6.0 Accuracy of the rLLNA

6.1 Performance Statistics

A critical component of a formal evaluation of the validation status of a test method is an assessment of the accuracy of the proposed tested method when compared to the current reference test method (ICCVAM 2003). This aspect of assay performance is typically evaluated by calculating:

- *Accuracy* (concordance): the proportion of correct outcomes (positive and negative) of a test method
- *Sensitivity*: the proportion of all positive substances that are classified as positive
- *Specificity*: the proportion of all negative substances that are classified as negative
- *Positive predictivity*: the proportion of correct positive responses among substances testing positive
- *Negative predictivity*: the proportion of correct negative responses among substances testing negative
- *False positive rate*: the proportion of all negative substances that are falsely identified as positive
- *False negative rate*: the proportion of all positive substances that are falsely identified as negative

The ability of the rLLNA to correctly identify potential skin sensitizers was compared to that of the traditional LLNA for 471 studies.[49] Of the 471 studies, 318 detected skin sensitizers and 153 detected non-sensitizers.[50] Classification of substances and complete data for each substance are located in **Annex III**. When studies for the substances tested more than once in the same vehicle were considered together to yield an overall skin sensitization classification, 465 unique substance–vehicle combination studies resulted. Of these, 315 detected sensitizers and 150 detected non-sensitizers.

Based on the available study data, the rLLNA has an accuracy of 98.7% (465/471), a sensitivity of 98.1% (312/318), a specificity of 100% (153/153), a false positive rate of 0% (0/153), and a false negative rate of 1.9% (6/318) when compared to the traditional LLNA. When substances tested more than once in the same vehicle were considered together, the resulting 465 studies give an accuracy of 98.7% (459/465), a sensitivity of 98.1% (309/315), a specificity of 100% (150/150), a false positive rate of 0% (0/150), and a false negative rate of 1.9% (6/315). The performance characteristics of the rLLNA as discussed in Kimber et al. (2006) are presented in **Table D-3**.

[49] Due to the small number of repeated studies (5%), all studies were treated independently for this accuracy evaluation. When the studies for the substances repeated in the same vehicle were considered together to yield an overall skin sensitization classification, there were 465 studies with unique substance–vehicle combinations.

[50] For two of the repeated studies (HCA and linalool alcohol), the LLNA obtained discordant results. In both cases, one study classified the substance as a non-sensitizer and the other classified it as a sensitizer. Review of the studies indicates differences in the highest dose levels tested. For each of the studies, the traditional LLNA and the rLLNA both classified the substance as a sensitizer or as a non-sensitizer.

Table D-3 Performance of the rLLNA in Predicting Skin Sensitizers Compared to the Traditional LLNA

Data	N	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive		False Negative	
		%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Kimber et al. (2006)	211	98.6	208/211	98.2	166/169	100	42/42	100	166/166	93.3	42/45	0	0/42	1.8	3/169
rLLNA	471	98.7	465/471	98.1	312/318	100	153/153	100	312/312	96.2	153/159	0	0/153	1.9	6/318
rLLNA - substances repeated in the same vehicle were considered together	465	98.7	459/465	98.1	309/315	100	150/150	100	309/309	96.2	150/156	0	0/150	1.9	6/315

Abbreviations: N = number of studies; No. = numbers used to calculate percentage

Kimber et al. (2006) proposed that a minimum testing concentration be considered for the purpose of judging the appropriateness of a non-sensitizing classification for a test substance. In their evaluation, Kimber et al. proposed testing a minimum concentration of 10% in a dose solution (2006). However, lack of sensitizing potential at 10% does not necessarily indicate that a substance will not elicit skin sensitization when tested at a higher concentration. In fact, 51 substances (16% [51/315]) within the current database were non-sensitizers at concentrations of 10%[51] but were sensitizers at higher concentrations (see **Annex IV**).

