ECVAM Workshop
Alternative Endpoints for the Local Lymph Node Assay
September 26, 2007
Ispra, Italy

Two Validation Studies on a Non-RI Modification of the Murine LLNA Assay Using ATP Measurement

1) Method of LLNA-DA and results of *intra*-laboratory studies

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Analysis Service Center
Kenji Idehara

Outline

- Method and *intra*-laboratory results

  Kenji Idehara

  What is LLNA-DA?
  Method
  Results of 31 well-known chemicals
  Performance

  —*Accuracy, EC3, Reproducibility*

- Inter-laboratory validation studies

  Takashi Omori

What is LLNA-DA?

《A non-RI modified method》

LLNA : Measure ^{3}H-TdR incorporation
to determine the endpoint of cell proliferation

LLNA-DA : Measure ATP content in the lymph node
to determine the cell number at the end of cell proliferation

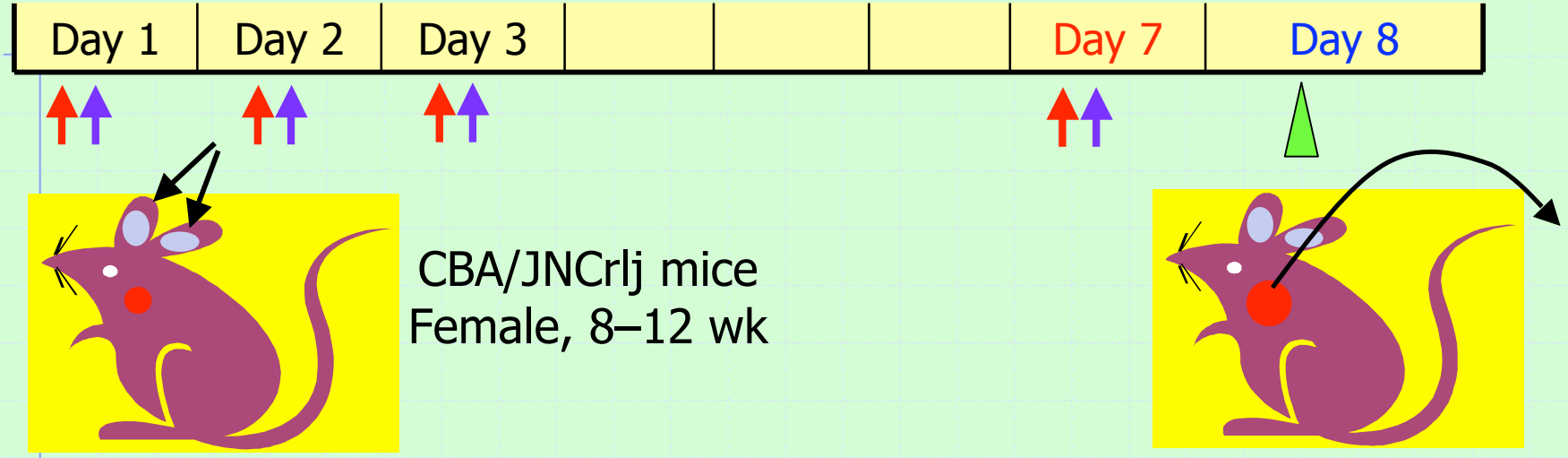
ATP: Adenosine triphosphate

Principal energy source for all living organisms.
ATP content is known to correlate with living cell number.
Bioluminescence is measured by luciferin-luciferase assay
to determine the ATP content.

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{Light}$$

LLNA-DA: LLNA modified by Daicel based on ATP content

Protocol of the LLNA-DA method



Days 1, 2, and 3, and Day 7

Application of chemicals or vehicle control: 25μL on the dorsum of both ears

Pretreatment with 1% SLS solution:
1 h before each application

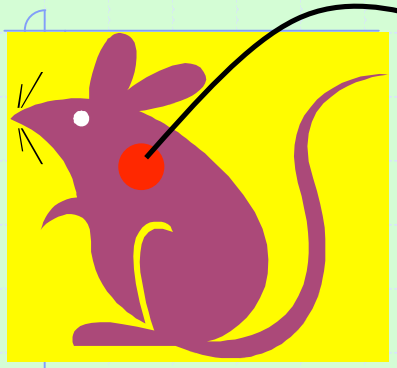
Day 8
(24–30 h after the last application)

Excision of auricular lymph nodes

Measurement of ATP content by luciferin-luciferase assay

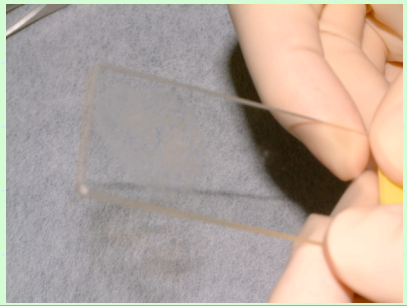
Procedure of measurement of ATP content

Excision of lymph nodes

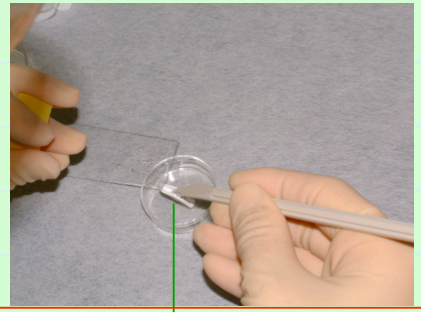


Lymph node weight (mg)

Preparation of cell suspension



Crush and spread Between 2 glass slides



Scrape and suspend in 1 ml PBS

Dilution with PBS (×100)

100μL

Crude cell suspension

20 s

Extraction of ATP

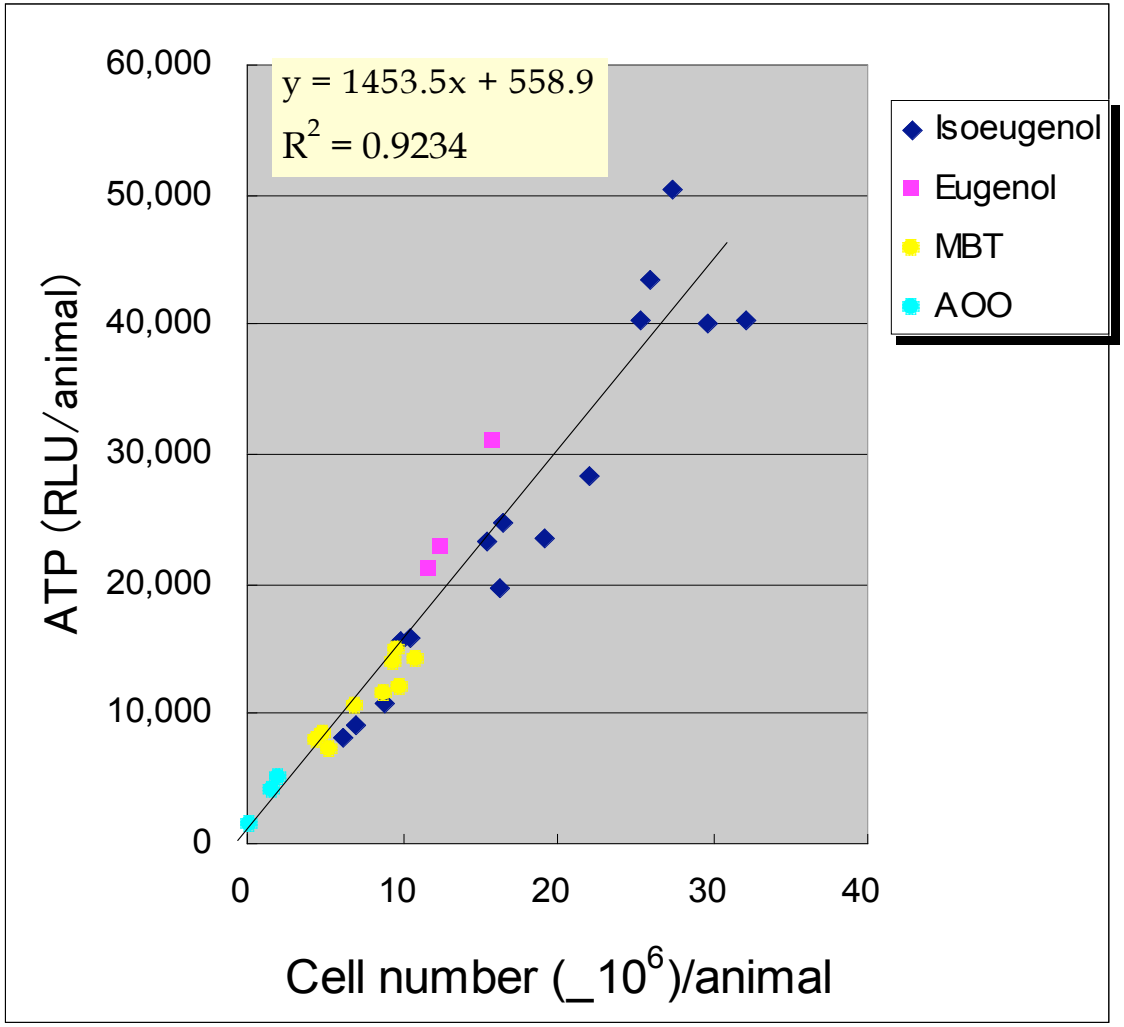
Luciferase

Measurement of ATP Relative light units (RLU)

10 s

Several measurement kits are easily available.
Measuring the ATP is very easy and rapid.
Measurements should be performed immediately after lymph node excision.

