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EURL Guidance document on the extension of quantitative confirmation methods

The contents of this document act as a guidance on how to implement the requirements of Commission Implementing Regulation 2021/808.

Contents

Version 2.0 – Table 2 updated for experimental design

1. Introduction

1.1. Scope

This guidance is the EURL interpretation of Commission Implementing Regulation 2021/808 which shall support laboratories in the practical implementation of the regulation's requirements. Laboratories operating under Commission Implementing Regulation 2021/808 are not obliged to follow this guidance minutely; different approaches are acceptable, if they offer a comparable level of validation data quality. This guidance applies to physico-chemical analytical methods (based on e.g. LC/MSMS, GC/MS, LC-DAD or LC-FLU detection) designed for confirmatory purposes, and may be used (with adaptations) for screening methods, as well. However, deviations from the general approach presented in this document may be necessary in order to accommodate for the specific requirements of certain detection modes.

1.2 Extension of method

Sometimes it becomes necessary to extend the scope of a previously comprehensively validated method (confirmatory or screening method) due to changes in legislation or due to the availability of new information. In these cases, an extension of the scope should be accomplished in an efficient and analytically sound way, ideally using a reduced number of samples compared to a full validation.

Some examples for reasons to extend the scope are:

1. Different matrix
   e. g. a change from bovine milk to sheep milk, change from porcine liver to bovine muscle
2. Additional analyte(s)
3. Extended concentration range
   e. g. if the RPA/MRL/ML/MMPR is changed
4. Changes in the method
   e. g. small change in extraction/purification (SPE cartridges of another supplier), a change of LC or GC column from one supplier to another, a new or another internal standard or a change of LC or GC system from one supplier to another.

Analytical methods which are fully validated either using the conventional or the alternative validation approach can often be extended by the analysis of a reduced amount of samples. Chapter 4 of Commission Implementing Regulation 2021/808 describes the requirements for the extension of analytical methods for the determination of pharmacologically active substances in food of animal origin and gives advice on the extension to new matrices, species, substances, and concentrations. This guidance strives to provide more detailed information for official control laboratories for the extension of analytical methods depending on the selected initial validation approach. It applies to analytical methods designed for screening, as well as for confirmatory purposes. For the purpose of this guidance document

the definitions given in Commission Implementing Regulation 2021/808 supplemented by the definitions given in the EURL Guidance document on confirmation method validation apply.

2. General considerations on the extension of methods

If a method is changed or extended the application of a reduced validation scheme is in general possible. Nevertheless, the type and number of modifications to be validated in a single reduced validation scheme should always be based on expert knowledge and previous experiences, e. g. a change in detection technique for example from HPLC-UV to LC-MS/MS would require a complete validation in any case.

The results of a successfully conducted reduced validation scheme can be accepted as a contribution to proving the fitness for purpose of the method. The modified method may henceforth be applied. Anyhow, to assure the validity of this assumption the method performance shall be monitored continuously and compared to the initially obtained validation parameters. Ideally, this ongoing method performance control is designed in a way that the missing data for a complete validation can be collected over time (i. e. a few data points in each analytical series). For guidance on ongoing method performance verification, refer to the respective guidance document. Also guidelines of the responsible accreditation body should be taken into account.

2.1. Analytes

Generally, the method extension to additional compounds is only possible for analytes which can be included in the analytical method without divergence from the method description. If these requirements are fulfilled, the application of a reduced validation scheme followed by the monitoring of the method performance through ongoing quality control is sufficient.

2.2. Concentration range

Due to changed requirements (e.g. the setting or revision of MRLs, MLs, MMPRs and RPAs) it may become necessary to adapt the concentration range for which a method is validated. For such a case, the application of a reduced validation scheme is acceptable. Again the monitoring of the method performance through ongoing quality control is required (see also general considerations given under 2).

2.3. Species and matrices

Generally, the method extension to additional species and/or matrices is only possible if they can be included without divergence from the method description. The inclusion of new matrices and/or species in an already validated analytical method should always be a case-by-case decision based on the knowledge and experiences gained so far with the method and preliminary experiments assessing potential matrix effects and interferences.

In cases where MRLs for a compound differ for certain matrices it is most likely difficult to adapt the method scope to the additional requirements since in this case two modifications (concentration range and matrix/species) have to be considered. If previous experiments or experience prove there are no difficulties regarding these modifications, a reduced validation can be applied. In case of difficulties, a full validation is recommended.

