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Front Office Food and Product Safety

Assessment of the toxicity of citrinin

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Summary

Citrinin is a mycotoxin and usually formed by fungi during storage of food. It is mainly found in grain but it can also be found in other products of plant origin like beans, fruits, vegetables and fruit juices, herbs and spices and tainted dairy products. In a recent biomonitoring study of multiple mycotoxins in the Belgian population low concentrations of citrinin were frequently detected.
The Netherlands Food and Consumer Product Safety Authority (NVWA) monitors the occurrence of mycotoxins in the Netherlands and advises the Dutch government on the food safety risks related to mycotoxins.
In 2012 the European Food Safety Authority (EFSA) has published an opinion where it was reported that due to limitations and uncertainties in the toxicity database (especially genotoxicity and carcinogenicity), the derivation of a health-based guidance value that would cover all possible adverse outcomes of citrinin was not considered appropriate.
Nonetheless, a health-based guidance value was set on the highest dosage of a 90-day general toxicity study (not covering specific endpoints, for example carcinogenicity, developmental effects) because no effects were reported.
In this report a new literature search was performed to find out whether new toxicity studies have been published (2011 to 2015). And secondly, if new toxicity studies could be used to derive a benchmark dose or a health-based guidance value.
The literature search produced 38 new toxicity articles on citrinin, where seven of these studies contained in vivo animal tests. Two of the seven studies were suitable for BMD analysis. The lowest BMDL of 48 µg/kg bw/day obtained from the endpoint 'decreased crown rump length' from the Singh study is considered as the appropriate point of departure for risk assessment. This BMDL is 2.4 times higher than the (conservative estimate of the) NOAEL determined by EFSA in 2012. There are no new scientific articles available on the in vivo genotoxicity or carcinogenicity of citrinin. A re-evaluation of an article published in 1983 on the

tumorigenicity of citrinin in rats revealed that the study was not suitable for BMD analysis. Therefore, we agree with EFSA's concern regarding the genotoxicity and/or carcinogenicity of citrinin and EFSA's request for a well-designed toxicological study in laboratory animals to further explore the carcinogenic potential of citrinin.

Keywords: mycotoxin, citrinin, health-based guidance value, benchmark dose (BMD), literature search, toxicity.

Background of the request of NVWA

In a recent biomonitoring study of multiple mycotoxins in the Belgian population three mycotoxins were detected in more than 50% of the urine samples of 394 volunteers (Heyndrickx et al., 2015). Besides deoxynivalenol (DON) and ochratoxin A (OTA), that were anticipated to be present in urine on the basis of intake and kinetic characteristics, also low concentrations of citrinin were frequently detected. Urine samples of 72% of the children (n=155) and 59% of the adults (n=239) contained citrinin, at maximum concentrations of 416 pg/ml (children) and 1494 pg/ml (adults).

In a mycotoxin-dedicated total diet study, carried out in the Netherlands in 2013/2014, citrinin was not detected in the food products examined (Lopez et al., 2015). Based on these results it has been decided to investigate whether such a high percentage of urine samples positive for citrinin could be found in the Dutch population. Therefore, urine samples collected among adults in a Dutch cohort (Doetinchem study) will be analysed for the presence of citrinin in 2016.

Another emerging issue regarding citrinin is the presence of citrinin in food supplements based on 'red yeast rice' (RYR). A maximum limit (ML) of 2000 µg citrinin/kg RYR supplement has been set and this ML will be reviewed in the coming year(s) (EU, 2014). Recent publications indicate that concentrations above the ML are found (Chen et al., 2015; Liao et al., 2014; Ostry et al., 2013).

In 2012 the European Food Safety Authority (EFSA) has published an opinion on citrinin and they have suggested the use of a 'level of no concern' for the nephrotoxicity of citrinin. This 'level of no concern' is based on a study with rats. No effects were found in the study therefore the highest dosage (20 µg/kg bw/day) was used to set a level of no concern of 0.2 µg/kg bw per day. The use of such a 'level of no concern' is unusual and EFSA has explicitly stated that: "based on the available data a concern for genotoxicity and carcinogenicity could not be excluded at the level of no concern for nephrotoxicity". Due to these and other limitations and uncertainties in the database, the derivation of a health-based guidance value that would cover all possible adverse outcomes of citrinin was not considered appropriate.

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Questions posed by NVWA

1. Carry out a literature search to find out whether new toxicity studies have been published since 2011 that can be used for the derivation of a benchmark dose (BMD) or a health-based guidance value (HBGV).
2. If new toxicity studies can be found then try to derive a BMD or a HBGV.
3. Examine if there is new information on the genotoxicity or carcinogenicity of citrinin and comment, if possible, on EFSA's concern regarding the genotoxicity and/or carcinogenicity of citrinin.

