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Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods

This document has been conceived as a technical guideline of the Commission Services. It does not represent the official position of the Commission. It does not intend to produce legally binding effects.

Only the European Court of Justice has jurisdiction to give preliminary rulings concerning the validity and interpretation of acts of the institutions of the EU pursuant to Article 267 of the Treaty.

Contents

Abbreviations/Definitions

DoR	Residue definition (Definition of Residue)
ERR	Extractable radioactive residue
HPLC	High performance liquid chromatography
LC-MS/MS	High performance liquid chromatography/tandem mass spectrometry
LOQ	Limit of quantification
LSC	Liquid scintillation counting
MRL	Maximum residue level
MS	Mass spectrometry
TLC	Thin layer chromatography
TRR	Total radioactive residue

1 Introduction

Revision 5 of this document includes Annex 2 containing questions and answers providing further precisions on the applicability of this Technical Guideline. The previous revision 4 became applicable on 23 February 2022. Since then, certain questions on applicability came up which are addressed in this revised revision 5. However, revision 5 does not contain any new elements.

Revision 5 of SANTE/2017/10632 was endorsed by the Member States in the SCoPAFF Phytopharmaceuticals, Pesticides Residues on 11 May 2023.

The extraction procedure is the crucial part of analytical methods and has great influence on the correct quantification of pesticide residues. The yield of extraction is affected by various factors, i.e. extraction time, extraction temperature, agitation as well as the type of the extraction solvent.

During routine validation of residue analytical methods recovery and precision are typically tested with fortified samples. High recovery and good precision are frequently observed in such tests. Nevertheless, this procedure cannot provide evidence for efficient extraction of incurred residues. The formation of conjugates or the incorporation into the plant matrix might be a reason that incurred pesticide residues are not easily accessible during extraction. For example, during proficiency tests with fortified samples that are aged with the aim to reflect incurred residues, it was noticed that the detected amount of a residue might depend on the water content of the crop even if freshly fortified samples showed good recovery in all cases [1]. It is not expected that the tests mimic the nature of (field) incurred residues. However, this observation might give evidence that the efficiency of the extraction process depends on the composition of the extraction solvent. The demonstration of the extraction efficiency cannot be performed by routine method validation with spiking experiments. It can only be assessed with samples bearing incurred residues [2].

The Commission Regulation (EU) No. 283/2013 [3] established data requirements for active substances of plant protection products in the EU. This regulation states in section 6.2 (Metabolism, distribution and expression of residues), that studies should “show the efficiency of extraction procedures for these components”. Although the regulation does not specifically include the need to address efficiency of the extraction procedures for monitoring methods, the Commission Communication document [4], fulfilling Point 6 of the Introduction of the Annex to

Regulation 283/2013, published a list of test methods and guidance documents relevant to the implementation of this regulation. The following guidance documents apply for post-approval control and monitoring purposes or for risk assessment:

- "Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes (SANTE/12830/2020)" [5]. Extraction efficiency addressed in point "3.10 Extraction efficiency".
- "OECD (2007) Guidance Document on Pesticide Residue Analytical Methods. Environment, Health and Safety Publications. Series on Testing and Assessment No. 72 and Series on Pesticides No. 39" [2]. Extraction efficiency addressed in point "Extraction efficiency / Radiovalidation".

2 Objective and scope

The purpose of this guidance document is to give advice on when and how to assess the suitability of the extraction procedures applied in pesticide residue analytical methods. However, it is not restricted to post-registration monitoring methods and is also applicable for residue analytical methods used for the quantification of residues in supervised field trials (also called pre-registration or data generation methods).

The guidance document has been developed primarily for the evaluation of extraction efficiency of analytical methods for plant matrices, but the basic principles are also applicable for products of animal origin.

Primarily the suitability of the extraction procedures should be evaluated based on information from metabolism studies performed with radiolabeled pesticides (if needed in combination with radio cross validation studies). This is especially the case for animal products since it is not expected that new animal metabolism studies or new animal feeding studies should be set up only in order to evaluate aspects of analytical methods and extraction efficiency. Therefore the possibilities for studying extraction efficiency must be fully considered in animal metabolism studies (and feeding studies, where applicable) when these studies are planned from the perspective of residues evaluation. However, the guidance does also provide suggestions regarding the design of new studies with respect to extraction efficiency and on how to approach cases where samples with incurred radiolabeled residues are no longer available.

Application of the guidance does not necessarily mean that new data will be required and should normally not result in the generation of new studies using radiolabeled substances.

Other factors which may affect extraction efficiency (e.g. particle size of the extracted homogenate, loss of analytes during cleanup or solvent evaporation) can be covered by validation experiments using samples fortified with non-radiolabeled pesticides. These factors should be evaluated based on recovery experiments during routine method validation and are not within the scope of this guidance document.

For internationally standardized multi-residue methods [7-10] a huge amount of validation data was already published. Nevertheless, these data are normally not generated by using sample materials with known concentrations of incurred residues. Consequently, an evaluation of the extraction efficiency is also necessary for the solvents and conditions used in multi-residue methods.

For plant extracts used as pesticides, the synthesis of radiolabeled reference compounds for metabolism studies can be difficult. In such case, the evaluation of the extraction efficiency might not be possible. At least all components included in the residue definition should be soluble in the extraction solvent.

3 Definitions

Cross-validation

Cross validation means the comparison of amounts of relevant residues extracted from samples with incurred residues using the solvent system of the monitoring method and the solvent system under the conditions applied during the metabolism studies.

Extraction

Extraction means here the physical process of dissolving an analyte by a solvent or partition into a solvent, i.e. transferring a substance from an (partly) insoluble matrix into a solvent. [11]

Extraction efficiency

The extraction efficiency of a solvent or solvent system is expressed as the ratio of extracted analyte(s) and the total amount of analyte(s) in a certain sample. In this context further losses during sample cleanup, evaporation or exchange of solvent during analysis are not considered here and need to be evaluated during method validation.

Marker compound(s)

Marker compound(s) refer, as far as possible, to a single compound or few compounds which typically represent the residue definition for monitoring. They are specific analytes which normally occur in large quantities, represent a substantial proportion of the total residue and are easy to measure (ideally by a multi-residue method). [12]

Non-extracted residue

The non-extracted residue is defined here as the residue remaining in the matrix after initial extraction with a solvent (prior to other exhaustive extraction steps such as acid or base hydrolysis). This may include also (mild) hydrolysis of extractable conjugates if included in the residue definition.

Radio-Cross-validation

When using samples with incurred residues from radiolabeled studies, radio-validation is the comparison of the relative amount of analytes/ radioactivity extracted by a residue analytical method with the relative amount extracted in the metabolism study. [2]

Residue definition (DoR) for dietary risk assessment

Comprises usually the parent compound and its toxicologically significant metabolites, taking into consideration both exposure and relative toxicities. [12]

Residue definition (DoR) for enforcement/monitoring

This definition may comprise a subset of the components included in the definition for dietary risk assessment. That subset would include ‘marker compounds’ which typically account for a substantial proportion of the residue. The definition of the residue for enforcement/monitoring

focuses on those analytes that (1) would indicate a possible misuse of the pesticide or (2) need to be analyzed for monitoring purposes and (3) is simple and can be detected and measured by a broad range of laboratories (i.e. residues that are easy to measure, ideally by a multiresidue method, usually occur in large quantities, and are common to all commodities in which residues are expected). [12]

Total radioactive residue (TRR)

The total radioactive residue comprises all radiolabeled compounds in the entire sample before extraction and usually measured by combustion. Alternatively, the total radioactive residue in metabolism studies can be determined after extraction by summing up the radioactivity in all extracts (determined by liquid scintillation counting) and the radioactivity in the remaining solids (determined by combustion).