2.4. Changes to the method

For all parameters which differ majorly from the details specified in the original method description and were not included in the initial validation study the necessity of a complete revalidation needs to be assessed by the expert. Whether a modification needs to be considered “major” or “minor” should be based on the extent of the change as well as an evaluation by an expert.

Changes which are presumed minor variations (e. g. different manufacturers of SPE cartridges, different instruments, changes in sample intake) can be tested in analytical series where the original and the modified method are applied on identical samples in parallel and directly compared or compared to the initial validation parameters. A full revalidation would only be necessary in cases where the modified method does not fulfil the requirements for approval of the series anymore.

3. Reduced validation using conventional approach

3.1. Experimental design

The fortification levels for a reduced validation are given in Table 1. In Table 2 the practical implementation of the extension of quantitative confirmatory methods using a reduced validation according to a conventional approach is described. Six batches of one matrix material are needed (A-F, for example, six different bovine muscle samples).

Table 1: Required fortification levels in a conventional reduced validation study.

Residue	*Level 1*	*Level 2*	*Level 3*
Unauthorised with RPA[1]	0.5[2]·RPA	1.0·RPA	1.5·RPA
Unauthorised	1.0·LCL	2.0·LCL	3.0·LCL
Authorised	0.1[3]·MRL/ML	1.0·MRL/ML	1.5·MRL/ML

[1] Analytes for which an MMPR has been established can be validated analogously to analytes for which an RPA has been established.
[2] Where 0.5 RPA is not reasonably achievable, this level can be replaced by the lowest reasonably achievable concentration between 0.5 and 1.0 RPA.
[3] Where 0.1 MRL/ML is not reasonably achievable, this level can be replaced by the lowest reasonably achievable concentration between 0.1 and 0.5 MRL/ML.

Table 2: Experimental design for reduced validation, levels as in Table 1.

Sample	Purpose	Batch	Level		
			Unauthorised ISO 11843	Unauthorised	Authorised
1	Calibration (matrix or standard solution)	A	0	0	0
2	Calibration (matrix or standard solution)	A	0+[1]	0+[1]	0+
3	Calibration (matrix or standard solution)	A	0++	0++	0++
4	Calibration (matrix or standard solution)	A	0+++	0+++	0+++
5	Calibration (matrix or standard solution)	A	0++++	0++++	0++++
6	Specificity	A	0	0	0
7	Specificity	B	0	0	0
8	Specificity	C	0	0	0
9	Specificity	D	0	0	0
10	Specificity	E	0	0	0
11	Specificity	F	0	0	0
12	CCα	A	Level 1	-	-
13	CCα	B	Level 1	-	-
14	CCα	C	Level 1	-	-
15	Trueness, repeatability at 0.1*MRL/ML	A	-	-	Level 1
16	Trueness, repeatability at 0.1*MRL/ML	B	-	-	Level 1
17	Trueness, repeatability at 0.1*MRL/ML	C	-	-	Level 1
18	Trueness, repeatability at 0.1*MRL/ML	D	-	-	Level 1
19	Trueness, repeatability at 0.1*MRL/ML	E	-	-	Level 1
20	Trueness, repeatability at 0.1*MRL/ML	F	-	-	Level 1
21	Trueness, repeatability, confirmation, CCα	A	Level 2	Level 1	Level 2
22	Trueness, repeatability, confirmation, CCα	B	Level 2	Level 1	Level 2
23	Trueness, repeatability, confirmation, CCα	C	Level 2	Level 1	Level 2
24	Trueness, repeatability, confirmation, CCα	D	Level 2	Level 1	Level 2
25	Trueness, repeatability, confirmation, CCα	E	Level 2	Level 1	Level 2
26	Trueness, repeatability, confirmation, CCα	F	Level 2	Level 1	Level 2
27	CCα	A	Level 3	-	-
28	CCα	B	Level 3	-	-
29	CCα	C	Level 3	-	-

[1] More '+' means higher fortification level

3.2. Criteria and calculation

After analysis and data processing the performance characteristics can be determined using statistical software tools. Criteria are related to Annex I of Commission Implementing Regulation 2021/808 (Table 3).

Table 3: Performance characteristics and the criteria to be applied in accordance with CIR 2021/808.

