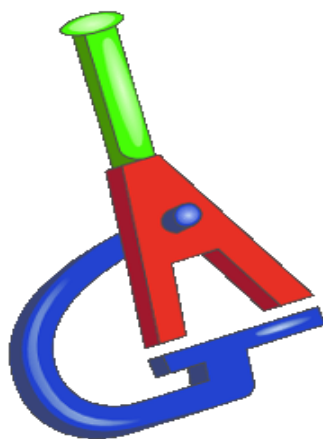




IAG-A5: Method for the Determination of Fruits and Seeds of *Ambrosia spp.* in Animal Feedstuff 2019



International Association of Feedstuff Analysis
Section Feedstuff Microscopy
2019



Ambrosia spp.: fruits and seeds



1. Objective and field of application

Aim of this method is to support the prohibition in Directive 2002/32/EC and, in that framework, to help to minimize the risk of distribution of seeds of *Ambrosia spp.*

The method is used for both qualitative and quantitative determination of whole fruits and seeds of *Ambrosia spp.* in raw material, non-pelleted and pelleted animal feedstuff.

2. Principle

Ambrosia spp. is determined by the visual identification of their fruits and seeds. Quantification is done by weighing and counting the amount of identified whole fruits and seeds in the sieved fractions of the sample.

3. Reagents

3.1 Depelletising:

Distilled Water

4. Equipment and accessories

- Stereo microscope (up to 70x magnification)
- Magnifier (up to 10x magnification)
- Analytical balance (accuracy 0,1 g)
- Analytical balance (accuracy 0,001 g)
- Sieves with different mesh size (1,5 mm and 4 mm)
- Reference material
- Additional laboratory equipment is listed in supporting documents (9)

5. Procedure

5.1 Preparation of the laboratory sample

5.1.1 Non-pelleted feed

The non-pelleted laboratory sample (at least 500g) is weighed and sieved. All three sieve fractions (lower 1,5mm, between 1,5 – 4,0mm and above 4,0mm) have to be analysed.

5.1.2 Pelleted feed

Pelleted feed has to be carefully depelleted in a first step according to IAG-Method A1 Sample preparation for macroscopic and microscopic analysis (9) under 5.2.2.2 or 5.2.2.3.



5.2 Identification by microscopy

5.2.1 Fruits and Seeds

Fruits and seeds of *Ambrosia spp.* are identified based on their characteristic features. The identification of fruits and seeds will be facilitated by comparison to reference material and existing descriptions (10).

Fruits and seeds of *Ambrosia spp.* observing under the stereomicroscope at magnification six times or more shows following characteristics:

The fruits are enclosed individually in the flower bracts which form a 1 up to 8 mm long and 1,5 up to 4 mm wide non-fleshy fruit presenting one apical thorn and several other spines placed as a crown. The characteristic rough skin is usually still present on the seeds isolated from the feed samples; but in some cases, the more or less naked and glossy seeds are found. By removing the pericarp you still will find the characteristic structures of the squarosed up to spiny crest with its terminal thorn. The seeds are coloured dark-brown to black.

The fruits of *Ambrosia artemisiifolia* ovate 2.0-3.5 mm long, 1.5-2.0 mm wide, narrowed at the base, with 5 to 7 small lateral thorns and one bigger thorn at the tip.

The fruits of *Ambrosia psilostachya* are similar to those of *Ambrosia artemisiifolia*, 2.5-3.0 mm long, 2.0-2.5 mm wide, but with lateral thorns not well developed or absent.

The fruits of *Ambrosia trifida* are 7-8 mm long, 3-4 mm wide and similar to those of *Ambrosia artemisiifolia*, with 4 to 8 small lateral thorns.

5.2.2 Pictures of *Ambrosia spp.*

All pictures made by stereomicroscope under 6x magnification, showing the different colours (Fig. 1-3) and size (Fig. 4-6) of the fruits of *Ambrosia spp.*, the seed naked and with parts of the pericarp (Fig.7) and some damaged seeds (Fig. 8).



Ambrosia spp.: Fig. 1-3: different colours (from dark to light brown) of fruits



Ambrosia spp.: Fig. 4-6: different size of fruits



Ambrosia spp.: Fig. 7: seeds, naked and with parts of the pericarp

Ambrosia spp.: Fig. 8: damaged seeds

5.3 Detection and quantification

The quantification of unground seeds and fruits of *Ambrosia spp.* is performed by using all three sieve fractions (lower 1,5mm, between 1,5 - 4,0 mm and above 4,0 mm).

