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:	9 samples	EW
Date of sample arrival:	Tuesday, May 12, 2009	EW
Sample condition at arrival:	***OK*** [X] ***Damaged*** [ ] ***Other*** [ ] *(see remarks)*	
Sample storage conditions:	Upon arrival, samples were stored at 4ºC, 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=3) and an *Immutines*-treated group (1 mL/m^2/week, green label, n=3) and an *Immutines*-treated group (1,5 mL/m^2/week, red label, n=3).
The control group consisted of untreated cucumbers. Control cucumbers were labeled C-1, C-2 and C-3. *Immutines*-treated cucumbers (1 mL/m^2) were labeled G-1, G-2 and G-3, and *Immutines*-treated cucumbers (1,5 mL/m^2) were labeled R-1, R-2 and R-3.

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:

Of each cucumber the total weight was determined.
From each cucumber, an equal-sized part (± 5 cm) was cut from the exact middle of the cucumber. The weight of each part was determined and also its volume was determined (*see results section*).
Each part was thoroughly grinded using a laboratory grinder. From each of the resulting samples, 2.5 gram was accurately weighed in labeled glass test-tubes.
To extract the hydrophilic contents from each sample, 10 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 samples were placed in a sonication bath for 15 min. Hereafter, all samples were thoroughly vortexed for 1 min, and placed back in the sonication 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.

		initials investigator
Date of sample preparation:	Tuesday, May 12, 2009	EW
Required assay:	**Hydrophilic ORAC assay**	EW
Date(s) of testing:	Wednesday 13 & Thursday 14 May, 2009	EW
Responsible investigator:	Dr. E. van den Worm 	EW
Report finalization date:	Thursday, June 4, 2009	EW
Remarks:	*- no remarks -*	EW

Short description of performed assay:

Samples provided by Hak Agrofeed BV were tested for their ***O**xygen **R**adical **A**bsorbance **C**apacity* (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 peroxyl radical generator. Kinetic fluorescence profiles were detected using an automated fluorescence reader (*Thermo Fluoroskan Ascent*). Fluorescence was monitored every minute for 1 hr. at 37ºC, 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 37ºC 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 ± Standard Deviation (S.D.).

Remarks:

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

TEST RESULTS:

Cucumber nr.	Total weight	Weight (part)	Volume (part)
C-1 (control)	366.18 g.	78.84 g.	78 cm^3
C-2 (control)	396.07 g.	93.85 g	92 cm^3
C-3 (Control)	291.51 g.	68.84 g.	66 cm^3
G-1 (1 mL/m^2)	344.22 g.	88.41 g.	88 cm^3
G-2 (1 mL/m^2)	350.12 g.	82.90 g.	82 cm^3
G-3 (1 mL/m^2)	282.66 g.	71.25 g.	72 cm^3
R-1 (1,5 mL/m^2)	333.06 g.	83.53 g.	82 cm^3
R-2 (1,5 mL/m^2)	395.42 g.	95.23 g.	94 cm^3
R-3 (1,5 mL/m^2)	339.59 g.	85.50 g.	82 cm^3

Samples	ORAC value (µmol TE / 100 g.)*	
Control cucumbers (C)	**127.5 ± 27.5 (mean ± S.D.)**	(n = 4)
***Immutines*-treated cucumbers (1 mL/m^2, G)**	**174.2 ± 27.5 (mean ± S.D.)**	(n = 4)
***Immutines*-treated cucumbers (1.5 mL/m^2, R)**	**170.0 ± 46.1 (mean ± S.D.)**	(n = 4)

** As stated previously, ORAC values are expressed as µmol TE per 100 g of test sample. If required, customer can extrapolate these ORAC values to µmol TE per cucumber or µmol TE per serving.*

NB: 1 µmol Trolox Equivalents (TE) equals 250 µg Trolox

	Antioxidant capacity (% of control)	Increase in antioxidant capacity (relative to control)
Control cucumbers (C)	**100**	**-**
***Immutines*-treated cucumbers (1 mL/m^2, G)**	**136.63 ± 21.5**	**36.6 %**
***Immutines*-treated cucumbers (1.5 mL/m^2, R)**	**133.3 ± 36.1**	**33.3 %**

Responsible Investigator:

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Appendix A

Antioxidant capacity of *Immutines* solution

To determine the possible contribution of the *Immutines* solution to the antioxidant capacity as determined in *Immutines-treated* cucumbers, the hydrophilic ORAC value of the *Immutines* solution is also determined.
The constituents of the reformulated *Immutines* are described in the ORAC Europe report of September 23, 2008.

The antioxidant capacity of the *Immutines* (expressed as hydrophilic ORAC value) is determined in the same way as previously described in this report (see pag. 4).

Sample	ORAC value (µmol TE / mL)*
***Immutines* solution**	**0.05 ± 0.0014 (n=3)**

** The hydrophilic ORAC value is expressed as µmol TE per mL Immutines. After re-calculation of the obtained ORAC value per mL, into µmol TE per 100 gram (to compare this value with the obtained ORAC values of the cucumbers), the hydrophilic ORAC value of the Immutines solution is:*

3.85 µmol TE / 100 g (relative density of *Immutines* solution: 1,3)