OECD GD 239 – Honey bee larvae *in vitro* testing and solvents: How to handle toxic solvents

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Introduction

By adopting the OECD guidance document 239 on *in vitro* bee larvae repeated exposure testing in the laboratory, a new guidance became final without taking into account several pending issues and unsolved problems. Important aspects to be taken into account are the use of solvents when testing practically insoluble compounds, as well as the confirmation of the homogenic distribution of substances within the feeding solutions.

Testing the active ingredient as technical compound instead of the corresponding formulation is requested from but not only limited to US and Canadian authorities for registration purposes. Indeed, several other authorities around the globe seem to follow that approach. Taking into consideration the high sensitivity and susceptibility to chemicals of the young larvae reared in the test, this leads to quite some problems in the practicability of the test itself.

Methodology

Material and test conditions:

- Test unit: grafting cells in 48 well plates
- Type of food: larval diet (Glucose, Fructose, Yeast extract and Gelée Royale) containing 2 % of solvent (acetone)
- Volume of food: 20, 30, 40 and 50 µL of food provided on D3, D4, D5 and D6, respectively

How to proceed:

- D1: Grafting
- D3, D4, D5 and D6: Feeding of larval diet containing 2 % acetone with or without test item
  ➔ **Evaporation of solvent (acetone) before feeding**
- D3 to D8 and D15: Mortality assessments
- D22: Emergence assessment

Figure 1: Larvae at D8

Figure 2: Pupae at D15

Results & Discussion

Evaporation	Type of test	Study n°	Mortality (%)		Hatching on D22 (%)
			D8	D15	
No	RF	1	34	n.a.	n.a.
		2	16	47	13
		3	25	100	0.0
		4	13	69	0.0
		5	69	n.a.	n.a.
		6	28	44	44
		7	19	100	0.0
	MT	8	10	27	60
		9	8.3	29	63
		10	31	85	15
		11	25	n.a.	n.a.
		12	29	n.a.	0.0
		13	8.3	23	65
		14	23	n.a.	n.a.
		15	19	n.a.	n.a.
Yes	RF	1	0.0	9.4	88
	MT	2	1.4	17	72
		3	2.1	19	71
		4	4.2	6.3	85
		5	2.1	4.3	92
		6	8.3	21	56
		7	0.0	6.3	90

Table 1: Solvent control mortalities with or without evaporation

The green cells corresponds to mortality or hatching values which meet the validity criteria whereas the red cells correspond to the values which do not meet the validity criteria.
Validity criteria for controls: mortality ≤ 15 % on D8 and emergence ≥ 70 % on D22.

Acetone induced mortality

As some substances which need to be tested are not soluble in water and as solubility issues increase in the highly saturated feeding solution, solvents are an unavoidable tool. The most common solvent used in chronic larvae studies is acetone. The mortality results of solvent control treatments suggest that acetone is inducing mortality in chronic larvae studies (Table 1). This impacts the results of studies and makes fulfilling the validity criteria of the solvent control treatment difficult. Indeed, only 27 % of studies conducted without evaporating the acetone present in the larval diet (n = 15) reached the validity criteria on D8. On D15, only 20 % of the studies were still valid but no study reached the validity criteria for the emergence on D22.

In order to limit mortality caused by acetone, the possibility of evaporating acetone from larval diets before feeding was investigated as it is a common practice adopted from other OECD guidelines (e.g. OECD 218/219, OECD 222 and OECD 232). When the acetone is evaporated prior to feeding the larval diet, mortality in the solvent control treatments decreases significantly. Indeed, by using the evaporation process, all of the conducted studies (n = 7) reached the D8 validity criteria, all were still valid on D15 and only one study did not reach the validity criteria on D22.

Solvent evaporation

In order to determine if the acetone present in larval diets is evaporated with the method used, the presence of acetone was analyzed in samples of larval diets before and after evaporation (Figure 3). The chromatograph of the sample taken before evaporation shows a peak at 5.5 min, indicative of the presence of acetone. On the contrary, the chromatograph of the sample taken after evaporation does not show a peak at 5.5 min, indicating the absence of acetone in the sample and thus in the larval diet.

