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Helsinki, 02 June 2023

Addressees
Registrants of JS_2459-10-1 as listed in Appendix 3 of this decision

Date of submission of the dossier subject to this decision
15/02/2019

Registered substance subject to this decision ("the Substance")
Substance name: trimethyl benzene-1,2,4-tricarboxylate
EC number/List number: 219-547-3

Decision number: Please refer to the REACH-IT message which delivered this communication (in format CCH-D-XXXXXXXXXX-XX-XX/F)

DECISION ON A COMPLIANCE CHECK

Under Article 41 of Regulation (EC) No 1907/2006 (REACH), you must submit the information listed below by ***9 March 2026***.

Requested information must be generated using the Substance unless otherwise specified.

Information required from all the Registrants subject to Annex VII of REACH

1. *In vivo* mammalian alkaline comet assay combined with *in vivo* mammalian erythrocyte micronucleus test, also requested below (triggered by Annex VII, Section 8.4., Column 2)

Information required from all the Registrants subject to Annex VIII of REACH

2. *In vivo* mammalian alkaline comet assay (triggered by Annex VIII, Section 8.4., Column 2; test method: OECD TG 489) combined with *in vivo* mammalian erythrocyte micronucleus test (test method: OECD TG 474) in rats, or if justified, in mice, oral route. For the comet assay the following tissues shall be analysed: liver, glandular stomach and duodenum. For the micronucleus test:
    - the aneugenic potential of the Substance must be assessed by using a centromere staining technique if the Substance induces an increase in the frequency of micronuclei in the OECD TG 474;
    - target tissue exposure must be demonstrated if the result of the OECD TG 474 is negative

The reasons for the request(s) are explained in Appendix 1.

Information required depends on your tonnage band

You must provide the information listed above for all REACH Annexes applicable to you in accordance with Articles 10(a) and 12(1) of REACH. The addressees of the decision and their corresponding information requirements based on registered tonnage band are listed in Appendix 3.

In the requests above, the same study has been requested under different Annexes. This is because some information requirements may be triggered at lower tonnage band(s). In such cases, only the reasons why the information requirement is triggered are provided

for the lower tonnage band(s). For the highest tonnage band, the reasons why the standard information requirement is not met and the specification of the study design are provided. Only one study is to be conducted; all registrants concerned must make every effort to reach an agreement as to who is to carry out the study on behalf of the others under Article 53 of REACH.

You are only required to share the costs of information that you must submit to fulfil your information requirements.

How to comply with your information requirements

To comply with your information requirements, you must submit the information requested by this decision in an updated registration dossier by the deadline indicated above. You must also **update the chemical safety report, where** relevant, including any changes to classification and labelling, based on the newly generated information.

You must follow the general requirements for testing and reporting new tests under REACH, see Appendix 4.

Appeal

This decision, when adopted under Article 51 of REACH, may be appealed to the Board of Appeal of ECHA within three months of its notification to you. Please refer to http://echa.europa.eu/regulations/appeals for further information.

Failure to comply

If you do not comply with the information required by this decision by the deadline indicated above, ECHA will notify the enforcement authorities of your Member State.

Authorised[1] under the authority of Mike Rasenberg, Director of Hazard Assessment

Appendix 1: Reasons for the request(s)
Appendix 2: Procedure
Appendix 3: Addressees of the decision and their individual information requirements
Appendix 4: Conducting and reporting new tests under REACH

[1] As this is an electronic document, it is not physically signed. This communication has been approved according to ECHA's internal decision-approval process.

Appendix 1: Reasons for the request(s)

Contents

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Reasons related to the information under Annex VII of REACH

1. *In vivo* mammalian alkaline comet assay combined with *in vivo* mammalian erythrocyte micronucleus test

1 Under Annex VII, Section 8.4., Column 2, an appropriate *in vivo* mammalian somatic cell genotoxicity study as referred to in Annex IX, point 8.4.4, must be performed in case of a positive result in any of the *in vitro* studies referred to in Annex VII, Section 8.4. The *in vivo* study must address the concerns raised by the *in vitro* study results, i.e. the chromosomal aberration concern or the gene mutation concern or both, as appropriate.

