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Assessment of the processing conditions which make the *Ambrosia* seeds non-viable

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Abstract

The European Commission requested EFSA to provide an assessment of the processing conditions which make *Ambrosia* seeds non-viable in feed materials and compound feed. This assessment also includes information on a reliable procedure to verify the non-viability of the seeds. *Ambrosia* seeds are known contaminants in feed with maximum levels set in the Directive 2002/32/EC. The manufacturing processes and processing conditions applied to the feed may affect the viability of the *Ambrosia* seeds. Therefore, the CONTAM Panel compared these conditions with conditions that have been shown to be sufficient to render *Ambrosia* seeds non-viable. The Panel concluded with a certainty of 99–100% that solvent extraction and toasting of oilseed meals at temperatures of 120°C with steam injection for 10 min or more will make *Ambrosia* seeds non-viable. Since milling/grinding feed materials for compound feed of piglets, aquatic species and non-food producing animals would not allow particles of sizes ≥ 1 mm (the minimum size of viable *Ambrosia* seeds) passing the grinding process it was considered very likely (with $\geq 90\%$ certainty) that these feeds will not contain viable *Ambrosia* seeds. In poultry, pig, and possibly cattle feed, particle sizes are ≥ 1 mm and therefore *Ambrosia* seeds could likely (66–90% certainty) survive the grinding process. Starch and gluten either from corn or wheat wet milling would not contain *Ambrosia* seeds with 99–100% certainty. Finally, ensiling fresh forages contaminated with *A. artemisiifolia* seeds for more than 3 months is very likely to render all seeds non-viable. The Panel concluded that a combination of the germination test and a subsequent triphenyl-tetrazolium-chloride (TTC) test will very likely (with $\geq 90\%$ certainty) verify the non-viability of *Ambrosia* seeds. The Panel recommends that data on the presence of viable *Ambrosia* seeds before and after the different feed production processes should be generated.

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- The names of the authors Jürgen Gropp and Gerhard Karrer which were inadvertently omitted
- The title of table 3 refers to sunflower and not rapeseed
- Data on soft seeds (rapeseeds, sunflower seeds) in annex B.1 which were inadvertently omitted

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Summary

Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed¹ establishes maximum levels for the presence of seeds of *Ambrosia* for feed materials and compound feed containing unground grains and seeds.

During oil production, in the oilseed cake the *Ambrosia* seeds are still to a certain extent present as intact seeds. However, interested stakeholder organisations suggested that the conditions of temperature, moisture, duration and use of solvent have made the seeds non-viable.

Therefore, EFSA was requested by the European Commission to provide an assessment of the processing conditions which make *Ambrosia* seeds non-viable. This assessment also included information on a reliable procedure to verify the non-viability of the seeds.

The genus *Ambrosia* L. (Asteraceae family) comprises of 46 accepted taxa at the species level. Of these species, 45 are native to the Americas while 1 species, *A. maritima*, is native to Europe, Africa and Southwestern Asia (Old World). Five *Ambrosia* species arrived in Europe, four of which became naturalised to various regions across the continent. Common ragweed (*A. artemisiifolia* L.) is the most successful invasive species in Europe while western ragweed (*A. psilostachya* DC.) is the second most successful invader.

In summer crops and stubble fields, common ragweed can produce high amounts of seeds that are either spread by agricultural machinery or crop seed containments. Detection of *Ambrosia* seeds in the above-mentioned context (crop seed, food, soil) is principally possible through examination by the naked eye. Microscope or magnifier can be used to increase the reliability of identifying ragweed seeds.

Viability of seeds is defined by embryos that can germinate. To determine this embryo status there are three approaches widely described in literature: (1) crush test; (2) germination test; and (3) triphenyl-tetrazolium-chloride (TTC) test. The ragweed seeds or any containment to be tested for germinable ragweed seeds should be stratified before any testing procedure by exposure to low temperature (optimum: $\leq 2^{\circ}\text{C}$) over a minimum of 4 weeks in darkness and moist condition, except if collected in late winter when they can be tested immediately. Crush test detects the presence of liquid in seeds. Germination tests detect germinability of seeds under optimal conditions. Changes in temperature and light simulating early spring conditions seem to provide highest germination efficacy. In the TTC-test respiring (living) seeds convert the colourless triphenyl-tetrazolium-chloride to a carmine-red coloured water-insoluble formazan by hydrogen transfer catalysed by the cellular dehydrogenases. Fully stained seeds are counted as 'viable'.

In the crush test, even non-viable seeds may contain liquid. In the germination test, a significant number of viable seeds may not germinate under the optimised germination test conditions. In the TTC test 'intermediate' (indistinct) colouration states of ragweed seeds may be observed making the viable/non-viable classification ambiguous. Therefore, it is essential to combine the germination test with subsequent TTC-test of the remaining seeds in order to test the survival of *Ambrosia* seeds. In general, the combination of germination test plus subsequent TTC will very likely ($\geq 90\%$ certainty) verify the non-viable cells.

In order to assess the processing conditions which make *Ambrosia* seeds non-viable in feed materials and compound feed the Panel on the Contaminants in the Food Chain (CONTAM Panel) compared these conditions with conditions that have been shown experimentally to be sufficient to render *Ambrosia* seeds non-viable.

For oilseed meals obtained during oil production the Panel concluded with a certainty of 99–100% that solvent extracted and oilseed meals toasted at temperatures of about 120°C with steam injection and for time of ≥ 10 min will not contain viable *Ambrosia* seeds. No evidence has been found indicating that *Ambrosia* seeds will be completely destroyed during the production processes of oilseed cakes/expeller for which solvent removal and toasting steps are not applied. Viable *Ambrosia* seeds may have a diameter down to 1 mm. Seeds of this magnitude could pass the grinding process (e.g. by hammer-mill and roller mill). Since milling/grinding feed materials for feed of poultry, pigs and possibly cattle would allow particles of ≥ 1 mm size passing the grinding process, it was considered likely (with 66–90% certainty) these feeds to contain viable *Ambrosia* seeds from the contaminated materials. When the feed materials are intended for use in feed for piglets, aquatic species and non-food producing animals, milling/ grinding would very likely prevent ($\geq 90\%$ certainty) the viability of *Ambrosia* seeds. Although no data are available it can be reasonably concluded with 99–100%

¹ OJ L 140, 30.5.2002, p. 10.

certainty, when considering the production process, that starch and gluten either from corn or wheat wet milling would not contain *Ambrosia* seeds at all. It should be noted that when pellets are pressed (more than 80% of compound feeds are pelleted), the pellet pressing performs a secondary crushing process which may further reduce the viability of the *Ambrosia* seeds. Regarding ensiling, it is very likely ($\geq 90\%$) that fresh forages contaminated with *A. artemisiifolia* seeds when ensiled for a minimum of 3 months will not contain viable seeds. In other heat treatments temperatures above 250°C are used in the dehydration of fresh alfa-alfa or temperatures up to 130°C and 200°C and moisture $> 17\%$ and up to 65% are applied in expanding and extruding feed materials, respectively. But no data are available allowing to unequivocally conclude that these processes will completely eliminate viability from *Ambrosia* seeds. However, the Panel assumes that viability will be strongly reduced under these conditions. Conditioning of feed materials before mixing is considered too mild (low temperature, short duration) to affect seed viability.

The Panel recommended that data on the presence of viable *Ambrosia* seeds before and after the different feed production processes should be generated.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed¹ establishes maximum levels for the presence of seeds of *Ambrosia* spp. for feed materials and compound feed containing unground grains and seeds.