Conclusion

1. The literature search produced 38 scientific articles published in the period 2011-2015. Seven of these studies contained in vivo animal tests where toxic effects of citrinin were investigated. Five studies were not included for a BMD analysis since the studies were performed with only one dosage or the route of administration was considered not relevant for oral exposure of humans. One immunotoxicity study was disregarded because the assessed parameters are not considered reliable predictors for immunotoxicity.
2. Two of the seven studies were suitable for a bench mark dose (BMD) analysis. The lowest BMDL of 48 µg/kg bw/day obtained from the endpoint 'decreased crown rump length' from the Singh study is considered as the appropriate point of departure for risk assessment. This BMDL is 2.4 times higher than the (conservative estimate of the) NOAEL determined by EFSA in 2012.
3. There are no new scientific articles available on the in vivo genotoxicity or carcinogenicity of citrinin. A re-evaluation of an article published in 1983 on the tumorigenicity of citrinin in rats revealed that the study was not suitable for BMD analysis. Therefore, we agree with EFSA's concern regarding the genotoxicity and/or carcinogenicity of citrinin and EFSA's request for a well-designed toxicological study in laboratory animals to further explore the carcinogenic potential of citrinin.

Introduction

Citrinin is a mycotoxin that is produced by different types of fungi belonging to the Aspergillus, Penicillium and Monascus genera. Citrinin is usually formed by fungi during storage. It is mainly found in grain but it can also be found in other products of plant origin like beans, fruits, vegetables and fruit juices, herbs and spices and tainted dairy products. High concentrations have been found in supplements based on 'red yeast rice' (REF). Citrinin often co-occurs with patulin and ochratoxin A because it can be produced by the same fungi (EFSA, 2012).
EFSA has assessed the toxicity of citrinin and came to the conclusion that citrinin is nephrotoxic and a no-observed-adverse-effect level (NOAEL) of 20 µg/kg body weight per day was identified from a 90-day study in male

Wistar rats (Lee et al., 2010 as cited by EFSA, 2012). In this study three concentrations were administered and no effects were observed at the highest dose tested of 20 µg/kg bw/day. Due to the limitations and uncertainties in the database, the derivation of a health-based guidance value was not considered appropriate but a 'level of no concern' for nephrotoxicity of 0.2 µg/kg bw per day was determined. However, based on the (limited) available data a concern for genotoxicity and carcinogenicity could not be excluded at the level of no concern for nephrotoxicity.

Approach

To investigate whether new articles have been published since the EFSA opinion was published in 2012 regarding the toxicity of citrinin the RIVM information specialists performed a literature search. The RIVM literature search used several databases: Medline, EMBASE, Chemical AbstractsPlus, TOXCENTER and Scopus. The search period used was from 2011 to October 2015. Only the generic search words "citrinin", "toxicity" and search words to exclude in vitro studies were used. The search produced 38 articles in total. Based on the titles and abstracts, a selection was made for those studies that contained in vivo animal tests where the toxic effects of citrinin were investigated. No studies on in vivo genotoxicity or chronic toxicity/carcinogenicity were available. The selection resulted in seven relevant short term repeated dose studies. Only two of these seven studies were considered suitable for bench mark dose (BMD) analysis (see also appendix I). The other five studies (which are presented and discussed to provide some more details in Appendix I) were not included for BMD analysis since the studies were performed with only one dosage or the route of administration was considered not relevant for oral exposure of humans. One immunotoxicity study was excluded because the assessed parameters were not considered reliable predictors for immunotoxicity (WHO, 2012). The reason for this was that it was unknown whether the observed changes in the immune organs and in serum were sufficient to impair the immune response.

Selected toxicity studies

As indicated above two studies were selected for BMD analysis. The first study from Singh et al. (2014) is a developmental toxicity study, the second study from Hayashi et al. (2012) is a 70 and 90 day toxicity study. Both studies were not performed according to GLP or OECD testing protocols.

1. In the study by Singh et al. (2014), Wistar rats were exposed to citrinin via the diet during a 10-week premating period and during mating and gestation. At the start of the study 25 animals/sex/dose were included. Four dose groups were evaluated, i.e. 0, 1, 3 or 5 mg/kg citrinin in the diet (equivalent to 0, 0.05, 0.15 and 0.25 mg/kg bw/d using a factor of 0.05[1]). After mating, 10 pregnant females per experimental group were housed in individual breeding cages until day 20 of pregnancy. Maternal

[1] Conversion factor used to estimate exposure expressed as mg/kg bw/d from dietary concentration. For rats this factor is 0.05 mg/kg bw/day per mg/kg feed (WHO/FAO, 240). For other species and for drink water, different factors are applied.

observations included number of corpora lutea, number of implants, resorptions and live and dead fetuses. In fetuses, the presence of skeletal and visceral malformations was evaluated. Maternal effects included increased water intake, dullness, rough hair coat, polyuria and reduced body weight. Mortality was not observed in the dams. Reproductive effects included reduced foetal bodyweight, reduced crown-rump length and increased number of malformations.