1.1. Triggering of the information requirement

2 Your dossier contains positive results for the *in vitro* gene mutation study in bacteria (key study, 2014) and *in vitro* cytogenicity test (key study, 2007) and *in vitro* gene mutation study in mammalian cells (key study, 2007) which raise the concerns for gene mutations and chromosomal aberrations.

3 Therefore, the information requirement is triggered.

4 The information provided, its assessment and the specifications of the study design are addressed under request 2.

Reasons related to the information under Annex VIII of REACH

2. *In vivo* mammalian alkaline comet assay combined with *in vivo* mammalian erythrocyte micronucleus test

5 Under Annex VIII, Section 8.4., Column 2, an appropriate *in vivo* mammalian somatic cell genotoxicity study as referred to in Annex IX, point 8.4, must be performed in case of a positive result in any of the *in vitro* studies referred to in Annex VII or VIII, Section 8.4. The *in vivo* study must address the concerns raised by the *in vitro* study results, i.e. the chromosomal aberration concern or the gene mutation concern or both, as appropriate.

2.1. Triggering of the information requirement

6 Your dossier contains positive results for the *in vitro* gene mutation study in bacteria (key study, 2014) and *in vitro* cytogenicity test (key study, 2007) and *in vitro* gene mutation study in mammalian cells (key study, 2007) which raise the concerns for gene mutations and chromosomal aberrations.

7 Therefore, the information requirement is triggered.

2.2. Information provided

8 You have provided:

(i) an *in vivo* mammalian erythrocyte micronucleus test with the Substance.

(ii) an *in vivo* unscheduled DNA synthesis study with the Substance.

2.3. Assessment of the information provided

2.3.1. The provided study (i) does not meet the specifications of the test guideline(s)

9 To be considered adequate, study (i) has to meet the requirements of OECD TG 474. Therefore, the following specifications required by the OECD TG 474 must be met:

a) the study includes a minimum of three dose level groups of treated animals, as well as a negative control group and a positive control group;

b) each group includes a minimum of 5 analysable animals;

c) the proportion of immature erythrocytes among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 500 erythrocytes for bone marrow and 2000 erythrocytes for peripheral blood;

d) at least 4000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes;

e) the proportion of immature erythrocytes among total (immature + mature) erythrocytes and the mean number of micronucleated immature erythrocytes are reported for each group of animals;

f) a clear negative outcome is concluded when the data available shows that bone marrow exposure to the Substance or its metabolite(s) occurred.

10 The study (i) is described as *in vivo* Mammalian Erythrocyte Micronucleus Test (equivalent to OECD Guideline 474). However, the following specifications are missing:

a) the number of groups of treated animals and the negative and positive control groups are not reported;

b) the number of animals per group is not reported;

c) the number of immature and mature erythrocytes scored per animal for determining the proportion of immature erythrocytes among total (immature + mature) erythrocytes for each animal is not reported;

d) the number of immature erythrocytes scored per animal for determining the incidence of micronucleated immature erythrocytes is not reported;

e) the proportion of immature erythrocytes among total (immature + mature) erythrocytes and the mean number of micronucleated immature erythrocytes are not reported for each group of animals;

f) you did not demonstrate that bone marrow exposure to the Substance, or its metabolite(s), occurred;

11 The information provided does not cover the specifications required by the OECD TG 474.

12 Therefore, study (i) cannot be used to address the chromosomal aberration concern identified *in vitro* and the information requirement is not fulfilled.

2.3.1. Study (ii) not adequate for the information requirement

13 The study (ii) is described as a UDS test. This study is not an *in vivo* somatic cell genotoxicity study addressing concerns for gene mutations.