Effect of evaporation on test items

Once it was confirmed that the acetone actually evaporated during the evaporation process, the possible impacts of the evaporation process on the test item were investigated. A homogeneity test was conducted with treated larval diets before and after evaporation. The diets were analytically measured in a three layer approach based on Whitmire et al., NonClinical Dose Formulation Analysis Method Validation and Sample Analysis, White Paper, The AAPS Journal, Vol 12, No. 4, 2010. No significant differences were found in the chromatographs and in the recovery values before and after evaporation. The test item was evenly distributed in all samples and all layers evaluated, therefore all larval diets were homogeneous before and after the evaporation (Figure 4). In addition, the recovery values in all layers were in the range of 82 to 112 % of the nominal concentration before evaporation and of 83 and 105 % after evaporation. This suggests that evaporation has no impact on the test item, its homogeneity and its concentration in the larval diets.

Conclusion

Solvents are sometimes needed to solubilize test substances that cannot be dissolved in water and saturated feeding solutions. As honey bee larvae are a quite sensitive test system, the toxicity of solvents should be investigated and solvents should be evaporated if they present toxic effects on larvae. The here presented methodology shows that solvents can be used as carriers to introduce test items in larval diets without inducing adverse effects on the test system. Further on, the test item itself remains unaffected by the method. In principle, this method can be used with any solvent that has a higher volatility than the actual test item.

