

Analysis report ORAC Europe BV

*initials
investigator*

Customer name:

Hak Agrofeed BV
att. Mr. B. Hak
Leemansstraat 2
4251 LD Werkendam
The Netherlands

Amount of samples
delivered:

63 samples

EW

Date of sample arrival:

Thursday, June 11, 2009

EW

Sample condition at arrival:

OK Damaged Other (see remarks)

Sample storage conditions:

Upon arrival, samples were stored at
4°C, in the dark.

EW

Remarks:

Samples were presented as
individually wrapped cucumbers.
Cucumbers were divided into three
separately labeled groups.

EW

Customer's description of samples:

The delivered cucumbers were divided into three groups: a control group (n=21), an *Immutines*-treated group (3 mL/m²/week, blue label, n=21), and an *Immutines*-treated group (4 mL/m²/week, red label, n=21).

The control group consisted of untreated cucumbers. Control cucumbers were labeled C-1 till C-21. *Immutines*-treated cucumbers (3 mL/m²/week) were labeled B-1 till B-21, and *Immutines*-treated cucumbers (4 mL/m²/week) were labeled R-1 till R-21.

All cucumbers were grown at the nursery of A.W. Vahl in IJsselmuiden, The Netherlands.

Further details about the cultivation procedures and additional information about *Immutines* are provided in the appendix of the ORAC Europe report of September 23, 2008.

ORAC Europe sample preparation:

Three groups of 21 cucumbers each were delivered (see above). All cucumbers were weighed individually (see appendix 1, page 8).

Each group of 21 cucumbers (control, blue label and red label) were divided into 3 separate groups of 7 cucumbers. These groups were labeled C1, C2, C3 (control group), B1, B2, B3 (3 mL/m²/week, blue) and R1, R2, R3 (4 mL/m²/week, red). Of each individual cucumber of these groups of 7, a part of exactly 100 gram was cut from the exact middle of the cucumber. Within each separate group, these 7 pieces were pooled and all parts were thoroughly grinded using a laboratory grinder. This procedure yielded a homogenate of 7x 100 gram cucumber per group of 7 cucumbers.

So in total, 9 groups are formed (3 groups within the control group, 3 groups within the blue group, and 3 groups within the red group).

to be continued on page 3

**ORAC Europe
sample preparation:**

continued from page 2:

All obtained homogenates were thoroughly mixed.

From each of the homogenates, 5.0 gram was accurately weighed in labeled glass test-tubes.

To extract the hydrophilic contents from each sample, 20 mL of an acetone/water/acetic acid solution (140:59:1, v/v) was added to each test-tube. This solution (abbreviated as AWA) is commonly used to extract hydrophilic constituents from food samples.

All sealed test-tubes with homogenates were placed in a ultrasonication bath for 15 min. Hereafter, all samples were thoroughly vortexed for 1 min, and placed back in the ultrasonication bath for another 15 min.

All tubes were vortexed again for another minute and finally, all tubes were centrifuged at 800 x g for 15 min.

Supernatants were carefully collected and stored in dark glass bottles at 4°C until further use in the hydrophilic ORAC assay.

**Determination
of dry weight:**

Three empty, glass round-bottom flasks of 500 mL were accurately weighed. To each flask, an exactly weighed amount of homogenate of each of the cucumber groups was added (C: control, B: 4 mL/m²/week and R: 4 mL/m²/week).

Each flask with homogenate was accurately weighed.

Prior to the actual freeze-drying, the homogenates in the flasks were frozen by slowly rotating the flasks in liquid nitrogen.

Then, the flasks were attached to a freeze-dryer and were freeze-dried for 36 hours.

Hereafter, the weights of the flasks with freeze-dried homogenates were again accurately determined.

Using the previously obtained weights of the flasks and the homogenates, the dry weight of every sample could be determined (see page 7).

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investigator*

Date of sample preparation:

Monday, June 15, 2009

EW

Required assay:

**Hydrophilic ORAC assay
Determination of dry weight**

EW

Date(s) of testing:

Tuesday, June 16 & Wednesday, June 17, 2009

EW

Responsible investigator:

Dr. E. van den Worm



EW

Report finalization date:

Friday, July 3, 2009

EW

Remarks:

- no remarks -

EW

Short description of performed assay:

Samples provided by Hak Agrofeed BV were tested for their **Oxygen Radical Absorbance Capacity** (ORAC), using the commonly accepted and well-validated hydrophilic ORAC assay with fluorescein as fluorescent probe and with AAPH (2,2'-azobis (2-methyl-propionamidine) dihydrochloride) as a physiological relevant peroxy radical generator. Kinetic fluorescence profiles were detected using an automated fluorescence reader (*Thermo Fluoroskan Ascent*). Fluorescence was monitored every minute for 1 hr. at 37°C, using an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

In the ORAC assay, Trolox (a water-soluble derivative of vitamin E) is used as an internal standard. Therefore, the results of the ORAC assay are expressed as μmol Trolox equivalents (TE) **per 100 g of test-sample**. This is a standard way of expressing ORAC values.