The entire sample (if necessary in fractions of principally 500 grams) as defined in Regulation (EC) 152/2009 amendment of Regulation (EU) 691/2013) is evaluated in order to avoid problems from inhomogeneity. The procedure includes weighing the sample and sieving using the sieves of 1,5 mm and 4 mm. All *Ambrosia spp.* seeds are selected from all three fractions. It can be helpful to spread a portion of the grains in a large flat bin. The fruits or seeds preferably form a layer of one fruit/seed thick. Every fruit/seed is inspected based on shape and colour and fruits are moved to a pile in the corner of the bin when matching the description of a fruit of *Ambrosia spp.* After a full examination of the sample the selected fruits per size fraction are counted and weighed, and the percentage is calculated.

5.4 Determination by Determinator

→ Annex 3



6. Calculation and report

6.1 Calculation

The amount of *Ambrosia spp.* seeds/fruits in mg/kg (ppm) feedstuff (original sample) is calculated using the following formula:

$$C = \frac{BC \times 1000}{E} \text{ [mg/kg]}$$

C = amount of *Ambrosia spp.* seeds/fruits in mg/kg feedstuff (ppm)

BC = selected *Ambrosia spp.* seeds/fruits in the laboratory sample or an aliquot of it [mg]

E = total weight of the laboratory sample or an examined aliquot of the laboratory sample [g]

6.2 Report

6.2.1 Negative result:

As far as was discernible using a microscope, unground *Ambrosia spp.* seeds/fruits were not found in the submitted sample.

6.2.2 Positive result:

As far as was discernible using a microscope C mg unground seeds/fruits of *Ambrosia spp.* per kg feedstuff were found in the submitted sample.

Optional: As far as was discernible using a microscope x unground seeds/fruits of *Ambrosia spp.* were found in the submitted sample.

7. Validation

For validation – if needed - results of the listed IAG-Ringtest can be used:

- 2010 IAG Ringtest Ambrosia in bird feed organized by Agroscope Posieux (CH)
- 2011 IAG Ringtest Ambrosia in bird feed organized by Agroscope Posieux (CH)
- 2012 IAG Ringtest Ambrosia in linseed organized by CVUA-RRW Krefeld (D)
- 2014 IAG Ringtest Ambrosia in bird feed organized by RIKILT Wageningen (NL)
- 2015 IAG Ringtest Ambrosia in bird feed organized by Agroscope Posieux (CH)



8. Remarks

- 8.1 Fragments of *Ambrosia spp.* seeds/fruits are not determined, because they are unable of further spreading.
- 8.2 This method also is suitable for the examination of raw material and food.
- 8.3 This method has been developed by the International Association of Feedstuff Analysis (IAG) – Section Feedstuff Microscopy.

9. Supporting document

IAG-Method A1 for Sample Preparation for Macroscopic and Microscopic Analysis in its actual Version

→ IAG-Homepage (www.iag-micro.org)

10. Literature

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ANNEX 1

1. General information about *Ambrosia spp.*

All the following general information and much more information about *Ambrosia spp.* can be found in the internet per example under:

→ https://en.wikipedia.org/wiki/Ambrosia_artemisiifolia (look also at the other languages)

- Common name is Ragweed.
- Ragweeds are flowering plants in the genus *Ambrosia* in the aster family, Asteraceae. There are a large number of species in genus *Ambrosia*.
- They are distributed in the tropical and subtropical regions of the New World, especially in North-, South- and the Middle of America.
- *Ambrosia spp.* fruits and seeds were verifiably imported to Europe by cereals and oilseed and are distributed mainly by the use of bird feeds.
- Plants of *Ambrosia spp.* can produce on average 3000 - 6000 fruits and seeds, which are germinable for many years and produce up to a billion of pollen.
- Concentrations of less than 10 pollen/m³ air can cause allergic reactions by human. Up to now there is no immediate risk of fruits and seeds of *Ambrosia spp.* found in feed for animals, but there are first indications that pollen of *Ambrosia spp.* are effectively allergenic to animals.
- Longevity: The seed of *Ambrosia spp.* has very little to moderate persistence in the soil. It takes less than 1 year for the seed bank to be reduced by 50% and about 10 years for 99% depletion.
- Dormancy: *Ambrosia spp.* seed requires winter chilling (overwintering) to remove primary dormancy.
- *Ambrosia spp.* can also invade agricultural land where it acts as a weed in a number of crops (in particular in sunflower, maize, soybean and cereals) and can cause significant decreases in yields. Additional agricultural problems may be the seedinhibiting substances for economic plants and the possibility to act as additional host for pests.