Correlation between of cell number and ATP



Wide dynamic range

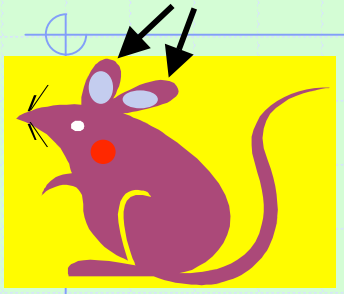
Detection range:
1,000～500,000 RLU

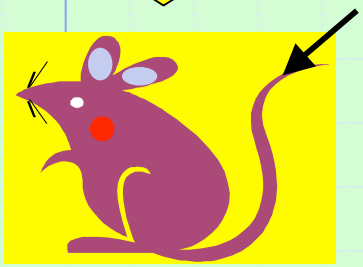
SI value: 1 to 20

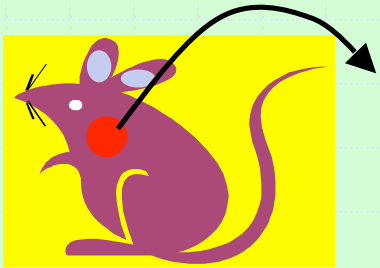
RLU:relative light units

ATP content (RLU) is linearly related to the cell number

Difference between LLNA & LLNA-DA

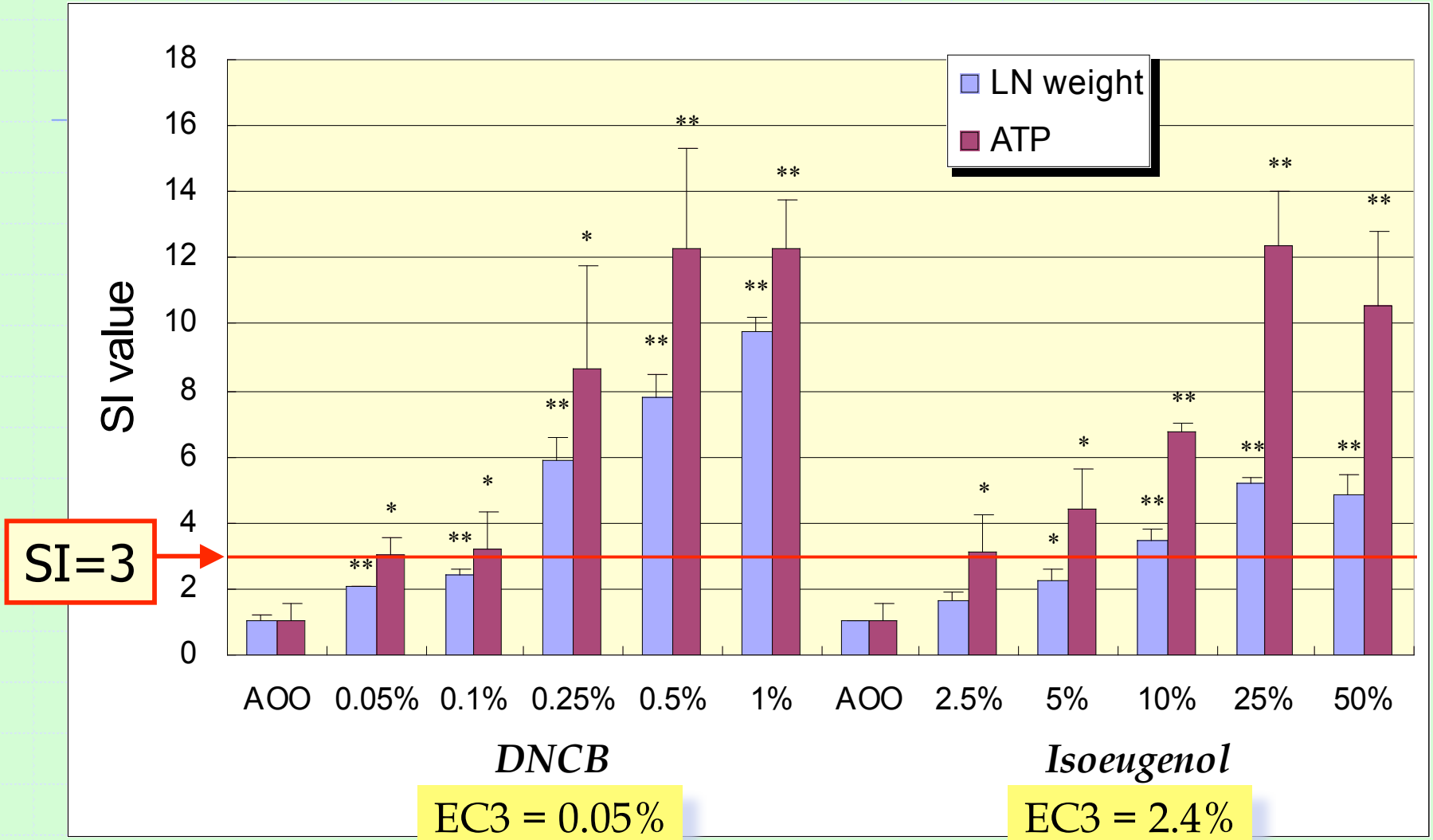






LLNA	LLNA-DA
Days 1, 2, and 3 Application	Days 1, 2, and 3, and Day7 Application +pretreatment with 1% SLS solution
Day 6 ^{3}H-**TdR** injection	
5 h later Excision of auricular lymph nodes Measurement of ^{3}H-TdR (Index of cell proliferation)	Day 8 Excision of auricular lymph nodes Measurement of ATP (Index of cell number)