Each sample was dissolved and diluted in freshly prepared 75mM sodium phosphate buffer (pH = 7.4) shortly before the experiment.

All reagents were freshly prepared prior to the experiment. All solutions were kept in the dark at 37°C except the AAPH solution which was kept on ice (in the dark) until use.

From the obtained experimental data, final ORAC values were calculated using the 'area under the curve' (AUC). The net AUC was obtained by subtracting the AUC of the blank from that of the sample. The relative ORAC value (expressed as Trolox equivalents) was calculated by extrapolation from the Trolox calibration curve (AUC_{Trolox} vs. [Trolox]). ORAC values are expressed as mean values \pm Standard Deviation (S.D.).

Remarks:

Before all dilution steps and final addition to test-plate, all sample dilutions were carefully vortexed.

TEST RESULTS (I):

Samples	ORAC value ($\mu\text{mol TE} / 100 \text{ g.}$)[*]	
Control cucumbers (C)	90.0 \pm 1.0 (mean \pm S.D.)	(n = 4)
<i>Immutines</i>-treated cucumbers (3 mL/m²/week, B)	123.3 \pm 15.8 (mean \pm S.D.)	(n = 4)
<i>Immutines</i>-treated cucumbers (4 mL/m²/week, R)	116.7 \pm 8.7 (mean \pm S.D.)	(n = 4)

** As stated previously, ORAC values are expressed as $\mu\text{mol TE}$ per 100 g of test sample. If required, customer can extrapolate these ORAC values to $\mu\text{mol TE}$ per cucumber or $\mu\text{mol TE}$ per serving.*

NB: 1 $\mu\text{mol Trolox Equivalent}$ s (TE) equals 250 $\mu\text{g Trolox}$

	Antioxidant capacity (% of control)	Increase in antioxidant capacity (relative to control)
Control cucumbers (C)	100	-
<i>Immutines</i>-treated cucumbers (3 mL/m²/week, B)	137 \pm 17.6	37.0 %
<i>Immutines</i>-treated cucumbers (4 mL/m²/week, R)	129.7 \pm 9.6	29.7 %

TEST RESULTS (II):

Determination of dry weight:

cucumbers:	empty flask	flask + homogenate (<u>before</u> freeze-drying)	flask + homogenate (<u>after</u> freeze-drying)	percentage dry weight
Control	158.06 g.	267.42 g.	162.51 g.	4.07% (100%)
Blue (3 mL/m ² / week, B)	163.54 g.	269.39 g.	168.03 g.	4.24% (+ 4.2%)
Red (4 mL/m ² / week, R)	155.62 g.	265.66 g.	160.42 g.	4.36% (+7.1%)

Responsible Investigator:

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(CEO, ORAC Europe BV)

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Appendix 1: weights of individual cucumbers per group (in grams):

Control group (n=21)

C1 (n=7): 367.95; 369.53; 429.11; 447.51; 394.08; 467.07; 396.31
Average weight: 410.22 gram

C2 (n=7): 451.65; 320.72; 362.69; 437.18; 363.45; 449.18; 409.36
Average weight: 399.18 gram

C3 (n=7): 383.34; 340.26; 319.05; 478.27; 337.69; 320.22; 366.26
Average weight: 363.58 gram

Immutines-treated group (3 mL/m²/week, blue) (n=21)

B1 (n=7): 300.21; 431.18; 433.50; 359.12; 390.75; 296.02; 421.78
Average weight: 376.08 gram

B2 (n=7): 412.92; 384.11; 366.53; 333.28; 329.31; 412.13; 453.59
Average weight: 384.54 gram

B3 (n=7): 466.26; 415.46; 389.46; 338.01; 344.12; 419.12; 424.18
Average weight: 399.52 gram

Immutines-treated group (4 mL/m²/week, red) (n=21)

R1 (n=7): 482.83; 389.68; 476.49; 407.34; 321.40; 468.17; 447.24
Average weight: 427.59 gram

R2 (n=7): 474.91; 520.91; 485.62; 525.33; 392.43; 497.13; 551.91
Average weight: 492.61 gram

R3 (n=7): 438.54; 351.02; 417.64; 407.88; 385.92; 466.60; 483.36
Average weight: 421.57 gram