2. In a 70-day and a separate 90-day study performed by Hayashi et al (2012), female BALB/c mice were orally exposed via drinking water to citrinin. After 70 days exposure to 0, 1.25 and 7.5 ppm (equivalent to 0, 0.156 and 0.938 mg/kg bw/d using a factor 0.125), no effects on body weight, food consumption or clinical signs were observed. Kidney, liver and ovary were investigated for organ weight and histopathology. Except for a slight increase in relative ovary weight, no other effects on these organs were observed. After 90 days exposure to 0, 15 and 30 ppm (equivalent to 0, 1.875 and 3.75 mg/kg bw/d using a factor 0.125) reduced body weight (week 1-2) and reduced water consumption (week 5) was observed. Further, the ovary weight (relative/absolute) was increased and the relative liver weight was reduced. Evaluation of the biochemical parameters showed that alanine aminotransferase (ALT) was increased, and blood urea nitrogen (BUN) was decreased at low dose while increased at high dose as compared to control. Evaluation of the ovaries revealed an increased number of large follicles.

BMD analysis

The BMD analysis on the selected dose-response data of citrinin was performed according to the guidance of the Scientific Committee (EFSA, 2009). It should be noted that the BMDLs do not cover genotoxic and carcinogenic effects.

BMD analysis was performed for the potentially critical endpoints of citrinin, i.e. all end-points showing a trend in their dose-response and which indicated an in- or decrease larger than 5% in mean response or risk (details on the dose-response analyses of all selected endpoints are available in a supplemental material which is available upon request). For each potentially critical endpoint the BMDLs were calculated for each of the accepted models. This procedure results in two or more BMDL values per endpoint, reflecting the differences between the models used. To calculate the BMDL to be used as point of departure, the following steps were followed:

- For each endpoint, the lowest BMDL obtained from all accepted models (see tables 1 and 2) was identified.
- The lowest BMDL from all studies and all endpoints was determined, which was then the overall BMDL.

This latter overall BMDL was considered as the appropriate point of departure for further risk assessment, e.g. to derive a health-based guidance value.

Table 1.The lowest BMDL and highest BMDU[1] obtained from all accepted models from the endpoints obtained from Singh et al (2012).

Response	**Benchmark response**	**BMDL (mg/kg bw/day)**	**BMDU (mg/kg bw/day)**
Resorptions	10%**	0.081	0.24
Total gross anomalies	10%**	0.17	0.35
Wrist drop	10%**	0.17	0.35
Stretched forelimb	10%**	0.23	0.60
Total visceral anomalies	10%**	0.064	0.34
Internal hydrocephaly	10%**	0.19	0.47
Microphtalmia	10%**	0.14	4.1
Enlarged renal pelvis	10%**	0.14	0.26
Total skeletal malformations	10%**	0.092	0.21
Incomplete ossification of skull bones	10%**	0.092	0.21
Absence of sternal bones	10%**	0.16	0.33
Sacral, caudal vertebrae, failure of ossification	10%**	0.22	0.58
Malformed phalanges of fore limbs / underdeveloped	10%**	0.20	0.46
Malformed phalanges of hind limbs	10%**	0.21	0.28
Foetal bw (g)	5%*	0.059	0.076
Crown rump length (cm)	5%*	0.048	0.12

[1] Upper confidence limit of the BMD; *change in response compared to background; ** extra risk[2]

2 Extra risk is the absolute increase in risk adjusted for the background risk. In other words, the increased risk above background for a dose (d) divided by the fraction of the population not responding at the background (i.e. at dose zero). Extra risk is calculated as follows: [f(d)–f(0)] / [1–f(0)], e.g. when the risk at dose (d) is 23.5% and the background response is 15% then the extra risk is [23.5 – 15]/[100 – 15] = 10% (for a more formalistic definition see EFSA, 2009).

Table 2. The lowest BMDL and highest BMDU[1] obtained from all accepted models from the endpoints obtained from Hayashi et al (2014)

Response	**Benchmark response**	**BMDL (mg/kg bw/day)**	**BMDU (mg/kg bw/day)**	**notes**
Relative ovary weight (% of bw)	5%*	0.49	1.6	90 day only
Absolute ovary weight (mg)	5%*	0.48	1.9	90 day only
Ovary: no. of small follicles/area	5%*	0.30	2.2	90 day only
Ovary: no. of large follicles/area	5%*	0.24	0.83	90 day only
Kidney: regenerating tubules, focal	10%**	0	2.3	90 day only, infinitively wide CI, data too poor to derive PoD

[1] Upper confidence limit of the BMD; * change in response compared to background; ** extra risk[2]

The BMDL of 48 µg/kg bw/day obtained from the endpoint 'crown rump length' (CRL) from the Singh study was considered as the appropriate point of departure. CRL is the length of the fetus from the top of its head to the bottom of its torso. A decreased CRL is an indication of growth restriction. This BMDL was 2.4 times higher than the NOAEL determined by EFSA. It should be kept in mind that the NOAEL was derived from a study in which three concentrations were administered and no effects were observed at the highest dose tested of 20 µg/kg bw/day.