4 Evaluation of existing data

4.1 Studies and samples used for evaluation

Usually, the evaluation of the extraction efficiency is based on studies conducted with radiolabeled pesticides. Samples from metabolism studies with primary crops or rotational crops (depending on the predominance of the considered analyte(s)) and with animals can be used. [13-15]

Non-radiolabeled samples collected from crop field trials or from food monitoring can be used for cross-validation studies to compare extraction efficiency of different solvents. For products of animal origin also non-radiolabeled feeding studies can be used for cross-validation experiments if available.

4.2 Matrix groups to be considered

Validation of pre- and post-registration analytical methods has to be conducted for a limited number of defined matrix groups. [2, 5]The extraction efficiency should be evaluated for all matrix groups or animal commodities for which residue analytical methods are required.

In principle, one example for each matrix group or respective commodity for post-registration methods andfor pre-registration methods is sufficient [5]. Bridging between high water content

and acidic matrices is acceptable for slightly acidic matrices, e.g. apple, tomato, grapes, but should be justified by the applicant. Generally, the selection of matrix groups depends on the availability of sample material from metabolism studies or samples with incurred residues.

For matrices which are difficult to analyse, in principle an evaluation of the extraction efficiency would be desired as well, but depends on the availability of radiolabeled sample material or samples with incurred residues.

A number of pesticides forms residues located at the surface of treated matrices only (>70% TRR). In such cases, the extraction process is rather a solution of the analyte than an extraction of incurred residues. For such situations, the non-submission of data for different matrix groups should be justified with reference to surface washing experiments when conducted in metabolism studies.

4.3 Selection of analytes

The efficiency of the extraction method should be evaluated for all analytes included in the residue definition for monitoring (relevant for post-registration methods) as well as in the residue definition for risk assessment (relevant for pre-registration methods) as soon as quantifiable concentrations occur.

If analytes included in the residue definition differ for a certain matrix, then the extraction efficiency should be evaluated for the corresponding analyte/matrix combination.

5 Principle of evaluation of extraction efficiency

As a first step in the evaluation of extraction efficiency, information about the extracted fraction of the TRR from studies with radiolabeled pesticides should be used. Extraction efficiency for a compound or several compounds is sufficient (1) if a large fraction of the TRR is extractable with the extraction solvent of the monitoring method and (2) the compounds included in the residue definition account for a large fraction in this extract. That means that compounds included in the residue definition should not be found in additional extraction steps or in the residues remaining. If compounds included in the residue definition are not observed in the primary metabolism, other available studies using radiolabeled pesticides should be used.

Generally, the evaluation of the extraction efficiency is only necessary for pesticides showing significant residues, i.e. residues at or above the limit of quantification (LOQ) of the analytical method. For compounds included in annex IV of the Regulation (EC) No. 396/2005 an evaluation of the extraction efficiency is not required.

The sample material with radiolabeled incurred residue is typically available for approval of active substances, only. For the evaluation of the extraction efficiency for additional matrices or for different solvents food samples containing incurred residues should be performed (cross-validation).

When setting up new metabolism studies, an additional extraction of treated commodities with solvent systems typically used for pre- and post-registration methods is proposed in order to facilitate the evaluation of extraction efficiency in future. A detailed description of such an "ideal" study is given in chapter 6. Most existing metabolism studies, however, do not follow the outline described in chapter 6. For these cases a stepwise approach is proposed to decide whether and how the extraction efficiency should be addressed. The decision trees give advice for the evaluation of existing studies. Slightly different decision trees apply for post-registration methods (see chapter 5.1) and for pre-registration methods (see chapter 5.2).

5.1 Decision tree for post-registration monitoring methods

The evaluation of the extraction efficiency is performed by using a stepwise approach. It is illustrated in the flow diagram shown in Fig. 1 and is explained in detail below this figure.

Generally, the trigger values mentioned in the decision tree should be applied for the complete residue definition for monitoring, i.e. the sum of all compounds included in the DoR.