Performance characteristic	**Criteria (CIR/2021/808)**	**Remarks**
Identification	See §1.2.4.2, Tables 3 and 4	
CCα	See §2.6, 1.2.1	-Authorized: higher than but as close as possible to the MRL/ML -Unauthorized: below RPA
Trueness	See §1.2.2.1, Table 1	
Repeatability	See §1.2.2.1, below Table 2	
Within-laboratory reproducibility	See §1.2.2.1, Table 2	Multiply repeatability with factor 1.5 to get within-laboratory reproducibility (see 1.2.2.2 under Table 2 in CIR/2021/808)

Specificity	See §2.3, point 3	
Calibration (additional)	See §2.8	Acceptable R^2 should be described

If presumed necessary, absolute recovery, matrix effect, stability and ruggedness can be determined for additional analytes/matrices.

4. Alternative validation approach

The alternative validation approach offers the advantage of enabling the analyst to accept or reject a method change based on statistical data obtained from directly relating the initial validation data with validation data obtained by implementing the modifications. For method alterations that do not result in significantly different results, i. e. which do not show significant factor effects, the changes or extensions are acceptable. Usually, factor effects up to 10 % are regarded as tolerable. However, expert knowledge also needs to be taken into account for this evaluation and the requirements outlined in Commission Implementing Regulation 2021/808 need to be met.

If a change is not acceptable, a full method validation for the modification is required. This holds for screening, as well as for confirmation methods validated using the alternative approach. However, if the method validation is not successful, the modified method might still be suitable as a qualitative method (i. e. a confirmation method, which does not fulfil the criteria of a quantitative method). A general approach for the extension of methods which have initially been validated using the alternative validation approach is to repeat a minimum of four experimental runs[4] of the original experimental plan applying the modified method. The runs to be replaced shall be chosen in such a way as to adequately represent all of the influencing factors considered in the initial study. An example is given in section 4.1. The general requirements as regards e. g. the number of concentration levels to be included in the validation study are identical to those for a full validation and are described in the *EURL Guidance document on confirmation method validation*. For the method extension study, a minimum of five different batches (one batch for each of the four runs plus one identical batch for all of the four required matrix calibrations) is required, although it is recommended to include the maximum of eight different batches (one batch for each of the four runs plus one batch for each of the four required matrix calibrations). It is considered favourable to perform no more than two of the four experimental runs required for the method extension within one week.

After conduction of the four experimental runs, the validation data are calculated by combining the four repeated runs with the complimentary runs from the initial validation data. The parameters thus obtained for the method extension study are evaluated for any statistically significant and relevant factor effects and are checked for compliance with the requirements of Commission Implementing Regulation (EU) 2021/808. As stated in section 2 the remaining four validation runs using the modified method conditions

[4] irrespective of the number of experimental runs in the initial validation

should be repeated e. g. as a part of the ongoing method performance verification process. The following sections point out specific requirements of certain method extensions and give examples.

4.1. Selecting experimental runs for a method extension study

In Table 4 an example of an experimental plan for a validation study for the determination of nitroimidazoles in plasma/serum and milk is given. The statistical evaluation did not imply a significant and relevant difference between any of the factors included in the validation study. In order to extend the method to the matrix muscle, the experimental runs no. 1-4 are repeated using muscle samples and the same factor-level combinations as in the initial validation study (Table 5). Please note how that results in all the other factor-level combinations appearing twice. It is not adequate to choose runs where the factor levels are unequally distributed: For the given example, it would not be acceptable to repeat e. g. the runs 01, 02, 07, 08 as only a sample amount of 2 g would be considered for the method extension study (Table 6).

Table 4: Experimental plan for a method validation study for the determination of nitroimidazoles in plasma/serum and milk.

Run	*Matrix*	*Operator*	*Amount of matrix*	*Storage of extract*	*Filtration*	*Final volume*
Run 01	milk	unfamiliar	2 g	2-3 days of storage at +4 °C	no	250 µL
Run 02	milk	familiar	2 g	immediate analysis	yes	150 µL
Run 03	milk	unfamiliar	1 g	2-3 days of storage at +4 °C	yes	150 µL
Run 04	milk	familiar	1 g	immediate analysis	no	250 µL
Run 05	plasma/serum	unfamiliar	1 g	immediate analysis	no	150 µL
Run 06	plasma/serum	familiar	1 g	2-3 days of storage at +4 °C	yes	250 µL
Run 07	plasma/serum	unfamiliar	2 g	immediate analysis	yes	250 µL
Run 08	plasma/serum	familiar	2 g	2-3 days of storage at +4 °C	no	150 µL

Table 5: Adequate experimental plan for a method extension validation study for the inclusion of an additional matrix (determination of nitroimidazoles in muscle).

