Determination of the dusting potential and endotoxin activity of three batches of Amino acid zootechnical mixture

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Contents

Summary

Three batches of a new product were provided by Metex-Noovistago France, for analysis of dustiness and endotoxin content. The product consists of a mixture of three amino acids (L-arginine (L-Arg), L-glutamine (L-Gln), L-threonine (L-Thr)) and a dry plant extract (grape extract of Vitis Vinifera spp. Vinifera). The area of application of the new product is to be used for animal nutrition.
Methods applied were conform to those proposed by EFSA (EFSA Journal 2012;10(1):2539)3, i.e. the Stauber-Heubach analysis determining the dust potential and light scattering to determine the particle size distribution. Endotoxin activities were determined by the Limulus amoebocyte lysate (LAL) test.
From the analysis of the particle size distribution and endotoxin content, classification of representative samples of the three batches of the product was achieved. More specifically from these experiments, the following conclusions are drawn:

- For the three batches a comparable amount of dust was generated in the Stauber-Heubach test. The three batches have similar, bimodal, particle size distributions consisting of relatively small particles with peak positions at few hundreds of nm and at 4-7 microns.
- All three batches can be classified as “moderate” for inhalable, and “high” for thoracic and respirable potentials.
- Endotoxin levels varied among the different batches and were relatively low, being 74.1, 51.3 and 104.9 EU/mg for respectively batch AA Mix#1, AA Mix#2 and AA Mix#3.

The data obtained can be used for the calculation of inhalation exposure under practical conditions in a factory.

1 Introduction

1.1 Background

A new product is produced by Metex-Noovistago (Metex),is intended to be used in animal nutrition and will be brought on the market in dry form. The new product is a mixture of three amino acids (L-arginine (L-Arg), L-glutamine (L-Gln), L-threonine (L-Thr)) and a dry plant extract (grape extract of Vitis Vinifera spp. Vinifera). In this report, the above described product will be referred as amino acid zootechnical mixture or 'the product'. Despite the purification steps of the manufacturing process, the product may contain some level of endotoxins to which workers may be exposed while they handle it. Therefore, additional information is sought on the particle size distribution of the product, its classification according to dustiness, and its endotoxin content. Three different batches of the amino acid zootechnical mixture in powder form were provided by Metex, to a third party - Research Diet Service. Wageningen Food & Biobased Research (WFBR) took samples from these batches and further distributed this to another third party-Delft Solid Solutions, where measurements for dustiness and particle size distribution were carried out. During the sampling moment WFBR took also part of each batch and used it for measurements for endotoxin content (performed by WFBR). The findings from both studies were analyzed by WFBR to draw results, conclusions and assemble them to the present report.

1.2 Objective

The objective of the study is to obtain and record data on the classification according to dustiness and endotoxin content of amino acid zootechnical mixture. Analyses were carried out on the product in powder form.

1.3 Sponsor

Sponsor of the present study is:Metex-Noovistago, Address: 32 rue Guersant, 75017, Paris France.

1.4 Study parameters

Study parameters are dustiness in mg/m^3 determined by the Stauber-Heubach method, particle size distribution, aerated and packed bulk density, endotoxin content and classification according to dustiness and endotoxin contents in the dust fractions of the feed product, amino acid zootechnical mixture.

1.5 Study outline

For this study a representative sample from each of the three different batches of amino acid zootechnical mixture as supplied by the sponsor were taken. The batches were stored at WFBR in a fridge at 4-7^{o}C as part of a more extensive study on stability. The samples were used for both classification of dustiness (in mg/m^3), according to the Stauber-Heubach method as proposed in EFSA Journal[3], and determination of the endotoxin content of the dust generated according to the Limulus amoebocyte lysate (LAL) assay. The results and conclusions are presented in this report.

2 Materials and methods of analysis

2.1 Test product

Three different batches of the amino acid zootechnical mixture were tested in this study. The code for each of the batches along with the corresponding iLes number as provided by Metex are presented in Table 2-1.

Table 2-1 Amino acid zootechnical mixture samples of the present study.

Sample	iLes number (Metex)
AA Mix #1	E00006442 001
AA Mix #2	E00006442 002
AA Mix #3	E00006442 003

Visual inspection of the samples shows that the three batches are fine powders with an off-white color containing dark specks.

Representative samples of a suitable size were taken from each batch namely AA Mix#1, AA Mix#2 and AA Mix#3 on 19 October 2021 for endotoxin determination, in order to prevent contamination during handling of the samples. Also representative samples of suitable size were taken on the same date, labelled and sent to a third party - Delft Solid Solutions (DSS) on 23 December 2021 for the classification according to dustiness by the Stauber-Heubach method. The remaining parts of the samples of the three batches of test product were stored as backup samples at a controlled temperature of a 4-7°C at WFBR.