Genotoxicity and carcinogenicity

As mentioned before no new studies on the in vivo genotoxicity or carcinogenicity were found. Therefore, the only available in vivo tumorigenicity study was re-evaluated (Arai and Hibino, 1983). This study was considered of limited relevance for the risk assessment of citrinin. They used only male F344 rats tested at one dose group and the number of rats/group (10) exposed for 80 weeks. Moreover, only the kidneys were evaluated by microscopical examination. The kidneys showed progressive histopathological changes and high incidences of adenomas. No such changes were observed in the control group. This study was limited to a maximum period of 80 weeks, which is somewhat shorter than the normal duration of a rodent carcinogenicity study, i.e. at least 2 years (104 weeks). Hence, given the observed high incidence of adenomas it cannot be excluded that carcinomas would have occurred, had the exposure time been increased to the full length of a carcinogenicity study. Nevertheless, given the above-mentioned limitations, the study was not sufficiently informative to be used as a basis for the estimation of the carcinogenic potency of citrinin. Summarised, RIVM agrees with EFSA that well-designed toxicological

studies in laboratory animal species to further explore the carcinogenic potential of citrinin are needed.

Conclusions and answers to the specific questions

1. The literature search produced 38 scientific articles published in the period 2011-2015. Seven of these studies contained in vivo animal tests where toxic effects of citrinin were investigated. Five studies were not included for a BMD analysis since the studies were performed with only one dosage or the route of administration was considered not relevant for oral exposure of humans. One immunotoxicity study was disregarded because the assessed parameters are not considered reliable predictors for immunotoxicity.
2. Two of the seven studies were suitable for a BMD analysis. The lowest BMDL of 48 µg/kg bw/day obtained from the endpoint 'crown rump length' from the Singh study is considered as the appropriate point of departure for risk assessment. This BMDL is 2.4 times higher than the (conservative estimate of the) NOAEL determined by EFSA in 2012.
3. There are no new scientific articles available on the in vivo genotoxicity or carcinogenicity of citrinin. A re-evaluation of an article published in 1983 on the tumorigenicity of citrinin in rats revealed that the study was not suitable for BMD analysis. Therefore, we agree with EFSA's concern regarding the genotoxicity and/or carcinogenicity of citrinin and EFSA's request for a well-designed toxicological study in laboratory animals to further explore the carcinogenic potential of citrinin.

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Appendix I. Overview of relevant in vivo toxicity studies on Citrinin during 2011-2015.

- In the study by Singh et al. (2014), Wistar rats were exposed to citrinin via the diet during a 10-week premating period and during mating and gestation. At start of the study 25 animals/sex/dose were included. Four dose groups were evaluated, i.e. 0, 1, 3 or 5 mg/kg citrinin in the diet (equivalent to 0, 0.05, 0.15 or 0.25 mg/kg bw/d using a factor of 0.05). After mating, 10 pregnant females per experimental group were housed in individual breeding cages until day 20 of pregnancy. Maternal observations included number of corpora lutea, number of implants, resorptions and live and dead fetuses. In fetuses, the presence of skeletal and visceral malformations was evaluated. Maternal effects included increased water intake, dullness, rough hair coat, polyuria and reduced body weight. Mortality was not observed in the dams. Reproductive effects included reduced foetal bodyweight, reduced crown-rump length and increased number of malformations.

This study can be used for a BMD analysis.

- Kumar et al (2014) investigated the potential of citrinin to induc oxidative damage and apoptosis in kidney. New-Zealand white rabbits were exposed via the diet to citrinin (0 or 15 mg/kg feed, equivalent to 0 or 0.45 mg/kg bw/d using a factor of 0.03). Also co-exposure with OTA was tested (not further evaluated here). No effects on oxidative damage (as measured by malondialdehyde levels) was observed for citrinin exposure both after 30 as well as after 60 days of exposure. An increase in the % of apoptotic cells was however observed upon citrinin exposure (30 or 60 days) as compared to controls. Further some morphological alterations were observed in the citrinin-treated animals (proximal convoluted tubular epithelium, chromatin margination and cytoplasmic condensation in interstitial cells at day 60; folding of cell membrane, forming membrane blebs).

Given that a single dose was investigated, this study was not included for a BMD analysis.