Run	*Matrix*	*Operator*	*Amount of matrix*	*Storage of extract*	*Filtration*	*Final volume*
Run 01_2	muscle	unfamiliar	2 g	2-3 days of storage at +4 °C	no	250 µL
Run 02_2	muscle	familiar	2 g	immediate analysis	yes	150 µL
Run 03_2	muscle	unfamiliar	1 g	2-3 days of storage at +4 °C	yes	150 µL
Run 04_2	muscle	familiar	1 g	immediate analysis	no	250 µL

Table 6: Inadequate experimental plan for a method extension validation study the inclusion of an additional matrix (determination of nitroimidazoles in muscle).

Run	*Matrix*	*Operator*	*Amount of matrix*	*Storage of extract*	*Filtration*	*Final volume*
Run 01_2	muscle	unfamiliar	2 g	2-3 days of storage at +4 °C	no	250 µL
Run 02_2	muscle	familiar	2 g	immediate analysis	yes	150 µL
Run 07_2	muscle	unfamiliar	2 g	immediate analysis	yes	250 µL
Run 08_2	muscle	familiar	2 g	2-3 days of storage at +4 °C	no	150 µL

If additional analytes should be included in an existing analytical method, all factor levels should be investigated twice. An example of a selection of adequate experimental runs based on the original validation given in Table 4 for such a case is given in Table 7.

Table 7: Example of an experimental plan for a method extension validation study for the inclusion of additional analytes (determination of nitroimidazoles in muscle).

Run	*Matrix*	*Operator*	*Amount of matrix*	*Storage of extract*	*Filtration*	*Final volume*
Run 01_2	milk	unfamiliar	2 g	2-3 days of storage at +4 °C	no	250 µL
Run 02_2	milk	familiar	2g	immediate analysis	yes	150 µL
Run 05_2	plasma/serum	unfamiliar	1 g	immediate analysis	no	150 µL
Run 06_2	plasma/serum	familiar	1 g	2-3 days of storage at +4 °C	yes	250 µL

4.2. Additional analytes

For a reduced validation scheme, four representative experimental runs of the original validation study should be chosen and repeated with the exact same factor-level combinations, now including the additional analyte(s). All factor-level combinations should appear twice (for an example see Table 7).

As there are no data from the initial validation for the analyte(s) to be added, at least four appropriate fortification levels and one blank level or five appropriate fortification levels should be investigated. It is acceptable to use sample material different from the material used in the initial validation (same matrices/species but different batches).

The results of the method extension study amend the corresponding results of the initial validation study and are sufficient for the calculation of all necessary parameters for the new analyte(s). All calculated parameters need to fulfil the criteria laid down in Commission Implementing Regulation 2021/808. Note that since the analyte is newly introduced into the method, it is not possible to compare the initial validation data and the newly calculated validation data for plausibility. However, data of structurally related analytes with properties similar to those of the newly added analyte(s), which have been included in the initial validation study may act as indicators if they are also considered in the method extension study. If the statistical data obtained in the method extension study implies that there are major matrix interferences hindering the correct quantification of the newly included analyte(s), it is necessary to develop and fully validate a novel analytical method.

4.3. Adaption of the concentration range

As with the alternative validation approach instead of distinct concentration levels a concentration range is validated, a revised legal limit is often already covered by the existing method validation data. In such a case, parameters like the critical concentrations can be recalculated for the new level of interest based on the data already available (see section 4.3.1 for examples). Should the required concentration levels for the revised legal limit not be covered by the initial validation, then four representative experimental runs of the original validation study should be repeated with additional fortification levels for an extension of the concentration range. The concentration levels of the original validation study can be included completely or only partly for the repetition of the experimental runs. The number of fortification levels which need to be investigated within the method extension study depends on the (in)congruity of the concentration range which has initially been validated and the concentration range needed to satisfy the requirements arising from the revised legal limit. If the concentration ranges of the initial validation study and the planned method extension study overlap, it should also be assessed whether the number of fortification levels within this overlap is sufficient for the method extension or whether additional fortification levels may be required. Therefore, the number of fortification levels necessary for a study aiming at an adaption of the validated concentration range is always a case by case decision based on expert opinion.

