

Validation Report

AlerTox ELISA Casein KIT3043/KT-5761

INDEX

| | |
|--------------------------------|----|
| 1. Scope | 2 |
| 2. Precision..... | 2 |
| A) Intra-Assay Variation | 2 |
| B) Inter-Assay Variation | 3 |
| 3. Recovery | 3 |
| 4. Analytical Sensitivity..... | 6 |
| 5. Linearity..... | 7 |
| 6. Cross-Reactivity | 9 |
| 7. Robustness | 10 |

1. Scope

The AlerTox ELISA Casein is designed for the determination of cow's milk casein (α , β , γ) 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.

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

| Replicate | Level 1 | Level 2 | Level 3 | |
|---------------|------------|------------|-------------|------------|
| 1 | 0.47 | 1.78 | 6.16 | |
| 2 | 0.46 | 1.79 | 5.57 | |
| 3 | 0.44 | 1.60 | 6.01 | |
| 4 | 0.43 | 1.82 | 6.02 | |
| 5 | 0.48 | 1.80 | 5.43 | |
| 6 | 0.42 | 1.75 | 5.41 | |
| 7 | 0.45 | 1.78 | 4.46 | |
| 8 | 0.41 | 1.59 | 6.10 | |
| 9 | 0.47 | 1.80 | 5.19 | |
| 10 | 0.44 | 1.80 | 5.24 | |
| 11 | 0.46 | 1.81 | 6.24 | |
| 12 | 0.47 | 1.90 | 5.93 | |
| 13 | 0.48 | 1.70 | 5.21 | |
| 14 | 0.44 | 1.75 | 5.53 | |
| 15 | 0.44 | 1.77 | 5.71 | |
| 16 | 0.39 | 1.64 | 4.81 | |
| 17 | 0.39 | 1.82 | 4.82 | |
| 18 | 0.44 | 1.83 | 4.62 | |
| 19 | 0.45 | 1.82 | 4.61 | |
| 20 | 0.48 | 1.85 | 4.71 | |
| Mean | 0.44 | 1.77 | 5.39 | |
| SD | 0.03 | 0.08 | 0.58 | RMS |
| CV [%] | 6.1 | 4.6 | 10.7 | 7.6 |

The coefficient of variation is ranging from 4.6% to 10.7% depending on the concentration.

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.

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

| Assay No. | Level 1 | Level 2 | Level 3 | |
|---------------|------------|------------|-------------|-------------|
| 1 | outlier | 1.55 | 4.02 | |
| 2 | 0.43 | 1.72 | 4.24 | |
| 3 | 0.45 | 1.60 | 4.70 | |
| 4 | 0.39 | 1.86 | 5.48 | |
| Mean | 0.42 | 1.68 | 4.61 | |
| SD | 0.03 | 0.14 | 0.64 | RMS |
| CV [%] | 7.5 | 8.1 | 14.0 | 10.3 |

The coefficient of variation is ranging from 7.5% to 14.0% depending on the concentration.

3. Recovery

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

Table 3: Recovery of various samples tested with the AlerTox ELISA Casein

Cookies

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| 0.6 ppm | 0.69 | 115 |
| 2 ppm | 1.86 | 93 |
| 6 ppm | 5.51 | 92 |
| | Mean | 100 |

Soy drink

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| 0.6 ppm | 0.61 | 102 |
| 2 ppm | 1.95 | 97 |
| 6 ppm | 4.95 | 83 |
| | Mean | 94 |

Sausage

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| 0.6 ppm | 0.61 | 102 |
| 2 ppm | 1.43 | 72 |
| 6 ppm | 4.05 | 68 |
| | Mean | 80 |

White wine

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| 0.6 ppm | 0.66 | 110 |
| 2 ppm | 2.03 | 102 |
| 6 ppm | 5.61 | 94 |
| | Mean | 102 |

Bread crumbs

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| 0.6 ppm | 0.53 | 88 |
| 2 ppm | 1.51 | 76 |
| 6 ppm | 4.55 | 76 |
| | Mean | 80 |

Orange juice

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| 0.6 ppm | 0.54 | 91 |
| 2 ppm | 1.63 | 82 |
| 6 ppm | 4.83 | 81 |
| | Mean | 84 |

Chocolate

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| 0.6 ppm | 0.59 | 99 |
| 2 ppm | 1.64 | 82 |
| 6 ppm | 4.73 | 79 |
| | Mean | 86 |

Mean recoveries are ranging from 80% to 102% depending on the sample matrix.