2.2 Dustiness by the Stauber-Heubach method

The Stauber-Heubach method (type II) differs from the classical Heubach method[1] (type I) in the way the coarse particle separator and filter housing are positioned in relation to the drum. They are positioned upwards at an angle of 110° and this configuration is referred to as Stauber-Heubach (or type II)[2]. This is further exemplified in Figure 2-1. To determine a reliable dustiness value, at least three independent measurements are performed with samples of each batch of the amino acid zootechnical mixture and the average is calculated. The used mass of product is approximately 50 g, weighed with an accuracy of at least 0.01 g. The dust generating drum is rotated at 30 rpm during 5 minutes and with an air flow rate of 4 L/min. In this period of time, a total of 20 L of air is being used. The dustiness is always reported with the experimental conditions.

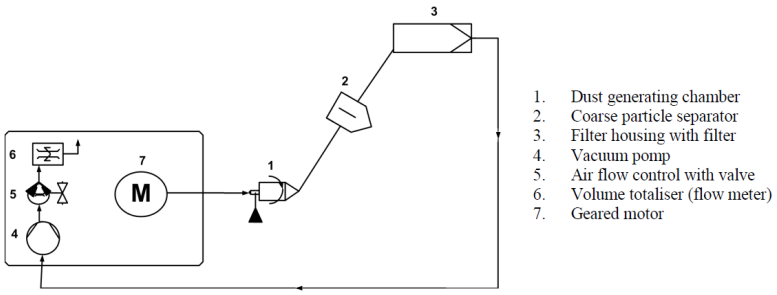

Figure 2-1 Schematic representation of the Stauber-Heubach set-up.[2]

2.3 Particle size analysis by laser diffraction

The particle size distributions are measured on the dust collected from the Stauber-Heubach measurements. Since the Stauber-Heubach method uses an air stream to transport the dust to the filter, the particle size distribution is also measured dry dispersed in air. The dust from the Stauber-Heubach analysis is gathered by tapping against the filters and samples are taken for measuring the particle size distribution. The particle size distribution via laser diffraction is measured with a Malvern Mastersizer 2000 in combination with a Scirocco 2000 sample dispersion unit for dispersing the samples in air.
In general small particles are cohesive and will form agglomerates. A venturi pressure of 2 bar is used to disintegrate any agglomerates and form primary particles to be measured by light scattering. The configuration for measuring in air has a measuring range from 0.1 µm up to 2000 µm. The optical model used is Lorentz-Mie taking into account the optical characteristics of the particles, in accordance with ISO 1332-1:2009[5]. In the method a particle refractive index of n=1.52 and an absorption index of 0.1 is used. The particle refractive index (1.52) is the common used value for particles of which the particle refractive index is unknown and the absorption index of 0.1 is typical for white to slightly colored powders. The validity of the optical characteristics is checked by the quality of the fit of the deconvoluted diffraction pattern and the measured diffraction pattern.

2.4 Bulk density

The Hosokawa powder tester is used to determine the aerated density, packed density and the compressibility. Fine powders are led through a sieve of 710 µm and samples with large particles are led through a sieve of approximately 3 mm to obtain a loosely packed powder bed. The powder is collected in a sample cup of exactly 100 cm^3, this is then referred to as aerated density. To go from an aerated to a packed bulk density, 180 taps were used to compact the sample material as documented in the respective ASTM standard[5].

2.5 Endotoxin determination

The use of LAL assay for the detection of endotoxin evolved from the observation by Bang[8] that a Gram-negative infection of limulus Polyphemus, the horseshoe crab, resulted in fatal intra-vascular coagulation. Levin and Bang[8,9] later demonstrated that this clotting was the result of a reaction between endotoxin and a clottable protein in the circulating amoebocytes of limulus. Following the development of a suitable anticoagulant for limulus blood, Levin and Bang[10] prepared a lysate from washed amoebocytes which was an extremely sensitive indicator of the presence of endotoxin. Solum[11,12] and Young, Levin and Prendergast[13] have purified and characterized the clottable protein from LAL and have shown the reaction with endotoxin to be enzymatic. The present LAL method utilizes the initial part of the LAL endotoxin reaction to activate an enzyme, which in turn releases p-nitroaniline (pNA) from a synthetic substrate, producing a yellow color.

The principle of endotoxin determination using the LAL is a two-step method:

$$\text{LAL Proenzyme} \xrightarrow{\text{Endotoxin}} \text{Enzyme}$$

$$\text{Substrate} + H_2O \xrightarrow{\text{Enzyme}} \text{Peptide} + \text{pNA}$$

The 'endpoint chromogenic LAL test is a quantitative test for gram-negative bacterial endotoxin. Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the LAL[13]. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyzes the release of pNA from the colorless substrate Ac-Ile-Glu-Ala-Arg-pNA. The free pNA is measured photometrically at 405–410 nm after the reaction is stopped with stop reagent. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1–1.0 EU/mL range (EU = endotoxin units).