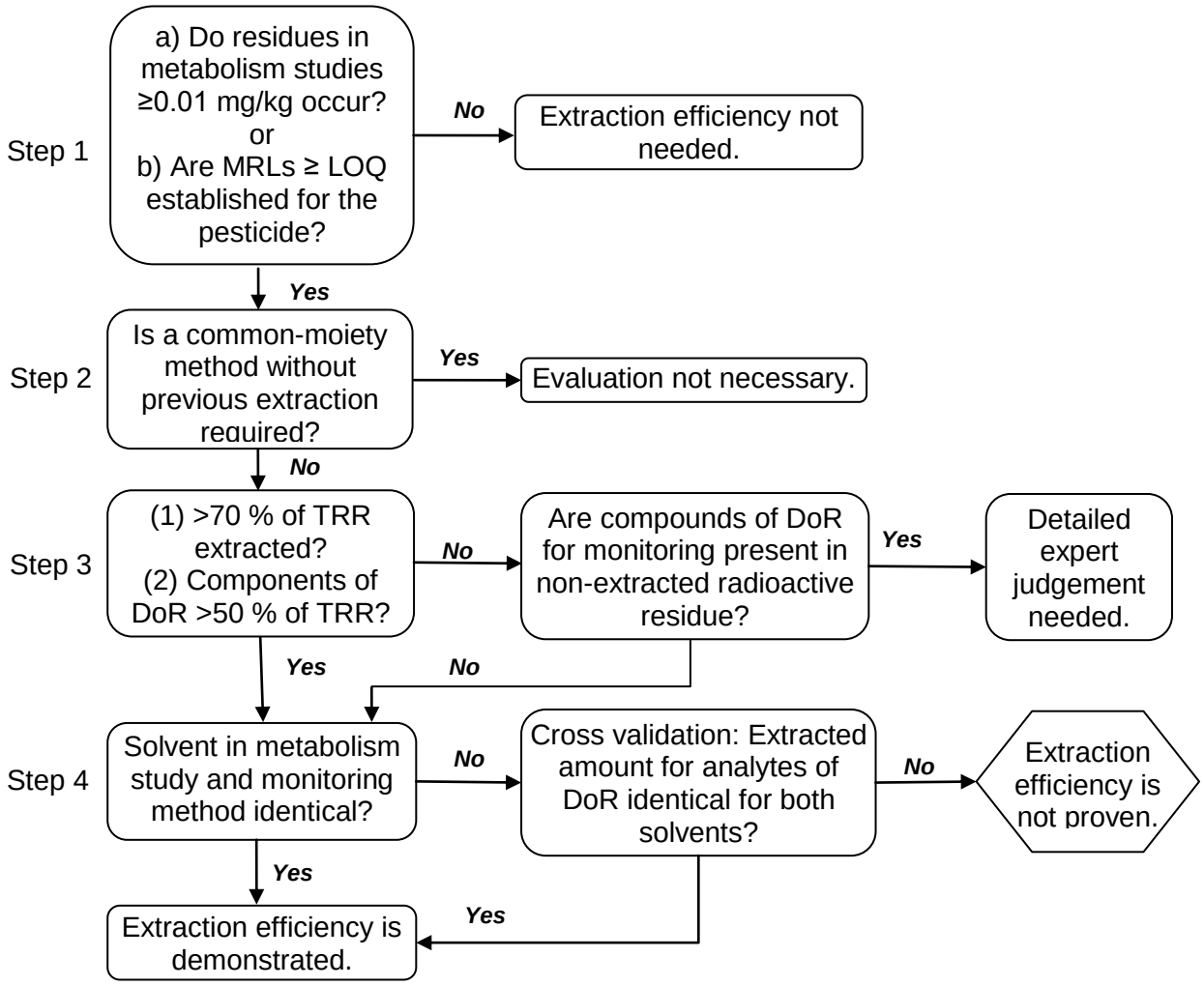

Fig. 1 Decision tree for post-registration methods

The choice of the criterion for step 1 depends on the availability of data, e.g. not in all cases MRLs are set or proposed at the time of preparation of a dossier.

Step 1a: Do residues ≥0.01 mg/kg occur in samples from metabolism studies (at 1N rate)?

According to the guidelines [13-15] for metabolism studies, no characterization of radioactive residues is needed for residues <0.01 mg/kg. Typically the metabolism studies are overdosed to produce residues which can be easily quantified or identified. If no residues are detected in these studies it is unlikely that residues will occur in residue trials. Therefore, this trigger values is also applicable to decide on the need to evaluate the extraction efficiency. For pesticides with residues not exceeding the

trigger in any crops belonging to a particular matrix group testing of extraction efficiency is not needed for this matrix group.

In rare cases where MRLs are regulated below the common default limit of 0.01 mg/kg (e.g. for some pesticides in baby food or for pesticides with concerns regarding the outcome of consumer intake (low ADI or ARfD), the trigger has to be adjusted accordingly. The trigger also needs to be adjusted for pesticides included in annex V of Reg. (EC) No 396/2005.

For feeding stuffs the extraction efficiency has only to be demonstrated if MRLs > LOQ are set. Extraction efficiency in food of animal origin has to be demonstrated for commodities showing residues >0.01 mg/kg in metabolism/feeding studies at 1N rate.

Step 1b: Are MRLs ≥LOQ established for the pesticide?

For pesticides or pesticide/commodity combinations without MRLs (e.g. compounds listed in annex IV of Regulation (EC) No. 396/2005 or for compounds without MRLs for animal matrices) there is no need for enforcement of a legal limit and consequently not need for consideration of extraction efficiency.

Step 2: Is a common moiety method without previous extraction required?

For some pesticides with a complex metabolism it is not possible to identify marker compounds that occur in all relevant matrices. In such cases, the residue definition for monitoring sometimes is based on a common moiety formed by chemical conversion of several compounds of the residue. If the chemical conversion is conducted without a previous separate extraction step, an evaluation of the extraction efficiency is not needed. If the same common moiety method is used for metabolism studies and for residue trials for at least 3 different analytical crop groups, an additional consideration of further crop groups is not necessary.

Step 3: (1) Are at least 70 % of the TRR extracted with the tested solvent system?

(2) Does the sum of radioactive residues for all components of the DoR for monitoring >50 % of the TRR?

If both conditions are fulfilled, usually more than 50 % of the components included in the DoR for monitoring will be extracted, which is considered acceptable.

In cases, where the components of the DoR represent <50 % of the TRR and/or <70 % of the TRR was extractable with the tested solvent system, extraction efficiency may be sufficient if the remaining (non-extracted) radioactivity

- is represented by radiolabels incorporated into biomolecules or integrated into the crop matrix, or
- cannot be liberated from the crop matrix without chemical conversion, e.g. using a strong acid or base only, or
- is attributable to identified metabolites, which are not included in the residue definition.

The justification of sufficient extraction efficiency based on these arguments (which then allows proceeding to step 4) has to be made in a conclusive way using experimental data from metabolism studies.

In cases, where the extraction process with the selected solvent is not exhaustive, compounds included in the residue definition could remain in the residue and are only become liberated in subsequent harsher extraction steps (e.g. acid or base hydrolysis). A possible reason for this could be binding of the analyte to the sample matrix leading to reduced accessibility for the selected solvent. Consequently, the selected solvent system may not be suitable. In this case a detailed expert statement is needed to justify that extractable residue levels are not underestimated.

Step 4: Is the solvent used in the metabolism study and in the monitoring method identical?

At this stage the extraction efficiency is considered as being sufficiently proven if the same solvent is used in metabolism studies and monitoring methods. Solvent mixtures are considered as being identical if their composition varies by not more than 20 vol.-%.

The extracted ratio (extraction efficiency) for compounds of the residue definition in a commodity is expressed as

$$extracted\ ratio = \frac{w[sum of\ analytes]_R}{w[sum of\ analytes]_M} * 100\% \qquad \text{equation 1}$$

Whereas:

w[*sum of analytes*]$_R$	Mass ratio of analyte or sum of analytes included in the residue definition for monitoring as determined by the residue analytical method, expressed as mg parent compound equivalents / kg or % of the TRR
w[*sum of analytes*]$_M$	Mass ratio of analytes or sum of analytes included in the residue definition for monitoring as determined in the metabolism study, expressed as mg parent compound equivalents / kg or % of the TRR

In cases where different solvent systems are used in metabolism studies and residue analytical methods, the following two options are proposed:

- Radio-cross-validation: Samples from metabolism studies with quantifiable TRR are available. The amount of compounds (included in the DoR for monitoring) in the extractable portion of the TRR using the solvent from the monitoring method is determined and then compared to the amount of analytes resulted by extraction using the original solvent of the metabolism study.

  In cases where the use of different solvent systems results in larger differences of the extracted amount of components included in the residue definition, other solvents have to be tested in a cross validation experiment:

- Cross-validation: Samples from field trials with quantifiable amounts of incurred residues are available. The extractable portions of the analytes of interest (included

in the DoR) using in parallel the solvent from the monitoring method and the solvent from the metabolism study are determined and compared to each other.