Results of extreme or strong sensitizers

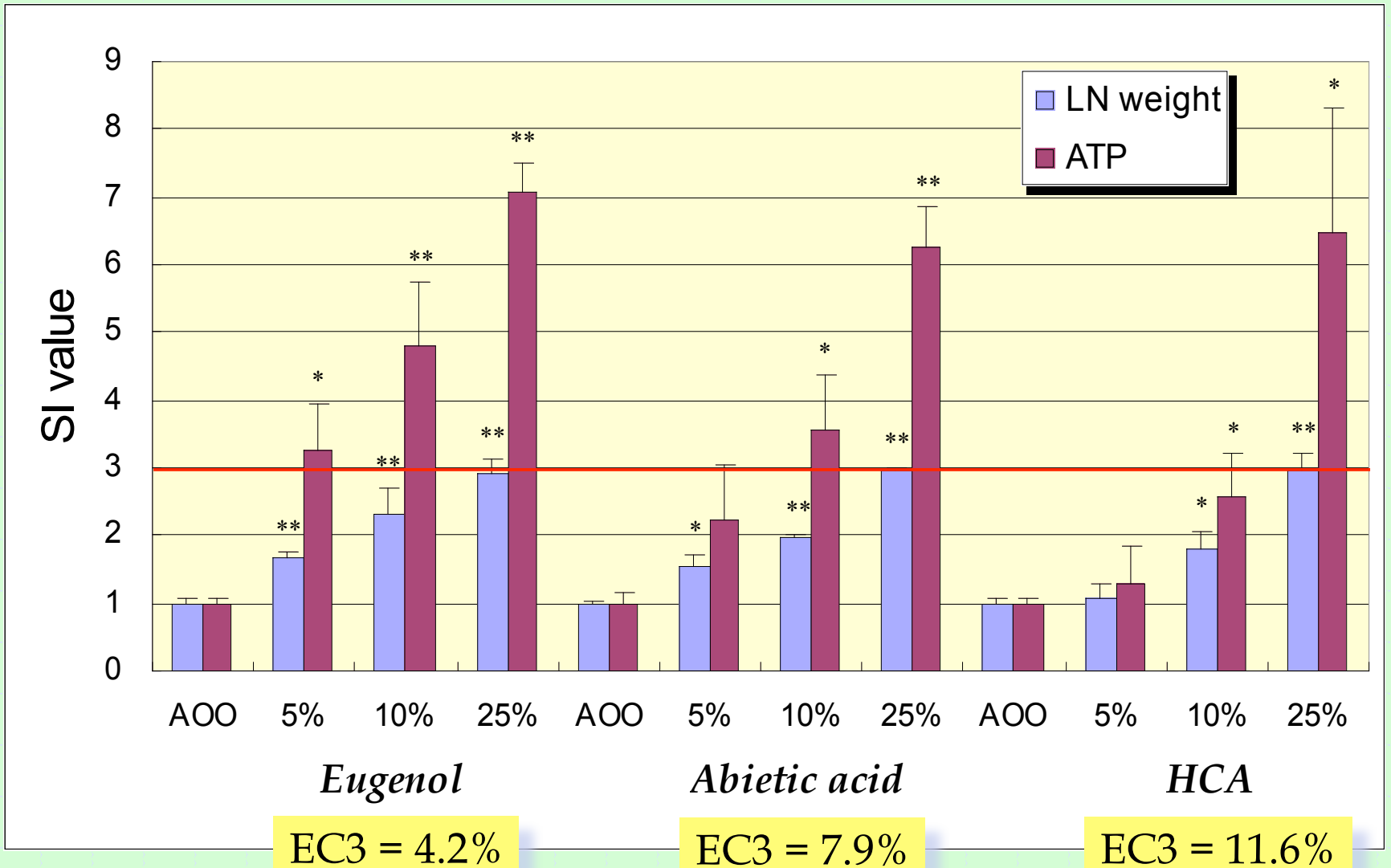


EC3 = 0.05%

EC3 = 2.4%

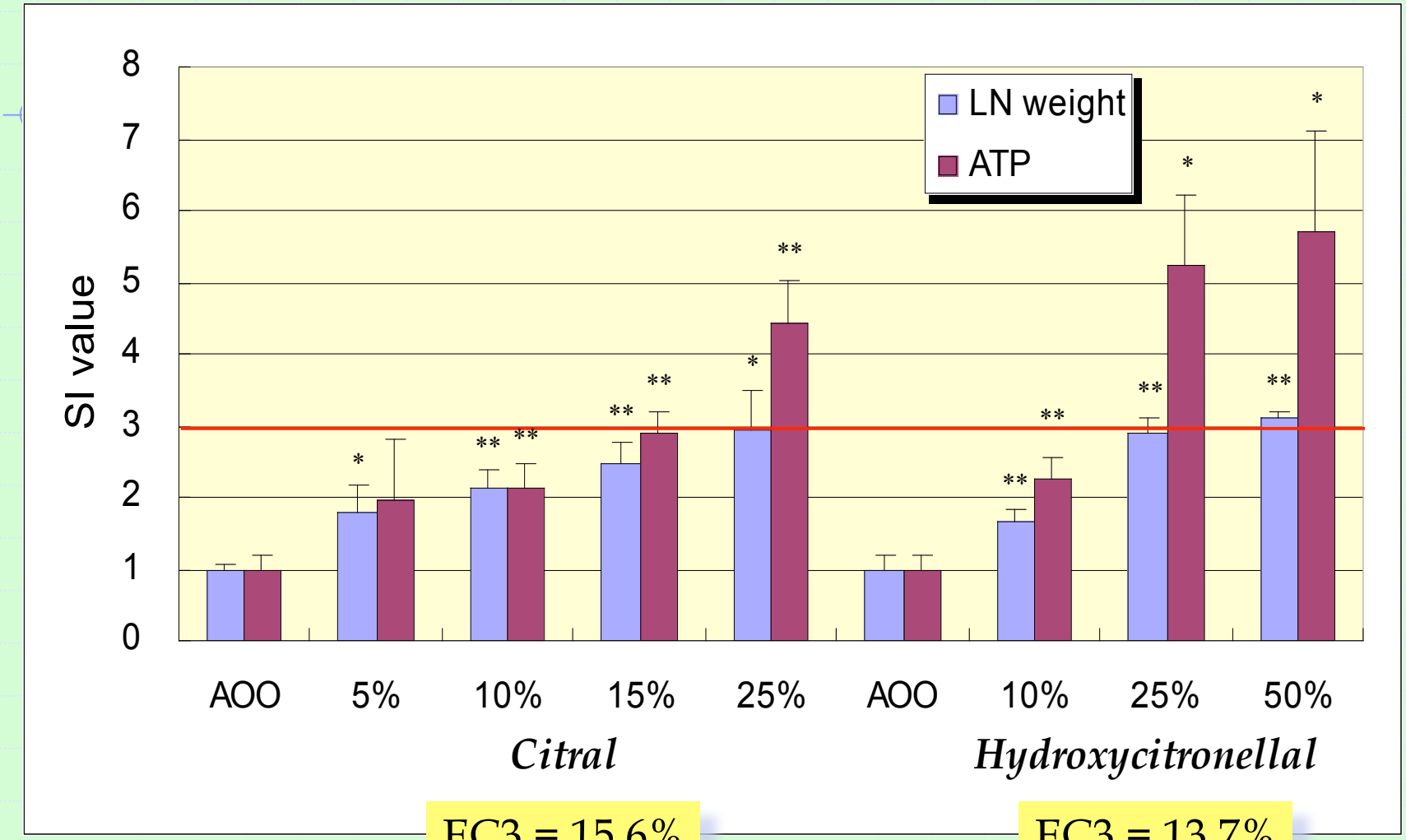
Data presented as mean ± S.D., *$p < 0.05$, **$p < 0.01$.
AOO: acetone/olive oil (4:1, v/v)
DNCB: 2,4-dinitrochlorobenzene

Results of moderate sensitizers



Data presented as mean ± S.D., * $p < 0.05$, ** $p < 0.01$
AOO: acetone/olive oil (4:1, v/v)
HCA: hexyl cinnamic aldehyde

Results of weak sensitizers

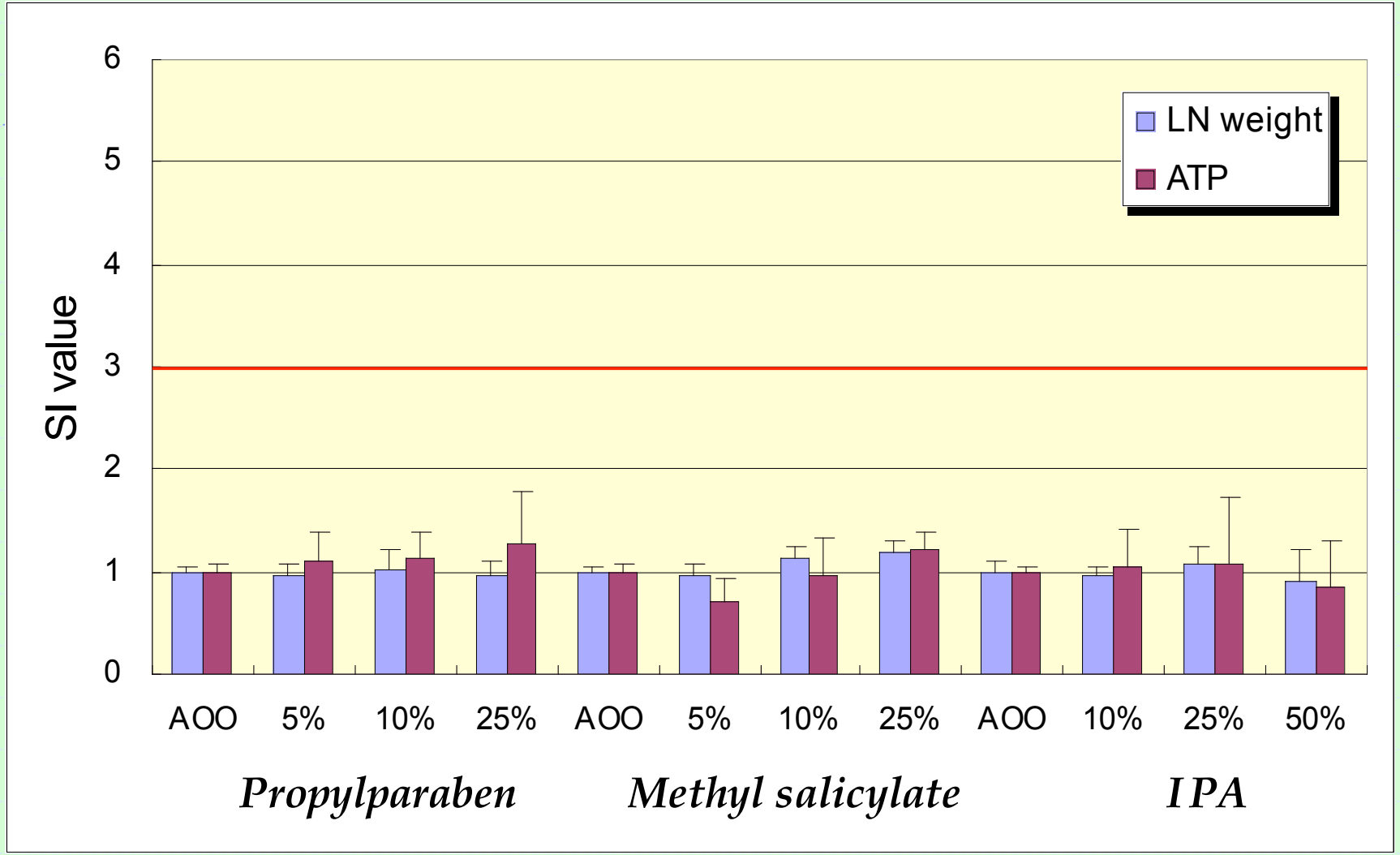


EC3 = 15.6%

EC3 = 13.7%

Data presented as mean ± S.D., *$p < 0.05$, **$p < 0.01$.
AOO: acetone/olive oil (4:1, v/v)

Results of weak sensitizers and irritants



Data presented as mean ± S.D., *$p < 0.05$, **$p < 0.01$.
AOO: acetone/olive oil (4:1, v/v)
IPA: isopropyl alcohol

Summary of results
31 well-known chemicals

Chemicals	LLNA-DA	*LLNA	*GPMT/BA	*HMT/HPTA
2,4-Dinitrochlorobenzene	+	+	+	
p -Phenylenediamine	+	+	+	+
Toluene diisocyanate	+	+		
Glutaraldehyde	+	+		
$K_2Cr_2O_7$	+	+	+	+
Pthalic anhydride	+	+	+	
Trimellitic anhydride	+	+		
Formaldehyde	+	+	+	+
Cinnamic aldehyde	+	+	+	+
Isoeugenol	+	+	+	+
$CoCl_2$	+	+	+	+
Eugenol	+	+	+	+
Resorcinol	+	+	-	+
Benzocaine	+	+/-	+	+/-
Abietic acid	+	+	+	+
Hexyl cinnamic aldehyde	+	+	+	
Mercaptobenzothiazol	-	+	+	+
Citral	+	+	+	+
Hydroxycitronellal	+	+	+	+
Imidazolidinyl urea	+	+	+	+
SLS	+	+	-	-
$NiSO_4$	-	-	+	+
Benzalkonium chloride	+	-	-	+
Propyl paraben	-	-	-	+/-
Diethylphthalate	-	-		
1-Bromobutane	-	-		
Methysalicylate	-	-	-	-
Chlorobenzene	-	-	-	
Lactic acid	-	-	-	
Hexane	-	-		-
Isopropanol	-	-	-	

GPMT: guinea pig maximization test
BA: Buehler assay
HMT: human maximization test
HPTA: human patch test allergen

* K. E. Haneke, *et al.*, *Reg. Toxicol. Pharmacol.*, (2001) **34**, 274–286.