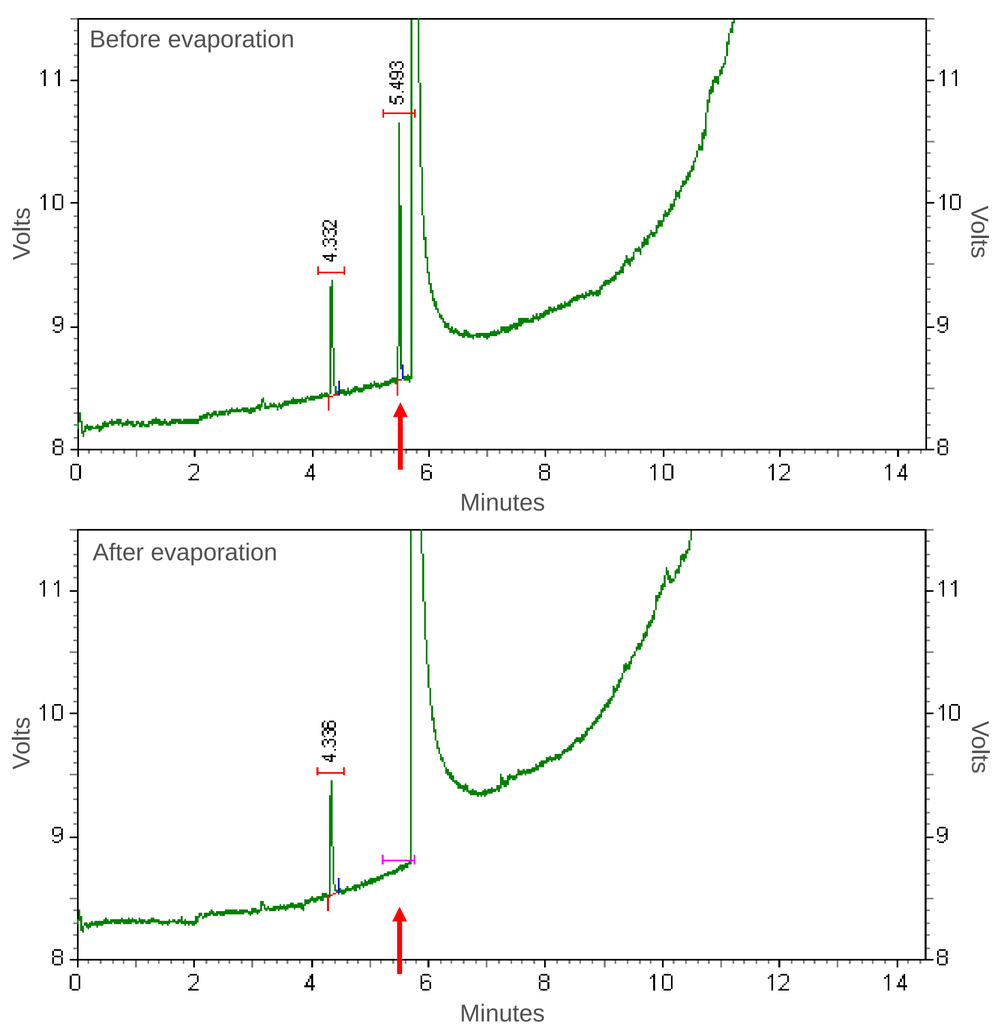

Figure 3: Presence of acetone in larval diet before and after the evaporation process
GC-FID Chromatograms of Diet C. Samples diluted with water/acetonitrile (1/1, v/v).
Acetone retention time (red arrow): 5.5 min

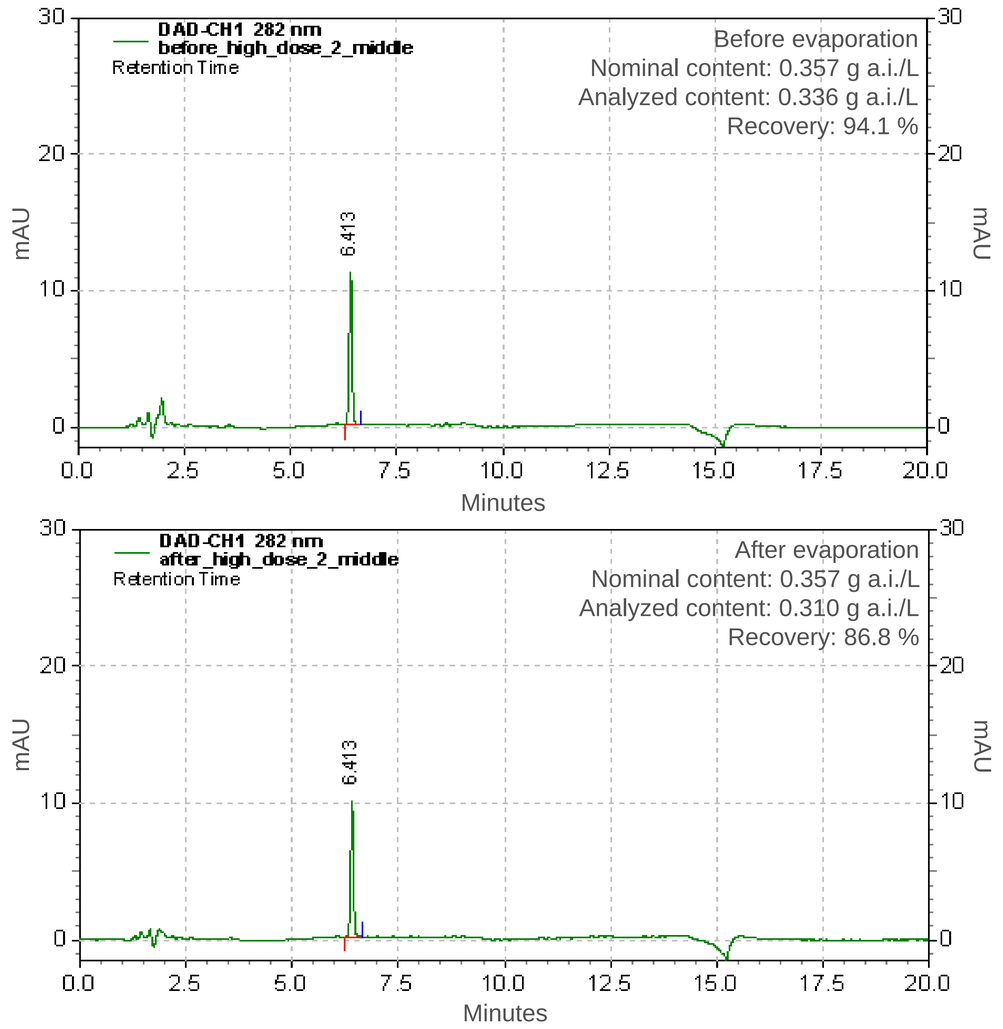

Figure 4: Concentrations of test item in larval diet before and after the evaporation process
HPLC/UV Chromatographs of Diet C. Samples diluted with water/acetonitrile (1/1, v/v).