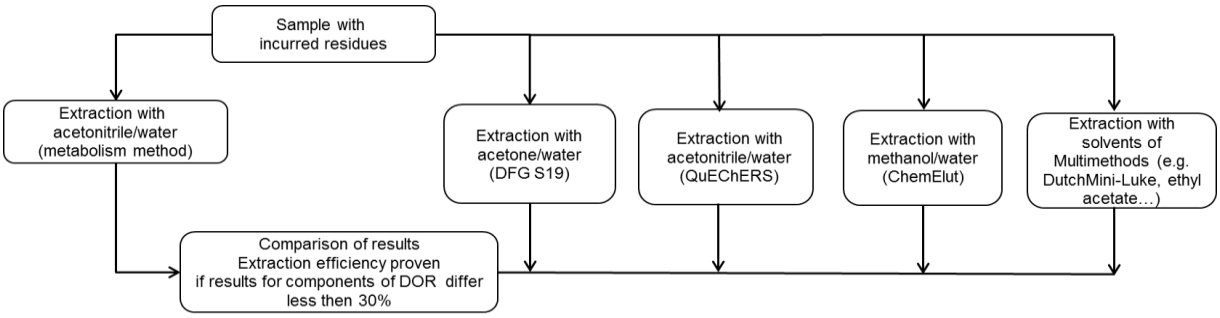

Fig. 2 Principle of cross-validation

Extraction efficiency will be considered as being sufficiently proven if the extracted portion of the TRR relates to the analyte of interest (included in the DoR) and if the residue amount (in case of cross-validation with non-radiolabelled incurred residues) differs by no more than 30 % (for residues >0.01 mg/kg) compared to the results obtained with the solvent of the metabolism study.

The extraction efficiency is not sufficient if cross validation experiments show that the extracted amount of components being part of the residue definition is significantly lower (< 70 %) when using the solvent of the proposed monitoring method.

A simple justification based on “similar” physical or chemical properties (e.g. density, dipole moment, dielectric constant, “polarity”) of the different solvents is not sufficient.

5.2 Decision tree for pre-registration methods

The proof of extraction efficiency of pre-registration methods is required in the corresponding guidance documents [2, 5]. The analytical methods used for pre-registration follow the same principles as post-registration methods. Hence, the decision tree is also applicable for evaluation of pre-registration methods.

The following exception is made: Step 1b (whether MRLs ≥LOQ are set) of the decision tree is omitted. Although for feeding stuffs currently no MRLs are set, a proof of the extraction

efficiency might be required where determinable residues occur that are quantified for use in the dietary burden calculation. In exceptional circumstances, the quantification of lower levels than 0.01 mg/kg in either food or feed may be required for risk assessment purposes, which has to be considered on a case by case basis. For justification of the remaining steps please refer to chapter 5.1. Please note that the DoR referred to in this decision tree is the DoR for risk assessment.

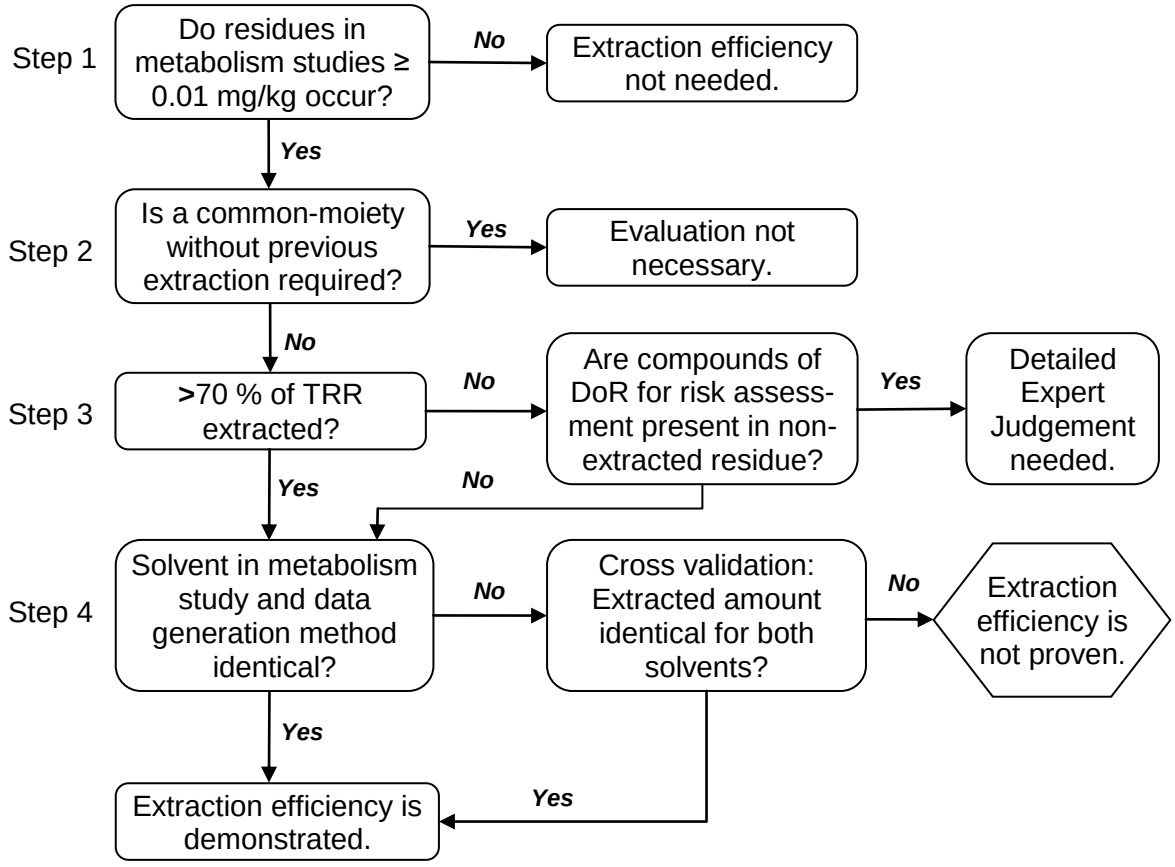

Fig. 3 Decision tree for pre-registration methods

6 Extraction efficiency as part of an "ideal" metabolism study

This section may be considered if new metabolism studies are planned.

For post-registration purposes (monitoring and MRL enforcement) multiresidue methods are preferably used [5]. These methods are capable of analysing several hundreds of compounds after a single extraction with typical solvents. For pre-registration purposes often single residue methods for pesticides are used to cover all analytes of the residue definition for dietary risk assessment.

The testing of these typical solvents for their extraction efficiency could be easily integrated in the experimental design of metabolism studies and is also recommended in the respective OECD test guidelines [13-15].

Metabolism studies are performed according to the appropriate guidelines [13-15] using radiolabeled test substances. At termination of the study, the extractable TRR is quantified, the metabolic pathway is proposed and the compounds of the residue are characterized, identified and quantified. A general extraction efficiency module is proposed as follows:

Crop samples available from the metabolism study should desirably be extracted with the following set of solvents which are typical for multiresidue (covering DoR for enforcement) and data generation methods (covering DoR for dietary risk assessment):

- Extraction with **acetonitrile/water (1/1, v/v)**, typically used for the European standard method EN 15662:2008 and AOAC method 207.01 (QuEChERS method) [9];
- Extraction with **acetone/water (2/1, v/v)**, for non-fatty crops or extraction with **acetonitrile/acetone** according to module E7 typically used for European standard method EN 12393:2013 (DFG S19 method) [7];
- Extraction with **methanol/water (2/1, v/v)** typically used for European standard method EN15637:2008 (ChemElut method) [8];
- Extraction with **ethyl acetate**, typically used for European standard method EN 12393:2013 (SweEt method) [10];
- Extraction with **solvent(s) of data generation method(s)**

The experiments may be performed as add-on within metabolism studies or in separate studies. Applicants should report results from extraction efficiency testing within a metabolism study also in the respective analytical chapter. For each analyte which is part of the DoR, the yield (in mg parent equivalents / kg or % of the TRR), released by acetonitrile/water (1/1, v/v), acetone/water (2/1, v/v), methanol/water (2/1, v/v), ethyl acetate and the solvent of the data generation method, is individually compared to the yield (in mg parent equivalents / kg or % of the TRR) obtained in the metabolism study. The extraction efficiency is calculated according to equation 1. The choice of the solvent may also depend on analytical properties of the analytes. If it is expected that certain solvents are not suitable for sufficient extraction because of their properties it is not necessary to test them. In such situations a justification should be provided by the applicant.