The concentration of endotoxin in a sample is calculated from the absorbance values of solutions containing known amounts of endotoxin standard.
Three checks were made to measure the endotoxin levels in the products received. The first two used the Pierce LAL chromogenic Endotoxin Quantitation Kit from Thermo scientific (catalog number Pierce 88282). The final test was performed using the LAL QCL-1000 Kit from Lonza (product number 50-647U). Both kits are identical in setup, only the Lonza kit can handle a larger number of samples to be determined. The kit provides a lyophilized stock of *E.coli* endotoxin. This is reconstituted in 1 ml of pyrogen free water. From this stock solution a standard series of dilution is made containing 1.0; 0.50; 0.25 and 0.1 EU/ml. This standard series of dilution was used to calculate the activity in a sample dilution. LAL Proenzyme solution and Chromogenic substrate solution were made according to the instructions of the manufacturer.

A sample or standard was mixed with the LAL Proenzyme solution and incubated at 37°C (±1°C) for 10 minutes to allow the activation of the proenzyme. A substrate solution was then mixed with the LAL-sample and incubated at 37°C (±1°C) for an additional 6 minutes. The reaction was stopped with stop reagent (25% acetic acid). If endotoxin is present in the sample, a yellow color will develop. The absorbance of the sample can be determined spectrophotometrically at wavelength 405–410 nm. Since this absorbance is in direct proportion to the amount of endotoxin present, the concentration of endotoxin can be calculated from the calibration curve. All samples and standards were measured in triplicate unless stated otherwise.

2.6 Estimation of inhalable, thoracic and respirable fractions

The dust obtained by the Stauber-Heubach method and analyzed by diffraction light scattering is calculated according to the inhalable, thoracic and respirable fractions using the DIN EN 481 standard[6]. However, this standard only provides tabulated values and formula based on the aerodynamic diameter of monodisperse particles. No guidelines are given, however, to estimate the dust potentials from a given size distribution as measured by light scattering. The estimation of the dust potential, based on size distribution data and using the tabulated aerosol fractions given in DIN EN 481, is presented here. On this reference (DIN EN 481) an explanatory schematic representation of the three fraction the fractions as a function of the aerodynamic diameter is provided and is presented on Figure 2-1.

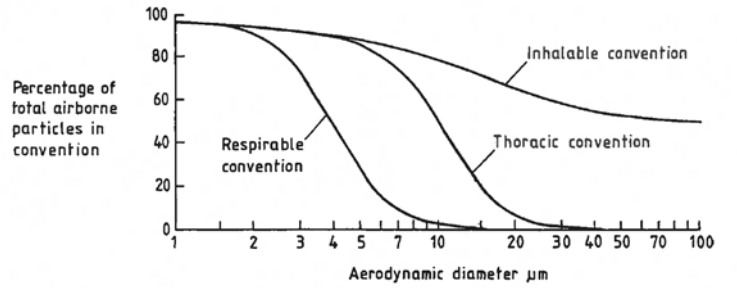

Figure 2-1 Inhalable, thoracic and respirable fractions as percentages of total airborne particles. Figure is presented in DIN EN 481.

The inhalable fraction (E_I) as a function of the aerodynamic diameter (D) is given by:

$$E_I = 50(1 + EXP(-0.06D)) \text{ (equation 2.1)}$$

DIN EN 481 also provides numerical approximations for the sigmoidal functions for the calculation of the thoracic and respirable fractions. However, it was found that sigmoidal expressions of the type as presented in equation 2.2 and calculated by regression software (Table Curve 2D v5.01.01) gave better correlations with the tabulated values than those given by DIN EN 481.

$$E_x = a + b(1 - (1 + EXP(\frac{D + dLN\left(2^{\frac{1}{e}} - 1\right) - c}{d})^e)) \text{ (equation 2.2)}$$

In this equation *x* corresponds on respectively thoracic (*t*) and respirable (*r*) and D for the particle diameter. The parameters a, b, c, d and e were fitted for thoracic and respirable fractions and are presented in Figure 2-1. Values for these parameters a, b, c, d and e are provided on Table 2-2.

Table 2-2 Parameters and correlations given in relation to the numerical data presented by DIN EN 481 of the sigmoidal equation 2.2, describing the thoracic and respirable fractions in relation to particle size diameter D.

Parameter	Thoracic	Respirable
A	101.6289	100.5392
B	-101.501	-100.641
C	9.92321	3.997753
D	2.749191	0.744899
E	0.681124	0.446015
R^2	0.999798	0.999953

The assumption here is to use the distributions given by equations 2.1 and 2.2 separately for the log based mean diameter in selected size bands as obtained from the size distribution data provided by diffraction light scattering (Table 2-3).

Table 2-3 Selected size bands for calculation of the inhalable, thoracic and respirable fractions.