These provisions were taking into account the outcome of the Scientific Opinion on the effect on public or animal health or on the environment on the presence of seeds of *Ambrosia* spp. in animal feed.² The Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) noted that *Ambrosia* seeds may contaminate feed materials containing maize, wheat, sunflowers, millet, peanuts, soybean, peas and beans and that transport of these feed materials to a processing plant or a feed miller without prevention measures to avoid dissemination into the environment and in particular the use of these contaminated seeds in bird feed (seeds) used for wild and ornamental birds may be an important route of ragweed dispersal.

The legislation also provides that in case unequivocal evidence is provided that the grains and seeds are intended for milling or crushing, there is no need to perform a cleaning of the grains and seeds containing non-compliant levels of seeds of *Ambrosia* spp. before milling or crushing on the condition that:

- the consignment is transported as a whole to the milling or crushing plant, and
- the milling or crushing plant is informed in advance of the presence of high level of *Ambrosia* spp. seeds in order to take additional prevention measures to avoid dissemination into the environment, and
- solid evidence is provided that prevention measures are taken to avoid dissemination of *Ambrosia* spp. seeds into the environment during transport to the crushing or milling plant, and
- the competent authority agrees to the transport, after having ensured that the abovementioned conditions are fulfilled.

In case these conditions are not fulfilled, the consignment must be cleaned before any transport into the EU and the screenings must be appropriately destroyed.

However, in the case seeds destined for crushing, in the oilseed cake the *Ambrosia* seeds are still to a certain extent present as intact seeds. However, evidence has been provided by interested stakeholder organisations demonstrating that the conditions of temperature, moisture, duration, and use of solvent have made the seeds non-viable.

Therefore, it is appropriate to request EFSA to assess the processing conditions which make *Ambrosia* seeds non-viable and to provide information on appropriate diagnostic tests proving the non-viability of the seeds.

1.1.2. Terms of Reference

In accordance with Art. 29 (1) of Regulation (EC) No 178/2002, the European Commission asks the European Food Safety Authority for an assessment of the processing conditions which make *Ambrosia* seeds non-viable. This assessment shall also include information on reliable procedure to test the non-viability of the seeds.

1.2. Interpretation of the Terms of Reference

The processing conditions applied to feed materials and compound feed will be assessed for their potential to inactivate *Ambrosia* seeds that are present as contaminants in these feeds. The terms used for processing conditions of feed, are in accordance with the definitions given in the glossary (Part B) of Commission Regulation (EU) No 68/2013³ on the Catalogue of feed materials as amended

² EFSA Panel on Contaminants in the Food Chain (CONTAM), EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) and EFSA Panel on Plant Health (PLH); Scientific Opinion on the effect on public or animal health or on the environment on the presence of seeds of *Ambrosia* spp. in animal feed. EFSA Journal 2010;8(6):1566, 37 pp. <https://doi.org/10.2903/j.efsa.2010.1566>. Available online: www.efsa.europa.eu

³ OJ L 029 30.1.2013, p. 1.

by Commission regulation (EU) 2022/1104⁴. The currently available procedures to test the viability of *Ambrosia* seeds are compared for their reliability.

1.3. Additional information

1.3.1. Summary of the previous EFSA assessment on *Ambrosia* seeds

EFSA has previously published a Scientific Opinion assessing the possible effect on public or animal health or on the environment on the further distribution of *Ambrosia* spp. in the European Union (EFSA, 2010). Regarding the effects on the environment, some indications were identified that *A. artemisiifolia* could become highly invasive in certain environmentally valuable habitats and might be linked to impoverishment of species richness. However, direct evidence was lacking that *Ambrosia* spp. cause extinction of plant species. Birdfeed was highlighted as potentially playing an important role in introducing *Ambrosia* to new, previously not infested areas because it contains significant quantities of unprocessed *Ambrosia* seeds. The contribution of other compound feed to the dispersion of *Ambrosia* was deemed to be negligible because of destruction of *Ambrosia* seeds during processing of animal feed material compounded for use in livestock. Finally, *Ambrosia* seeds do not appear to survive the ensiling process and so the largest contribution of weed seed in animal diets is therefore likely to arise from contaminated hay and grain.

2. Data and Methodologies

The assessment on the conditions making *Ambrosia* seeds non-viable was developed applying a structured methodological approach, which implied developing *a priori* the protocol or strategy of the assessment and performing each step of the risk assessment in line with the strategy and documenting the process. The protocol in Annex A for this assessment contains the method that was proposed for all the steps of the risk assessment process, including any subsequent refinements/changes made.

2.1. Data

Feed business operators have submitted information describing feed processing conditions in general (Annex B). In addition, relevant information was identified by extensive literature searches (Annex C). During the development of the assessment, additional publications were collected by applying a 'snowballing approach'.

2.2. Methodologies

The assessment is conducted in line with the principles described in the EFSA guidance on transparency in the scientific aspects of risk assessment (EFSA, 2009) and on Uncertainty Analysis in Scientific Assessments (2018a) from the EFSA Scientific Committee, as appropriate and as depicted in the protocol of the Annex A.

3. Assessment

3.1. Characteristics and nature of *Ambrosia* spp.

The genus *Ambrosia* L. comprises of 46 accepted taxa at the species level.⁵ Only *A. maritima* is supposed to be native to the Old World (Europe, Africa, Southwestern Asia), all other species are native to the Americas (Payne 1962). Some *Ambrosia* species were dispersed by man unintentionally from their home countries to other parts of the world (Montagnani et al., 2017). Five species arrived in Europe and four of them became naturalised to different parts on the continent. Common ragweed (*A. artemisiifolia* L.) is the most successful invader in Europe, but also in Asia, Australia and South America. The second most successful species for invasion to Europe is western ragweed (*A. psilostachya* DC.) whose geographical range in Europe is even broader, being established from Sweden and Finland in the North to southern Italy and Spain (Karrer et al., 2023). Giant Ragweed (*A. trifida* L.) is established only in few regions (Russia, Italy, the Czech Republic) and forms elsewhere unstable

⁴ OJ L 177, 4.7.2022, p. 4–74.

⁵ <https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:327075-2#children>

populations based on repeated introductions of seeds (Montagnani et al., 2017). Finally, slim leaf bur ragweed (*A. tenuifolia* Spreng.) is fully established at a few places in Spain, Southern Italy, Southern France and Romania (Karrer et al., 2021) but only temporarily introduced in few harbours. Plant identification is easily possible by use of the characters given in Karrer et al. (2016b).

Belonging to the plant family Asteraceae, the introduced ragweed species produce characteristic hard-coated and mostly one-seeded fruits (achenes – commonly called 'seeds', Figure 1). These seeds have no specific dispersal syndrome and drop down from the mother plant after ripening. Seeds are dispersed either by animals (birds or mammals) or – more effectively – by running water, soil transport or plant translocation (Gebben, 1965; Bassett and Crompton, 1975, 1982; Harrison et al., 2003; Karrer, 2011; Essl et al., 2015). Specifically, the annual weed common ragweed seeds are spread very effectively by man (adhered to machinery or harvested crops and hay) in its native as well as invasive range (Vitalos and Karrer, 2009; Karrer, 2014; Essl et al., 2015).

Common ragweed as well as the rare giant ragweed are commonly part of the soil seedbank in arable fields (Gebben, 1965; Karrer, 2011; Essl et al., 2015). In ecosystems without noteworthy soil perturbation (meadows, fallows) the seeds stay at the soil surface and tend to germinate in the subsequent year by 95–99% (Karrer, 2011; Kazinczi and Pál-Fám, 2018; Dong et al., 2020). In contrast, when seeds are buried into deeper soil layers due to ploughing of arable fields, they keep their strong dormancy and can survive for decades (Darlington and Steinbauer, 1961; Gebben, 1965; Willemsen, 1975; Baskin and Baskin, 1977; Karrer, 2016a; Karrer et al., 2016a; Kazinczi and Pál-Fám, 2018).