The four experimental runs selected for a method extension study are performed including the new concentration levels, evaluated and combined with the data of the corresponding four experimental runs of the initial validation. As with a full validation, the parameters calculated with regards to the revised legal limit need to fulfil the criteria laid down in Commission Implementing Regulation 2021/808. The monitoring of the method performance through ongoing quality control is required and ideally the four remaining experimental runs required for a complete validation are repeated in this context (see also section 2).

4.3.1. Example cases for changes to the validated concentration ranges

Table 8 gives three examples of methods for the determination of an MRL compound and the respective concentration ranges for which they were initially validated. If the MRL is changed, there are several possibilities on how to proceed with the method extension, depending on the initially validated concentration range.

In *Example A* the MRL used to be 500 µg/kg and is then lowered to 20 µg/kg. Neither the new MRL itself, nor any of its multiples[5] for which validations is required (2(-10) µg/kg, 20 µg/kg, 30 µg/kg) is

[5] MRL compounds need to be validated at 0.1-0.5*MRL, 1.0*MRL and 1.5*MRL. A method validated using the alternative validation approach needs to be validated for a concentration range covering these levels.

covered by the initial validation. It is therefore necessary to extend the method to concentrations below the initially validated concentration range. Since the previous and the new MRL differ so greatly in this example, it might be advisable to include several concentration levels in the range of the required levels in order to obtain statistically sound and more realistic values for the validation parameters.

In *Example B* the original MRL of 500 µg/kg is lowered to 250 µg/kg. Therefore, the concentration range which needs to be covered by the method for this new MRL is mostly identical to the existing method which was validated for 50-750 µg/kg. Even though it is already possible to calculate a decision limit based on experimental data, it is recommended to amend the method with lower concentration levels so as to also cover the 0.1 multiple of the revised MRL (should this concentration level be analytically achievable).

In *Example C* the MRL was not adjusted as drastically as in the other examples but only from 500 µg/kg to 400 µg/kg. Due to the layout of the initial validation study, the concentration range which needs to be validated for the revised MRL is already covered by the existing method and no method extension study is necessary. Using the existing data, the relevant parameters can be recalculated for the new level of interest.

Table 8: Example cases for changes in the legal limit requiring an adaption of the validated concentration range.

	Example A	*Example B*	*Example C*
Previous MRL	500	500	500
Minimum validated concentrations to be covered by the concentration range (0.1 (-0.5), 1.0, 1.5*MRL)	50 (-250), 500, 750	50 (-250), 500, 750	50 (-250), 500, 750
concentration range in the initial validation	50-750	50-750	10-750
Revised MRL	20	250	400
Minimum validated concentrations to be covered by the concentration range (0.1 (-0.5), 1.0, 1.5*MRL)	2 (-10), 20, 30	25 (125), 250, 375	40 (-200), 400, 600
Exemplary concentration range for the revised validation	0-50	(20-750) revision recommended	No revision required

4.4. Additional species and matrices

To some extent different matrices and species can already be included as factors in the experimental design for the initial validation study which is the preferred procedure due to the increased statistical confidence. A prerequisite for such an approach is always the extensive testing of the different matrices/species in preliminary experiments. This is also a requirement for the extension of existing methods to new matrices and species especially when using a reduced validation scheme. Care should also be taken to not introduce too many changes at the same time. The decision which changes to implement simultaneously should always be justified with preliminary experiments and based on expert opinion.

For the extension of a method to additional matrices/species using a reduced validation scheme, a factor included in the initial validation which exhibited no or no significant effect is replaced by this new factor (e. g. matrix or species). Four appropriate experimental runs of the initial validation study are repeated with the new factor level, evaluated and combined with the data of the previous four runs. If the assessment of the factorial effects shows only a minor or an acceptable difference between the two new factor levels (e. g. less than 10 % difference between pork and poultry meat) the extension of the method to these new parameters is possible.

Alternatively, the performance data of the repeated experimental runs may be calculated separately from the original validation data and then compared to the data of the initial validation. For both scenarios, the constraints given in the guidance on method validation using the alternative validation approach apply.