The extraction tests performed with the proposed solvents provide in most cases the information necessary for assessing the extraction efficiency. The final monitoring method does not need to be known at that point in time.

Extraction efficiency is considered as being sufficiently addressed if the extracted yields of analytes included in the residue definition differ by not more than 30 % for residues >0.01 mg/kg compared to the results obtained with the solvent of the metabolism study.

7 Application

The procedure for evaluating extraction efficiency concerns the **data requirements** for:

- new active substance approval and renewal of active substances.
- new product authorisations and renewal of product authorisations.
- applications for new MRLs under Article 6 of Regulation (EC) No 396/2005 [6].
- MRL reviews and focused MRL assessments in accordance with Article 12 and Article 43, respectively, of Regulation (EC) No 396/2005.

Both in the old (Reg. (EC) No 544/2011) and the new data requirements (Reg. (EC) No 283/2013 [3]) provisions are foreseen that the efficiency of the extraction procedures in the metabolism studies should be demonstrated.

According to SANTE/2020/12830 [5] the description of the analytical method should include data on extraction efficiency. The extraction procedures used in the methods for risk assessment and post-approval control and monitoring purposes for the determination of residues in food/feed of plant and animal origin should be verified.

Revision 3 of SANTE 2017/10632 became applicable on 23 November 2019, two years after its endorsement by the Member States in the SCoPAFF, section Phytopharmaceuticals, Pesticides Residues on 22 November 2017. However, the obligation to check extraction efficiency was not new at that time. Moreover, the recommendation to test the extraction efficiency of other solvents as part of the metabolism study is only a suggestion in this guideline, not an obligation. However, by performing the described extraction tests in the framework of the metabolism

studies, the requirement of demonstrating extraction efficiency will be in most cases fulfilled simultaneously for both the pre- and post-registration analytical methods, avoiding additional tests at a later stage.

For dossiers submitted in the context of the **approval or the renewal of approval of active substances**, the Extraction Guidelines apply as from 23 November 2019 (date of submission of approval or renewal dossier).

For **renewal of product authorisations or for new product authorisations** or extension of uses **for which no change of the MRL is needed,** the data requirements used for the latest renewal or approval should be considered. This means that no additional proof of extraction efficiency is required if it had not been required in the renewal of approval/approval procedure itself. Extraction efficiency should be addressed if for a product authorization a different analytical methodology (in methods for risk assessment and/or monitoring) is used, compared to that of the approval/renewal procedure of the active substance.

However, the uncertainty associated with the absence of the demonstration of extraction efficiency should be highlighted in the conclusion of the registration report.

For **applications for new MRLs under Art. 6 of Reg. (EC) No 396/2005**, the extraction efficiency needs to be demonstrated in line with the requirements described in this document since 23 November 2019. Since clarifications of applicability were requested and introduced by revision 4 of these Technical Guidelines, dossiers submitted after 22 February 2022 (date of endorsement by the Member States of revision 4 in the SCoPAFF, section Phytopharmaceuticals, Pesticides Residues of 22 February 2022), will be dealt with as follows:

- within the scientific check of new MRL applications submitted under Article 6 of Regulation (EC) No 396/2005, EFSA will ask for informal clarifications/ for additional information from the applicant and/or the Rapporteur Member State/Evaluating Member State

- where such additional information of extraction efficiency cannot be provided or not be fully provided following such a request, EFSA will not stop the clock of the ongoing

assessment, but will highlight the additional uncertainty associated with the absence of the information in the Reasoned opinion.

For the **Art. 12 MRL reviews and Art. 43 MRL assessments**, the data requirements from the latest approval or renewal should be considered, so proof of extraction efficiency for pre- and post-registration analytical methods in line with this document, will only be required, if it was required for the latest approval or renewal. In case that for the Art. 12 MRL reviews and Art. 43 MRL assessments crops out of the scope of the approval or renewal process are considered, additional proof of extraction efficiency might be required but not necessarily. In those cases, no new studies should be requested and tentative MRLs with footnotes requiring additional data within 2 years could be set. Alternatively, uncertainties could be highlighted by EFSA in those cases where information on extraction efficiency was insufficient. However, new studies will be needed on the occasion of a new MRL application or at the next renewal of approval procedure of the respective substance.

As regards the data requirements for pesticides residues, the details of application remain case-by-case decisions. They will in most cases be applicable for data requirements such as residue field trials and feeding studies. In exceptional cases they might also be applicable for other data requirements, e.g. analytical methods only used for storage stability studies if those are not part of residue field trials or feeding studies.

For the analytical **quality control and method validation procedures for pesticides residues in food and feed** (SANTE/11312/2021 or a more recent version of this document) the demonstration of the extraction efficiency is crucial. For monitoring methods where possible solvents and extraction conditions need to be used for which extraction efficiency was demonstrated during the studies for approval or renewal of an active substance or for the application for a new MRL.

References

[1] Poulsen, M.E., Christensen, H.B., Herrmann, S.S., Accred Qual Assur (2009) 14:477-485, doi: 10.1007/s00769-009-0555-2

[2] OECD: Series On Testing And Assessment No. 72; Series On Pesticides No. 39: Guidance Document On Pesticide Residue Analytical Methods; ENV/JM/Mono(2007)17

(http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282007%2917&doclanguage=en)

[3] Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with regulation (EC) no 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market

(http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:093:0001:0084:EN:PDF)

[4] Commission communication in the framework of the implementation of Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market (2013/C 95/01)

(http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2013:095:0001:0020:EN:PDF)

[5] Guidance document on pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes, SANTE/2020/12830;

(https://ec.europa.eu/food/system/files/2021-02/pesticides_mrl_guidelines_2020-12830.pdf)

[6] Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC, OJ L 70, 16.3.2005, p. 1–16.

[7] Methods M and N in EN 12393:2013, Foods of plant origin - Multiresidue methods for the determination of pesticide residues by GC or LC-MS/MS

see also A. Lozano, B. Kiedrowska J. Scholten, M. de Kroon, A. de Kok, A. R. Fernández-Alba: Miniaturisation and optimisation of the Dutch mini-Luke extraction method for implementation in the routine multi-residue analysis of pesticides in fruits and vegetables; Food Chemistry (2016) 192: 668–681 NL Method

[8] EN15637:2008: Foods of plant origin- Determination of pesticide residues using LC-MS/MS following methanol extraction and cleanup using diatomaceous earth

[9] EN 15662:2008: Foods of plant origin- Determination of pesticide residues using GC-MS and/or LC-MS/(MS) following acetonitrile extraction/partioning and cleanup by dispersive SPE – QuEChERS-method)

[10] Method P in EN 12393:2013, Foods of plant origin - Multiresidue methods for the determination of pesticide residues by GC or LC-MS/MS;
see also T. Philström, G. Blomquist, P. Friman, U. Pagard, B. Österdahl: Analysis of pesticide residues in fruit and vegetables with ethyl acetate extraction using gas and liquid chromatoggraphy with tandem mass spectrometry detection; Anal Bioanal. Chem. (2007) 389:1773-1789 SWeET

[11] IUPAC 1: IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997)., doi:10.1351/goldbook.S05752.