Fresh seeds of *A. artemisiifolia* are not yet dormant (Willemsen and Rice, 1972; Karrer, 2011) and need some weeks for ripening in autumn including cool temperatures to develop innate dormancy (primary dormancy) (Payne and Kleinschmidt, 1961; Willemsen, 1975). Primary dormancy can be broken by low winter temperatures over several weeks followed by fluctuating moderate warm spring temperatures and starting long-day light conditions (Willemsen and Rice, 1972; Pickett and Baskin, 1973; Willemsen, 1975). Breaking dormancy was most successful in several germination experiments when applying cold-wet stratification and some scarification (Gebben, 1965; Willemsen, 1975; Karrer, 2011; Onen et al., 2020; Hall et al., 2021). Germination behaviour did not differ significantly between different regions in European experiments (Ortmans et al., 2016a,b; Onen et al., 2020). However, differences in preferred temperature ranges for germination was detected when native American seed lots and European seed origins were compared (Leiblein-Wild et al., 2014). Commonly, seeds overwintering on the soil surface tend to germinate at these conditions to almost 100%.

Buried *Ambrosia* seeds experience low temperature fluctuations without light but higher CO₂ concentrations; therefore, these seeds switch to secondary dormancy that cannot be broken just as successful as after the first dormancy period (Davis, 1930; Bazzaz, 1968, 1970). Secondary dormancy can be broken by re-stratification up to 50% (Bazzaz, 1970; Baskin and Baskin, 1980; Guillemain and Chauvel, 2011). Anyway, several seeds stay dormant even after such second stratification. This seems to a species-specific trait of this R-strategist⁶ to leave some seeds in the soil seed bank for future seasons awaiting suitable conditions for germination and establishment (Grime, 2001; Karrer, 2016b).

The maximum age of surviving *A. artemisiifolia* seeds was documented to be 39 years by Toole and Brown (1946) from the long-term burial experiment by Duvel (1905). Fumanal et al. (2008), Karrer et al. (2016b), Kazinczi and Pál-Fám (2018) and Hall et al. (2021) documented up to 95% survival in seeds buried up to 9 years depending on burial duration as well as seed age and geographical origin.

Until today, almost only seeds of *A. artemisiifolia* were detected in Europe as a contaminant in crop seeds for agricultural use or food industry (Karnkowski, 2000; EFSA, 2007a; Karrer, 2011). EFSA (2007b) documented crop lots from Belgium and Holland to be contaminated with few *A. trifida* seeds.

Three main habitat types are most prone to infection by invasive *Ambrosia* species:

- 1) Agricultural fields: This holds for Southern, Middle and Eastern Europe (from Southern France, Northern Italy, North-eastern Germany (Brandenburg and Saxony), all lowland areas of Austria and Slovenia, Northern and continental Croatia, Northern Bosnia and Herzegovina, Southern Slovakia, Northern Romania, whole of Hungary and most of Serbia, Southern Ukraine, Russia and Moldova). In summer crops and stubble fields, common ragweed can produce high amounts of seeds that are either spread by agricultural machinery (Karrer, 2014) or crop seed containments (Karnkowski, 2000; Nawrath and Alberternst, 2010; Karrer, 2011).

⁶ Species who invest all resources into reproductive units.

- 2) Roadsides: Roadsides in Europe and North America are cultivated by regular cuts one to four times a year and suffer from tearing up the turf by machinery and accidents. This favours the establishment of R-strategist plants (Grime, 2001) like common ragweed (Joly et al., 2011; Karrer, 2011; Simard and Benoit, 2011; Milakovic et al., 2014). Further spread along roadsides is caused by contaminated mowing machinery (Vitalos and Karrer, 2009) and passing vehicles (Karrer, 2011; Lemke et al., 2019). Soils of infected road verges are commonly very contaminated by ragweed (Karrer, 2011, Milakovic and Karrer, 2016).
- 3) Construction areas: Any construction work related to open soil habitat types offers perfect conditions for the annual *A. artemisiifolia*. After a few years these soils are heavily contaminated by ragweed in the soil seed bank (Bohren et al., 2006; Buttenschøn et al., 2010; Karrer, 2011; Alberternst and Nawrath, 2016).

3.2. Methods of analysis of the *Ambrosia* seeds and their viability

3.2.1. Detection of *Ambrosia* seeds

The probability of detection of a contaminated product depends on the quantity and quality of the test material, and on the weed seed characteristics (and thus on the weed species) (EFSA, 2007b). Detection of *Ambrosia* seeds in the above-mentioned context (crop seed, food, soil) is principally possible through examination by the naked eye. In case of older seeds, the pericarp gets partially lost and the hard-coated seed *sensu strictu* is to be seen and can be misidentified with *Carex* or *Persicaria* nutlets. Microscope or magnifier can be used to increase the reliability of identifying ragweed seeds.

Karrer et al. (2016c) provides a protocol for standardised sampling and handling of *Ambrosia* seed material or substrates possibly containing *Ambrosia* seeds. Storage of soil seedbank samples for a longer period should be avoided. At least, storage under wet conditions leads to decay of ragweed seeds within a few months/years. If soil cores are stored after drying of samples the survival rates tend to be higher (maximum of 5 years for quantitative analyses).

3.2.2. Optical identification of the seeds

Seeds of *Ambrosia* species invasive to Europe look strikingly different (Figures 1–3).

Easy to identify are seeds of *Ambrosia trifida* having very big seeds (0.5–1.1 cm) in length with 2–3 not very sharp spiny appendices (Figure 1).



Figure 1: Seeds of *Ambrosia trifida* (from Karrer et al., 2016c). The ruler showing mm scale applies to the right image only

A. artemisiifolia seeds can be expected in all samples collected from Central Eastern and Southern Europe (excluding the Mediterranean coastline). Commonly, the seeds have a distinct and sharp terminal spine and subordinated lateral slender spines (Figure 2). The length has been reported to

vary from 2.5 to 5 mm (Hall et al., 2021), the width varies from 1.5 and 3.0 mm (lowest value: 1 mm^{7,8}). The seed weight also varies a lot: Hall et al. (2021) showed mean weights of *A. artemisiifolia* seeds between 3.7 and 8.8 mg differing by years of sampling (min: 1.0 and max. 13.5 mg). Ortmans et al. (2016b) found that seed mass varied with population origins (in Western Europe) and with mother plants from 2.1 to 12.7 mg. On tall mother plants on average smaller seeds were counted compared to small individuals with fewer but bigger seeds. Interestingly, Leiblein-Wild et al. (2014) found that in their experiments, the single seed mass of seed lots from the native range were on average lower than those in the invasive European range what was also correlated with higher frost tolerance of the European origins.



Figure 2: Seeds of *Ambrosia artemisiifolia* (from Karrer, 2016c). The scale grid indicates 1 cm between the thick orange lines and thin lines show 2 mm increments

Ambrosia psilostachya is documented as agricultural weed only from southern France (Fried et al., 2015; Karrer et al., 2023) but was never reported as crop seed contamination. The seeds are of the same size like in *A. artemisiifolia* but the spines are less distinct or even missing in several cases (Figure 3).



Figure 3: Seeds of *Ambrosia psilostachya*. (© Chauvel 2016)

During transportation or deposition below ground, the pericarp can get lost (Figure 4). *Ambrosia* nuts are rounded in transect whereas *Persicaria* or *Carex* nutlets are triangular. Often empty seed coats can be found but must be neglected. They can be segregated by soft touch with a pincer.

