the five time points, the 6 aliquots comprise of n=2 raw bovine milks, n=2 pasteurised bovine milk and n=2 bovine milk powder, at each time point

Note, the samples used at each time point are the same i.e., same two raw bovine milks for T0, T1, T2 etc.

In this example, each of the 6 different bovine milk samples are different from each other, in order to better capture any effect between samples. The study may be carried out using 6 aliquots of the same sample, for each time point</td></tr>
<tr><td>Vessels</td><td>Polypropylene centrifuge tubes with polypropylene caps</td></tr>
<tr><td>Analytical Method</td><td>Dedicated quantitative confirmatory method for the determination of 17-β-Estradiol, 17-α-Estradiol, Ethinylestradiol in milk.</td></tr>
</table>

Implementation

- Aliquot n=5 aliquots of each of the six different samples
- Divide the 30 aliquots into five sets, with each set containing n=1 aliquot of the six blank matrix samples. Each of the five sets of samples will be used for each stability time point.
- Label each of the five sets of samples according to the relevant time point i.e., T0 (0 days), T1 (7 days), T2 (28 days), T3 (42 days) and T4 (56 days).
- Fortify the n=6 samples designated as T4 (56 days) with the analytes of interest at the required concentration. Note: do not add internal standard at this stage. These samples will correspond to time point T4 (56 days). Vortex the sample for 1 min. and leave the samples for approx. 30 min to allow the solvent to evaporate.
- Store the T4 fortified samples and the four sets of unfortified samples in the freezer at ≤ -20°C.
- After 14 days, remove the next set of n=6 samples assigned as time point T3 (42 days). Allow to thaw and fortify the n 6 samples with the analytes of interest at the required concentration. Note: do not add internal standard at this stage. Once fortified, vortex for 1 min. and leave the samples for approx. 30 min to allow the solvent to evaporate. Return the samples to the freezer to store at ≤ -20°C.
- After a further 14 days, remove the next set of samples assigned as time point T2 (28 days). Allow to thaw and fortify the n=6 samples with the analytes of interest at the required concentration. Note: do not add internal standard at this stage. Once fortified, vortex for 1 min. and leave the samples for approx. 30 min to allow the solvent to evaporate. Return the samples to the freezer to store at ≤ -20°C.
- After a further 21 days, remove the next set of samples assigned as time point T1 (7 days). Allow to thaw and fortify the n=6 samples with the analytes of interest at the required concentration. Note: do not add internal standard at this stage. Once fortified, vortex for 1 min. and leave the samples for approx. 30 min to allow the solvent to evaporate. Return the samples to the freezer to store at ≤ -20°C.
- Finally, after a further 7 days, remove all stability samples. Allow the samples to thaw by leaving to stand at room temperature.
- Fortify the remaining set of n=6 samples assigned as time point T0 (0 days), with the analytes of interest at the required concentration. Once fortified, vortex for 1 min. and leave the samples for approx. 30 min to allow the solvent to evaporate.
- Fortify all 30 stability samples with internal standard solution and extract the sample in accordance with the relevant procedure.

Data Evaluation

- Evaluate the results at each concentration level:
  - calculate the mean response ratio (area of analyte divided by area of internal standard) of the n=6 test portion for each time point T0, T1, T2, T3 and T4.
  - calculate the percentage difference of the n=6 results obtained for the samples stored under the conditions to be investigated, relative to the mean of the n=6 results obtained for the samples stored under reference conditions (i.e., T0)
  - assess whether the criterion for the maximum acceptable deviation is met for the investigated storage conditions. For matrix stability, in order to be considered stable, the deviation at a given time point of the n=6 replicates, must be within ± within-laboratory reproducibility (swR) percentage.

  and

  - assess whether the analyte of interest can be confirmed at the investigated temperature-time combinations (Table 2)

Table 6: Summary of matrix stability for each analyte in milk matrix, based on the relative percentage difference between the mean response factor of the n=6 samples at each of the given storage times (7, 28, 42 and 56 days) relative to T0= 0d.

Analyte	Percentage Relative Difference (relative to T0) (%)					Within-laboratory reproducibility, swR (%)
	T0 0 days	T1 7 days	T2 28 days	T3 42 days	T5 56 days	
Ethinylestradiol	0.0	-1.7	-2.1	-1.7	-2.7	± 5.7
Alpha-Estradiol	0.0	-3.1	-1.7	-2.9	-3.4	± 5.5
Beta-Estradiol	0.0	-1.7	-1.1	-2.4	-3.8	± 4.2

Conclusion

The relative percentage difference between the mean response factor of each analyte, at each storage time point, relative to T0 (0 days storage), is less than the within laboratory reproducibility for each respective analyte. It has been demonstrated that all analytes are stable in bovine milk matrix for at least 56 days storage at ≤ -20°C.

Annex 2 Stability Database Order of Business

in preparation

Annex 3 Stability Data Submission Form

in preparation ASAP

References

[1] *Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC. Commission Implementing Regulation (EU) 2021/808*, **2021**.

[2] V. Gold, A. McNaught, *The IUPAC Compendium of Chemical Terminology*, International Union of Pure and Applied Chemistry (IUPAC), Research Triangle Park, NC, **2025**.

[3] International Organization for Standardization, *Reference materials. Selected terms and definitions*.

[4] A. Lamberty, H. Schimmel, J. Pauwels, *Fresenius J Anal Chem* **1998**, *360*, 359.

[5] *Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Commission Regulation (EU) No 37/2010*, **2009**.

[6] International Organization for Standardization, *Reference materials - Approaches for characterization and assessment of homogeneity and stability*.

[7] EURL, *EURL guidance document on the quality control during routine analysis (ongoing method performance verification). Version 1.2*, **2023**.