14 This is an indicator test that detects some DNA repair mechanisms (measured as unscheduled DNA synthesis in liver cells). However, as reminded in the Guidance on IRs & CSA, R.7a, Section R.7.7.6.3 (page 571-572), the UDS test is sensitive to some (but not all) DNA repair mechanisms and not all gene mutagens are positive in the UDS test. The sensitivity of the UDS test has been questioned (Kirkland and Speit, 2008 [1]) and its lower predictive value towards rodent carcinogens and/or *in vivo* genotoxicants has been confirmed in comparison with the TGR assay and comet assay (EFSA, 2017 [2]). Therefore, a negative result in a UDS assay alone is not a proof that a substance does not induce gene mutation. Moreover, though a positive result in the UDS assay can indicate exposure of the liver DNA and induction of DNA damage by the substance under investigation, it is not sufficient information to conclude on the induction of gene mutation by the substance.

[1] Kirkland D and Speit G (2008) Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*. Mutat Res 654:114-32.

[2] EFSA Scientific Committee, Hardy A, Benford D, Halldorsson T, Jeger M, Knutsen HK, More S, Naegeli H, Noteborn H, Ockleford C, Ricci A, Rychen G, Silano V, Solecki R, Turck D, Younes M, Aquilina G, Crebelli R, Gurtler R, Hirsch-Ernst KI, Mosesso P, Nielsen E, van Benthem J, Carfì M, Georgiadis N, Maurici D, Parra Morte J and Schlatter J, 2017. Scientific Opinion on the clarification of some aspects related to genotoxicity assessment. EFSA Journal 2017;15(12):5113, 25 pp. https://doi. org/10.2903/j.efsa.2017.5113.

15 Based on the above, the study is not adequate for addressing the gene mutation concern identified *in vitro*.

16 Therefore, the information requirement is not fulfilled.

17 ECHA considers that an appropriate *in vivo* follow-up genetic toxicity study is necessary to address the concerns identified *in vitro*.

18 In your comments to the draft decision, you indicate your intention to collect the full study report of the *in vivo* studies (i) and (ii) and to evaluate the need to perform the *in vivo*

mammalian alkaline comet assay combined with *in vivo* mammalian erythrocyte micronucleus test.

19 However, the *in vivo* study must address the concerns raised by the *in vitro* study results, *i.e.* the chromosomal aberration concern or the gene mutation concern or both, as appropriate. In the case where you would be able to submit valid robust study summaries, study (i) would only address the concern of the *in vitro* cytogenicity study and study (ii) would not address the concern of the gene mutation. Therefore, the intention you describe in your comments would in any case not resolve the concern for gene mutation necessary to consider the information provided as compliant.

2.4. Test selection

20 The positive *in vitro* results available in the dossier indicate a concern for both chromosomal aberration and gene mutation.

21 The *in vivo* mammalian erythrocyte micronucleus test ("MN test", OECD TG 474) and the *in vivo* mammalian alkaline comet assay ("comet assay", OECD TG 489) can be combined in a single study (see OECD TG 474 paragraph 37c; OECD TG 489 paragraph 33; Guidance on IRs & CSA, Section R.7.7.6.3). While the MN test can detect both structural chromosomal aberrations (clastogenicity) and numerical chromosomal aberrations (aneuploidy), the comet assay can detect primary DNA damage that may lead to gene mutations and/or structural chromosomal aberrations. A combined study will thus address both the identified concerns for chromosomal aberration as well as gene mutation.

22 The combined study, together with the results of the *in vitro* mutagenicity studies, can be used to make definitive conclusions about the mechanism(s) inducing *in vivo* mutagenicity and lack thereof. Furthermore, the combined study can help reduce the number of tests performed and the number of animals used while addressing (structural and numerical) chromosomal aberrations as well as gene mutations.

23 Therefore, the comet assay combined with the MN test is the most appropriate study for the Substance.

2.5. Specification of the study design

24 According to the test method OECD TG 489, rats are the preferred species. Other rodent species can be used if scientifically justified. According to the test method OECD TG 474, the test may be performed in mice or rats. Therefore, the combined study must be performed in rats, or if justified, in mice.

25 Having considered the anticipated routes of human exposure and adequate exposure of the target tissue(s) performance of the test by the oral route is appropriate.

