

Validation Report

AlerTox ELISA Lupine

KIT3057/KT-5914

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1. Scope

The AlerTox ELISA Lupine is designed for the determination of lupine in food. The present report describes the validation process and its results.

2. Precision

A) Intra-Assay Variation

The intra-assay variation was determined by testing three controls of various concentration levels in 20fold replicates.

| Replicate | Level 1 | Level 2 | Level 3 | |
|-----------|---------|---------|---------|-----|
| 1 | 5.7 | 20 | 34 | |
| 2 | 6.1 | 18 | 36 | |
| 3 | 6.2 | 22 | 38 | |
| 4 | 6.2 | 19 | 37 | |
| 5 | 6.3 | 19 | 34 | |
| 6 | 6.3 | 20 | 35 | |
| 7 | 7.0 | 20 | 36 | |
| 8 | 5.7 | 16 | 31 | |
| 9 | 5.4 | 20 | 35 | |
| 10 | 5.7 | 20 | 39 | |
| 11 | 6.0 | 21 | 34 | |
| 12 | 5.6 | 21 | 35 | |
| 13 | 6.0 | 17 | 34 | |
| 14 | 6.2 | 19 | 32 | |
| 15 | 6.2 | 20 | 28 | |
| 16 | 6.1 | 18 | 29 | |
| 17 | 5.3 | 18 | 29 | |
| 18 | 5.8 | 20 | 30 | |
| 19 | 6.0 | 22 | 33 | |
| 20 | 6.3 | 21 | 31 | |
| Mean | 6.0 | 20 | 33 | |
| SD | 0.4 | 1.5 | 3.0 | RMS |
| CV [%] | 6.1 | 7.8 | 9.1 | 7.8 |

Table 1: Intra-assay variation of the AlerTox ELISA Lupine

The coefficient of variation is ranging from 6.1% to 9.1% depending on the concentration.

RMS = Root Mean Square



B) Inter-Assay Variation

The inter-assay variation was determined by testing three controls of various concentration levels in four different test runs of the same kit lot.

| Assay No. | Level 1 | Level 2 | Level 3 | |
|-----------|---------|---------|---------|-----|
| 1 | 4.6 | 13 | 27 | |
| 2 | 5.1 | 13 | 22 | |
| 3 | 4.6 | 14 | 23 | |
| 4 | 4.6 | 14 | 25 | |
| Mean | 4.7 | 13 | 24 | |
| SD | 0.2 | 0.5 | 2.2 | RMS |
| CV [%] | 5.1 | 4.1 | 9.3 | 6.6 |

Table 2: Inter-assay variation of the AlerTox ELISA Lupine

The coefficient of variation is ranging from 4.1% to 9.3% depending on the concentration.

3. Recovery

For recovery experiments different sample matrices were spiked with lupine to obtain various final concentrations after performing all sample pre-treatment steps. Tested samples and results were as follows.

| Table 3: Recover | y of various san | nples tested with the | AlerTox ELISA Lu | pine |
|------------------|------------------|-----------------------|------------------|------|
|------------------|------------------|-----------------------|------------------|------|

| Biscuit | | |
|--------------|---------------|--------------|
| Target Value | Actual | Recovery [%] |
| | Concentration | |
| 5 ppm | 5.9 | 119 |
| 15 ppm | 16.7 | 111 |
| 30 ppm | 32.7 | 109 |
| | Mean | 113 |

Orange Juice

| Target Value | Actual Concentration | Recovery [%] |
|--------------|-------------------------|--------------|
| 5 ppm | 6.1 | 122 |
| 15 ppm | 15.1 | 100 |
| 30 ppm | 26.4 | 88 |
| | Mean | 104 |



Sausage **Target Value Recovery** [%] Actual Concentration 5 ppm 5.3 105 15 ppm 14.7 98 30 ppm 28.5 95 Mean 99

Ketchup

| Target Value | Actual Concentration | Recovery [%] |
|--------------|-------------------------|--------------|
| 5 ppm | 5.4 | 109 |
| 15 ppm | 14.0 | 93 |
| 30 ppm | 27.4 | 91 |
| | Mean | 98 |

Croquette

| Target Value | Actual Concentration | Recovery [%] |
|--------------|-------------------------|--------------|
| 5 ppm | 6.4 | 127 |
| 15 ppm | 16.3 | 109 |
| 30 ppm | 28.9 | 96 |
| | Mean | 111 |

Mean recoveries are ranging from 98% to 113% depending on the sample matrix.