4.5. Changes to the method

The validation of minor modifications of analytical methods can be accomplished by the approach described in the previous sections (repetition of four runs of the initial validation with modified factor levels). Alternatively, the method suitability can be assessed by comparing the results of both the original and the modified method applied to 6-8 matrix materials fortified to at least two concentration levels. If available, reference materials should also be analysed. Such a comparison should ideally be carried out on two or more independent occasions.

If a significant performance difference is observed or if matrix interferences appear problematic, it is necessary to carry out a full validation for the modified method.

For changes in the measuring instrument, the same methodology has to be followed. However, it is ideal to already include a second instrument as a factor in the experimental plan for the initial validation.

4.6. Calculation

The calculation of the performance characteristics is described in the *EURL Guidance document on method validation*.

4.7. Example

Following preliminary experiments and previous validations, a method for the determination of nitroimidazoles in plasma/serum and muscle was developed and validated for the species pig and turkey. The factors and factor levels included in the validation are given in Table 9, the experimental plan is given in Table 10.

Table 9: Factors and factor levels included in a validation study for an analytical method for the determination of nitroimidazoles.

factor		*level A*	*level B*
I	kind of matrix	muscle	plasma/serum
II	species	pig	turkey
III	operator	unfamiliar	familiar
IV	amount of matrix	2 g	1 g
V	storage of final extract	2-3 days of storage at +4 °C	immediate analysis
VI	filtration	no	yes (100 kDa)
VII	final volume	250 µL	150 µL

Table 10: Experimental plan for the initial validation study for an analytical method for the determination of nitroimidazoles.

Run	*Kind of matrix*	*Species*	*Operator*	*Amount of matrix*	*Storage of extract*	*Filtration*	*Final volume*
Run 01	muscle	pig	unfamiliar	2 g	2-3 days of storage at +4 °C	no	250 µL
Run 02	muscle	pig	familiar	2 g	immediate analysis	yes	150 µL
Run 03	muscle	turkey	unfamiliar	1 g	2-3 days of storage at +4 °C	yes	150 µL
Run 04	muscle	turkey	familiar	1 g	immediate analysis	no	250 µL
Run 05	plasma/serum	pig	unfamiliar	1 g	immediate analysis	no	150 µL
Run 06	plasma/serum	pig	familiar	1 g	2-3 days of storage at +4 °C	yes	250 µL
Run 07	plasma/serum	turkey	unfamiliar	2 g	immediate analysis	yes	250 µL
Run 08	plasma/serum	turkey	familiar	2 g	2-3 days of storage at +4 °C	no	150 µL

All proportional and constant deviations associated with the factor levels (Table 13) were acceptable, with maximum deviations of around 2 %. The recoveries, as well as the reproducibility standard deviations were also in an acceptable range (Table 14). As all the remaining requirements of Commission Implementing Regulation 2021/808 were also fulfilled, the method was fully validated for pig and turkey muscle and plasma/serum.

Additional experiments showed that the method also worked reliably for the matrix bovine milk. Therefore, a method extension study for this matrix was planned. The factors included in the method extension study are given in Table 11 and the experimental plan is shown in Table 12.

Table 11: Factors and factor levels included in a method extension study for an analytical method for the determination of nitroimidazoles. Note that the initial factor II "species" was disregarded for the extension.

factor		*level A*	*level B*
I	matrix	milk	plasma/serum
~~II~~	~~species~~	~~pig~~	~~turkey~~
III	operator	unfamiliar	familiar
IV	amount of matrix	2 g	1 g
V	storage of final extract	2-3 days of storage at +4 °C	immediate analysis
VI	filtration	no	yes (100 kDa)
VII	final volume	250 µL	150 µL

Table 12: Experimental plan for the method extension study for an analytical method for the determination of nitroimidazoles in milk, muscle and plasma/serum. Runs 05 to 08 were not repeated but represent the runs from the initial validation study which were used for the statistical calculations.