1.1 Plants (Fig. 9)

The hairy stems are green to light pinkish red (Fig. 11). The leaves are up to 15cm long and 10cm across, and are opposite or alternate along the stems. They are deeply pinnatifid, broadly lanceolate (in outline), and usually much wider at the base than the tip (Fig. 10). Many of the upper stems terminate in one or more cylindrical spikes of flowers. The small flowers are initially green, but later turn yellowish green or brown as they mature and develop into achenes. The male flowers producing a fine yellow pollen that is easily carried by the wind. Numerous seeds are produced by the female flowers, which can remain viable many years.



Ambrosia spp.: Pictures of plant, leaves and stem (Fig. 9-11)

1.1.1 *Ambrosia artemisiifolia*

Ambrosia artemisiifolia is an annual weed. It reproduces by seeds. One plant may develop 30.000 to 40.000 seeds. Seeds can keep their vitality 5 to 14 years. On heavily infested plots, the population rate can reach till 500 plants/m². Seeds germinate in warm and well-aerated soil. The plants secrete phytotoxic substances.

Ambrosia artemisiifolia grows 20-180 cm tall, sometimes reaching 250 cm. The stem is upright, angular, much branched. The whole plant is hairy. The leaves are pinnatifid, 5-10 cm long. The root is vertical, and can reach a depth of 4 m. Male and female flowers are borne in separate few-flowered heads, the male heads in long inflorescences at the tips of the shoots, and the female ones in the axils of the leaves and at the base of the male inflorescence.

1.1.2 *Ambrosia psilostachya*

Ambrosia psilostachya is a perennial weed. It reproduces mainly by rhizomes. Few seeds are formed, but they are important in spreading the weed over long distances.

Ambrosia psilostachya has an upright stem reaching 100 cm or more. The lower leaves are opposite, and the upper alternate. The leaves are pinnatifid, petiolate, 5-12 cm long. The stem, leaves and branches are covered with dense hairs, giving the plant a grey-green colour. Inflorescences are similar to those of *Ambrosia artemisiifolia*, but male inflorescence is denser, 7 to 15 cm long and may contain 50 to 100 heads of flowers.

1.1.3 *Ambrosia trifida*

Ambrosia trifida is an annual weed. It reproduces by seeds. Its biology is similar to that of *Ambrosia artemisiifolia*, but it is more frost-resistant, develops faster, and its mature seeds appear earlier.

Ambrosia trifida has an upright stem reaching 100 cm and more (exceptionally 300 cm). The leaves are opposite, 3-palmately lobed (lower leaves may be 5-palmately lobed, higher leaves may be non-lobed), petiolate. The inflorescences are similar to those of *Ambrosia artemisiifolia*, but the male inflorescence is longer (up to 20 cm) and the female heads are larger (2-4 mm in diameter).



ANNEX 2

Tetrazoliumtest for *Ambrosia spp.*

Materials:

- Seeds and Fruits of *Ambrosia spp.*

If there are a lot of them, choose randomly, i.e., do not exclude small or light ones that you might suppose to be less viable.

Reagents:

- 1% TTC-solution (ca. 100ml)
- Distilled water
- Tap water

Implementation:

Fruits/seeds (diaspora) of *Ambrosia spp.* are imbibed in tap water at room temperature overnight (i.e., for ca. 12 -15hours). The fruits/seeds are cut open with a surgical scalpel or similar instrument in a vertical line (top to base). The bigger part of the fruit/seed is used for testing, the other part is discarded. The fruit/seed halves are put into a glass and filled up with TTC solution. Glasses are tightly closed and put to react at 30°C for 6 hours in darkness. Because TTC is light sensible, avoid unnecessary light input. If it is not possible to keep on with the protocol after these 6h, the closed glasses can be stored in a refrigerator (~6-8°C) over night. TTC solution is poured off and halves are rinsed under distilled water. Under a dissecting microscope the seedhalves are removed from the integument (outer shell).

Seeds are counted in 3 classes:

- a) stained (=alive),
- b) not stained resp. no fully developed embryo present (=dead),
- c) intermediate cases that are only lightly or partly stained.

For the decision on intermediate see below.

The staining of different tissues in the seed may have different implications for the interpretation of the test. A dead (= not stained) radicula in an otherwise stained seed will mean that the seed is dead (Fig. 12).

For the sake of simplicity and ease of judgment, all seeds that are completely stained shall be deemed alive, seeds without any trace of staining will be deemed dead and the rest intermediate.



Ambrosia spp.: Fig 12.: Tetrazoliumtest: red seeds alive, uncoloured seeds dead



ANNEX 3

Determination by Determinator

Some more supporting information for identification can be provided by Determinator, where an unpublished module on Ambrosia fruit identification is available. There are advantages using an expert system, opportunities for training and sample documentation among them, but this module needs validation and approval up to now.