According to the 1999 ICCVAM-recommended LLNA protocol, the maximum concentration tested should be "the highest achievable level while avoiding overt systemic toxicity and/or excessive local irritation." Similar text is included in OECD TG 429 (2002). Thus, setting a minimum testing concentration is not advised because the maximum soluble concentration that avoids systemic toxicity and/or excessive local irritation may be less than 10% with a non-sensitizing result.

6.2 Discordant Results

In the current analysis, six substances yielded false negative results in the rLLNA. The discordant substances were 2-methyl-2H-isothiazol-3-one, C19-azlactone, azithromycin, camphorquinone, nickel sulfate, and a substance designated as non-ionic surfactant 2. A review of the data for the false negatives indicates that the traditional LLNA classification of the substances as skin sensitizers was based on a low- or mid-dose level that produced an SI ≥ 3, while the highest dose level tested produced an SI < 3 (see **Table D-4**). Because the rLLNA evaluates only the highest dose level tested, all six substances were identified as non-sensitizers (i.e., false negatives). Four of the six substances that resulted in false negatives using the rLLNA compared to the traditional LLNA came from LLNA studies that used pooled data. Graphs of the dose-response curves for these six substances are provided in **Figure D-1**.

Table D-4 Traditional LLNA Data for Substances Identified as False Negatives by the rLLNA

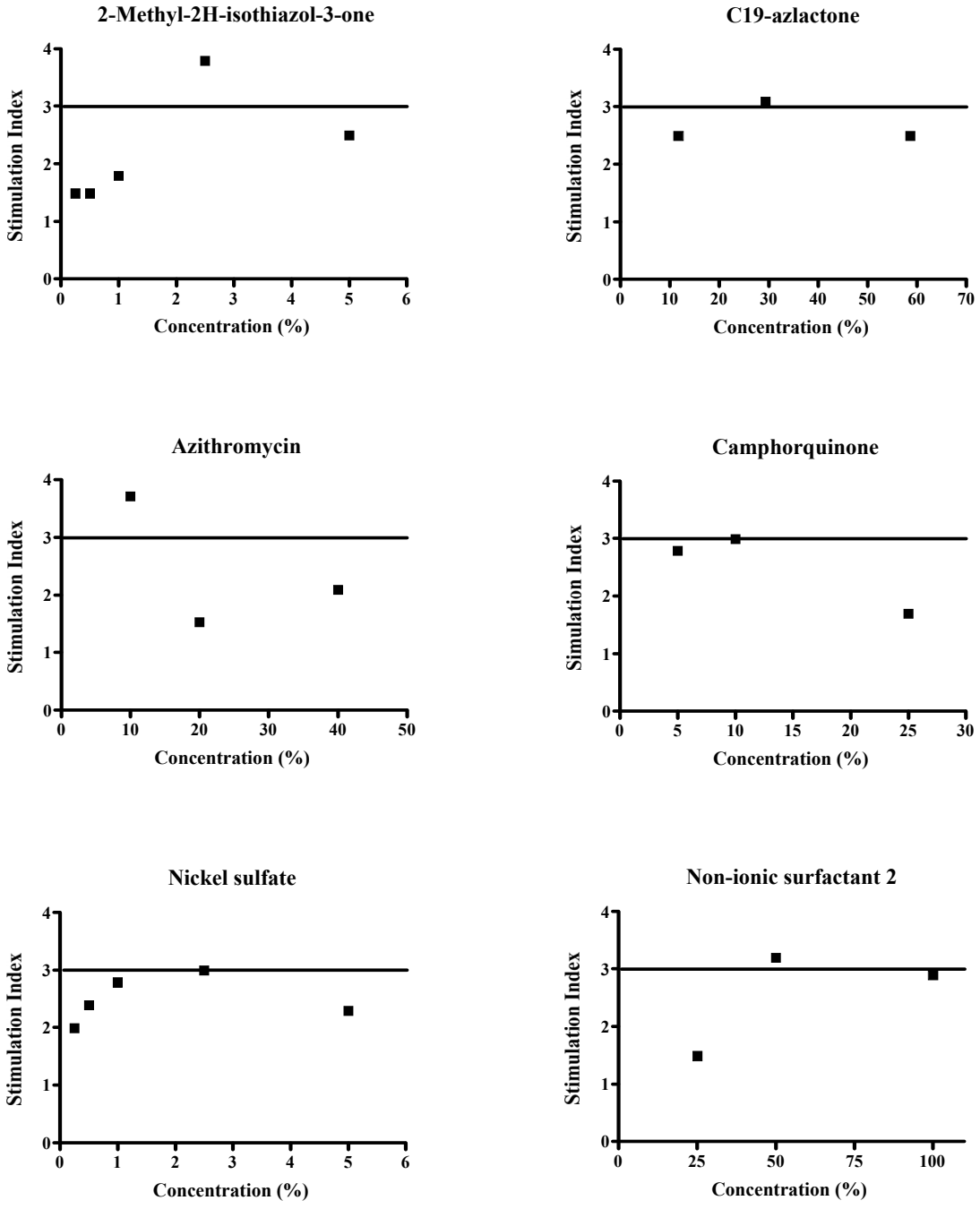
Substance	Vehicle	EC3	Traditional LLNA Data (Low- or Mid-Dose Level)		Traditional LLNA Data (Highest Dose Level)	
			Dose (%)	SI	Dose (%)	SI
2-methyl-2H-isothiazol-3-one	AOO	1.9	2.5	3.8	5	2.5
C19-azlactone	AOO	26	29.33	3.1	58.67	2.5
Azithromycin	Acetone	NC[1]	10	3.7	40	2.1
Camphorquinone	AOO	10	10	3.0	25	1.7
Nickel sulfate	Pluronic L92 (1%)	2.5	2.5	3.0	5	2.3
Non-ionic surfactant 2	AOO	47.1	50	3.2	100	2.9