- In a 70-day and a separate 90-day study performed by Hayashi et al (2012), female BALB/c mice were orally exposed via drinking water to citrinin. After 70 days exposure to 0, 1.25 or 7.5 ppm (equivalent to 0, 0.156 or 0.938 mg/kg bw/d using a factor 0.125), no effects on body weight, food consumption or clinical signs were observed. Kidney, liver and ovary were investigated for organ weight and histopathology. Except for a slight increase in relative ovary weight, no other effects on these organs were observed. After 90 days exposure to 0, 15 or 30 ppm (equivalent to 0, 1.875 or 3.75 mg/kg bw/d using a factor 0.125) reduced body weight (week 1-2) and reduced water consumption (week 5) was observed. Further, the ovary weight (relative/absolute) was increased and the relative liver weight was reduced. Evaluation of the biochemical parameters showed that alanine aminotransferase (ALT) was increased, and blood urea nitrogen (BUN) was decreased at low dose while increased

at high dose as compared to control. Evaluation of the ovaries revealed an increased number of large follicles.

This study can be used for a BMD analysis.

- Qingqing et al. (2012) investigated the effect of citrinin on the male reproductive system in mice. Male mice (Kunming strain) were exposed to citrinin once daily during a 7 day premating period via intraperitoneal administration in dose levels of 0, 0.0625, 0.625 or 6.25 mg/kg bw/d (as reported). Twenty-four hours after the last administration, each male was placed in an individual cage with untreated super-ovulated females of the same strain. Successfully mated females were sacrificed on GD15 and evaluated for number of foetuses. The male animals were sacrificed on the same day and evaluated for weight of reproductive organs, semen quality, sperm production and testosterone concentration. Increased relative organ weights (100% * organ weight/body weight) were observed for testis (low/mid/high dose), epididymis (mid/high), seminal vesicle (low/mid/high), preputial gland (low/mid/high). The percentage of live spermatozoa in citrinin-treated animals was significantly lower than control animals and the percentage of abnormal spermatozoa were increased at mid and high dose. Total epididymis sperm counts and serum testosterone were significantly reduced at low, mid and high dose. Females mated with citrinin-treated males showed lower pregnancy rates. No embryos were found in females mated with males exposed to the high dose citrinin.

Given that the route of administration was considered not relevant for oral exposure of humans, this study was not included for a BMD analysis.

- Singh et al. (2012) investigated the effect of citrinin on apoptosis in liver, kidney and spleen in pregnant Wistar rats and their foetuses. Pregnant femal es were treated with citrinin orally via the diet (0 or 10 mg/kg feed, equivalent to 0.5 mg/kg bw/d using a factor of 0.05) on GD 6-20. Dams were sacrificed on day 60 post-treatment, and selected organs (kidney, liver, spleen) were evaluated for apoptosis. Also foetal kidney and liver were evaluated. Degenerative changes of the kidney were observed in citrinin-treated dams. An increased percentage of apoptotic cells of kidney, liver and spleen were found in citrinin-treated dams. Evaluation of foetal kidney cells also revealed apoptosis.

Given that a single dose was investigated, this study was not included for a BMD analysis.

- Singh et al. (2011) investigated the effect of citrinin on the immune response in pregnant Wistar rats. Pregnant femal es were treated with citrinin orally via the diet (0 or 10 mg/kg feed, equivalent to 0.5 mg/kg bw/d using a factor of 0.05) on GD 6-20. A reduced humoral and cell-mediated immune response was observed in citrinin-treated dams.

Given that a single dose was investigated, this study was not included for a BMD analysis.

- Islam et al. (2012) studied the immunomodulatory effects of citrinin in mice. Mice were orally exposed for 14 days at the dose of 0, 1, 5 or 10 mg/kg body weight. Systemic effects on the immune system were assessed by measuring several immune parameters in the spleen and

serum, whereas local effects on immune system of the small intestines were evaluated in the mesenteric lymph nodes (MLN) and Peyer's Patches.
Citrinin modulated the immune cell populations in the spleen and MLN. In the spleen and MLN a dose dependent decrease of the number of macrophages and B cells was found. Citrinin increased T cells subsets in the MLN, but not in the spleen. In the Peyer's Patches the number of cytotoxic T cells was increased and the number of B cells decreased after exposure to citrinin. Furthermore, IgM antibody levels in serum were significantly lower in mice exposed to citrinin compared to control mice. Other antibodies (IgA, IgG and IgE) were not affected. In all immune organs studied, citrinin induced apoptosis in immune cells and the immunomodulatory effects induced by citrinin may be explained by this. In this study, general immune assays were used to assess immune modulation by citrinin. According to the WHO Guidance for Immunotoxicity Risk Assessment for Chemicals (WHO, 2012), the assessed parameters are not considered reliable predictors for immunotoxicity. The reason for this is that it is unknown whether the observed changes in the immune organs and in serum are sufficient to impair the immune response.

Given that the measured parameters cannot be used to derive an effect level this study was not included for a BMD analysis.