⁷ <https://inspection.canada.ca/plant-health/seeds/seed-testing-and-grading/seeds-identification/Ambrosia-artemisiifolia/eng/1472605115589/1472605116050>

⁸ Keil DJ, 2012. *Ambrosia artemisiifolia*, in Jepson Flora Project (eds.) Jepson eFlora. Available online: https://ucjeps.berkeley.edu/eflora/eflora_display.php?tid=805 [Accessed: 16 May 2023].



Figure 4: Uncoated nut of *Ambrosia artemisiifolia* (Karrer et al., 2016c). © G Karrer

3.2.3. Testing viability

Viability of seeds is defined by living embryos that can germinate (Baskin and Baskin 2001). To find out this embryo status there are three approaches widely known in literature (Karrer et al., 2016b; Hall et al., 2021): (1) crush test; (2) germination test; and (3) triphenyl-tetrazolium-chloride (TTC) test. Methods for regular viability tests have been published by Karrer et al. (2016c) and the IAFA (2019).

Basically, the ragweed seeds or any containment to be tested for germinable ragweed seeds should be stratified before any testing procedure by exposure to low temperature (optimum: $\leq 2^{\circ}\text{C}$) over minimum 4 weeks in darkness and moist condition. Such treatment optimises the germinability of seeds. Seeds from the soil seedbank sampled in late winter or spring can be treated directly because of natural stratification in the field. Wet sieving would stimulate germination.

Crush test: can be applied to assess whether seeds contain live cells by squeezing out potential liquid of seed that have been cut in half. This liquid can also be detected from squeezing dead embryos.

Germination test: Conditions used for testing germinability of seeds vary and depends on seed dormancy and options to break dormancy. Various germination experiments with ragweeds gave evidence that changes in temperature and light simulating early spring conditions provided highest germination efficacy (i.e. Davis, 1930, Gebben, 1965, Bazzaz, 1968, 1970, Pickett and Baskin, 1973, Baskin and Baskin, 1977, Karrer, 2011, Leiblein-Wild et al., 2014, Karrer, 2016, Karrer et al., 2016c, Onen et al., 2020). Karrer et al. (2016a) provided a widely used manual specifically adapted to *Ambrosia* seeds. Germination can be confirmed by a 'visible radicle'. Seeds that do not germinate should be tested further by triphenyl-TTC staining for viability.

TTC-test: A manual for ragweed seed TTC-testing was published by Starfinger and Karrer (2016) and adapted by Hall et al. (2021). This procedure is very similar to that promoted by the International Association of Feedingstuff Analysis (IAFA, 2019) and an adaptation of a common cell viability assay for *in vitro* tissue culture applications. Viable seeds convert the colourless triphenyl-tetrazolium-chloride to a carmine-red coloured water-insoluble formazan by hydrogen transfer reaction catalysed by the cellular dehydrogenases. Fully stained seeds are counted as 'viable'. Most essential for an effective germination process is the primary root tip to be fully stained. This is needed for the escape of the embryo from the hard seed coat. The joint experiments performed along with the HALT *AMBROSIA* project as well with the SMARTER lab teams (Hall et al., 2021) showed that several 'intermediate' (indistinct) colouration states of ragweed seeds could be observed. Furthermore, the different labs were not able to classify the staining consistently although the same manual was used.

Seeds of 3–5 years of age tend to have delayed germination and are not able to develop further forming a well-developed primary root with root hairs. In case of fresh seeds (< 1 year of age), Karrer et al. (2016c) found them to develop rather normally.

Consequently, not fully stained ('intermediate') seeds should be counted 'alive' when seeds are fresh but counted 'dead' when more than 1 year in age. In case of soil seedbank samples of natural populations, regularly several seed cohorts from different years are present, and intermediate seeds must be evaluated 'dead' in the ecological sense.

In several studies (Vitalos and Karrer, 2008; Karrer, 2011; Milakovic and Karrer, 2016; Karrer et al., 2016a), authors found a significant number of viable seeds not germinating under the optimised germination test conditions. A differing but low percentage of seeds stayed dormant. In these cases, it is essential to combine the germination test with an a posteriori TTC-test of the remaining seeds. A sequence of such sophisticated analytical steps is shown in Figure 5 for the example of soil seed bank analyses (Karrer et al., 2016c). For most ecological applications the viability rate in the wide sense (number of germinated plus TTC-positive seeds/number of seeds tested) is important to detect from samples (i.e. soil samples) (see also Gosling (2003)). In case of contamination of crop seed with ragweed, commonly the number of visible seeds is related to the weight unit of the tested seed lot. Such measures are the basis of the Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed (EC, 2002). Feed contaminants are currently not tested for germinability or viability.

An open question is the appropriate sample size (number of tested seeds to ensure 100% non-viability). The current evidence from Hall et al. (2021) showed that viability rates of 100 seeds tested each by germination plus TTC test or by TTC stand-alone test were not 100% congruent, while sample sizes of 1000 would be sufficient.

In case of plants with very small seeds lacking a thickened coat (like ragweeds have), Evans blue (EB) test and fluorescein diacetate (FDA) test were also used for testing seed viability. However, these techniques were never applied to *Ambrosia* seeds due to their hard coat.

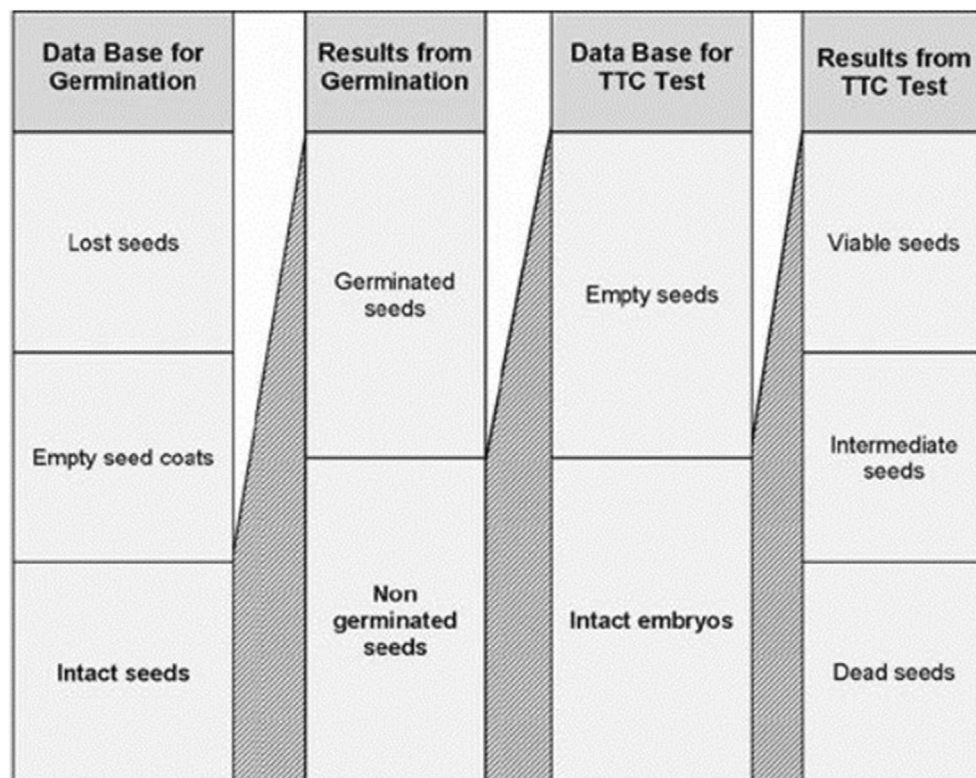


Figure 5: Hierarchy of *Ambrosia artemisiifolia* seed qualities tested for viability (Karrer et al., 2016c)

3.2.4. Limits of detection and quantification, and minimum number of samples

Limits of detection (LOD) and quantification (LOQ) are not relevant in the context of detection of counted distinct units like ragweed seeds in ecological experiments. At least in ecology these measures are not used in this statistical context.