26 In line with the test method OECD TG 489, the test must be performed by analysing tissues from liver as primary site of xenobiotic metabolism, glandular stomach and duodenum as sites of contact. There are several expected or possible variables between the glandular stomach and the duodenum (different tissue structure and function, different pH conditions, variable physico-chemical properties and fate of the Substance, and probable different local absorption rates of the Substance and its possible breakdown product(s)). In light of these expected or possible variables, it is necessary to analyse both tissues to ensure a sufficient evaluation of the potential for genotoxicity at the site of contact in the gastro-intestinal tract.

27 According to the test method OECD TG 474, in order to demonstrate the ability of the study to identify clastogens and aneugens, you must include two concurrent positive controls, one known clastogen and one known aneugen (OECD TG 474, paragraph 25, Table 1).

28 The combination of the OECD TGs 489 and 474 should not impair the validity of and the results from each individual study. Careful consideration should be given to the dosing, and tissue sampling for the comet analysis alongside the requirements of tissue sampling for the mammalian erythrocyte micronucleus test (see OECD TG 489, e.g. Bowen et al. 2011 [1]).

[1] Bowen DE *et al.* (2011) Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the comet assay and the flow-cytometric peripheral blood micronucleus test. *Muta Res*;722:7–19.

2.5.1. Assessment of aneugenicity potential

29 If the result of the *in vivo* MN test is positive, i.e. your Substance induces an increase in the frequency of micronuclei, you must assess the aneugenic potential of the Substance. In line with the OECD TG 474 (paragraph 42), you should use one of the centromere labelling or hybridisation procedures to determine whether the increase in the number of micronuclei is the result of clastogenic events (i.e. micronuclei contain chromosome fragment(s)) and/or aneugenic events (i.e. micronuclei contain whole chromosome(s)).

2.5.2. Investigation of target tissue exposure

30 The applicable test method OECD TG 474 states that "If there is evidence that the test substance(s), or its metabolite(s), will not reach the target tissue, it may not be appropriate to use this test". Additionally, a negative test result can be considered reliable only if "Bone marrow exposure to the test substance(s) occurred".

31 Therefore, to ensure that the data generated are adequate for hazard identification, you must take blood samples at appropriate times and measure plasma levels of the Substance and/or its metabolites (OECD TG 474, paragraph 40), unless exposure of the bone marrow can be demonstrated through other means, e.g. by showing a depression of immature to mature erythrocyte ratio (OECD TG 474, paragraph 48).

32 If the Substance is negative in this test, but it is not possible to demonstrate that bone marrow exposure to the Substance occurred, then ECHA will consider any remaining uncertainty concerning the mutagenic potential of the Substance and whether to request any further information.

2.5.3. Germ cells

33 You may consider collecting the male gonadal cells from the seminiferous tubules in addition to the other aforementioned tissues in the comet assay, as it would optimise the use of animals. You can prepare the slides for male gonadal cells and store them for up to 2 months, at room temperature, in dry conditions and protected from light. Following the generation and analysis of data on somatic cells in the comet assay, you should consider analysing the slides prepared with gonadal cells.

34 This type of evidence may be relevant for the overall assessment of possible germ cell mutagenicity including classification and labelling according to the CLP Regulation.

2.5.4. Cross-linking properties

35 You are reminded that you may decide to take into account the potential cross-linking properties of the Substance in the experimental setup of the comet assay and perform a modified comet assay in order to detect cross links. Therefore, you may consider preparing and analysing two sets of slides: one set of slides submitted to the standard experimental conditions (as described in the OECD TG 489); the other set of slides submitted to modified experimental conditions that enable the detection of DNA crosslinks. The modified experimental conditions may utilise one of the following options: (1) increase of

electrophoresis time, e.g. as described in reference 23 [2] in the OECD TG 489; (2) treatment of isolated cells (either in suspension or embedded in the slides) with a chemical (e.g. MMS); or (3) treatment of isolated cells (either in suspension or embedded in the slides) with ionising radiation (options 2 and 3 are described e.g. in references 36-39 [3-6] in the OECD TG 489 or Pant et al. 2015 [7]). In order to ensure the robustness of the test result a specific positive control group of animals would be needed.