Size band number (-)	Lower size (µm)	Upper size (µm)	LN based mean diameter D_i (µm)
1	0.25	0.5	0.35
2	0.5	1	0.71
3	1	2.5	1.6
4	2.5	5	3.5
5	5	10	7.1
6	10	25	15.8
7	25	50	35.4

The inhalable fraction E_{Ii} of particles in size band *i* with a mean size D_i is given by:

$$E_{Ii} = V_i E_I(D_i) \qquad \text{(equation 2.3)}$$

In which E_I refers to equation 2.1. The total inhalable fraction is then calculated by summing all fractions in the different size bands:

$$E_I = \sum_{i=1}^{7} V_i E_I(D_i) \qquad \text{(equation2.4)}$$

Likewise the thoracic and respirable fractions are calculated by:

$$E_x = \sum_{i=1}^{7} V_i E_X(D_i) \qquad \text{(equation 2.5)}$$

in which E_X refers to equation 2.2 using the parameters from Table 2-2.
The above described approach is an improved development of the EFSA DIN EN 481 and was used as such for determining the corresponding inhalable, thoracic and respirable fractions of the samples under investigation.

3 Results

3.1 Stauber-Heubach dust

Details of the Stauber-Heubach analyses of the different batches of amino acid zootechnical mixture, measured as independent triplicates for AA Mix #2 and AA Mix #3, and four fold for AA Mix#1 are presented in appendix A. The average values obtained are presented in Table 3-1 and graphically shown in Figure 3-1.

Table 3-1 Average values of the Stauber-Heubach dust values of the different batches of amino acid zootechnical mixture.

Batch	Dust (mg/kg)	per m^3 air (mg/m^3)	R.S.D. (%)
AA Mix #1	2957	7391	15.1
AA Mix #2	3271	8187	4.6
AA Mix #3	3092	7729	10.5

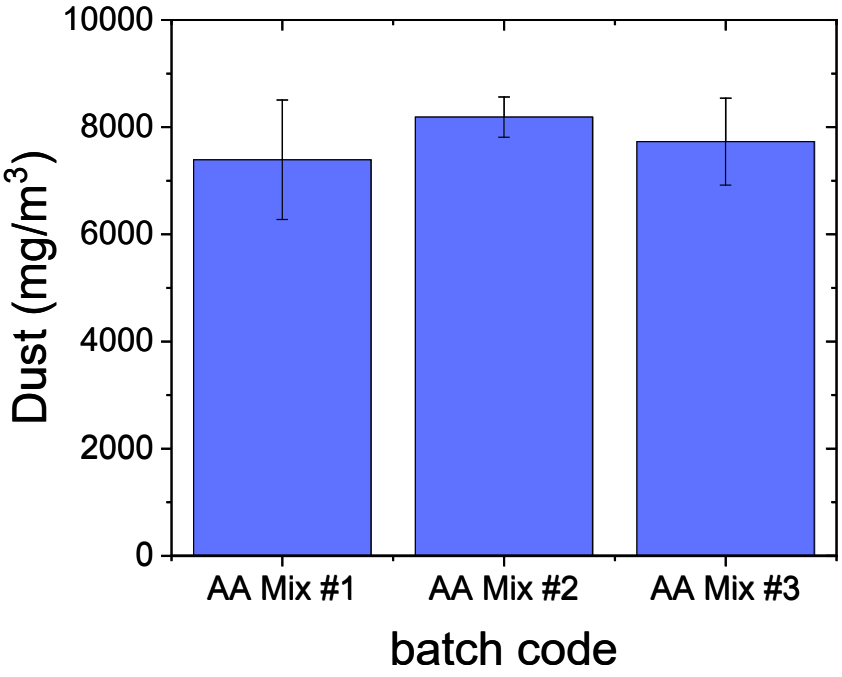

Figure 3-1 Dust values of the three batches of amino acid zootechnical mixture obtained by the Stauber-Heubach method, the bars indicate the standard error on 95% confidentiality base

Dust levels between the three different batches do not have significant differences, though the variances between the triplicates determined were large. Overall the dust variations ranged between 7300 and 8100 mg/m^3.

3.2 Particle size distribution in the dust

Details of the particle size data obtained from the collected dusts of the different amino acid zootechnical mixture batches are presented in Appendix B. Table 3-2 and Table 3-3 respectively summarizes the average size parameters and cumulative percentages.

Table 3-2 Summary of the particle size distribution data of the dust collected over the three batches of amino acid zootechnical mixture.