There are no reliable tests for the number of samples to get representative figures for the contamination of feed containments with *Ambrosia* seeds. In case of tests to ensure low or no contamination of crop seeds imported to different countries, every country follows its own rules. In the case of Austria, several crop seeds harvested for sowing of crops were tested for ragweed contamination for some years (Karrer, 2011).

3.3. Effect of processing on the viability of *Ambrosia* seeds

3.3.1. Occurrence of *Ambrosia* seeds in feed material and compound feed

There are no recent supranational data about contamination of specific feed materials with *Ambrosia* seeds available. In a German study (BELV-BLV, 2008), 31, 22, and 3.5% of tested samples of sunflower, millet and sorghum, respectively, were contaminated with *Ambrosia* seeds. In Austria, Hackl and Baumgarten, 2011 found that only 0.086% of seeds used for growing crops were contaminated with *A. artemisiifolia*. This traded seed material came mostly from Hungary but few also from Austria, Czech Republic and Germany. *Ambrosia* seeds can also be present in animal bedding or in spilled feed (feed that is lost during handling) and may bypass the animal and directly enter the manure stream. Both weed seed sources may result in manure containing viable weed seeds. Van Denderen (2008) found that 65% of mixed feed samples (hay) were contaminated with ragweed. Only few samples showed high levels of contamination with up to 194 seeds per 0.5 l of feed.

Directive 2002/32/EC on undesirable substances in animal feed limits the maximum content of *Ambrosia* in feed materials and compound feed containing unground grains and seeds to 50 mg/kg feed (with exception of 200 mg/kg for millet and sorghum not directly fed to animals).

The introduction of a contamination limit for ragweed in 2011 by the EC resulted in a reduction in spread of ragweed via bird feed. The CONTAM Panel concluded in 2010 that bird feed (seeds) used for wild and ornamental birds may be an important route of ragweed dispersal especially in non-infested areas. Vitalos and Karrer (2008) estimated that only 10% of bird feed samples contain significant numbers of ragweed seed, and that only 2% of them were viable. They concluded that the role of bird seed was probably overestimated as a cause of further ragweed spread. However, Monty et al. (2022) found that 13 out of 42 samples of bird feed from Belgium contained common ragweed seeds. Bird feed contained as an average 0.75 ± 0.265 seeds/kg of which 82.4% were viable. Although the legal limit seems to be respected, the bird feed trade still represents a substantial pathway for spreading ragweed.

3.3.2. Conditions to reduce *Ambrosia* seeds viability

Specific tests on the survival of ragweed seeds under control treatments were conducted in the EU-project HALT AMBROSIA (07.0322/2010/586350/SUB/B2).⁹ Some of the results, relevant for the current assessment, were reported below.

Starfinger and Söltner (2016) reported that heat treatment success on reducing ragweed seed viability strongly depends on laboratory conditions:

- Dry seeds may have survival rates of 80% after 96 h exposure to 60°C.
- Moist and wet seeds are reliably killed after 36 h at 50°C or after 24 h at 55°C.
- Both viability and the ability of seeds to survive heat is reduced in older seeds.

The studies by Starfinger and Söltner (2016) were made with only 20 seeds at each term and lacked replicates. According to Karrer et al. (2016c), Karrer et al. (2016b) and Kazinczi and Kerepesi (2016), the origin and the circumstances of sampling and storage until the start of the experiment can have a strong influence on viability tests. Therefore, these figures give only a rough indication about the cut-off temperature for making *Ambrosia* seeds non-viable.

Biomass for silage or composting can include ripened seeds that might be dispersed with the residues from biogas or composting plants. Few experiments were performed to follow the fate of ragweed seeds during these processes that are linked to influences of increased temperature. Starfinger and Söltner (2016) used an experimental biogas fermenter to test the viability of *Ambrosia* seeds during a period of 3 months of treatment under 37°C. After 3 months, silage ragweed seeds were dead by 100%. In the variant using batches with seeds in the fermenter, it took 8 days to make all ragweed seeds non-viable (Starfinger and Söltner, 2016).

In an Austrian experiment (Gansberger, 2011, Leonhardt et al., 2010), the exposure of ragweed seeds to a simulated biogas fermenter reduced germinability to zero after 3 days. Unfortunately, the authors desisted from subsequent TTC-tests of the non-germinated seeds.

⁹ <https://circabc.europa.eu/sd/a/d50b8080-6629-4ef5-9f3c-468b81a927b7/B%20Biogas%20fuel%20of%20ragweed%20seed%20contaminated%20material.pdf>

In general, anaerobic digestion in biogas plants tends to make ragweed seeds non-viable very effectively due to the anaerobic conditions and moistening of weed seeds (Westermann and Gerowitt, 2013).

In a very recent paper (Hall et al., 2023, submitted for publication), a sophisticated design provided more detailed results on the effects of high temperature on the survival rates of ragweed seeds. The authors found that wet seeds were in general more sensitive to any heat treatment than dry seeds. Simple air heating of seeds resulted in 100% dead seeds if the seeds were wetted before and exposed to 100°C or 90°C for 12 h. Exposed to 60°C, wet seeds kept viability to 55% even after 48 h heating. In case of dry seeds, no factor combination of temperature and duration could completely make all seeds non-viable. Even at 100°C, 1.2% of the seeds stayed viable after 48 h of heating.

Hot steam treatment (above 100°C) resulted in 100% killing only after an exposure of 5 min minimum for both dry and wet seeds. At the use of hot water (at 100°C), 100% of the seeds were non-viable already after 5 min in case of dry seeds and after 1 min already in case of wetted seeds.

3.3.3. Feed treatment and its influence on the survival of viable *Ambrosia* seeds

Feed business operators treat feed materials intended to become a constituent of a compound feed in different ways to ensure optimal nutrient bioavailability, animal health and homogeneity of mixed feed.

Some feed materials in particular the oil seeds are fed as products derived thereof. The lipid fraction of oils seeds (e.g. of soybean, rape seed, cottonseed, peanut, sunflower, oil pumpkin) is removed by pressing, solvent extraction or a combination of both methods to obtain valuable oils for human nutrition, the remaining products (oils seed cakes, expeller, solvent extracted oil meals) are valuable protein sources in animal nutrition. An exception is the infrared toasting of soybean which allows feeding of the full fat soybean for poultry and pigs. These feed materials are to a certain extent heat treated (for optimising lipid extraction, removal of the solvent, but also for reduction or elimination of anti-nutrients) before use by feed compounders.

Mechanical treatments such as grinding/milling (reducing the particle size of solid feed materials in a dry or wet process) are applied to more or less all feed materials. These processes would reduce the particle size of feed materials or allow fractionation of nutrients.

Different manufacturing processes applied to a feed material before mixing may affect the viability of *Ambrosia* seeds. In particular, treatments by mechanical power or heat, in some cases also applied to compound feed, will be considered below. It is also to be noted that preservation of high moisture crop forages (e.g. dehydration, ensiling) will be also addressed.