[12] OECD: Series On Testing And Assessment No. 63 and Series On Pesticides No. 31: Guidance Document On The Definition of Residue (As Revised in 2009); ENV/JM/MONO(2009)30; http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?doclanguage=en&cote=ENV/JM/MONO%282009%2930

[13] OECD Guidelines For Testing Of Chemicals, Section 5: Test Guideline No. 501: Metabolism in Crops, 25 Jan 2007

[14] OECD Guidelines For Testing Of Chemicals, Section 5: Test Guideline No. 503: Metabolism in Livestock, 25 Jan 2007

[15] OECD Guidelines For Testing Of Chemicals, Section 5: Test Guideline No. 502: Metabolism in Rotational Crops, 08 Jan 2007

Annex 1: Typical case studies

Example 1: Both criteria of step 3 of the decision tree are fulfilled and the suitability of a different solvent in the monitoring method is shown by cross validation.

Pesticide A is used as a fungicide in tomatoes and grapes. The residue definition for monitoring comprises the parent compound only.

In the analytical methods for monitoring an extraction procedure either with acetone/water, (2/1, v/v) according to EN 12393 (Method N)/DFG S19 multiresidue method or with acetonitrile/water (2/1, v/v) was used.

The metabolism study was performed in wine grapes following spray application of ^{14}C-labelled pesticide A. The TRR in grapes corresponds to 1.86 mg/kg of pesticide A. Grapes were surface washed with acetonitrile followed by homogenization and three extraction steps with acetonitrile/water (8/2, v/v).

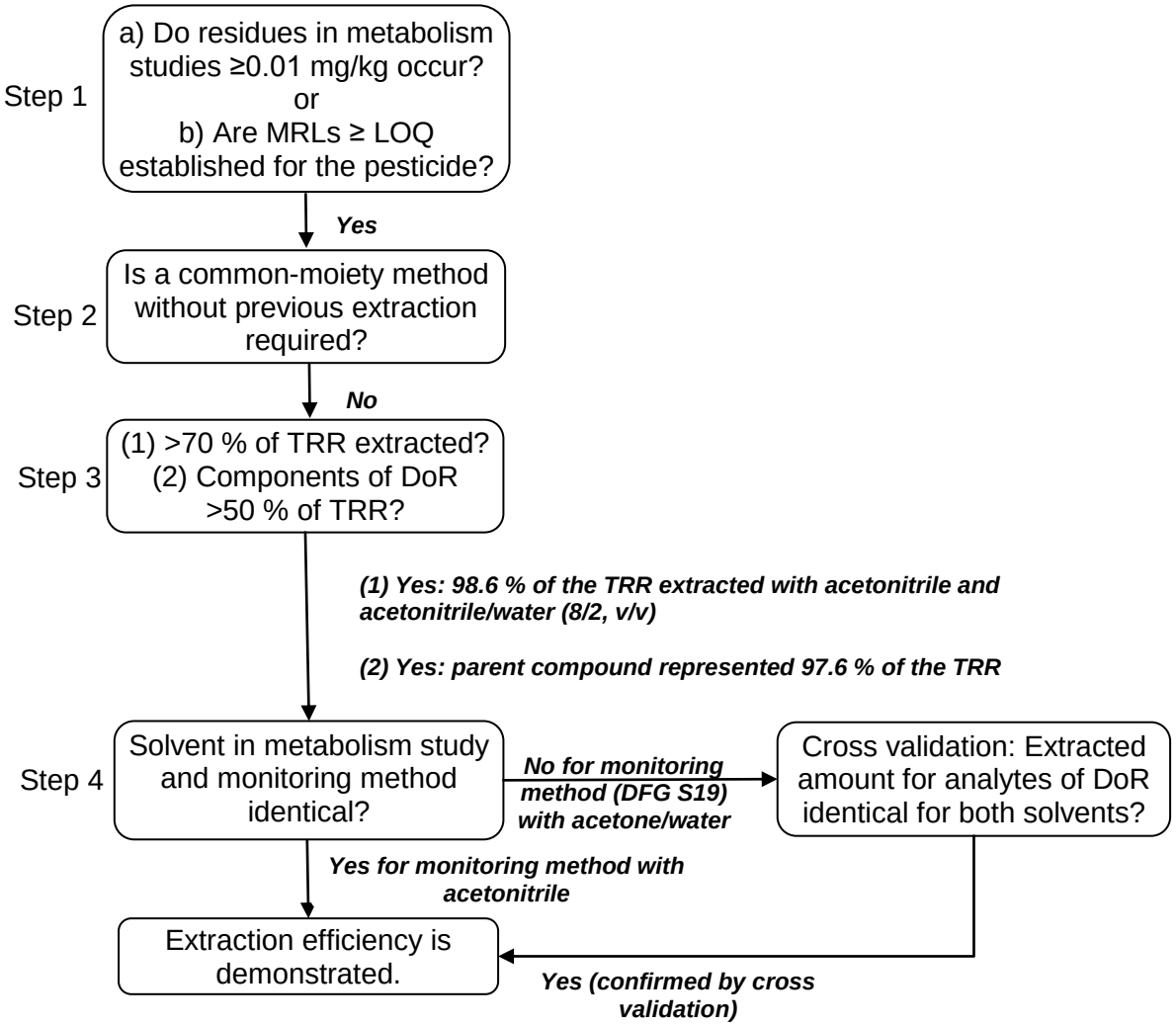

Fig. 4: Decision tree for typical case study 1

The radioactivity was measured by liquid scintillation counting (LSC) and the identification was made by HPLC, MS and TLC.

During initial surface wash of grapes 80 % of the TRR was removed by acetonitrile. The extraction steps by acetonitrile/water recovered additionally 18.6 % of the TRR. The major compound in the combined organic extracts (97.6 % of the TRR) was the parent compound. Only 1.4 % of the TRR of grapes was not extractable.

Because the results comply with both criteria of step 3, the efficiency of acetonitrile/water (8/2, v/v) as extraction solvent is considered as being proven.

For MRL enforcement and monitoring a method with a different solvent was proposed. This method is based on the extraction procedure according to DFG S19 method using acetone/water (2/1, v/v) as the extraction solvent.