Batch	D_0 µm	D_{10} µm	D_{50} µm	D_{90} µm	D_{100} µm	D[4.3] µm	Span
AA Mix #1	0.1	2.0	6.9	14.8	30.9	7.8	1.8
AA Mix #2	0.1	1.2	5.5	13.2	30.1	6.5	2.2
AA Mix #3	0.1	1.6	6.2	13.3	27.3	6.9	1.9

The definitions in table 3.2 are as follows:
- D_0: The minimum particle size.
- D_{10}: Distribution Percentile, 10% of the particle volume is smaller than the table value.
- D_{50}: Distribution Percentile, median particle size.
- D_{90}: Distribution Percentile, 90% of the particle volume is smaller than the table value.
- D_{100} Distribution Percentile, 100% of the particle volume is smaller than the table value, the end of the distribution
- D [4,3]: Volume weighted mean diameter $(\Sigma n_i D_i^4)/(\Sigma n_i D_i^3)$.
- Span: Distribution width, calculated as $D_{90} - D_{10} / D_{50}$

Table 3-3 Percentages below a certain particle size of the collected dust of the three batches of the amino acid zootechnical mixture.

Size (µm)	0.1	0.5	1	5	10	25	50
Batch	Vol%	Vol%	Vol%	Vol%	Vol%	Vol%	Vol%
AA Mix #1	0.0	3.9	6.6	32.5	72.0	99.5	100.0
AA Mix #2	0.0	6.2	9.3	45.6	80.0	99.7	100.0
AA Mix #3	0.0	5.2	7.9	39.0	77.4	99.9	100.0

As described earlier the dusts of the three batches AA Mix were similar. From the above described results also their particle size distribution appears to be similar having a bimodal distribution consisting of a large peak around 5-8 µm and a smaller one around 200-400 nm as can be seen from table 3.2 and 3.3 and figures B-1 and B-2 in Annex 2.

3.3 Bulk density

Bulk densities and compressibility obtained for the three batches of the amino acid zootechnical mixture are presented in Table 3-4.

Table 3-4 Bulk density values of the three batches of amino acid zootechnical mixture.

Batch	Aerated bulk density kg/m³	Packed bulk density kg/m³	Compressibility %
AA Mix 1	613	809	24.2
AA Mix 2	632	812	22.1
AA Mix 3	623	797	21.8

3.4 Classification according to dustiness

The volume fractions in the different size bands from the particle size distributions as determined by diffraction light scattering are presented in appendix C. Using these volume fractions the inhalable, thoracic and respirable fractions in each size band and the total fractions, calculated as presented in paragraph 2.6, are also presented in appendix C. The calculated total fractions obtained for the different amino acid zootechnical mixture batches are given in Table 3-5.

Table 3-5 Calculated fractions of the inhalable, thoracic and respirable dust from the three different batches of the amino acid zootechnical mixture.

Batch	E_I inhalable %	E_t thoracic %	E_r respirable %
AA Mix #1	82.51	63.73	29.70
AA Mix #2	84.71	70.80	37.43
AA Mix #3	83.63	67.91	32.34

Table 3-6 presents the dust potentials of the three batches of the amino acid zootechnical mixture calculated as the product of the different fractions presented in Table 3-5, and the average dust values (mg/m^3) obtained by the Stauber-Heubach analyses.

Table 3-6 Calculated inhalable, thoracic and respirable dust potentials of the three batches of the amino acid zootechnical mixture.

Batch	inhalable mg/m^3	Thoracic mg/m^3	respirable mg/m^3	RSD %
AA Mix #1	6098	4710	2195	15.1
AA Mix #2	6935	5796	3065	4.6
AA Mix#3	6464	5249	2499	10.5

The dust potentials determined are classified according to NEN-1551-2[7] as presented in Table 3-7, note that these classifications are given in mg/kg[1].

Table 3-7 Classification table for dustiness measurements with rotating drum (NEN 15051-2[7]).

Dustiness Category	Inhalable mass fraction mg/kg	Thoracic mass fraction mg/kg	Respirable mass fraction mg/kg
Very low	<300	<80	<10
Low	300-650	80-300	10-60
Moderate	>650-3000	>300-1000	>60-250
High	>3000	>1000	>210

Table 3-8 presents the dust potentials from Table 3-6 recalculated in mg/kg and their corresponding classifications.

Table 3-8 Calculated inhalable, thoracic and respirable dust potentials of the three batches of the amino acid zootechnical mixture in mg/kg, and their classifications.

	inhalable mg/kg	thoracic mg/kg	respirable mg/kg
AA Mix #1	2440 (moderate)	1885 (high)	878 (high)
AA Mix #2	2771 (moderate)	2316 (high)	1224 (high)
AA Mix#3	2586 (moderate)	2100 (high)	1000 (high)

[1] No classification in relation to Stauber-Heubach analysis could be found. After consulting experts in the field the classification in relation the drum method is presented here.

3.5 Endotoxin analysis

Results were obtained from the three batches of the amino acid zootechnical mixture could be interpolated within the standard range. Out of the spiked samples an average recovery of 98% was found giving an inhibition factor of 1.01. These results are presented in Table 3-9.

Table 3-9 Endotoxin contents determined in the three batches of the amino acid zootechnical mixture.

Batch	Specific endotoxin amount in product EU/g	error in percentage of endotoxin amount in product %
AA Mix #1	74.1	1.96
AA Mix#2	51.3	3.92
AA Mix #3	104.9	5.42

A relatively high variation was obtained between the batch AA Mix#3 with AA Mix#2 with a factor of 2 difference while AA Mix#1 batch was providing values in between the other two.