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Glutaraldehyde	+	+		
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Pthalic anhydride	+	+	+	
Trimellitic anhydride	+	+		
Formaldehyde	+	+	+	+
Cinnamic aldehyde	+	+	+	+
Isoeugenol	+	+	+	+
$CoCl_2$	+	+	+	+
Eugenol	+	+	+	+
Resorcinol	+	+	-	+
Benzocaine	+	+/-	+	+/-
Abietic acid	+	+	+	+
Hexyl cinnamic aldehyde	+	+	+	
Mercaptobenzothiazol	-	+	+	+
Citral	+	+	+	+
Hydroxycitronellal	+	+	+	+
Imidazolidinyl urea	+	+	+	+
SLS	+	+	-	-
$NiSO_4$	-	-	+	+
Benzalkonium chloride	+	-	-	+
Propyl paraben	-	-	-	+/-
Diethylphthalate	-	-		
1-Bromobutane	-	-		
Methysalicylate	-	-	-	-
Chlorobenzene	-	-	-	
Lactic acid	-	-	-	
Hexane	-	-		-
Isopropanol	-	-	-	

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Pthalic anhydride	+	+	+	
Trimellitic anhydride	+	+		
Formaldehyde	+	+	+	+
Cinnamic aldehyde	+	+	+	+
Isoeugenol	+	+	+	+
$CoCl_2$	+	+	+	+
Eugenol	+	+	+	+
Resorcinol	+	+	-	+
Benzocaine	+	+/-	+	+/-
Abietic acid	+	+	+	+
Hexyl cinnamic aldehyde	+	+	+	
Mercaptobenzothiazol	-	+	+	+
Citral	+	+	+	+
Hydroxycitronellal	+	+	+	+
Imidazolidinyl urea	+	+	+	+
SLS	+	+	-	-
$NiSO_4$	-	-	+	+
Benzalkonium chloride	+	-	-	+
Propyl paraben	-	-	-	+/-
Diethylphthalate	-	-		
1-Bromobutane	-	-		
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Lactic acid	-	-	-	
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Isoeugenol	+	+	+	+
$CoCl_2$	+	+	+	+
Eugenol	+	+	+	+
Resorcinol	+	+	-	+
Benzocaine	+	+/-	+	+/-
Abietic acid	+	+	+	+
Hexyl cinnamic aldehyde	+	+	+	
Mercaptobenzothiazol	-	+	+	+
Citral	+	+	+	+
Hydroxycitronellal	+	+	+	+
Imidazolidinyl urea	+	+	+	+
SLS	+	+	-	-
$NiSO_4$	-	-	+	+
Benzalkonium chloride	+	-	-	+
Propyl paraben	-	-	-	+/-
Diethylphthalate	-	-		
1-Bromobutane	-	-		
Methysalicylate	-	-	-	-
Chlorobenzene	-	-	-	
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Trimellitic anhydride	+	+		
Formaldehyde	+	+	+	+
Cinnamic aldehyde	+	+	+	+
Isoeugenol	+	+	+	+
$CoCl_2$	+	+	+	+
Eugenol	+	+	+	+
Resorcinol	+	+	-	+
Benzocaine	+	+/-	+	+/-
Abietic acid	+	+	+	+
Hexyl cinnamic aldehyde	+	+	+	
Mercaptobenzothiazol	-	+	+	+
Citral	+	+	+	+
Hydroxycitronellal	+	+	+	+
Imidazolidinyl urea	+	+	+	+
SLS	+	+	-	-
$NiSO_4$	-	-	+	+
Benzalkonium chloride	+	-	-	+
Propyl paraben	-	-	-	+/-
Diethylphthalate	-	-		
1-Bromobutane	-	-		
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Performance of LLNA-DA (vs. LLNA)

		LLNA	
		Positive	**Negative**
LLNA-DA	**Positive**	DNCB *p*-Phenylenediamine Cinnamaldehyde Isoeugenol Eugenol Abietic acid Imidazolidinyl urea Trimellitic anhydride Phthalic anhydride Glutaraldehyde Formaldehyde Hydroxycitronellal $K_2Cr_2O_7$ $CoCl_2$ TDI HCA Citral Resorcinol SLS **19**	Benzalkonium chloride **1**
	Negative	Mercaptobenzothiazol **1**	1-Bromobutane Diethyl phthalate Propylparaben Methyl salicylate Chlorobenzene $NiSO_4$ Hexane IPA Lactic acid **9**

Comparison	No. of comparisons	Sensitivity	Specificity	Positive predictivity	Negative predictivity	Accuracy
LLNA-DA vs. LLNA	30	**95%** (19/20)	**90%** (9/10)	**95%** (19/20)	**90%** (9/10)	**93%** (28/30)

Performance of LLNA-DA (vs. GPMT/BA)

		GPMT/BA	
		Positive	**Negative**
LLNA-DA	**Positive**	2,4-Dinitrochlorobenzene *p*-Phenylenediamine Phthalic anhydride Formaldehyde Cinnamic aldehyde Isoeugenol Eugenol Abietic acid Hydroxycitronellal Imidazolidinyl urea Benzocaine $K_2Cr_2O_7$ $CoCl_2$ HCA Citral **15**	Resorcinol SLS Benzalkonium chloride **3**
	Negative	Mercaptobenzothiazol $NiSO_4$ **2**	Propylparaben Methyl salicylate Chlorobenzene Lactic acid IPA **5**

Comparison	No. of comparisons	Sensitivity	Specificity	Positive predictivity	Negative predictivity	Accuracy
LLNA-DA vs. GPMT/BA	25	**88%** (15/17)	**63%** (5/8)	**83%** (15/18)	**71%** (5/7)	**80%** (20/25)

GPMT: guinea pig maximization test
BA: Buehler assay

Performance of LLNA-DA (vs. HMT/HPTA)

		HMT/HPTA	
		Positive	**Negative**
LLNA-DA	**Positive**	*p*-Phenylenediamine Formaldehyde Cinnamic aldehyde Isoeugenol Eugenol Resorcinol Abietic acid Citral Hydroxycitronellal Imidazolidinyl urea Benzalkonium chloride $K_2Cr_2O_7$ $CoCl_2$ **13**	SLS **1**
	Negative	Mercaptobenzothiazol $NiSO_4$ Propylparaben **3**	Methyl salicylate Hexane **2**

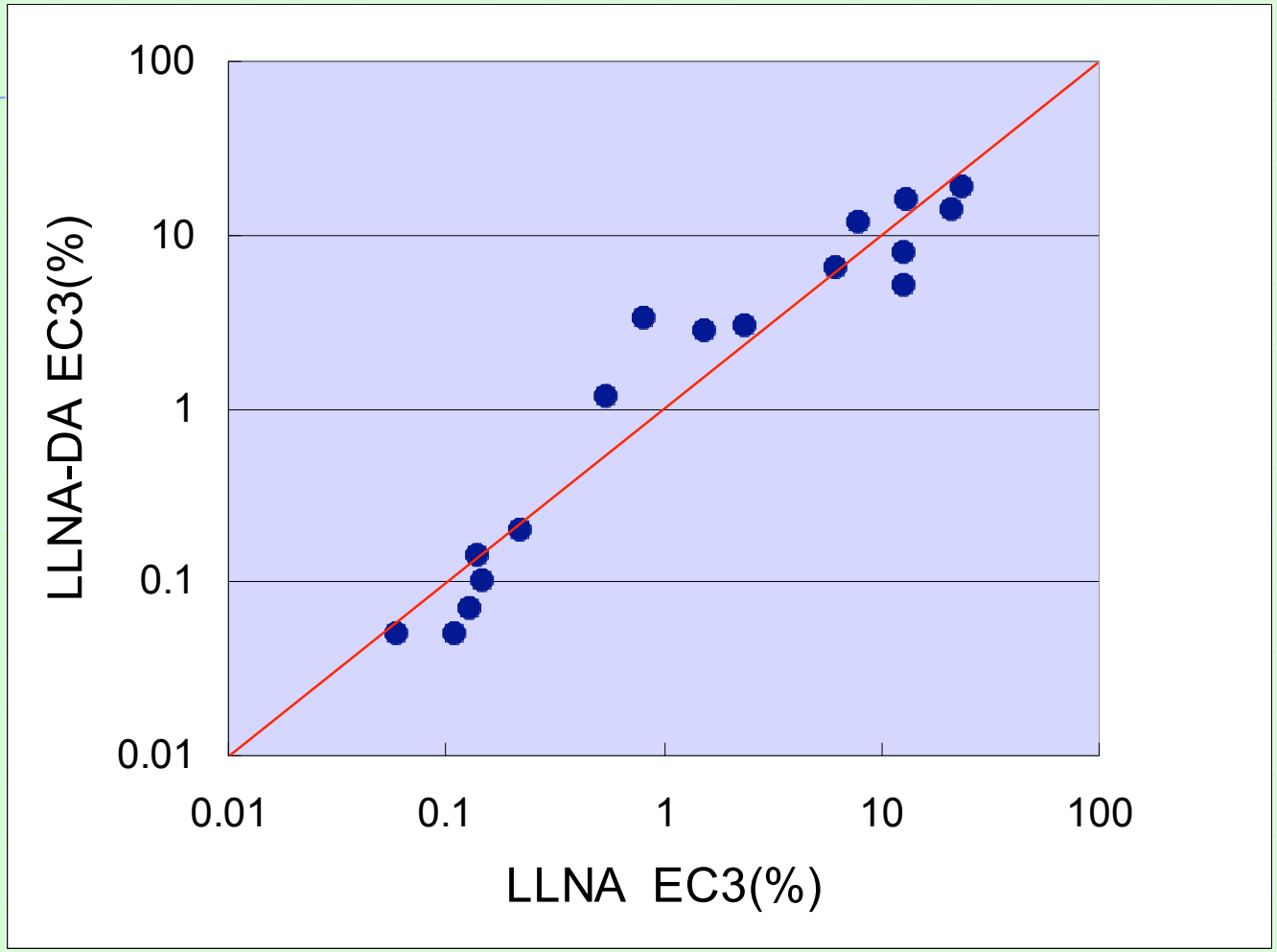
Comparison	No. of comparisons	Sensitivity	Specificity	Positive predictivity	Negative predictivity	Accuracy
LLNA-DA vs. HMT/HPTA	19	**81%** (13/16)	**67%** (2/3)	**93%** (13/14)	**40%** (2/5)	**79%** (15/19)