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Appendix II. BMD analysis of two studies on Citrinin

Introduction

The benchmark dose (BMD) analysis on the selected dose-response data of citrinin is performed according to the guidance of the Scientific Committee (EFSA, 2009). An overview of how to derive the appropriate lower confidence bound of the BMD (BMDL) from these dose-response data and choices to be made is provided.
The following information is provided:

A. Summary tables of the data for the endpoint(s) for which the BMD analysis is reported.
B. The value of the benchmark response (BMR) chosen, and the rationale for it.
C. The software used, including version number.
D. Settings and assumptions of the model fitting procedure.
E. An outline presenting the models used and the log-likelihoods, information on fitting and accepting models, and the BMDLs for the accepted models.
F. At least one plot of a fitted model to the data for the critical endpoint(s), including the one for the lowest BMDL.
G. Conclusion regarding the appropriate point of departure for citrinin, i.e. the selected BMDL.

These points are discussed below.

A. *Summary tables of the data for the endpoints for which the BMD analysis is reported.*
   The publications of Singh et al. (2014) and Hayashi et al. (2012) were screened on endpoints which show a trend in their dose-response relationship larger than 5% in mean response or risk. The reported data corresponding to these endpoints are copied into a spreadsheet. The obtained dose-response data from the Singh and Hayashi studies are presented in Tables 1 and 2.
   It was examined whether the available studies with only one dose group (plus a control) could be analysed in combination with other studies with one or more dose groups (using covariate analysis). However, this was not possible due to the varying experimental setups (species, route, exposure duration, etc.).
B. *The value of the BMR chosen, and the rationale for it.*
   Derivation of a reference point (i.e. BMDL) using the BMD approach requires the selection of a specified value for the benchmark response (BMR). Ideally, the BMR would reflect an effect size that is negligible or non-adverse. For continuous data the BMR is defined as a percent change in the average magnitude of the response variable as compared to the predicted background response. The recommended default value is a BMR of 5%. For quantal data the default BMR is defined as an extra

risk[3] of 10% (EFSA, 2009). These default BMRs may be modified based on toxicological considerations.
For the BMD analysis of the effects of citrinin the above defaults are applied. Deviation from the defaults is not warranted because there are no toxicological considerations to do so.

C. *The software used, including version number.*
For the analysis of the citrinin data we used PROAST version 61.2, which runs in R version 3.2.0 (www.cran.r-project.org).

D. *Settings and assumptions of the model fitting procedure*
Different models which fit the data equally well can result in different BMDLs, reflecting model uncertainty. To take this aspect of uncertainty into account, various models need to be fitted to the same dataset.
For continuous data the results of the minimal models of both the exponential family of models and the Hill family are derived (EFSA 2009). For quantal data, a set of models is applied, see EFSA (2009) for more details. Other settings are applied as described in the EFSA guidance.

E. *An outline presenting the models used and the log-likelihoods, information on fitting and accepting models, and the BMDLs for the accepted models.*
Relevant output of the model runs can be found in the supplemental material section S1 for data from Singh and section S2 for data from Hayashi. The supplemental material is available upon request.

F. *At least one plot of a fitted model to the data for the critical endpoint(s), including the one for the lowest BMDL*
For illustrative purposes only the graphs providing the lowest relevant BMDL are presented in figure 1. All other graphs can be found in the supplemental material.
The graphs show the response of crown rump length on the y-axis and the dose on the x-axis. The dose is plotted on a log-scale for better readability. The controls are plotted at an arbitrary low dose. In each dose group the small circles represent the (geometric) mean, and the whiskers show the (95%) confidence intervals of the means. Two circles are plotted in each dose group indicating the males and females. The solid line indicates the fitted exponential and Hill models. The horizontal dashed line indicates the BMR and the vertical dashed line the corresponding BMD.

G. *Conclusion regarding the appropriate point of departure, i.e. BMDL, of citrinin.*
The BMD analysis is performed for the potentially critical endpoints of citrinin. For each potentially critical endpoint the BMDLs are obtained for each of the accepted models. This procedure results in multiple BMDL values per endpoint, reflecting the differences between the models

[3] Extra risk is the absolute increase in risk adjusted for the background risk. In other words, the increased risk above background for a dose (d) divided by the fraction of the population not responding at the background (i.e. at dose zero). Extra risk is calculated as follows: [f(d)–f(0)] / [1–f(0)], e.g. when the risk at dose (d) is 23.5% and the background response is 15% then the extra risk is [23.5 – 15]/[100 – 15] = 10% (for a more formalistic definition see EFSA, 2009)Extra risk is calculated as follows: [f(d)–f(0)] / [1–f(0)], e.g. when the risk at dose (d) is 23.5% and the background response is 15% then the extra risk is [23.5 – 15]/[100 – 15] = 10% (for a more formalistic definition see EFSA, 2009)

used. To obtain the appropriate point of departure (or BMDL), the following steps are followed:

- For each endpoint, determine the lowest BMDL obtained from all accepted models (see Table 3 and 4)
- Determine the lowest BMDL from the selected endpoints, of the selected studies, which is then the overall BMDL.