3.6 Dust and endotoxin fractions

The inhalable, thoracic and respirable dust fractions were calculated as described in paragraph 2.5 making use of the volume fractions in the different size bands obtained by particle size analysis, as derived from the data presented in Table 3-5. The calculated dust fractions for the three different batches of amino acid zootechnical mixture are presented in Table 3-6. According to Appendix 1 in the EFSA Guidance[3] the exposure to the "active substance", being endotoxin, is taken to be the concentration of "active substance" in the dust multiplied by the dust concentration:

Active substance in air (EU/m^3) = dust (g/m^3) x active substance in dust (EU/g).

Based on this calculation the relative activities of the endotoxins related to the dust potentials of the three batches of the amino acid zootechnical mixture are given in Table 3-10.

Table 3-10 Calculated inhalable, thoracic and respirable endotoxins related to the dust potentials for the three batches of the amino acid zootechnical mixture

Batch	Inhalable EU/m^3	Thoracic EU/m^3	Respirable EU/m^3	RE (p=0.05) %
AA Mix #1	452	438	204	16
AA Mix #2	356	290	153	9
AA Mix #3	678	3905	1860	12

4 Conclusions

Representative samples of amino acid zootechnical mixture (three different batches: AA Mix#1, AA Mix#2 and AA Mix#3) were provided by Metex-Noovistago, France. Light scattering experiments and Limulus amoebocyte lysate (LAL) tests were performed on these samples. From the analyses both the classification of dustiness (in mg/m^3), according to the Stauber-Heubach method, and the determination of the endotoxin content was determined. For the endotoxin content, the method which has been developed previously, has been used for the current analysis.
Based on the above described experiments the following conclusions are drawn:

- The three batches AA Mix#1, AA Mix#2 and AA mix#3 were comparable in amount of dust generated in the Stauber-Heubach test and in the size distribution of the dust particles. All samples seem to have a based bimodal size distribution consisting of relatively small particles of with peak positions at few hundreds of nm and at 4-7 microns.
- All three batches can be classified as “moderate” for inhalable, and “high” for thoracic and respirable potentials.
- Endotoxin levels varied among the different batches, but were relatively low in all three cases, being 74.1, 51.3 and 104.9 EU/g for respectively batch AA Mix#1, AA Mix#2 and AA Mix#3.

The data obtained can be used for the calculation of inhalation exposure under practical conditions in a factory.

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Annex 1 Stauber-Heubach dustiness

Table A-1 presents the data of the Stauber-Heubach analysis of the amino acid zootechnical mixture batches determined as independent triplicates.

Table A-1 Stauber-Heubach analysis of amino acid zootechnical mixture batches determined as independent triplicates.

	Relative humidity (%)	**Sample weight (g)**	**Dust (g)**	**Dust (mg/kg)**	**Dust per m³ air (mg/m³)**	**RSD (%)**
AA Mix #1	49	49.49	0.1282	2591	6478	
	48	49.60	0.1274	2568	6420	
	45	49.82	0.1599	3209	8021	
	51	50.07	0.1732	3459	8647	
Average				2957	7391	15.1
AA Mix #2	55	48.54	0.1513	3117	7792	
	53	48.97	0.1604	3276	8191	
	57	49.52	0.1694	3421	8552	
Average				3271	8187	4.6
AA Mix #2	56	50.41	0.1419	2815	7038	
	54	50.45	0.1519	3011	7526	
	55	49.85	0.1719	3449	8623	
Average				3092	7729	10.5

Annex 2 Particle size analysis

Figure B-1 and Figure B-2 present the differential and cumulative size distributions of the dust collected by the Stauber-Heubach measurements from the three batches of the amino acid zootechnical mixture. All measurements were performed once at 1 bar venture pressure.