HMT: human maximization test
HPTA: human patch test allergen

Summary of EC3 value

Chemical name	LLNA-DA EC3%	LLNA EC3%
2,4-Dinitrochlorobenzene	0.05	0.03 - 0.09
p -Phenylenediamine	0.07	0.06 - 0.2
Toluene diisocyanate	0.05	0.11
Glutaraldehyde	0.10	0.10 - 0.20
$K_2Cr_2O_7$	0.14	0.14
Trimellitic anhydride	0.20	0.22
Formaldehyde	1.16	0.4 - 0.7
Cinnamic aldehyde	2.98	1.7 - 3.1
Isoeugenol	2.46, 2.35, 3.40	1.3 - 1.8
$CoCl_2$	3.27	0.82
Eugenol	4.50, 5.09, 5.59	13
Resorcinol	6.44	6.3
Benzocaine	6.57	+/-
Abietic acid	7.90	11.0 - 14.7
Hexyl cinnamic aldehyde	11.6	4.0 - 11.9
Citral	15.6	13
Hydroxycitronellal	13.7	20 - 23
Imidazolidinyl urea	18.8	23.9

Correlation of EC3 (LLNA vs. LLNA-DA)



EC3 values of LLNA-DA and original LLNA are almost in agreement

Reproducibility of EC3 values based on ATP content

Isoeugenol

Concentration (%)	SI value (ATP) ± S.D.		
	Exp. 1	Exp. 2	Exp. 3
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54		1.22 ± 0.13
1	2.28 ± 0.60		2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98
5	3.39 ± 0.69	4.39 ± 1.25	
10	5.68 ± 1.19	6.77 ± 0.23	
EC3	**3.40%**	**2.35%**	**2.46%**

2.74% ± 0.58% CV: 21%

Eugenol

Concentration (%)	SI value (ATP) ± S.D.		
	Exp. 1	Exp. 2	Exp. 3
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	**5.09%**	**5.59%**	**4.50%**

5.06% ± 0.55% CV: 11%

Reproducibility of EC3 values based on ATP content

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2.5	**2.78 ± 0.17**	**3.11 ± 1.15**	**3.01 ± 0.98**
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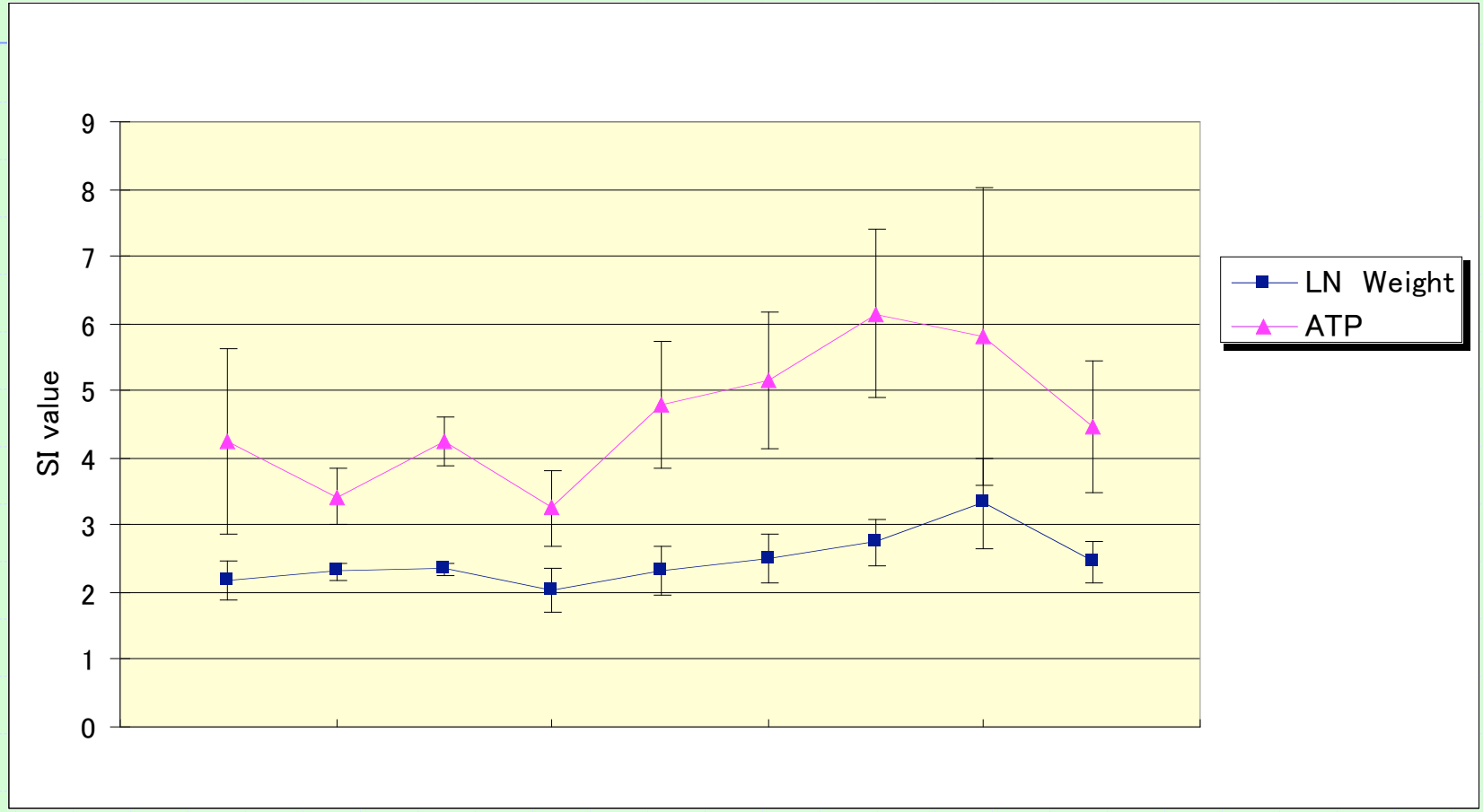
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	**5.09%**	**5.59%**	**4.50%**

5.06% ± 0.55% CV: 11%

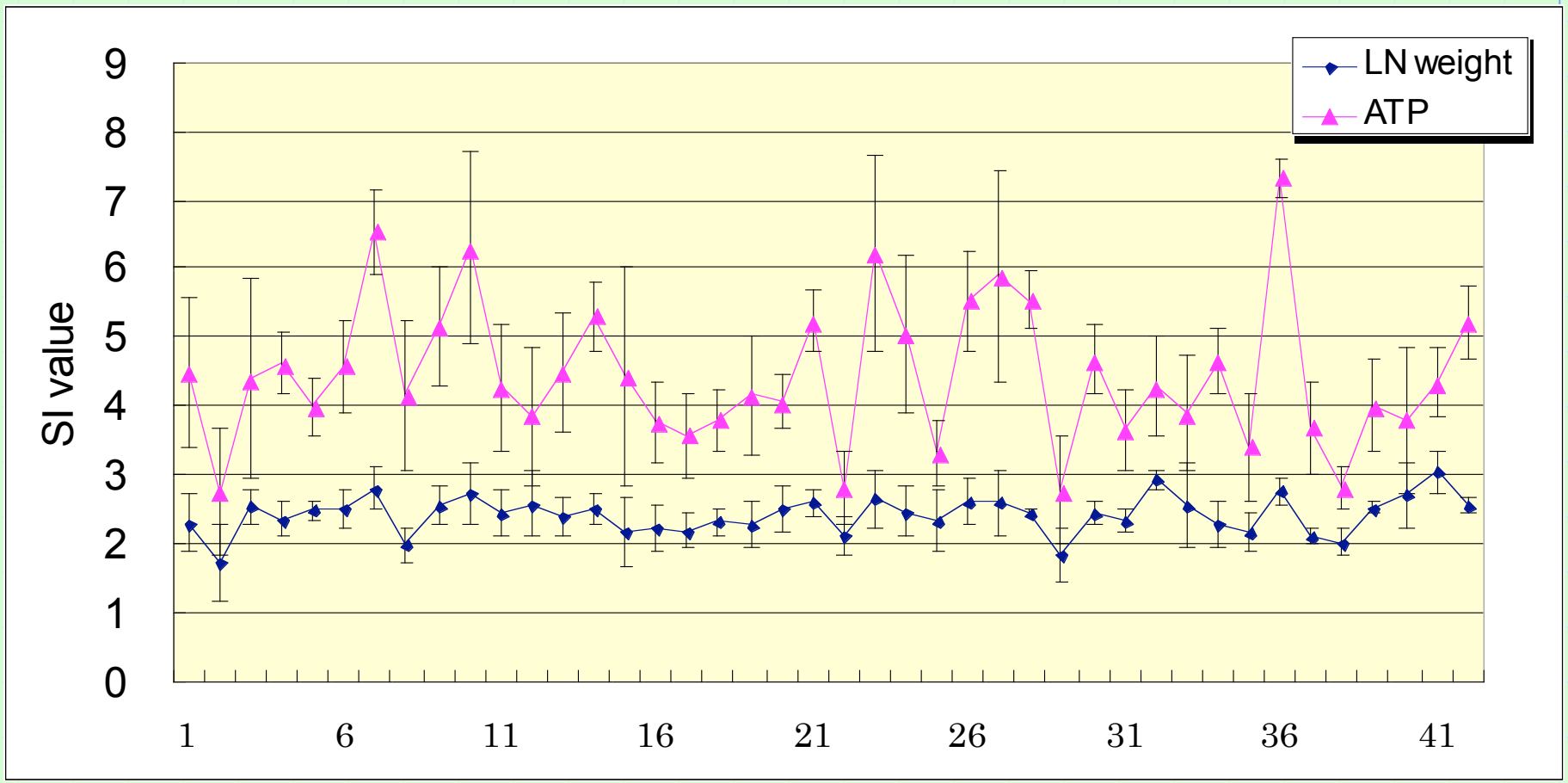
Reproducibility of SI value (10% eugenol/AOO)