Abbreviations: AOO = acetone: olive oil (4:1 by volume); EC3 = estimated concentration needed to produce a stimulation index of 3; NC = not calculated; SI = stimulation index

[1] Data was not calculated because extrapolation between points that bracket an SI of 3 could not be done.

[51] An initial dose was tested at a concentration of 10% or greater and resulted in an SI < 3, while a subsequent higher concentration resulted in an SI ≥ 3.

Figure D-1 Dose-Response Curves for Substances Identified as Sensitizers by the Traditional LLNA but as Non-Sensitizers by the rLLNA



Note: The horizontal line in each figure indicates a stimulation index of 3, which is the threshold for a positive response in the LLNA. Points on or above this line would indicate a positive (sensitizer) response, while points below this line would indicate a negative (non-sensitizer) response.

Table D-5 provides a summary of the available physicochemical properties of these substances and the vehicle used.

Table D-5 Summary of Available Physicochemical Properties for False Negatives, as Identified by the rLLNA

Substance	CASRN	Vehicle	Molecular Weight (g/mol)	K_{OW}[1]
2-Methyl-2H-isothiazol-3-one	2682-20-4	AOO	115.15	0.68[2]
C19-azlactone	—	AOO	379.63	5.21[2]
Azithromycin	83905-01-5	Acetone	748.99	3.24[3]
Camphorquinone	465-29-2	AOO	166.22	2.15[2]
Nickel sulfate	7786-81-4	Pluronic L92 (1%)	154.76	-0.17[3]
Non-ionic surfactant 2	—	AOO	—	—

Abbreviations: AOO = acetone: olive oil (4:1 by volume); CASRN = Chemical Abstracts Service Registry Number

[1] K_{OW} represents the octanol-water partition coefficient (expressed on log scale).

[2] K_{OW} calculated by the method of Moriguchi et al. (1994) and provided in Gerberick et al. (2005).

[3] K_{OW} calculated by the method of Meylan and Howard (1995) and obtained from the web site http://www.srcinc.com/what-we-do/databaseforms.aspx?id=385