4. Analytical Sensitivity

a) Matrix-independent Sensitivity

For determination of the analytical sensitivity, matrix-independent sample diluent was assayed in 24fold replicates. After identification of possible outliers the OD mean and standard deviation was calculated. The corresponding concentration of the OD_{mean} + 3x standard deviation was defined as limit of detection. This results in limit of detection according to the following table:



| Replicate | Sample diluent [OD] |
|--------------------|---------------------|
| 1 | 0.070 |
| 2 | 0.063 |
| 3 | 0.056 |
| 4 | 0.054 |
| 5 | 0.086 |
| 6 | 0.066 |
| 7 | 0.081 |
| 8 | 0.071 |
| 9 | 0.074 |
| 10 | 0.079 |
| 11 | 0.060 |
| 12 | 0.069 |
| 13 | 0.074 |
| 14 | 0.068 |
| 15 | 0.072 |
| 16 | 0.071 |
| 17 | 0.084 |
| 18 | 0.068 |
| 19 | 0.072 |
| 20 | 0.075 |
| 21 | 0.070 |
| 22 | 0.072 |
| 23 | 0.068 |
| 24 | 0.070 |
| Mean | 0.071 |
| SD | 0.008 |
| Limit of Detection | 0.21 ppm |

Table 4a: Matrix-independent analytical sensitivity of the AlerTox ELISA Lupine

b) Matrix-dependent Sensitivity

For determination of the analytical sensitivity, matrix-dependent lupine free biscuit orange juice, sausage, ketchup and croquette samples were assayed in 24fold replicates. After identification of possible outliers the OD mean and standard deviation were calculated. The corresponding concentration of the $OD_{mean} + 3x$ standard deviation was defined as limit of detection. This results in limits of detection according to the following table:



| Replicate | Biscuit | OrangeJuice | Sausage | Ketchup | Croquette |
|---------------------|----------|--------------------|-----------|----------|-----------|
| | matrix | matrix [OD] | matrix | matrix | matrix |
| | [OD] | | [OD] | [OD] | [OD] |
| 1 | 0.121 | 0.356 | 0.089 | 0.094 | 0.099 |
| 2 | 0.125 | 0.321 | 0.090 | 0.066 | 0.091 |
| 3 | 0.114 | 0.336 | 0.083 | 0.081 | 0.102 |
| 4 | 0.120 | 0.342 | 0.078 | 0.070 | 0.098 |
| 5 | 0.106 | 0.330 | 0.068 | 0.077 | 0.081 |
| 6 | 0.105 | 0.330 | 0.073 | 0.080 | 0.083 |
| 7 | 0.112 | 0.341 | 0.073 | 0.098 | 0.106 |
| 8 | 0.098 | 0.291 | 0.063 | 0.079 | 0.087 |
| 9 | 0.106 | 0.334 | 0.085 | 0.083 | 0.113 |
| 10 | 0.102 | 0.303 | 0.084 | 0.070 | 0.102 |
| 11 | 0.113 | 0.320 | 0.076 | 0.082 | 0.096 |
| 12 | 0.113 | 0.367 | 0.079 | 0.074 | 0.105 |
| 13 | 0.127 | 0.333 | 0.084 | 0.085 | 0.084 |
| 14 | 0.105 | 0.321 | 0.067 | 0.078 | 0.091 |
| 15 | 0.096 | 0.332 | 0.077 | 0.074 | 0.093 |
| 16 | 0.116 | 0.298 | 0.066 | 0.062 | 0.080 |
| 17 | 0.095 | 0.344 | 0.093 | 0.080 | 0.104 |
| 18 | 0.131 | 0.317 | 0.083 | 0.079 | 0.104 |
| 19 | 0.115 | 0.320 | 0.074 | 0.074 | 0.091 |
| 20 | 0.114 | 0.305 | 0.074 | 0.072 | 0.089 |
| 21 | 0.125 | 0.301 | 0.073 | 0.068 | 0.082 |
| 22 | 0.115 | 0.321 | 0.069 | 0.073 | 0.104 |
| 23 | 0.113 | 0.325 | 0.081 | 0.078 | 0.092 |
| 24 | 0.125 | 0.309 | 0.062 | 0.071 | 0.093 |
| Mean | 0.113 | 0.325 | 0.077 | 0.077 | 0.095 |
| SD | 0.010 | 0.018 | 0.009 | 0.008 | 0.009 |
| Limit of | 0.25 ppm | 0.69 ppm | 0.16 ppm | 0.06 ppm | 0.23 ppm |
| Detection | | | | | |
| LOD _{mean} | | | 0.278 ppm | | |