4.99 ± 1.35, CV: 27% , n=9

Data presented as mean ± S.D.
AOO: acetone/olive oil (4:1, v/v)

Reproducibility of SI value (15% HCA/AOO)



4.45 ± 1.05, CV: 24%, n=42

Data presented as mean ± S.D.
AOO: acetone/olive oil (4:1, v/v)

Summary

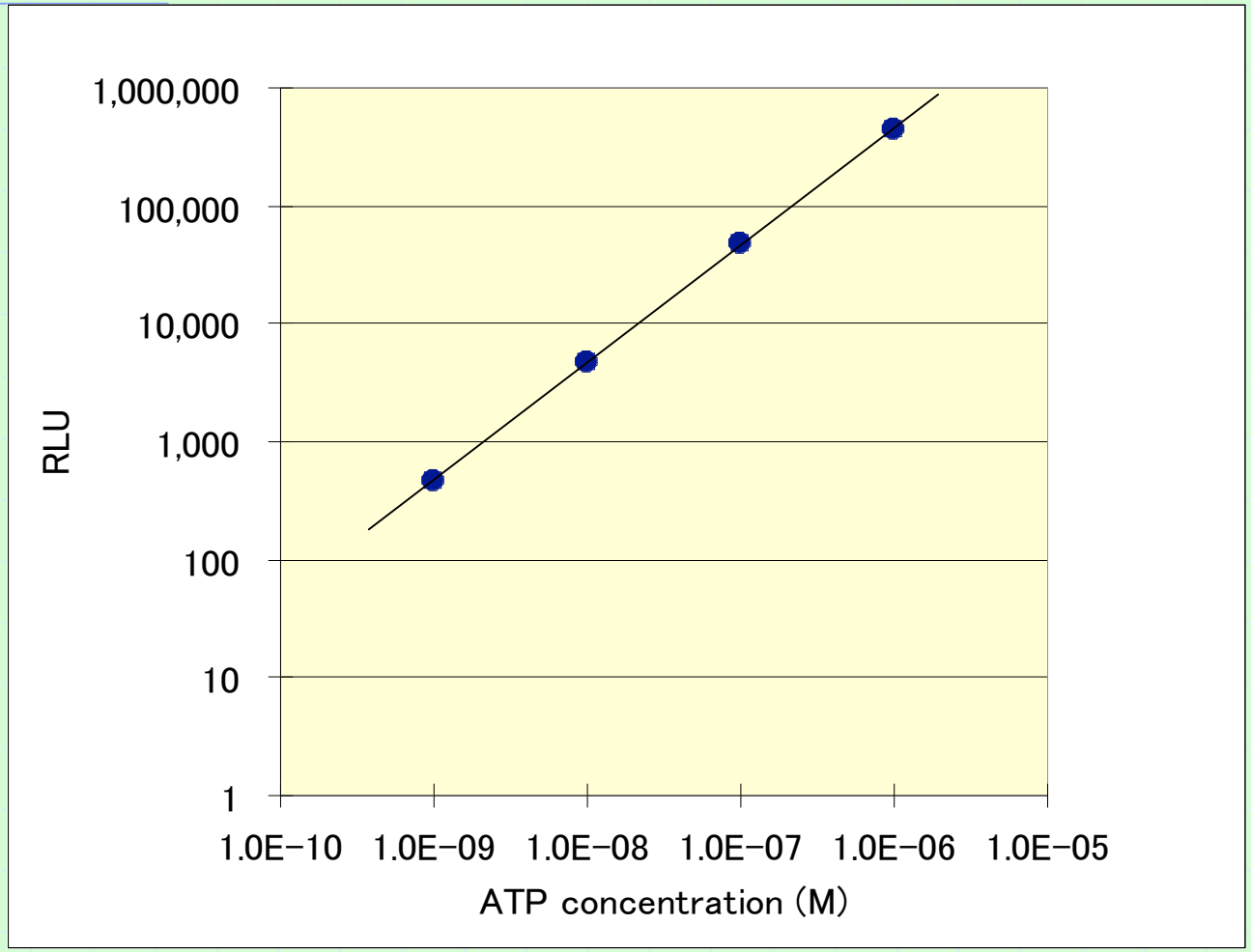
- We developed a modified LLNA method with a non-RI endpoint (LLNA-DA)
- In LLNA-DA, we measure the ATP content as the endpoint.

  -Luciferin-luciferase reaction-
- Simple operation to determine the ATP content and availability of a wide dynamic range

- Performance of LLNA-DA is similar to that of original LLNA.
- EC3 of LLNA-DA is almost equal to that of LLNA.

Reference information

Correlation of ATP concentration with relative light unit (RLU)

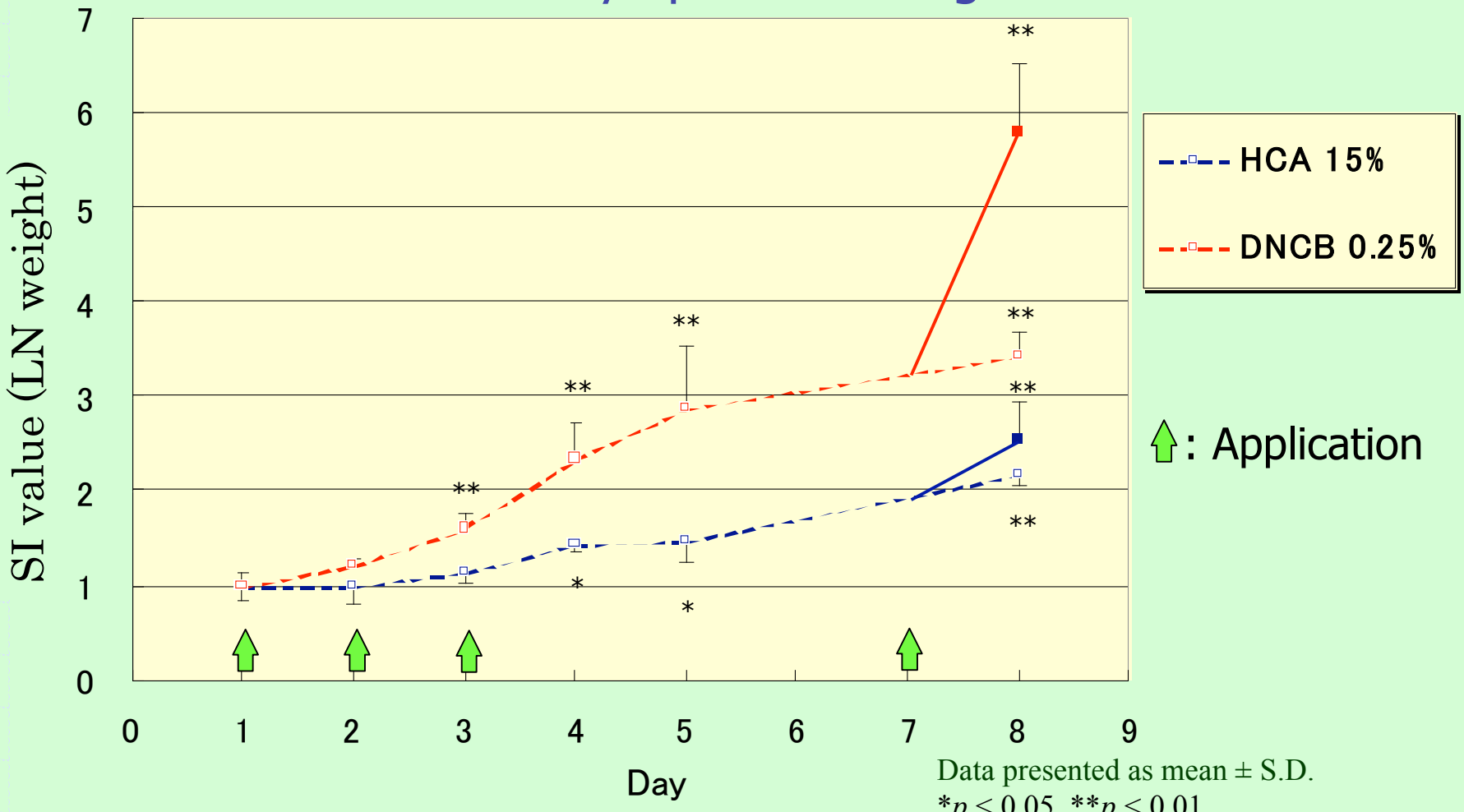


Correlation of ATP concentration with relative light unit (RLU)