This latter overall BMDL is considered as the appropriate point of departure for further risk assessment, e.g. to derive a health-based guidance value. In this particular case study with the BMDL of 0.048 mg/kg bw/day obtained from the endpoint crown rump length from the Singh study is considered as the appropriate point of departure. It should be noted that effects on the foetuses could be secondary to the maternal toxicity. However, since information on maternal effects is limited (only maternal body weight was measured), effects on foetuses was considered relevant and the BMDL on decreased crown rump length the most appropriate.

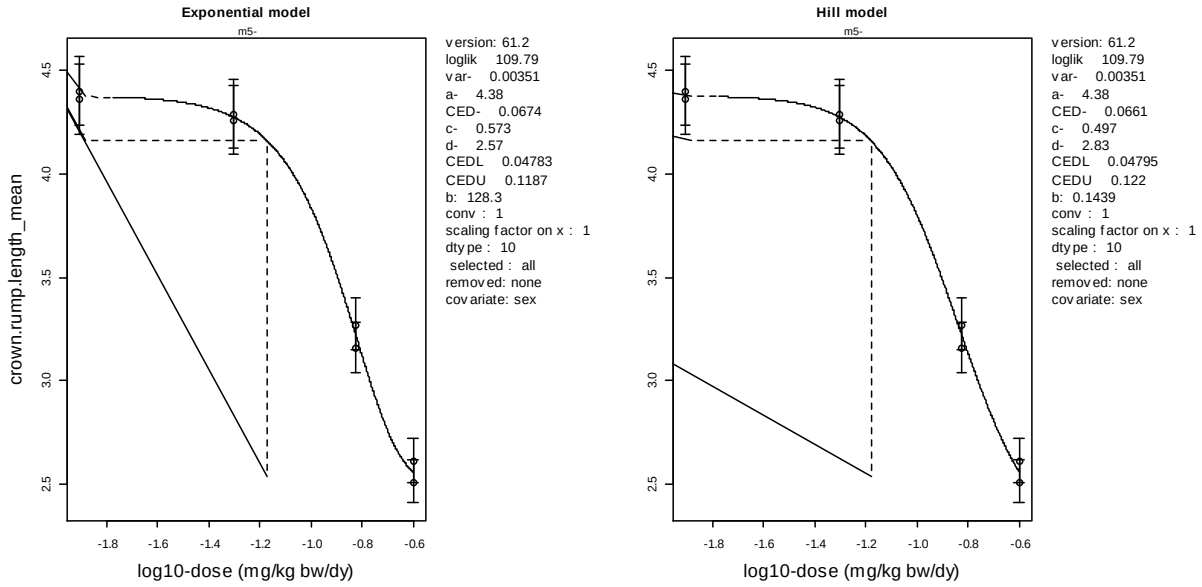

Figure 1. The response of crown rump length (cm) against the log_{10} dose. The left panel shows the optimal fitted curve of the exponential model family (E5), and the right panel shows the optimal fitted curve of the Hill model family (H5). See main text above for further description of the graphs.

Table 1: Dose-response data from Singh (2014)

Dose (mg/kg bw/d)	sex	foetal bw mean (g)	foetal bw SD	foetal bw sample size	crown rump length mean (cm)	crown rump length SD	crown rump length sample size
0	m	4.58	0.1	10	4.4	0.05	10
0.05	m	4.26	0.08	10	4.29	0.06	10
0.15	m	3.41	0.06	10	3.27	0.04	10
0.25	m	2.45	0.34	9	2.63	0.33	9
0	f	4.34	0.05	10	4.36	0.04	10
0.05	f	4.24	0.05	10	4.26	0.06	10
0.15	f	3.14	0.04	10	3.16	0.04	10
0.25	f	2.33	0.05	9	2.53	0.32	9

Table 1 continued

Dose (mg/kg bw/d)	Resorpt ions	Resorp-tions sample size	malformed foetuses total	malformed foetuses total sample size	wrist drop	wrist drop sample size	stretched forelimb	stretched forelimb sample size	malformed foetuses visceral	malformed foetuses viscera sample size
0	5	111	0	106	0	106	0	106	0	55
0.05	8	100	1	92	1	92	0	92	2	45
0.15	11	96	3	85	3	85	1	85	3	45
0.25	21	91	10	70	10	70	5	70	7	35

Table 1 continued

Dose (mg/kg bw/d)	internal hydrocephaly	internal hydrocephaly sample size	Micro-phtalmia	Micro-phtalmia sample size	enlarged renal pelvis	enlarged renal pelvis sample size	cryporchid testes	cryporchid testes sample size	malfor-med foetuses skeletal	malformed foetuses skeletal sample size
0	0	55	0	55	0	55	0	55	0	51
0.05	0	45	1	45	0	45	1	45	1	47
0.15	1	45	1	45	3	45	1	45	3	40
0.25	4	35	5	35	6	35	4	35	9	35