[2] Nesslany et al. (2007) in vivo comet assay on isolated kidney cells to distinguish genotoxic carcinogens from epigenetic carcinogens or cytotoxic compounds. Muta Res;630(1-2):28-41.
[3] Merk and Speit (1999) Detection of crosslinks with the comet assay in relationship to genotoxicity and cytotoxicity. *Environ Mol Mutagen*;33(2):167-72.
[4] Pfuhler and Wolf (1996) Detection of DNA-crosslinking agents with the alkaline comet assay. *Environ Mol Mutagen*;27(3):196-201.
[5] Wu and Jones (2012) Assessment of DNA interstrand crosslinks using the modified alkaline comet assay. *Methods Mol Biol*;817:165-81.
[6] Spanswick *et al.* (2010) Measurement of DNA interstrand crosslinking in individual cells using the Single Cell Gel Electrophoresis (Comet) assay. *Methods Mol Biol*;613:267-282.
[7] Pant K et al. (2015) Modified in vivo comet assay detects the genotoxic potential of 14-hydroxycodeinone, an α,β; -unsaturated ketone in oxycodone. Environ Mol Mutagen;56(9):777-87.

References

The following documents may have been cited in the decision.

Guidance on information requirements and chemical safety assessment (Guidance on IRs & CSA)

Chapter R.4 Evaluation of available information; ECHA (2011).
Chapter R.6 QSARs, read-across and grouping; ECHA (2008).
Appendix to Chapter R.6 for nanoforms; ECHA (2019).
Chapter R.7a Endpoint specific guidance, Sections R.7.1 – R.7.7; ECHA (2017).
Appendix to Chapter R.7a for nanomaterials; ECHA (2017).
Chapter R.7b Endpoint specific guidance, Sections R.7.8 – R.7.9; ECHA (2017).
Appendix to Chapter R.7b for nanomaterials; ECHA (2017).
Chapter R.7c Endpoint specific guidance, Sections R.7.10 – R.7.13; ECHA (2017).
Appendix to Chapter R.7a for nanomaterials; ECHA (2017).
Appendix R.7.13-2 Environmental risk assessment for metals and metal compounds; ECHA (2008).
Chapter R.11 PBT/vPvB assessment; ECHA (2017).
Chapter R.16 Environmental exposure assessment; ECHA (2016).

Guidance on data-sharing; ECHA (2017).
Guidance for monomers and polymers; ECHA (2012).
Guidance on intermediates; ECHA (2010).
All guidance documents are available online: https://echa.europa.eu/guidance-documents/guidance-on-reach

Read-across assessment framework (RAAF)

RAAF, 2017 Read-across assessment framework (RAAF); ECHA (2017).
RAAF UVCB, 2017 Read-across assessment framework (RAAF) – considerations on multi- constituent substances and UVCBs; ECHA (2017).

The RAAF and related documents are available online:
https://echa.europa.eu/support/registration/how-to-avoid-unnecessary-testing-on-animals/grouping-of-substances-and-read-across

***OECD Guidance documents* (OECD GDs)**

OECD GD 23 Guidance document on aquatic toxicity testing of difficult substances and mixtures; No. 23 in the OECD series on testing and assessment, OECD (2019).
OECD GD 29 Guidance document on transformation/dissolution of metals and metal compounds in aqueous media; No. 29 in the OECD series on testing and assessment, OECD (2002).
OECD GD 150 Revised guidance document 150 on standardised test guidelines for evaluating chemicals for endocrine disruption; No. 150 in the OECD series on testing and assessment, OECD (2018).
OECD GD 151 Guidance document supporting OECD test guideline 443 on the extended one-generation reproductive toxicity test; No. 151 in the OECD series on testing and assessment, OECD (2013).

Appendix 2: Procedure

This decision does not prevent ECHA from initiating further compliance checks at a later stage on the registrations present.