Table 4b: Matrix-dependent analytical sensitivity of the AlerTox ELISA Lupine

The calculated limit of detection is ranging from 0.21ppm to 0.278 ppm of lupine. Note that the derived limits of detection are strictly dependent on the coefficient of variation and may thus vary in every individual test. The data for sample diluent and matrices respectively were <u>not</u> determined in the same test runs. The LOD was therefore set to 0.3 ppm.

The lowest positive standard (2 ppm) was defined as limit of quantification to assure that all important matrices like milk, egg, wheat, rye, oats and barley result in concentrations lower than this value.



5. Linearity

Linearity was determined by spiking biscuit, orange juice, sausage, ketchup and croquette samples with lupine and testing subsequent dilutions of the resulting extracts. For calculation of the linearity the highest concentration was defined as reference value (100%) and further dilutions were expressed in per cent of this reference after consideration of the dilution factor.

Table 5: Matrix dependent linearity of the AlerTox ELISA Lupine

| Biscuit | | |
|--------------|------------------------|--------------|
| Target Value | Concentration [ppm] | Recovery [%] |
| 30 ppm | 32.7 | 100 |
| 15 ppm | 14.7 | 90 |
| 7.5 ppm | 8.6 | 105 |
| 3.8 ppm | 4.0 | 98 |
| 1.9 ppm | 1.6 | 78 |
| | Mean [%] | 93 |

Orange Juice

| Target Value | Concentration [ppm] | Recovery [%] |
|--------------|------------------------|--------------|
| 30 ppm | 27.1 | 100 |
| 15 ppm | 17.1 | 126 |
| 7.5 ppm | 8.3 | 123 |
| 3.8 ppm | 3.7 | 108 |
| 1.9 ppm | 1.2 | 70 |
| | Mean [%] | 107 |

Sausage

| Target Value | Concentration [ppm] | Recovery [%] | | | |
|--------------|------------------------|--------------|--|--|--|
| 30 ppm | 28.4 | 100 | | | |
| 15 ppm | 14.6 | 103 | | | |
| 7.5 ppm | 7.3 | 103 | | | |
| 3.8 ppm | 3.8 | 107 | | | |
| 1.9 ppm | 1.6 | 91 | | | |
| | Mean [%] | 101 | | | |



| Ketchup | | |
|--------------|------------------------|--------------|
| Target Value | Concentration [ppm] | Recovery [%] |
| 30 ppm | 27.4 | 100 |
| 15 ppm | 14.3 | 104 |
| 7.5 ppm | 7.8 | 114 |
| 3.8 ppm | 4.0 | 117 |
| 1.9 ppm | 1.5 | 90 |
| | Mean [%] | 106 |

| Croquette | | |
|--------------|------------------------|--------------|
| Target Value | Concentration [ppm] | Recovery [%] |
| 30 ppm | 28.9 | 100 |
| 15 ppm | 16.1 | 112 |
| 7.5 ppm | 7.5 | 103 |
| 3.8 ppm | 3.8 | 106 |
| 1.9 ppm | 1.7 | 95 |
| | Mean [%] | 104 |

For different matrices the mean linearity is ranging from 93% to 107%. The linearity is independent of the specific concentration and may only be affected by the intra-assay and inter-assay variation.