Table 1 continued

Dose (mg/kg bw/d)	incompl ossificat ion of the skull	incompl ossificati on of the skull sample size	absence sternal bones	absence sternal bones sample size	way outstret ched ribs	way outstret-ched ribs sample size	sacral caudal verte-brae failure of ossificati on	sacral caudal verte-brae failure of ossificati on sample size	malformed phalanges forelimbs	malformed phalanges forelimbs sample size	malformed phalanges hindlimb	malformed phalanges hindlimb sample size
0	0	51	0	51	0	51	0	51	0	51	0	51
0.05	1	47	0	47	0	47	0	47	0	47	0	47
0.15	3	40	2	40	0	40	0	40	0	40	0	40
0.25	9	35	5	35	2	35	3	35	4	35	5	35

Table 2: Dose-response data from Hayashi (2012)

Dose (mg/kg bw/d)	Duration (wk)	relative ovary weight (% body weight)	relative ovary weight sd	relative ovary weight sample size
0	70	0.034	0.007	15
0.15625	70	0.038	0.009	15
0.9375	70	0.041	0.013	15
0	90	0.035	0.009	15
1.875	90	0.042	0.008	15
3.75	90	0.044	0.007	15

Table 2 continued

Dose (mg/kg bw/d)	absolute ovary weight (mg)	absolute ovary weight sd	absolute ovary weight sample size	relative liver weight (% body weight)	relative liver weight sd	rel. liver weight sample size	Alanine aminotransferase (ALT) (IU/L)	ALT sd	ALT sample size	Aspartate amino-transfe-rase (ASAT)	ASAT sd	ASAT sample size
0	7.93	2.3	15	4.73	0.35	15	28.7	4.9	7	56.4	9.7	7
1.875	9.61	2.1	15	4.52	0.33	15	40.9	15.5	8	79.4	45.5	8
3.75	9.83	1.7	15	4.66	0.16	15	39	9.8	8	71.9	19.2	8

Table 2 continued

Dose (mg/kg bw/d)	small follicle area ovary	small follicle ovary sd	small follicle ovary sample size	large follicle area ovary	large follicle ovary sd	large follicle ovary sample size	Kidney regen. tubul. focal	Kidney regen. tubul. Focal sample size	Kidney PCNA positive tubular cells	Kidney PCNA positive tubular cells sd	Kidney PCNA positive tubular cells sample size
0	3.23	1.64	15	1.9	1.03	15	2	15	2.4	1	15
1.875	3.98	2.18	15	2.78	0.95	15	7	15	2.58	1.76	15
3.75	4.24	1.25	15	2.93	0.97	15	8	15	2.78	1.72	15

Table 3: The lowest BMDL and highest BMDU[1] obtained from all accepted models from the endpoints obtained from Singh et al (2014)

Response	**Benchmark response**	**BMDL mg/kg bw/day**	**BMDU mg/kg bw/day**
Maternal body weight (g) on GD 0	5%*	0.073	0.084
Maternal body weight (g) on GD 20	5%*	0.11	0.12
Resorptions	10%**	0.081	0.24
Total gross anomalies	10%**	0.17	0.35
Wrist drop	10%**	0.17	0.35
Stretched forelimb	10%**	0.23	0.60
Total visceral anomalies	10%**	0.064	0.34
Internal hydrocephaly	10%**	0.19	0.47
Microphtalmia	10%**	0.14	4.1
Enlarged renal pelvis	10%**	0.14	0.26
Total skeletal malformations	10%**	0.092	0.21
Incomplete ossification of skull bones	10%**	0.092	0.21
Absence of sternal bones	10%**	0.16	0.33
Sacral, caudal vertebrae, failure of ossification	10%**	0.22	0.58
Malformed phalanges of fore limbs / underdeveloped	10%**	0.20	0.46
Malformed phalanges of hind limbs	10%**	0.21	0.28
Foetal bw (g)	5%*	0.059	0.076
Crown rump length (cm)	5%*	**0.048**	0.12

[1] Upper confidence limit of the BMD

*change in response compared to background

** extra risk[3]

Table 4: The lowest BMDL and highest BMDU[1] obtained from all accepted models from the endpoints obtained from Hayashi et al (2012)

Response	**Benchmark response**	**BMDL mg/kg bw/day**	**BMDU mg/kg bw/day**	**notes**
Relative ovary weight (% of bw)	5%*	0.49	1.6	90 dy only
Absolute ovary weight (mg)	5%*	0.48	1.9	90 dy only
Ovary: no. of small follicles/area	5%*	0.30	2.2	90 dy only
Ovary: no. of large follicles/area	5%*	0.24	0.83	90 dy only
Kidney: regenerating tubules, focal	10%**	0	2.3	90 dy only, infinitively wide CI, data too poor to derive PoD

[1] Upper confidence limit of the BMD

*change in response compared to background

** extra risk[3]

References

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