ECHA followed the procedure detailed in Articles 50 and 51 of REACH.

The compliance check was initiated on 14 March 2022.

The deadline of the decision is set based on standard practice for carrying out OECD TG tests. It has been exceptionally extended by 12 months from the standard deadline granted by ECHA to take into account currently longer lead times in contract research organisations.

ECHA notified you of the draft decision and invited you to provide comments.

ECHA took into account your comments and did not amend the request.

In your comments on the draft decision, you requested an extension of the deadline to provide information from 30 to 36 months from the date of adoption of the decision. You provided an extract of an email exchange with one CRO. In his email, the CRO states that, based on current capacities, the in-life (range finder) can start *c.a.* 4 weeks upon the test item receipt and that the whole study from signature of study plan to draft report will take *c.a.* 7 months. The CRO explains that the extended setup (3 organs comet plus bone marrow) is expected to be available in 2024. ECHA therefore considers that it should be possible to provide the information requested in this decision by the set deadline.On this basis, ECHA has not modified the deadline to provide the information.

ECHA notified the draft decision to the competent authorities of the Member States for proposals for amendment.

As no amendments were proposed, ECHA adopted the decision under Article 51(3) of REACH.

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Appendix 3: Addressee(s) of this decision and their corresponding information requirements

In accordance with Articles 10(a) and 12(1) of REACH, the information requirements for individual registrations are defined as follows:

- the information specified in Annex VII to REACH, for registration at 1-10 tonnes per year (tpa), or as a transported isolated intermediate in quantity above 1000 tpa;
- the information specified in Annexes VII and VIII to REACH, for registration at 10-100 tpa;

Registrant Name	Registration number	Highest REACH Annex applicable to you
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

Where applicable, the name of a third party representative (TPR) may be displayed in the list of recipients whereas ECHA will send the decision to the actual registrant.

Appendix 4: Conducting and reporting new tests for REACH purposes

1. Requirements when conducting and reporting new tests for REACH purposes

1.1. Test methods, GLP requirements and reporting

(1) Under Article 13(3) of REACH, all new data generated as a result of this decision must be conducted according to the test methods laid down in a European Commission Regulation or to international test methods recognised by the Commission or ECHA as being appropriate.

(2) Under Article 13(4) of REACH, ecotoxicological and toxicological tests and analyses must be carried out according to the GLP principles (Directive 2004/10/EC) or other international standards recognised by the Commission or ECHA.

(3) Under Article 10(a)(vi) and (vii) of REACH, all new data generated as a result of this decision must be reported as study summaries, or as robust study summaries, if required under Annex I of REACH. See ECHA Practical Guide on How to report robust study summaries[2].

(4) Under the introductory part of Annexes VII/VIII/IX/X to REACH, where a test method offers flexibility in the study design, for example in relation to the choice of dose levels or concentrations, the chosen study design must ensure that the data generated are adequate for hazard identification and risk assessment.

1.2. Test material

Before generating new data, you must agree within the joint submission on the chemical composition of the material to be tested (Test Material) which must be relevant for all the registrants of the Substance.

(1) Selection of the Test material(s)

The Test Material used to generate the new data must be selected taking into account the following:

- the variation in compositions reported by all members of the joint submission,
- the boundary composition(s) of the Substance,
- the impact of each constituent/ impurity on the test results for the endpoint to be assessed. For example, if a constituent/ impurity of the Substance is known to have an impact on (eco)toxicity, the selected Test Material must contain that constituent/ impurity.

(2) Information on the Test Material needed in the updated dossier

- You must report the composition of the Test Material selected for each study, under the "Test material information" section, for each respective endpoint study record in IUCLID.
- The reported composition must include all constituents of each Test Material and their concentration values

With that detailed information, ECHA can confirm whether the Test Material is relevant for the Substance and whether it is suitable for use by all members of the joint submission.

[2] https://echa.europa.eu/practical-guides

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Technical instructions on how to report the above is available in the manual on How to prepare registration and PPORD dossiers (https://echa.europa.eu/manuals).