3.3.3.1. Heat treatment of oilseeds for the production of soybean oil meal and rapeseed and sunflower expeller

Before extraction, the soybean seeds undergo different treatments such as cooking, crushing, flaking and dehulling, which are aimed at increasing oil extraction and soybean meal quality. Cooking the seeds has positive effects on: moisture conditioning of seeds and easing dehulling. Crushing and flaking operations promote solvent extraction by changing the permeability of the soybean flakes. Dehulling is a facultative process that separates the oil-rich kernel from hulls which represents 8% of the seed and are mainly fibrous.

In the solvent extraction process, soybeans are cracked, dehulled (optional), heated, flaked and passed (or not) through a kind of extruder called an expander. The expander produces a porous pellet with increased cell rupture and greater density. This makes oil extraction by solvent easier (usually hexane but extraction with ethanol or with mixtures of hexane and ethanol are also possible). The extracted flakes are further dried to eliminate the solvent, then toasted and ground.¹⁰

EFSA requested feed business operators (see Section 6 Documentation provided to EFSA and Annex B) to provide information on feed production details. For instance, in the case of soybean oil meal, where the oil is extracted via a solvent, during initial flaking with a duration of 30 min, the soybeans are exposed to a temperature of 60°C while moisture is reduced to 8–10%. For oil extraction via hexane, the flakes are treated for 30 min at 60°C, at atmospheric pressure where moisture is 8–10%. The final production step consists of solvent removal and toasting. For toasting, direct steam is injected, temperature ranges between 68°C and 105°C for 20–30 min and moisture is reduced from 20% to 10%.

¹⁰ Heuzé V, Tran G and Kaushik S, 2020. *Soybean meal*. Feedipedia, a programme by INRAE, CIRAD, AFZ and FAO. Available online: <https://www.feedipedia.org/node/674> [Accessed: 4 March 2020] 18:25.

EFSA received information from oilseed business operators on the treatment (oil removal) not only on soybeans but also on rapeseed and sunflower seeds. The information is summarised in Tables 1, 2, 3 and provided in Annex B in more detail.

Table 1: Conditions used for soybean oil extraction and soybean meal production using hexane

Method		Drying	Tempering	Cleaning	Cracking	Dehulling + sieving	Conditioning	Cracking	Flaking	Extraction	Solvent removal
Cold dehulling	Temperature (°C)	65	20	20	20	20	60	60	60	55–65	70–120
	Time (min)	5–15	0.5–3 days	5	1	5	10–20	1	3	30–90	10
	Moisture		Low	Low	Low	Low	Medium ⁽¹⁾	No	100 ⁽²⁾	Low	No
		Pre-heating	Heating	Cleaning	Dehulling + sieving	Cracking	Flaking	Extraction		Solvent removal	
Hot dehulling	Temperature (°C)	60–70	85	60	60	60	60	55–65		70–120	
	Time (min)	20–30	5	1	5	1	3	30–90		10–20	
	Moisture	Medium ⁽¹⁾	Low	Low	Low		Low	No		No	

(1): Evaporating from seed.

(2): Direct steam injection.

The data for the production of rapeseed and sunflower seed are summarised in Tables 2 and 3.

Table 2: Conditions used for oil removal from rapeseed by expelling

Method		Sieving	Pre-heating	Flaking	Cooking	Pressing	Extraction	Solvent removal	Toasting	Drying	Cooling
Pressing + hexane extraction	Temperature (°C)	20	50	60	90–110	90–110	65	70–120	120	90	10–30
	Time (min)	2	5	1	10–20	3	60–90	10–20	15–30	10	10
	Moisture	Low	Low	Low	Medium ⁽¹⁾	Low	No	No	100 ⁽²⁾	Low	No
		Sieving	Conditioning	Pressing	Cooking	Pressing	Extraction	Solvent removal	Toasting	Drying	Cooling
Pressing, first step cold	Temperature (°C)	20		50–70	90–110	90–110					10–30
	Time (min)	2		3	10–20	3					5
	Moisture	Low		Low	Medium ⁽¹⁾	Low					Low
Pressing, first step hot	Temperature (°C)	20	90–110	90–110	90–110	90–110					10–30
	Time (min)	2	10–20	3	10–20	3					5
	Moisture	Low	Low	Low	Medium ⁽¹⁾	Low					Low

	Sieving	Cooking	Pressing	Extrusion	Pressing	Extraction	Solvent removal	Toasting	Drying	Cooling
Pressing, cold, extruder	20		50–70	135	120–135					10–30
	2		3	1	3					5

(1): Evaporating from seed.

(2): Direct steam injection.

Table 3: Conditions used for oil removal from sunflower by expelling

Method		Sieving	Dehulling	Flaking, if applied	Cooking	Pressing	Extraction	Solvent removal	Toasting	Drying	Cooling
Pressing + hexane extraction	Temperature (°C)	20	20	20	70–90	90–100	55–65	70–120	120	90	10–30
	Time (min)	2	15	1	10–20	3	60–90	10–20	15–30	10–20	10–20
	Moisture	Low	Low	Low	Medium ⁽¹⁾	Low	No	No	100 ⁽²⁾	Low	No
		Sieving	Dehulling	Pressing	Cooking	Pressing	Cooking	Pressing	Toasting	Drying	Cooling
Pressing, first step cold	Temperature (°C)	20	20	40–70	80–100	80–100					10–30
	Time (min)	2	15	3	10–20	3					5–15
	Moisture	Low	Low	Low	Medium ⁽¹⁾	Low					Low
Pressing, first step hot	Temperature (°C)	20	20		80–100	80–100	80–100	80–100			10–30
	Time (min)	2	15		10–20	3	10–20	3			5–15
	Moisture	Low	Low		Medium ⁽¹⁾	Low	Medium ⁽¹⁾	Low			Low

(1): Evaporating from seed.

(2): Direct steam injection.

Final toasting (120°C for 15–30 min with direct steam injection), drying (90°C for 10–20 min, low moisture) and cooling (10–30°C for 10–20 min) are not different between the two products obtained by different dehulling processes.

In summary, solvent extracted meals of oil seeds are treated several times to temperatures up to 90°C for some minutes, highest temperature (up to 120°C) is applied for removal of the solvent (the residual solvent should be minimised) and toasting. No specific pressure is allowed. Moisture does not exceed the natural dimension (about 10–20%), except when conditioning is applied (to increase oil extraction efficiency) and steam is injected for solvent removal/toasting.

A somewhat different picture results from oil removal by mechanical pressure, most commonly by an expeller (continuous pressing in screw like machinery, where friction causes heat). Conditioning temperatures before pressing are higher (70–100°C) and may last up to 20 min, both processes may be applied twice, so that treatment at high temperatures could last between 15 and 50 min. But also here, humidity of the treated product remains low, since water is not introduced in the process.

It should be noted, that for both final products, the oil seed expeller and the oil seed extraction meal, same mechanical treatments like flaking and/or crushing may be applied.

3.3.3.2. Mechanical treatment (grinding feed materials)

Dry milling is the physical or mechanical process of pounding or grinding grains to separate the endosperm from the pericarp and the germ. Grinding mills use pin, hammer or disk mills or a combination (multi-stage grinding) depending on the manufacturer. The endosperm is recovered from dry milling in several sizes called grits (0.65–5.8 mm particles), meal (0.17–0.65 mm particles) and flour (< 0.17 mm particles).

The intended particle size of feed materials used for a compound feed varies depending on target species. Information was provided by feed business operator¹¹ on the intended particle size obtained/intended by grinding of feed materials in feed for different target animals. The submitted values in Table 4 are approximate average values. There may also be individual cases where other sizes are used. The fineness is always a compromise between finer (can be pressed better into pellets, better digestion and thus better digestibility) and coarser (better stomach health for some animal species, energy savings in grinding). The exact settings used in each case can also depend on the customer's wishes regarding pellet texture.