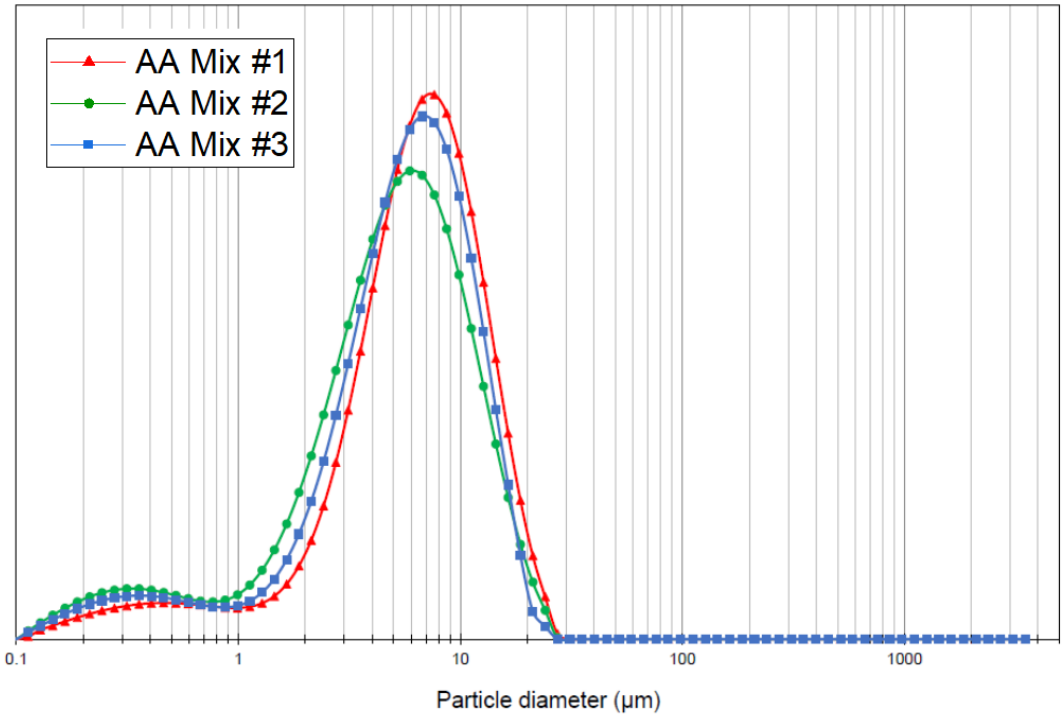

Figure B-1 Differential particle size distribution of the dust collected from the Stauber-Heubach measurements; (–)AA Mix #1, (–) AA Mix #2 , (–)AA Mix #3.

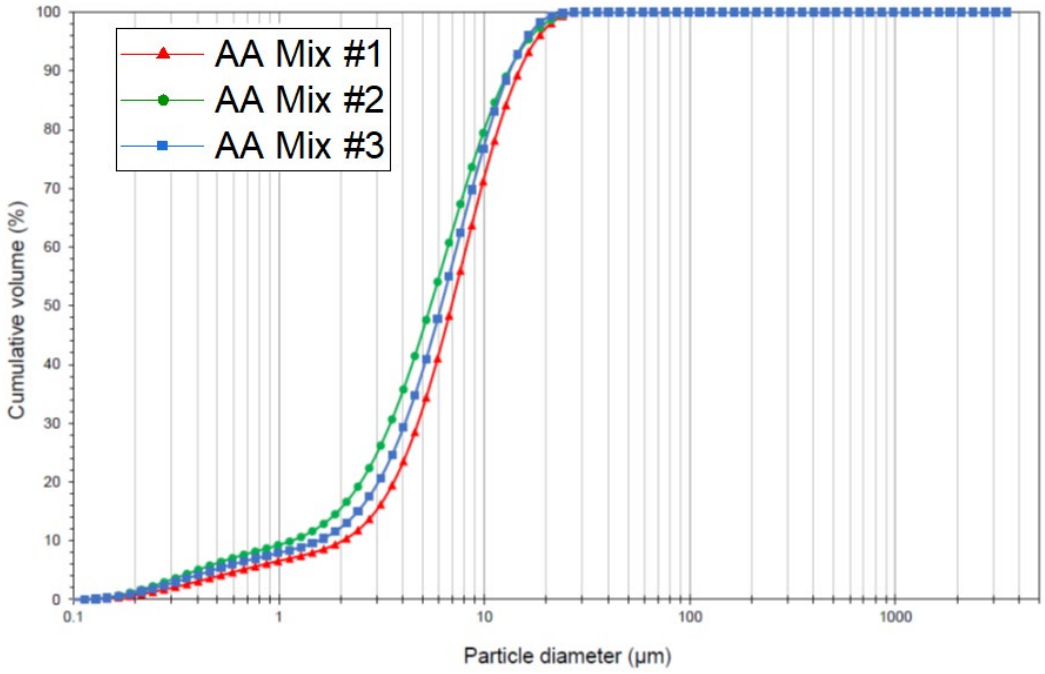

Figure B-2 Cumulative particle size distribution of the dust collected from the Stauber-Heubach measurements; (–)AA Mix #1, (–) AA Mix #2 , (–)AA Mix #3.

Table B-1 Summary of the particle size distribution data of the dust collected over the three amino acid zootechnical mixture batches.