In a separate cross-validation study the extraction of incurred (aged) residues in grapes using the solvent of the metabolism study (acetonitrile/water (8/2, v/v) was compared to the residues extracted by acetone/water (2/1, v/v) according to DFG S19 method. The residues of pesticide A in grape samples were quantified by LC-MS/MS. Using the solvent of the metabolism study the residue of pesticide A was 0.219 mg/kg. After extraction according to DFG S19 method with acetone/water (2/1, v/v) the residue of pesticide A was 0.198 mg/kg. The extraction efficiency expressed as ratio of extracted pesticide A using the acetone/water method and extracted pesticide A using the acetonitrile/water method is 0.198 mg/kg / 0.219 mg/kg *100 % = 90 %. Consequently, the results of the cross-validation study confirm the suitability of the extraction solvent of the DFG S19 method for high acid commodities (e.g. grapes). This conclusion can also be extrapolated to high water content commodities (e.g. tomatoes).

Example 2: Both criteria of step 3 of the decision tree are not fulfilled**.**

Pesticide B is used as an insecticide in a variety of plant commodities.

The residue definition for monitoring comprises the parent compound only.

In the proposed monitoring method an extraction with acetonitrile/water (1/1, v/v) according to EN 15662:2008 (QuEChERS method) is used.

The metabolism study was performed in lettuce plants with foliar application of ^{14}C-labelled pesticide B. The TRR was quantified by oxidative combustion of the milled samples. The TRR in lettuce samples corresponds to 4.39 mg/kg of pesticide B. The lettuce was homogenized and extracted with acetonitrile/water (1/1, v/v). The radioactivity was measured by LSC and the identification was made by HPLC and LC-MS/MS. Further extraction steps were performed by alkaline methanolic extraction and acidic hydrolysis Residues remaining after these steps were quantified by oxidative combustion.

The results of quantification and identification are given in the following table.

Table 1: Distribution of parent and metabolites in lettuce when dosed with ^{14}C-labelled pesticide B

Metabolite Fraction	lettuce	
	%TRR	mg/kg
Acetonitrile/water (1/1, v/v) extract	**64.5**	**2.834**
Parent compound	16.0	0.705
Metabolites 1, 2, 3, 4, 5, 6	37.6	1.652
Other compounds	10.9	0.481
Basic organic extract	**8.9**	**0.389**
Parent compound	< 0.1 %	0.001
Metabolites 1, 2, 3, 4, 5, 6	1.9	0.09
Other compounds	7.1	0.31
Acid hydrolysate	**17.1**	**0.752**
Parent compound	< 0.1 %	0.001
Metabolites 1, 2, 3, 4, 5, 6	3.4	0.15
Other compounds	13.4	0.589
Remaining residue	**9.5**	**0.418**

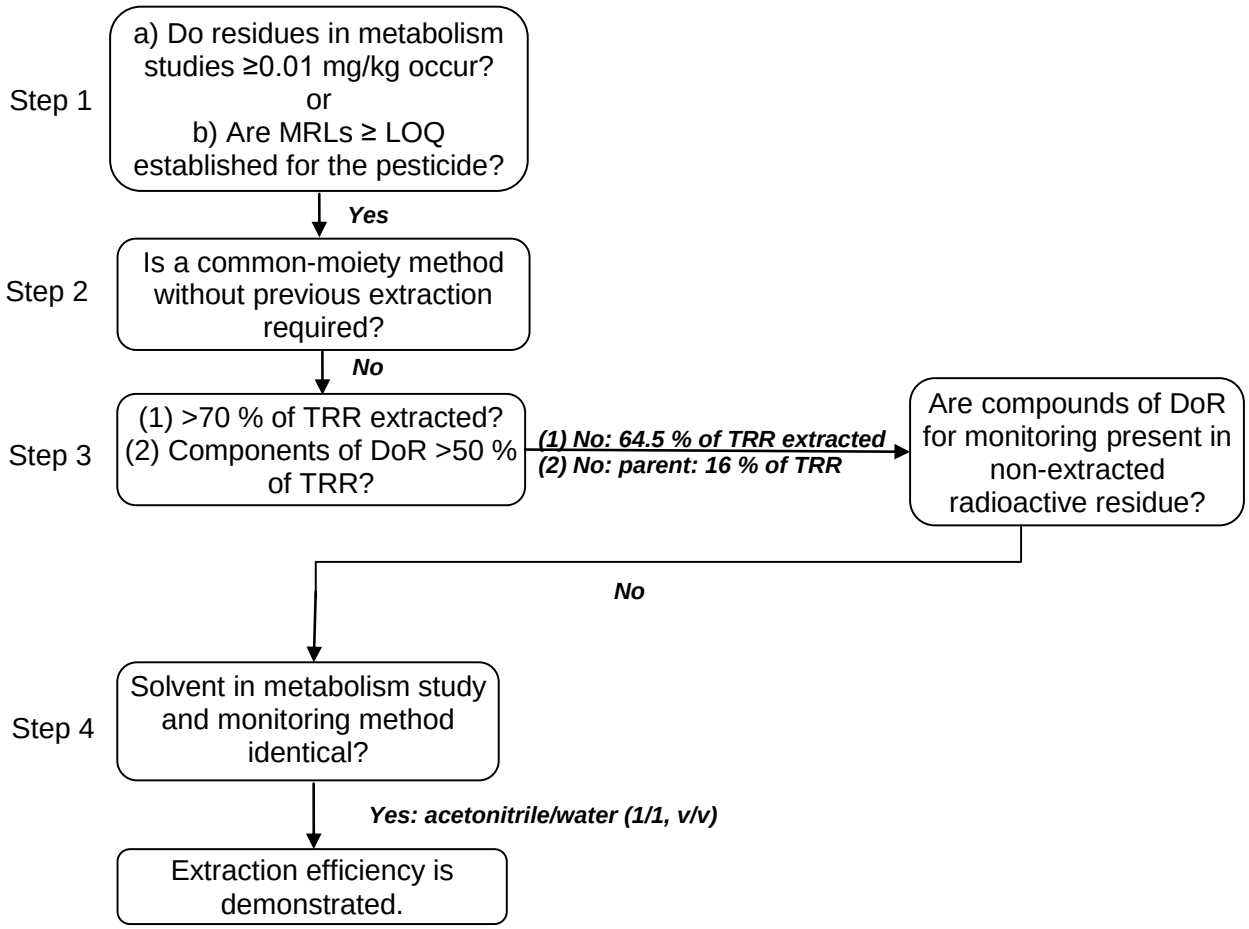

Fig. 5 Decision tree for typical case study 2

In the neutral organic extract, 2.83 mg/kg parent equivalents corresponding to 64.5 % of the TRR, were extractable. The parent compound accounted for 0.705 mg/kg or 16 % of the TRR. Six metabolites were identified in the organic extract accounting for 37.6 % of the TRR altogether. These metabolites are not part of the residue definition for monitoring. Unidentified extracted components accounted for 10.9 % of the TRR altogether. According to the stepwise approach as given in chapter 5.1 none of the criteria of step 3 was fulfilled.

Additional extraction steps using alkaline methanolic extraction followed by an acidic hydrolysis step were performed. In total 26 % of the TRR (1.141 mg/kg) were recovered during these extraction steps. No parent compound was detected in the alkaline extract or in the acidic hydrolysate. The residue remaining after hydrolysis accounted for 9.5 % of the TRR. It was confirmed that the majority of residues remaining after hydrolysis (>5 % of the TRR) was associated with lignin.

Summarizing the results of these experiments, the radioactive components in the neutral organic extract were nearly completely characterized.