In an additional validation experiment different rosé and red wines were spiked with casein to obtain various final concentrations after performing all sample pre-treatment steps. All measurements were done in 4fold replicates. The samples were measured directly after the dilution with extraction buffer, without incubation in the water bath. Tested samples and results were as follows.

Table 3a: Recovery of various rosé and red wines tested with the AlerTox ELISA Casein

Syrah, Rosé

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| unspiked | 0.01* | --- |
| 1.0 ppm | 0.8 | 80 |
| 2.5 ppm | 2.01 | 80 |
| | Mean | 80 |

Corbières, Red

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| unspiked | 0.02* | --- |
| 1.0 ppm | 0.88 | 88 |
| 2.5 ppm | 2.02 | 81 |
| | Mean | 85 |

Merlot, Red

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| unspiked | 0.00 | --- |
| 1.0 ppm | 1.01 | 101 |
| 2.5 ppm | 2.61 | 104 |
| | Mean | 103 |

Cabernet Sauvignon, Red

| Target Value | Actual Concentration | Recovery [%] |
|--------------|----------------------|--------------|
| unspiked | 0.03* | --- |
| 1.0 ppm | 0.8 | 80 |
| 2.5 ppm | 2.01 | 80 |
| | Mean | 80 |

* The measured value is not a real quantitative value, because it is far below LOQ and also below LOD. But it is also not a matrix effect. The value could be caused by microscopic small particles, which are floating in the wine.

Mean recoveries of the tested wines are ranging from 80% to 103% depending on the sample matrix

4. Analytical Sensitivity

For determination of the analytical sensitivity sample diluent was assayed in 21fold replicates. After identification of possible outliers the OD mean and standard deviation was calculated. The corresponding concentration of the $OD_{\text{mean}} + 3x$ standard deviation was defined as limit of detection. This results in limits of detection according to the following table:

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

| Replicate | Sample diluent [OD] |
|---------------------------|---------------------|
| 1 | 0.199 |
| 2 | 0.184 |
| 3 | 0.163 |
| 4 | 0.163 |
| 5 | 0.176 |
| 6 | 0.188 |
| 7 | 0.166 |
| 8 | 0.207 |
| 9 | 0.159 |
| 10 | 0.154 |
| 11 | 0.157 |
| 12 | 0.261 |
| 13 | 0.189 |
| 14 | 0.170 |
| 15 | 0.173 |
| 16 | 0.164 |
| 17 | 0.157 |
| 18 | 0.157 |
| 19 | 0.156 |
| 20 | 0.157 |
| 21 | 0.154 |
| Mean | 0.174 |
| SD | 0.025 |
| Limit of Detection | 0.04 ppm |

The calculated limit of detection is 0.04 ppm of casein and was set to 0.05 ppm.

The lowest positive standard (0.20 ppm) was defined as limit of quantification to assure that all important matrices like wheat, oats, rye, barley, cocoa, orange, soy and pork-meat result in concentrations lower than this value.

5. Linearity

Linearity was determined by spiking soy drink, sausage, white wine, orange juice, cookies, bread crumbs and chocolate samples with casein 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 Casein ELISA

Soy drink

| Target Value | Concentration [ppm] | Recovery [%] |
|--------------|---------------------|--------------|
| 6 ppm | 4.39 | 100 |
| 3 ppm | 2.07 | 94 |
| 1.5 ppm | 1.37 | 125 |
| 0.75 ppm | 0.63 | 116 |
| 0.375 ppm | 0.34 | 125 |
| | Mean [%] | 115 |

Sausage

| Target Value | Concentration [ppm] | Recovery [%] |
|--------------|---------------------|--------------|
| 6 ppm | 5.15 | 100 |
| 3 ppm | 2.47 | 96 |
| 1.5 ppm | 1.36 | 106 |
| 0.75 ppm | 0.63 | 98 |
| 0.375 ppm | 0.32 | 100 |
| | Mean [%] | 100 |