Table 4: Intended mean particle size of feed materials foreseen in compound feed for different target animals (µm, Min: minimum, Max: maximum)

	Poultry layer	Poultry broiler	Piglet	Pig	Cattle	Pet food	Aquatic species
Min (µm)	1,200	1,000	350	800	500	300	200
Max (µm)	1,800	1,200	500	1,000	700	400	300

3.3.3.3. Wet milling

Pure starch from maize or wheat is industrially obtained by the wet-milling process. The separated gluten proteins of both cereals are also a result of this process. The main feature of the process is to soak the kernels to soften them before milling. Screening, centrifuging and washing are used to separate the constituents. The following short description follows articles published by Sayaslan (2004) for wheat products and by Jackson and Shandera Jr (1995) for maize products. The principles of wet-milling in the commercial production of starch and gluten protein are similar for both cereals. Wet-separation of proteins and starch from both cereals is based on their water insolubility, density and particle size.

The first step after cleaning consists of steeping the cereals in mildly acidified or neutral aqueous environment (sometimes heated). The germ is then separated from the other parts of the cereal by slow grinding. Upon wetting, proteins in the endosperm form particles that are larger in size but less dense than starch granules. The different 'swimming' behaviour allows separation of protein and starch by physical processes (centrifuge, hydrocyclone (a multiple starch washing procedure) or screen). Different feed materials result from wet-milling: 98% pure starch, gluten with about 60% (maize) or 80% (wheat) protein containing gluten meal, germ meal, germ extraction meal, germ oil and gluten feed (steep water solids with cereal fibre).

¹¹ Documentation provided to EFSA No. 3.

3.3.3.4. Other heat treatments

Conditioning

Before mixing, feed materials are often also conditioned (short time heat treated). The temperatures applied for 10–20 s are between 85°C and 95°C for poultry feed and between 75°C and 85°C for pigs and cattle feed. Pelleting of the compound feed would result in a further mainly superficial heat treatment for a few seconds between 60°C and 85°C. Temperature is between 17°C and finally (by cooling) < 14°C.

Dehydration of alfalfa leading to alfalfa meal¹²

Lucerne (Alfalfa) is a common feed material (*Medicago sativa* L. and *Medicago x varia* Martyn plants or parts thereof) and is used as a natural source of protein for all animal species. The main preservation process of alfalfa is dehydration of the fresh plant's nutritional qualities.

During dehydration, the moisture content of wilted alfalfa is reduced by 75% to 50% in a rotary drum dryer. Within the drum, the wet chops are dried from an initial moisture content of about 30–70% (by weight, wet basis) to about 6–12%. Typical combustion gas temperatures are between 250°C and 600°C in dryer entrance (French data) or between 154°C and 816°C at the inlet and 60–95°C at the outlet (US data).

On leaving the rotary drum dryer, the alfalfa is crushed and may be pelleted.

3.3.3.5. Combined heat and mechanical treatment (e.g. expansion, extrusion)

Other manufacturing procedures for compacting the compound feed are expansion and extrusion. Expansion is defined by Commission Regulation (EU) 2022/1104 as a thermal process, during which the product's internal water content, abruptly steamed, leads to the breaking-up of the product; extrusion is defined as a thermal process, during which the product's internal water content is rapidly evaporated leading to the breaking-down of the product, combined with specific shaping of the product by passing through a defined orifice.

Expansion can be applied to a compound feed for poultry, pigs and cattle for 5–10 s at pressure up to 50 bar and developing temperatures of 105, 115 and 130°C, respectively. Feed materials may be expanded at lower temperature (80–110°C) and pressure (20–40 bar). Moisture of the product during the process is > 17%.

During extrusion, higher temperatures (up to 200°C) and pressures (40–100 bar) can be obtained, at an intermediate moisture up to 65%, but feed is exposed to the entire process only for a few seconds.

3.3.3.6. Ensiling – preservation of forages

Simard and Lambert-Beaudt (2016) investigated the survival of weed seeds when ensiled together with crops. For simulating the ensiling process, the authors used mini-silos. Seven weed species (100 seeds each of (Common ragweed (*Ambrosia artemisiifolia* L.), Canada fleabane [*Conyza canadensis* (L.) Cronquist], and kochia [*Kochia scoparia* (L.) Shrad.], Redroot pigweed (*Amaranthus retroflexus* L.) and velvetleaf) inserted in nylon bags) were placed at random locations in mini-silos together with maize or alfalfa. Seed survival was measured at start, 1, 3 and 6 months after start of ensiling. There were five replicates per storage time for all treatments (including control group without seeds). Viability was tested by germination, ungerminated seeds by standard tetrazolium staining.

Samples of both silage types with *A. artemisiifolia* inserted taken after 1 month contained still viable seeds (< 10%). But 1 month viability of the other seed species varied considerably (1–55% in alfalfa silage, up to 80% in maize silage). After 3- or 6-month storage no viable seeds could be identified for *A. artemisiifolia* in both crops. Viability of the other seeds was also zero or close to null.

3.3.4. Survival of *Ambrosia* seeds in differently processed feeds

- Oilseed meals obtained during oil production

The temperatures of about 120°C are applied to oilseeds in a humid environment by steam injection for ≥ 10 min during solvent removal and toasting (see Section 3.3.3.1). These conditions

¹² Information Sources: Désialis, a collective approach by alfalfa and sugar beet pulp producers to develop sales of their French dehydrated products intended for animal nutrition, and United States Environmental Protection Agency (<https://www.epa.gov/default/files/documents>).

have been shown to be sufficient to render *Ambrosia* seeds non-viable (see Section 3.3.2). Consequently, it can be concluded that solvent extracted and toasted oilseed meals will not contain viable *Ambrosia* seeds.

For oilseed cakes/expeller for which solvent removal and toasting steps are not applied, evidence is lacking indicating that *Ambrosia* seeds will be completely destroyed during the production processes of oilseed cakes/expeller.

- Milling/grinding

Grinding/milling produces feed materials with various mean particles sizes intended for the different animal species (Table 4). Smallest, however, still viable *Ambrosia* seeds may have a diameter of 1 mm. Seeds of this magnitude could survive the grinding process (e.g. by hammer-mill and roller mill) of feed materials for poultry, pigs and possibly cattle, depending on the maximum particle size of the milled product. When the feed materials are intended for use in feed for piglets, aquatic species and non-food producing animals, grinding would prevent viable *Ambrosia* seeds.

It should be noted that when pellets are pressed (more than 80% of compound feeds are pelleted), the pellet pressing performs a secondary crushing process which may further reduce the viability of the *Ambrosia* seeds.

- Wet milling

Although no data are available, it can be reasonably concluded when considering the production process, that starch and gluten either from maize or wheat wet milling would not contain *Ambrosia* seeds at all.

- Ensiling

Fresh forages contaminated with *A. artemisifolia* seeds when ensiled for a minimum of 3 months will not contain viable seeds (see Section 3.3.3.6). The effect is obtained under anaerobic conditions at a slightly acidic pH value in a wet environment.

- Other heat treatments

Concerning conditioning of feed materials before mixing, the conditions used are considered too mild (low temperature, short duration) to affect seed viability.

Although very high temperatures (above 250°C) are used in the dehydration of fresh alfa-alfa or expanding and extruding feed materials in a humid environment (temperatures up to 130°C and 200°C and moisture > 17% and up to 65%, respectively), no data are available allowing to unequivocally conclude that this process will completely eliminate viability from *Ambrosia* seeds. However, the Panel assumes that viability will be strongly reduced under these conditions.