Batch	D_0 µm	D_{10} µm	D_{50} µm	D_{90} µm	D_{100} µm	D[4.3] µm	Span
AA Mix #1	0.1	2.0	6.9	14.8	30.9	7.8	1.8
AA Mix #2	0.1	1.2	5.5	13.2	30.1	6.5	2.2
AA Mix #3	0.1	1.6	6.2	13.3	27.3	6.9	1.9

The definitions in table B-1 are as follows:

- D_{10}: Distribution Percentile, 10% of the particle volume is smaller than the table value.
- D_{50}: Distribution Percentile, median particle size.
- D_{90}: Distribution Percentile, 90% of the particle volume is smaller than the table value.
- D_{100} Distribution Percentile, 100% of the particle volume is smaller than the table value, the end of the distribution
- D [4,3]: Volume weighted mean diameter $(\Sigma n_i D_i^4)/(\Sigma n_i D_i^3)$.
- Span: Distribution width, calculated as $D_{90} - D_{10} / D_{50}$

Table B-2 Percentages below a certain particle size of the collected dust of the amino acid zootechnical mixture batches.

Size (µm) Batch	0.1 Vol%	0.5 Vol%	1 Vol%	5 Vol%	10 Vol%	25 Vol%	50 Vol%
AA Mix #1	0.0	3.9	6.6	32.5	72.0	99.5	100.0
AA Mix #2	0.0	6.2	9.3	45.6	80.0	99.7	100.0
AA Mix #3	0.0	5.2	7.9	39.0	77.4	99.9	100.0

Figure B-1 and Figure B-2 show that the three batches have similar particle size distributions

Annex 3 Calculations from particle size

From the particle size distribution data, the inhalable, thoracic and respirable fractions are calculated. Table C-1 presents the volume fractions in the different size bands, selected on a log base, obtained from the light scattering data and given in Table B-2.

Size (µm)	**0.1**	**0.5**	**1**	**5**	**10**	**25**	**50**
Batch	**Vol%**	**Vol%**	**Vol%**	**Vol%**	**Vol%**	**Vol%**	**Vol%**
AA Mix #1	0.0	3.9	6.6	32.5	72.0	99.5	100.0
AA Mix #2	0.0	6.2	9.3	45.6	80.0	99.7	100.0
AA Mix #3	0.0	5.2	7.9	39.0	77.4	99.9	100.0

Table C-1 Volume fractions and log mean sizes from the different size bands obtained from the light scattering data for different batches of amino acid zootechnical mixture as presented in Table B-2.

Size band number	**Lower size**	**Upper size**	**LN based mean diameter D_i**	**Batch AA Mix #1 V_i**	**Batch AA Mix#2 V_i**	**Batch AA Mix #3 V_i**
I	**microns**	**microns**	**microns**	**Vol%**	**Vol%**	**Vol%**
1	0.25	0.5	0.35	3.9	6.2	5.2
2	0.5	1	0.71	2.7	3.1	2.7
3	1	2.5	1.6	11.4	8.7	6.1
4	2.5	5	3.5	14.5	27.6	25
5	5	10	7.1	39.5	34.4	38.4
6	10	25	15.8	27.5	19.7	22.5
7	25	50	35.4	0.5	0.3	0.1

Using these volume fractions the inhalable, thoracic and respirable fractions in each size bands and the total fractions are calculated as presented in paragraph 2.5 were calculated.

Table C-2 Estimated inhalable, thoracic and respirable fractions of dust from amino acid zootechnical mixture batch AA Mix #1 obtained by the Stauber-Heubach analysis.

Size band number	**LN based mean diameter D_i**	**E_I Inhalable**	**E_t Thoracic**	**E_r Respirable**
i	**Microns**	**%**	**%**	**%**
1	0.35	3.9	3.8	3.9
2	0.71	2.6	2.6	2.7
3	1.6	10.9	11.0	10.8
4	3.5	13.1	13.2	8.9
5	7.1	32.7	28.8	3.5
6	15.8	19.1	4.3	0.0
7	35.4	0.3	0.0	0.0
Total		82.5	63.7	29.7

Table C-3 Estimated inhalable, thoracic and respirable fractions of dust from amino acid zootechnical mixture batch AA Mix #2 obtained by the Stauber-Heubach analysis.

Size band number *i*	LN based mean diameter D_i Microns	E_I Inhalable %	E_t Thoracic %	E_r Respirable %
1	0.35	6.1	6.1	6.2
2	0.71	3.0	3.0	3.1
3	1.6	8.3	8.4	8.2
4	3.5	25.0	25.2	17.0
5	7.1	28.4	25.1	3.0
6	15.8	13.7	3.0	0.0
7	35.4	0.2	0.0	0.0
Total		84.7	70.8	37.4

Table C-4 Estimated inhalable, thoracic and respirable fractions of dust from amino acid zootechnical mixture batch AA Mix #3 obtained by the Stauber-Heubach analysis.

Size band number *i*	LN based mean diameter D_i Microns	E_I Inhalable %	E_t Thoracic %	E_r Respirable %
1	0.35	5.1	5.1	5.2
2	0.71	2.6	2.6	2.7
3	1.6	5.8	5.9	5.8
4	3.5	22.6	22.8	15.4
5	7.1	31.8	28.0	3.4
6	15.8	15.6	3.5	0.0
7	35.4	0.1	0.0	0.0
Total		83.6	67.9	32.3

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