6. Cross-Reactivity

For the following foods no cross-reactivity (results < LOQ) were detected:

Table 6: Non-cross-reactive food matrices in the AlerTox ELISA Lupine

| Milk | Tomato | Hazelnut | Macadamia nut |
|-----------|-----------------|------------|---------------|
| Egg | Pork | Almond | Chestnut |
| Wheat | Sunflower seed | Cacao | Peach |
| Rye | Pumpkin seed | Potato | Cherry |
| Oats | Pine seed | Leech | Plum |
| Barley | Cashew | Pecan | Pea |
| Rice | Peanut | Brazil nut | Bean |
| Corn | Guar gum | Coconut | |
| Buckwheat | Locust bean gum | Walnut | |
| Sesame | Chicken | Pistachio | |



The following cross-reactivities could be determined:

| Food | Cross-reactivity [%] |
|----------------|----------------------|
| Chickpea | 0.0003 |
| Soy, unroasted | 0.07 |
| Soy, roasted | 0.0009 |
| Soy lecithin* | 0.002 |
| Lentil | 0.0004 |
| Beef* | 0.0003 |

Table 7: Cross-reactive food matrices in the AlerTox ELISA Lupine

*) Validated in an additionally validation run.

7. Robustness

Robustness was determined by variation of different handling parameters as defined in the instruction manual. The results were compared with the results of samples analyzed according to the intended method. An un-spiked biscuit sample and a sample spiked with 5 ppm of lupine were analyzed respectively.

A) Variation of extraction temperature

The extraction temperature, defined as 60 °C, was changed to 40 °C and 70 °C, respectively.

| Table 0. valiation of extraction temperature in the Alerrow LetoA Laping | Table 8: | Variation of | extraction | temperature | in the <i>i</i> | AlerTox B | ELISA Lu | pine |
|--|----------|--------------|------------|-------------|-----------------|-----------|----------|------|
|--|----------|--------------|------------|-------------|-----------------|-----------|----------|------|

| Sample | Result 60 °C | Result 40 °C | Result 70 °C |
|---------------|--------------|--------------|--------------|
| Biscuit 0 ppm | 0.15 ppm | 0.13 ppm | 0.22 ppm |
| Biscuit 5 ppm | 4.47 ppm | 4.73 ppm | 4.18 ppm |

Under consideration of the intra-assay and inter-assay variations, the results do <u>not</u> differ significantly.

B) Variation of extraction time

The extraction time, defined as 15 min, was changed to 10 min and 20 min. respectively.



| Table 9: Variation of extraction | time in the AlerTox ELISA Lupin | е |
|----------------------------------|---------------------------------|---|
|----------------------------------|---------------------------------|---|

| Sample | Result 15 min | Result 10 min | Result 20 min |
|---------------|---------------|---------------|---------------|
| Biscuit 0 ppm | 0.15 ppm | 0.26 ppm | 0.15 ppm |
| Biscuit 5 ppm | 4.47 ppm | 5.13 ppm | 5.41 ppm |

Under consideration of the intra-assay and inter-assay variation, the results do <u>not</u> differ significantly.

C) Drift

In contrast to the test procedure as defined in the instruction manual the incubation time of the samples was extended and reduced by 5 minutes compared to the calibrators (20 min).

Table 10: Drift in the AlerTox ELISA Lupine

| Sample | Result 20 min | Result 15 min | Result 25 min |
|---------------|---------------|---------------|---------------|
| Biscuit 0 ppm | 0.15 ppm | 0.02 ppm | 0.05 ppm |
| Biscuit 5 ppm | 4.47 ppm | 2.85 ppm | 6.55 ppm |

The results differ significantly. Drift in extensive test runs should be avoided by pipetting calibrators once before the samples and once after the samples, using the mean value for calculation.