3.4. Uncertainty

The aim of the uncertainty analysis was to identify and quantify uncertainties affecting the assessment of the viability of *Ambrosia* seeds and combine them to assess the overall certainty of the main conclusions, as recommended in the EFSA Guidance on uncertainty analysis (EFSA Scientific Committee, 2018). The uncertainty was quantified for the conclusions for which data were available. For quantification the overall uncertainty by expert judgement, the subjective probability scale recommended for harmonised use in EFSA was applied. The uncertainties were prioritised and not quantified if of low priority. The experts opinion was elicited for the overall uncertainty associated with the final outcomes through expert group judgement taking into account the identified sources of uncertainty.

3.4.1. Identification of sources of uncertainty

In a first step, the individual sources of uncertainties were identified, discussed and prioritised for quantification (Table 5). Subsequently, high priority uncertainties were quantified by expert judgement.

Table 5: Prioritisation of uncertainties for their impact on the final conclusions

Section	Question	Source of uncertainty	Prioritisation of uncertainties for quantification
3.1 Characteristics and nature of <i>Ambrosia</i> spp.	Minimum size of <i>Ambrosia</i> seeds	Minimum size has been set to 1 mm	Low
3.2 Methods of analyses	Methods available to detect non-viable seeds	Combination of germination and TTC	Low
3.3 Effect of processing on the viability of <i>Ambrosia</i> seeds	Levels of contamination	Limited data are available, however, the levels of contamination are considered low.	Low
3.3.2 Conditions to reduce <i>Ambrosia</i> seeds viability	Temperature and time	100 °C for ≥ 10 min in hot steam	Low
3.3.3 feed treatment	Conditions for the different steps	Sufficient information	Low
	Milling/Grinding	Particle size ranges of feed materials foreseen in compound feed for different target animals are available only for the mean and not for the 95th percentile.	High
Overall assessment	Is experimental testing of <i>Ambrosia</i> seeds viability representative of <i>Ambrosia</i> seed survival during feed manufacturing processes.	<i>Ambrosia</i> seeds viability has not been tested in batches simulating commercial feeds. However, the Panel considered the data obtained with <i>Ambrosia</i> seeds to adequately represent their survival under feed manufacturing processes.	Low

The lack of data on the range of the particle size at the 95th percentile was considered as a source of high uncertainty regarding the role of grinding/milling on the viability of *Ambrosia* seeds. Data were available for the range of the mean particle sizes for several animal feeds. In order to estimate the range of the particle sizes at the highest percentile the experts considered the publication by Lyu et al. (2020, 2021), who studied the particle size distributions of different grinding processes. From the data presented, the experts considered that the range of the 95th percentile could be estimated by applying a factor of 1.5 to the upper range of the mean values.

To obtain a final assessment of overall uncertainty, the experts made judgements on the certainty for the following conclusions:

- What is the probability that solvent extracted and oilseed meals toasted at temperatures of about 120°C with steam injection and for time of ≥ 10 min does not contain viable *Ambrosia* seeds?
- What is the probability that feed materials submitted to grinding/milling for poultry, pigs and cattle contain viable *Ambrosia* seeds?
- What is the probability that feed materials submitted to grinding/milling for piglets, aquatic species and non-food producing animals contain viable *Ambrosia* seeds?
- What is the probability that ensiled fresh forages contaminated with *A. artemisiifolia* seeds for more than 3 months contain viable *Ambrosia* seeds?
- What is the probability that the combination of germination test plus subsequent TTC identify the non-viable cells?

Consensus probabilities have been obtained as expressed in the final conclusions below.

4. Conclusions

4.1. Processing conditions which make *Ambrosia* seeds non-viable

Oilseed meals obtained during oil production

- It can be concluded with a certainty of 99–100% that solvent extracted and oilseed meals toasted at temperatures of about 120°C with steam injection and for time of ≥ 10 min will not contain viable *Ambrosia* seeds.
- No evidence has been found indicating that *Ambrosia* seeds will be rendered non-viable during the production processes of oilseed cakes/expeller for which solvent removal and toasting steps are not applied.

Milling/grinding

- Viable *Ambrosia* seeds may have a diameter down to 1 mm. Seeds of this magnitude could survive the grinding process (e.g. by hammer-mill and roller mill). Since milling/grinding of materials for poultry, pigs and possibly cattle feeds would allow particle sizes ≥ 1 mm to pass the grinding process, it was considered likely (with 66–90% certainty) these feeds to contain viable *Ambrosia* seeds from contaminated materials.
- When the feed materials are intended for use in feed for piglets, aquatic species and non-food producing animals, milling or grinding would very likely prevent ($\geq 90\%$ certainty) the viability of *Ambrosia* seeds.
- It should be considered that when pellets are pressed, the pellet pressing performs a secondary crushing process which may further reduce the viability of the *Ambrosia* seeds.

Wet milling

- It can be concluded with 99–100% certainty that starch and gluten either from corn or wheat wet milling would not contain *Ambrosia* seeds. This is an assumption based on the conditions during the production process although no data are available to confirm.

Ensiling

- It is very likely ($\geq 90\%$) that fresh forages contaminated with *A. artemisiifolia* seeds when ensiled for a minimum of 3 months will not contain viable seeds.

Other heat treatments

- Conditioning of feed materials before mixing is considered too mild (low temperature, short duration) to affect seed viability.

Combined heat and mechanical treatments

- Although very high temperatures (above 250°C) are used in the dehydration of fresh alfa-alfa or expanding and extruding feed materials in a humid environment (temperatures up to 130°C and 200°C and moisture $> 17\%$ and up to 65%, respectively), no data are available allowing to unequivocally conclude that this process will eliminate viability from *Ambrosia* seeds. However, the Panel assumes that viability will be strongly reduced under these conditions.

4.2. Reliable procedures to verify the non-viability of the seeds

- The germination test alone cannot reliably detect all viable seeds due to the possible presence of dormant seeds.
- Non-viable seeds will not be stained in the TTC test, when this is properly applied. However, old seeds may be intermediately stained in the TTC test and they can eventually be viable or non-viable.
- No single test is fully sufficient to check the viability of *Ambrosia* seeds.
- The combination of germination test plus subsequent TTC will very likely ($\geq 90\%$ certainty) verify the non-viable cells.

5. Recommendations

It is recommended that data on the presence of viable *Ambrosia* seeds before and after the different feed production processes are to be generated.

6. Documentation as provided to EFSA

- 1) Conditions in feed production. April 2023. FEDIOL.
- 2) Documentation provided to EFSA: Thermal stress during processing. April 2023. B + B engineering/DGF.
- 3) Particle size. April 2023. Research Institute of Feed Technology (IFF).
- 4) Wheat milling. April 2023. Starch Europe.

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Abbreviations

CO ₂	carbon dioxide
CONTAM Panel	Panel on Contaminants in the Food Chain
EB	Evans blue test
FDA	fluorescein diacetate test
FEDIOL	EU vegetable oil and proteinmeal industry association
FEEDAP	Feed Applications unit
IAFA	International Association of Feedingstuff Analysis
IFF	International Research Association Feed Technology
LoD	limits of detection
LoQ	limits of quantification
pH	potential of hydrogen
TTC test	triphenyl tetrazolium chloride test

Annex A – Protocol for the Assessment of the processing conditions which make the *Ambrosia* seeds non-viable

Annex B – Information provided by the Food/Feed Business Operators

Annex C – Literature search and selection for relevance of studies

Annexes A–C are available under the Supporting Information section on the online version of the scientific output.