Reference information

Effect of fourth application on Day 7

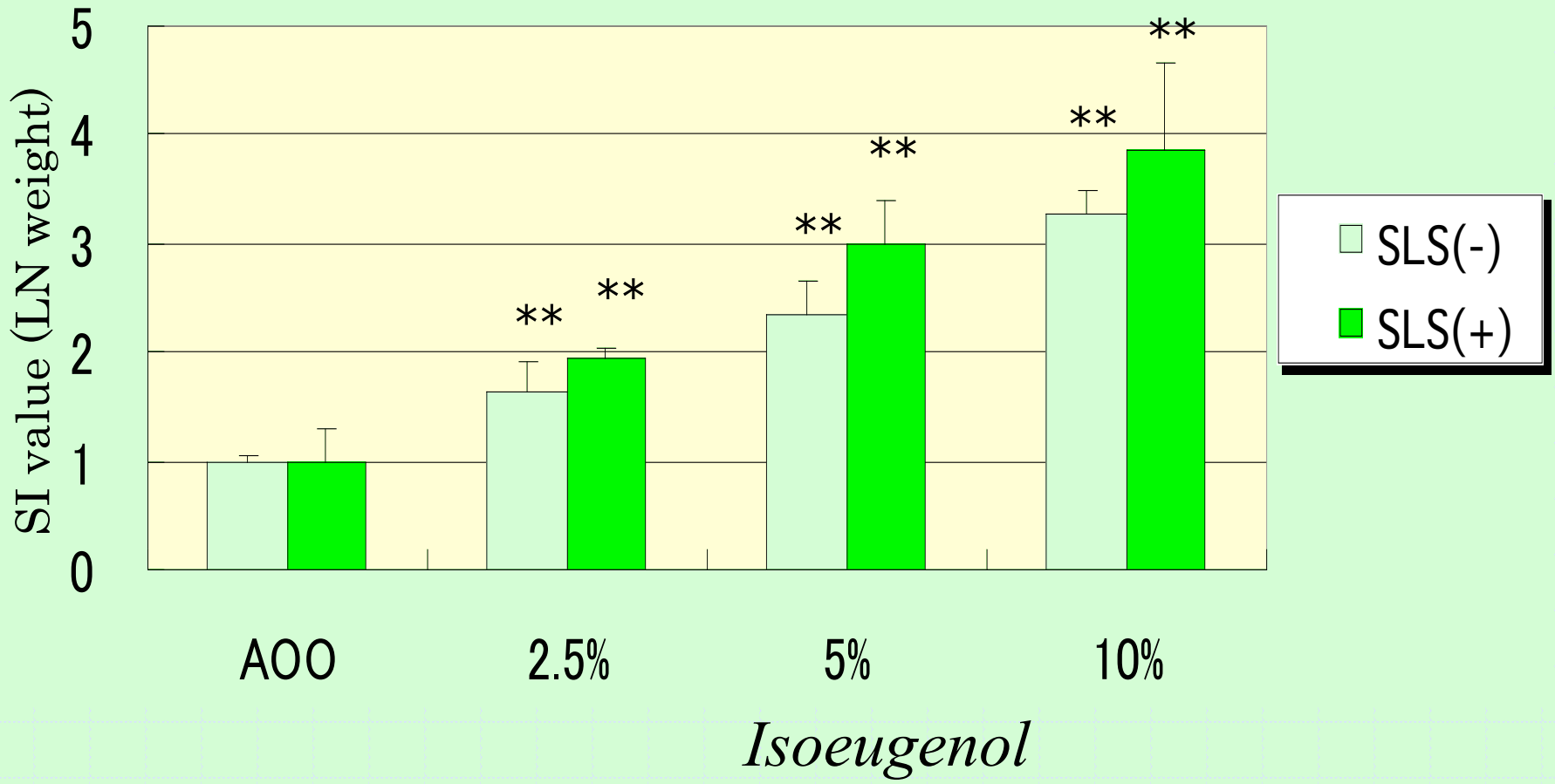
—Variation in lymph node weight—



Data presented as mean ± S.D.
*$p < 0.05$, **$p < 0.01$.
HCA: hexylcinnamaldehyde
DNCB: 2,4-dinitrochlorobenzene

Reference information

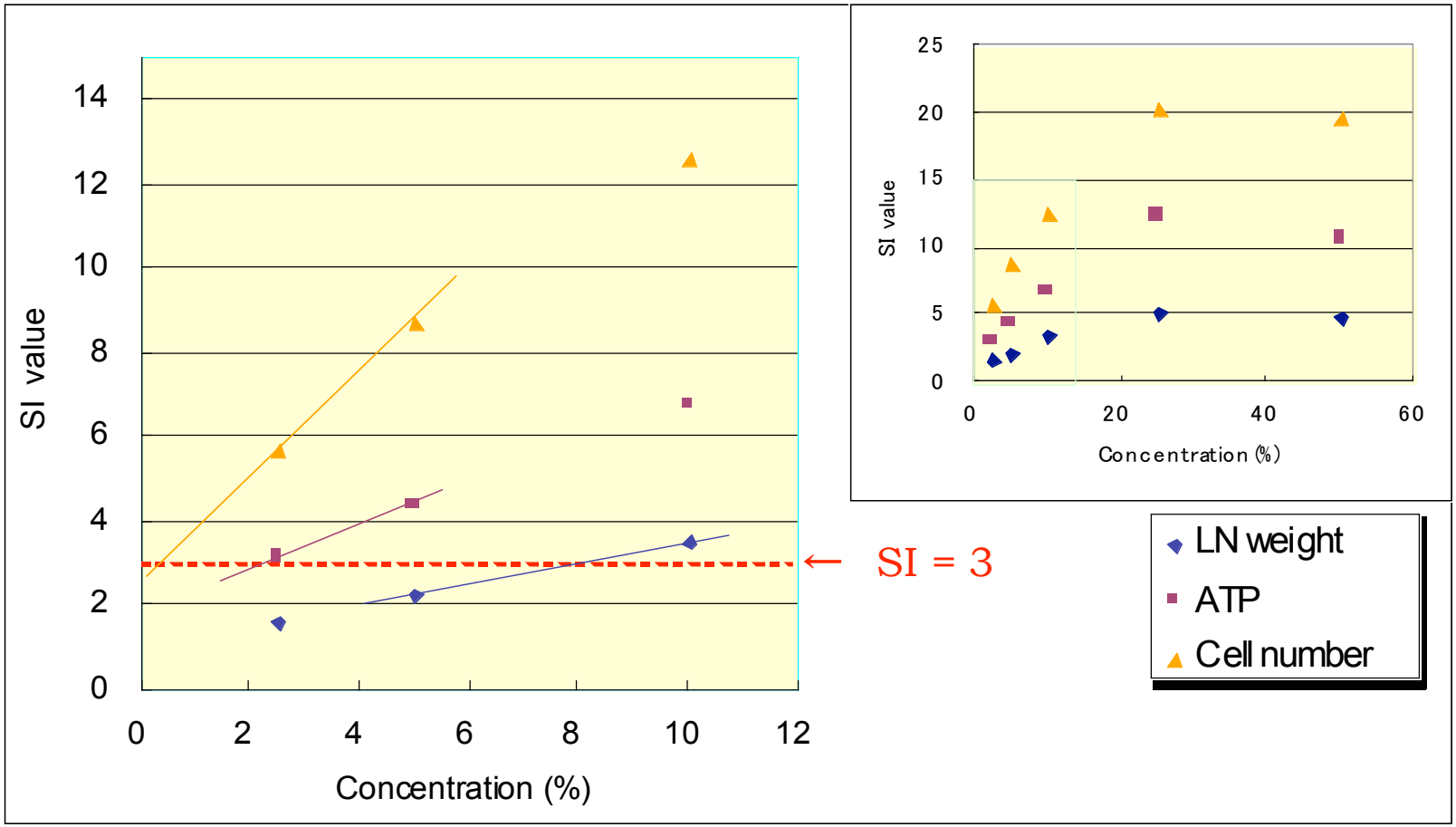
Effect of pretreatment with 1% SLS solution



Data presented as mean ± S.D.
*$p < 0.05$, **$p < 0.01$.

Reference information

Variation in EC3 by difference of endpoints for isoeugenol



EC3 = 8.02% (LN weight)

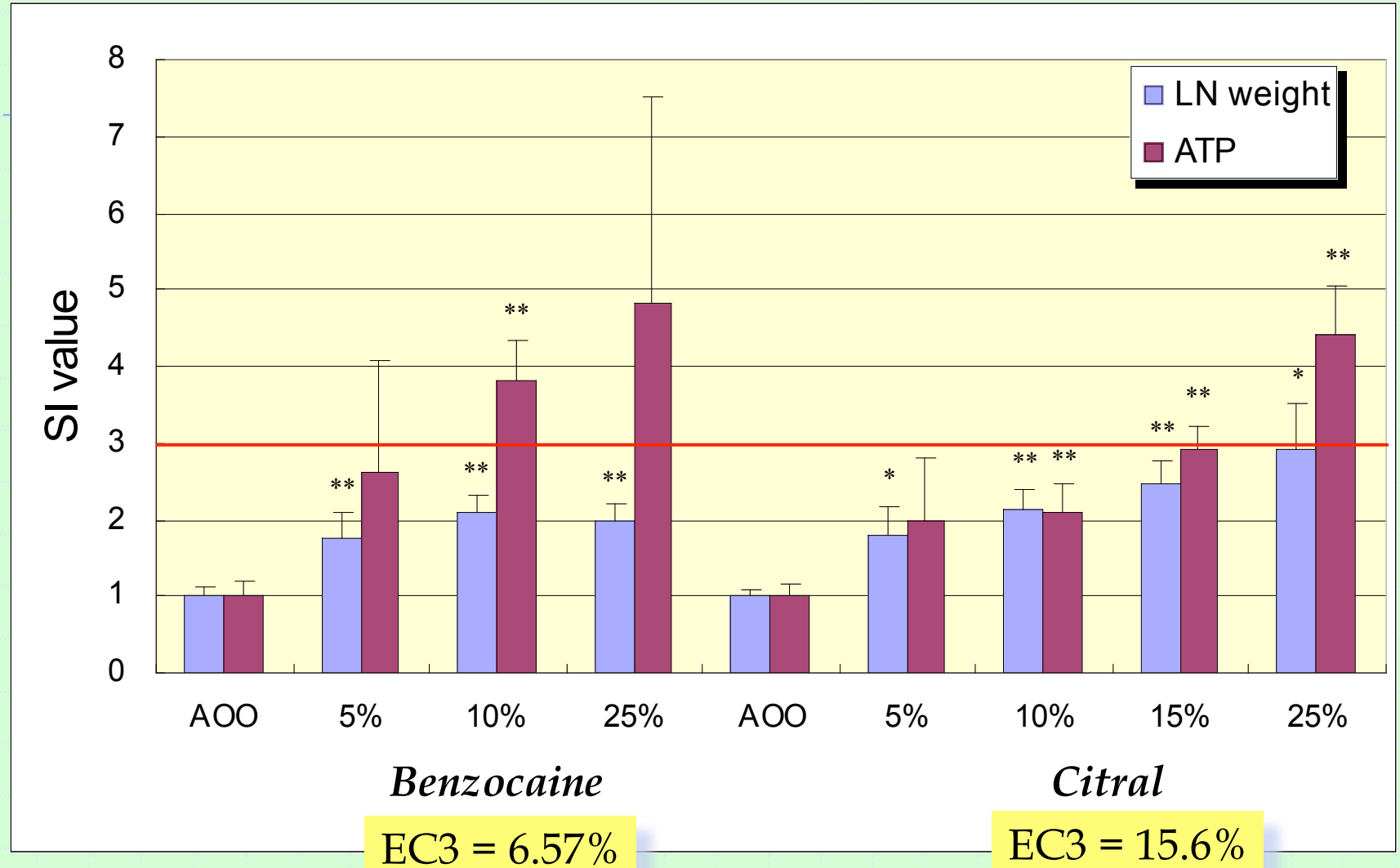
EC3 = 2.28% (ATP)