Although the extracted amount of the total residue (<70 % of the TRR) and the amount of parent compound (16 % of the TRR) were quite low, the extraction efficiency by using acetonitrile/water (1/1, v/v) as the solvent is considered as being acceptable for crops with high water content.

Example 3 – Extraction efficiency is tested as part of an ideal study.

In metabolism studies wheat plants, carrots and lettuce were treated with pesticide C which readily forms metabolite D. The residue definition for monitoring comprises metabolite D only.

The results of the extraction procedure in the metabolism studies were compared to the amounts of extracted radioactive residues obtained with typical solvents of multiresidue methods. Samples of plant matrices with radiolabeled residues were collected from metabolism studies. The analytical results are given in the following tables.

Table 2: Results of extraction of radiolabeled samples using different solvents

		Metabolism study [1]		QuEChERS (solvent: acetonitrile)		DFG S19 (solvent: acetone/water, 2/1, v/v)	
	TRR [mg/kg]	ERR [2] [mg/kg] (% of TRR)	Metabolite D [mg/kg]	ERR [mg/kg] (% of TRR)	Metabolite D [mg/kg]	ERR [mg/kg] (% of TRR)	Metabolite D [mg/kg]
Wheat straw	60	17.2 (29)	0.335	5.56 (9)	0.181	8.74 (15)	0.076
Carrot root	0.232	0.173 (75)	0.038	0.037 (16)	0.022	0.121 (52)	0.030
Lettuce	0.297	0.243 (82)	0.119	0.202 (68)	0.136	0.181 (61)	0.108

[1] extraction solvent: 3 x methanol followed by 2 x water

[2] ERR: extractable radioactive residue

Table 3: Extraction efficiency of metabolite D for different solvents

	QuEChERS (solvent: acetonitrile)	DFG S19 (solvent: acetone/water, 2/1, v/v)
	Extraction efficiency (%)	Extraction efficiency (%)
Wheat straw	54	23
Carrot root	58	79
Lettuce	114	91

The extracted radioactivity was quite low (29 % of the TRR) for wheat straw. The metabolite D was quantified in the extract in low amounts only (0.335 mg/kg correspond to 0.6 % of the TRR).

The extraction efficiency for solvents of the multiresidue methods is 54 % and 23 % for QuEChERS method and DFG S19 method, respectively. Therefore, both solvents are not suitable for extraction of incurred residues in straw.

The extracted radioactivity in carrot roots was higher than in straw (75 % of the TRR). Nevertheless, the metabolite D was quantified at only 16 % of the TRR with the solvent of the metabolism study.

Using solvents of the multiresidue methods, the extraction efficiency is only acceptable for DFG S19 method (79 %). For the extraction using the QuEChERS method the extraction efficiency is only 58 %. According to the proposed trigger value this extraction procedure is therefore not suitable and an underestimation of levels of incurred residues is expected.

For lettuce, the extraction efficiency for both tested multiresidue methods is higher than 70 % and therefore acceptable.

Annex 2 Collection of frequently asked questions and answers

The guidance on the evaluation of the extraction efficiency has been applied since 2017 for the assessment of the extraction procedure of residue analytical methods used for risk assessment and monitoring. Since then, practical experiences have revealed some aspects, which were not sufficiently addressed in this guidance. These issues have been discussed in various expert meetings. To support harmonized decisions, most frequent questions were compiled in this Annex and answers provided.

Question 1a: How to approach the situation if available metabolism studies do not cover all analytical matrix groups, or if differences in crop groups for metabolism and analytical matrix groups result in a matrix group not being covered? For example, the metabolism study was performed on citrus fruits and the new MRL application/ representative uses under renewal are e.g., on avocado. Citrus and avocado fall in the same metabolism group (fruits), but not in the same analytical method matrix group (high acid vs high oil content).

Question 1b: According to the guidance document, it is desirable that extraction efficiency is proven for matrices, which are difficult to analyze. How to deal with these matrices when samples with radiolabeled incurred residues are not available?

Answer: Both questions refer to the general problem that an appropriate metabolism study is not available for the corresponding analytical sample matrix. In this case, the extraction efficiency cannot be evaluated according to the procedures outlined in Fig. 3 and Fig. 5 of the guidance. The following options are proposed to approach this problem:

Option 1:

- This option should be used if metabolism studies are available for at least three analytical matrix groups and the metabolic pathway is identical.
- An indirect cross-validation with samples of the missing sample matrix containing incurred residues from trials or monitoring samples should be performed with (a) the extraction solvent(s) of the metabolism studies and (b) different extraction solvents suitable for the residue analysis for the sample matrix in question. Extraction efficiency will be considered as being sufficiently proven if the extracted amounts of the analyte of interest differ by no more than 30% (for residues >0.01 mg/kg). Using this option is connected to some uncertainties, as the metabolic pathway in the (analytical) sample is assumed to be identical to the one in known metabolism studies.

Option 2:

- This option should be used if option 1 is not applicable, e.g. if less than three metabolism studies are available and/or the metabolic pathways differ or the extraction procedure is not applicable for routine residue analysis.
- It is proposed to compare the extracted amount from samples with incurred residues from field trials or monitoring samples by using at least three different extraction solvents (preferred for multi-residue methods). This procedure results in a relative extractability, demonstrating which solvent is the most suitable one for extraction of incurred residues. The uncertainty connected to this option is the lack of reference values from samples with

radiolabeled incurred residues. The results will only provide a ranking of the most suitable extraction solvents.

If none of the two options is applicable due to analytical problems (e.g. low procedural recoveries), a justification should be provided.

Question 2: An applicant has no proprietary rights for a metabolism study. How to proceed?

Answer: If the information on the extraction efficiency of a certain matrix group/solvent combination or the solvent used in a metabolism study etc. is publicly available (e.g. draft (re)assessment report), the applicant can refer to this information. In case the provided information on solvents and matrix groups matches those used by the applicant, no further testing is required. In case no information on extraction solvents used in the metabolism study and in residue analytical methods is available, it is recommended to approximate extraction efficiency by performing an indirect cross validation with samples containing incurred residues from field trials or monitoring samples and testing different solvents.

Question 3: The extraction solvent of the monitoring method for fatty matrices differs from the solvent used in the metabolism study, but there are no uses on fatty matrices. How to proceed?

Answer: The evaluation of extraction efficiency is not required for sample matrices not covered by the intended/registered uses.

Question 4: The residue definition includes metabolites, which are not covered by the metabolism studies (e.g. formed during processing). How should extraction efficiency be evaluated in such a case?

Answer: In this case, direct testing of samples with incurred radiolabeled analytes is not possible. An indirect testing of samples containing incurred residues with different solvent systems of (multi)-residue methods should be performed instead. This procedure results in a relative extractability, demonstrating which solvents are the most suitable ones for extraction of the metabolite in question from incurred residues The procedure might be not successful for polar metabolites, which are only extractable by polar solvent systems or for compounds, which are only extractable in a special pH-range. A detailed justification should be provided.

Question 5: Is the evaluation of extraction efficiency necessary for analytical methods for honey?

Answer: Honey is considered as a relatively simple matrix without possible incorporation or residues bound to matrix components. No separate studies to address the extraction efficiency in honey are needed.