Run	*Matrix*	*Operator*	*Amount of matrix*	*Storage of extract*	*Filtration*	*Final volume*
Run 01_2	milk	unfamiliar	2 g	2-3 days of storage at +4 °C	no	250 µL
Run 02_2	milk	familiar	2 g	immediate analysis	yes	150 µL
Run 03_2	milk	unfamiliar	1 g	2-3 days of storage at +4 °C	yes	150 µL
Run 04_2	milk	familiar	1 g	immediate analysis	no	250 µL
Run 05	plasma/serum	unfamiliar	1 g	immediate analysis	no	150 µL
Run 06	plasma/serum	familiar	1 g	2-3 days of storage at +4 °C	yes	250 µL
Run 07	plasma/serum	unfamiliar	2 g	immediate analysis	yes	250 µL
Run 08	plasma/serum	familiar	2 g	2-3 days of storage at +4 °C	no	150 µL

As the method extension was to be carried out only for bovine milk, the factor II “species” was disregarded in the extension study. Since neither of the two matrices included in the initial validation study exhibited a relevant effect on the method parameters, in principle either runs 01 to 04 or runs 05 to 08 could have been repeated with the new matrix milk. Had the factor “matrix” not been included in the initial validation study, it would have been necessary to replace any of the remaining factors, which do not significantly influence the method parameters, with the added factor “matrix” with only one factor level – “milk”. As in this example none of the factors significantly influence the validation parameters, as gathered from the values calculated for the constant and proportional deviation, any one of the original factors could have been replaced with the newly introduced factor “milk”.

After conduction of the four modified experimental runs 01_02 to 04_02 in random order the validation data were calculated by combining the results of these experimental runs with the data of runs 05 to 08 of the initial validation study (matrix plasma/serum). The results for the proportional and constant deviation are all in the range of 0.1-2 % and since the general requirements of Commission Implementing Regulation (EU) 2021/808 were also fulfilled, the method is valid for the additional matrix milk (Table 13, Table 14).

A comparison of the deviations calculated from the original validation data and from the method extension study data shows that the values are mostly within the same range. This further underlines that the chosen factors are not inherently critical to the method performance. Larger but not relevant differences are apparent for the factor matrix (plasma/serum and muscle: 0.133, 0.275; plasma/serum and milk: 1.690, 1.984) and the factors filtration (plasma/serum and muscle: -2.264, -2.036; plasma/serum and milk: -0.132, -0.109) and volume of the final extract (plasma/serum and muscle: 2.327, 2.244; plasma/serum and milk: 0.178, 0.300). The data for the recovery, the CCα and the reproducibility standard deviation also only differ slightly between the initial validation and the method extension study (Table 14). As all the requirements are fulfilled, the method can be applied to plasma/serum and muscle of pig and turkey, as well as to bovine milk. The method performance should be continuously monitored and ideally, the required quality control samples should be planned in such a way as to generate data for the experimental runs 05 to 08, thereby obtaining a complete set of validation data (eight experimental runs) also for bovine milk.

Table 13: Proportional and constant deviation of the factor levels in the initial validation and the 4-run method extension for the determination of metronidazole.

		Initial validation for muscle and plasma/serum		*Method extension for milk and plasma/serum*	
Factor	*Level*	*Proportional deviation*	*Constant deviation*	*Proportional deviation*	*Constant deviation*
matrix	plasma / serum(+); milk/muscle(-)	0.133	0.275	1.690	1.984
species	pig (+); turkey (-)	1.126	0.980		
operator	unfamiliar (+); familiar(-)	-2.556	-1.178	-2.246	-1.154
amount of matrix	2 g(+); 1 g(-)	0.982	0.129	0.675	0.124
storage of extract	direct analysis(+); 2-3 days of storage(-)	-0.225	-0.012	0.285	0.143
filtration	yes (+); no(-)	-2.246	-2.036	-0.132	-0.109
volume	200 ul final volume(+); 120 ul final volume(-)	2.327	2.244	0.178	0.300

Table 14: Validation parameters for select nitroimidazoles for the determination in plasma/serum and muscle of pig and turkey and bovine milk.

	Initial validation for muscle and plasma/serum			*Method extension for milk and plasma/serum*		
Analyte	*CC_α*	*Recovery [%] at CC_α*	*Relative reproducibility standard deviation s_R [%] at CC_α*	*CC_α*	*Recovery [%] at CC_α*	*Relative reproducibility standard deviation s_R [%] at CC_α*
Dimetridazole	0.153	108.5	10.2	0.131	109.2	7.4
HMMNI	0.163	106.8	13.5	0.200	98.2	18.1
Metronidazole	0.072	107.0	10.7	0.068	104.7	9.0
MNZOH	0.153	100.2	12.0	0.159	103.2	11.6
Ronidazole	0.109	93.1	18.9	0.081	102.2	13.4