White wine

| Target Value | Concentration [ppm] | Recovery [%] |
|--------------|---------------------|--------------|
| 6 ppm | 4.60 | 100 |
| 3 ppm | 2.79 | 121 |
| 1.5 ppm | 1.44 | 126 |
| 0.75 ppm | 0.83 | 144 |
| 0.375 ppm | 0.33 | 116 |
| | Mean [%] | 127 |

Orange juice

| Target Value | Concentration [ppm] | Recovery [%] |
|---------------------|----------------------------|---------------------|
| 6 ppm | 5.61 | 100 |
| 3 ppm | 2.96 | 106 |
| 1.5 ppm | 1.49 | 106 |
| 0.75 ppm | 0.74 | 105 |
| 0.375 ppm | 0.36 | 102 |
| | Mean [%] | 105 |

Cookies

| Target Value | Concentration [ppm] | Recovery [%] |
|---------------------|----------------------------|---------------------|
| 6 ppm | 4.15 | 100 |
| 3 ppm | 1.89 | 91 |
| 1.5 ppm | 1.24 | 119 |
| 0.75 ppm | 0.66 | 127 |
| 0.375 ppm | 0.24 | 94 |
| | Mean [%] | 108 |

Bread crumbs

| Target Value | Concentration [ppm] | Recovery [%] |
|---------------------|----------------------------|---------------------|
| 6 ppm | 3.38 | 100 |
| 3 ppm | 1.70 | 101 |
| 1.5 ppm | 1.14 | 134 |
| 0.75 ppm | 0.55 | 129 |
| 0.375 ppm | 0.27 | 130 |
| | Mean [%] | 123 |

Chocolate

| Target Value | Concentration [ppm] | Recovery [%] |
|---------------------|----------------------------|---------------------|
| 6 ppm | 4.87 | 100 |
| 3 ppm | 2.74 | 112 |
| 1.5 ppm | 1.34 | 110 |
| 0.75 ppm | 0.79 | 129 |
| 0.375 ppm | 0.44 | 144 |
| | Mean [%] | 124 |

For different matrices the mean linearity is ranging from 100% to 127%. 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) could be detected:

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

| | | | |
|------------|-----------|-----------|-----------------|
| Wheat | Corn | Cacao | Pork meat |
| Rye | Rice | Sesame | Chicken meat |
| Oats | Egg | Sucrose | β-Lactoglobulin |
| Barley | Soy | Beef meat | Orange |
| White wine | Rosé wine | Red wine | |

The following cross-reactivity could be determined:

| Food | Cross-reactivity [%] |
|-------------|-----------------------------|
| Ewe's milk | < 1.2 |
| Goat's milk | < 1.1 |

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 unspiked white wine sample and a sample spiked with 2 ppm of casein were analyzed respectively.

Variation of extraction temperature

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

Table 8: Variation of extraction temperature in the AlerTox ELISA Casein

| Sample | Result 60 °C | Result 40 °C | Result 70 °C |
|------------------|--------------|--------------|--------------|
| White wine 0 ppm | 0 ppm | 0 ppm | 0 ppm |
| White wine 2 ppm | 2.10 ppm | 2.35 ppm | 2.10 ppm |

Under consideration of the intra-assay and inter-assay variations as stated in chapter 2 the results do not differ significantly.

Variation of extraction time

The extraction time, defined as 15 min, was changed to 5 min. 10 min and 20 min. respectively.

Table 9: Variation of extraction time in the AlerTox ELISA Casein

| Sample | Result 15 min | Result 10 min | Result 20 min |
|------------------|---------------|---------------|---------------|
| White wine 0 ppm | 0 ppm | 0 ppm | 0 ppm |
| White wine 2 ppm | 2.23 ppm | 2.26 ppm | 2.05 ppm |

Under consideration of the intra-assay and inter-assay variation as stated in chapter 2, the results do not differ significantly.

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 Casein ELISA

| Sample | Result 20 min | Result 15 min | Result 25 min |
|------------------|---------------|---------------|---------------|
| White wine 0 ppm | 0 ppm | 0 ppm | 0.2 ppm |
| White wine 2 ppm | 2.10 ppm | 1.75 ppm | 3.10 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.

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