EC3 = 0.31% (Cell number)

EC3 = 1.3 - 3.3%
(Original LLNA)

Reference information

Results of moderate and weak sensitizers



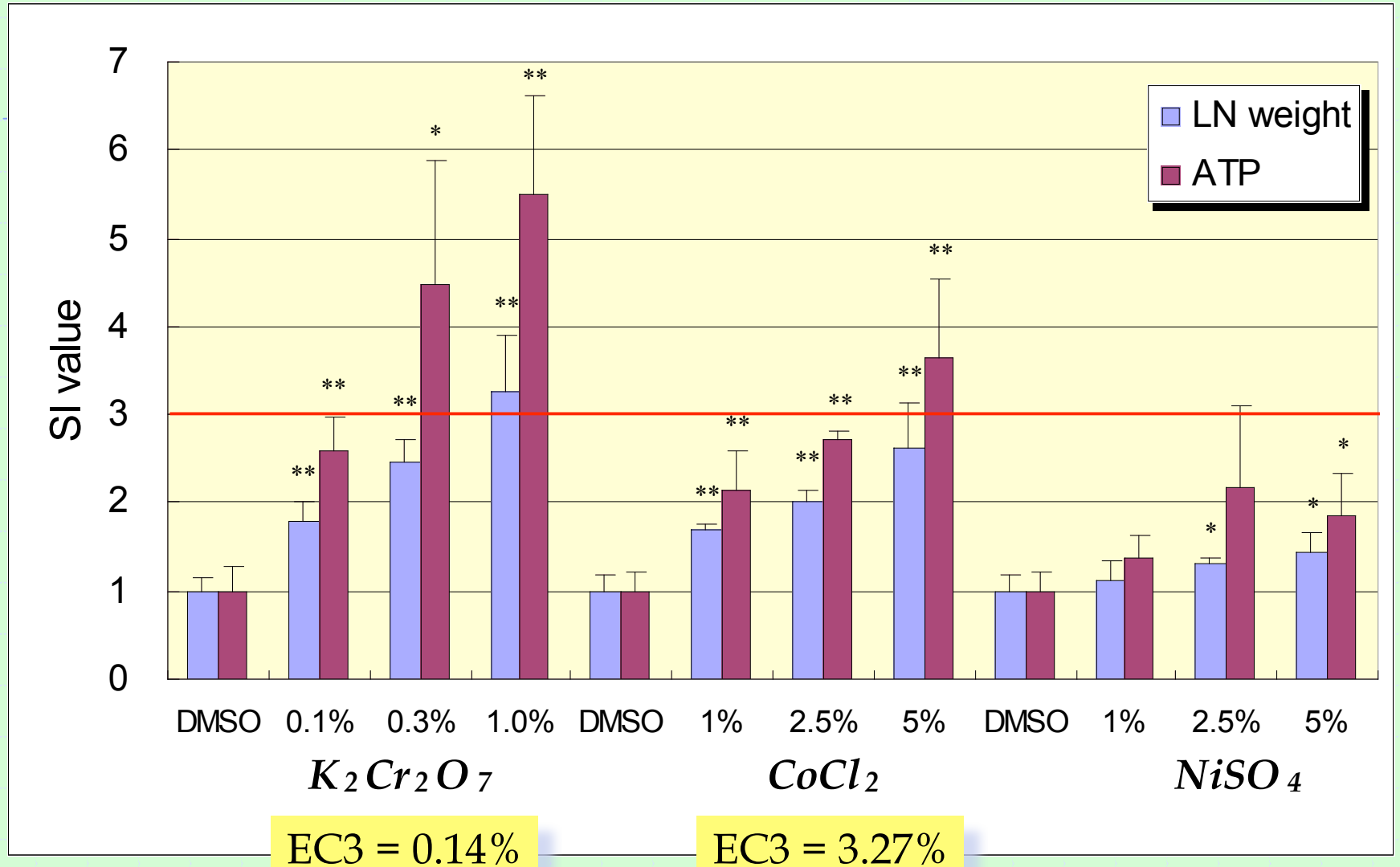
EC3 = 6.57%

EC3 = 15.6%

Data presented as mean ± S.D., *$p < 0.05$, **$p < 0.01$.
AOO: acetone/olive oil (4:1, v/v)

Reference information

Results of metal salts



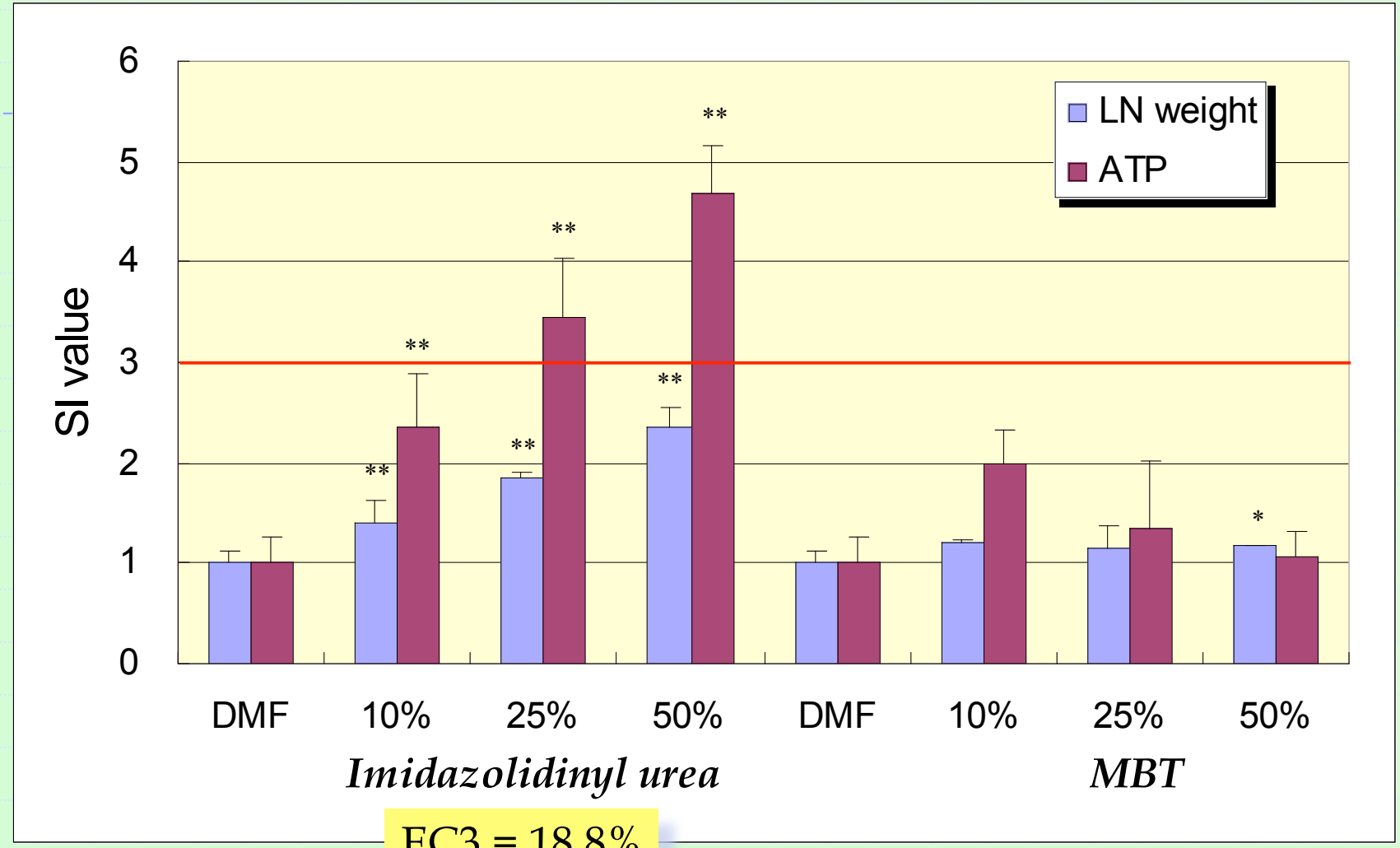
EC3 = 0.14%

EC3 = 3.27%

Data presented as mean ± S.D., $*p < 0.05$, $**p < 0.01$.
DMSO: dimethylsulfoxide

Reference information

Using DMF as vehicle

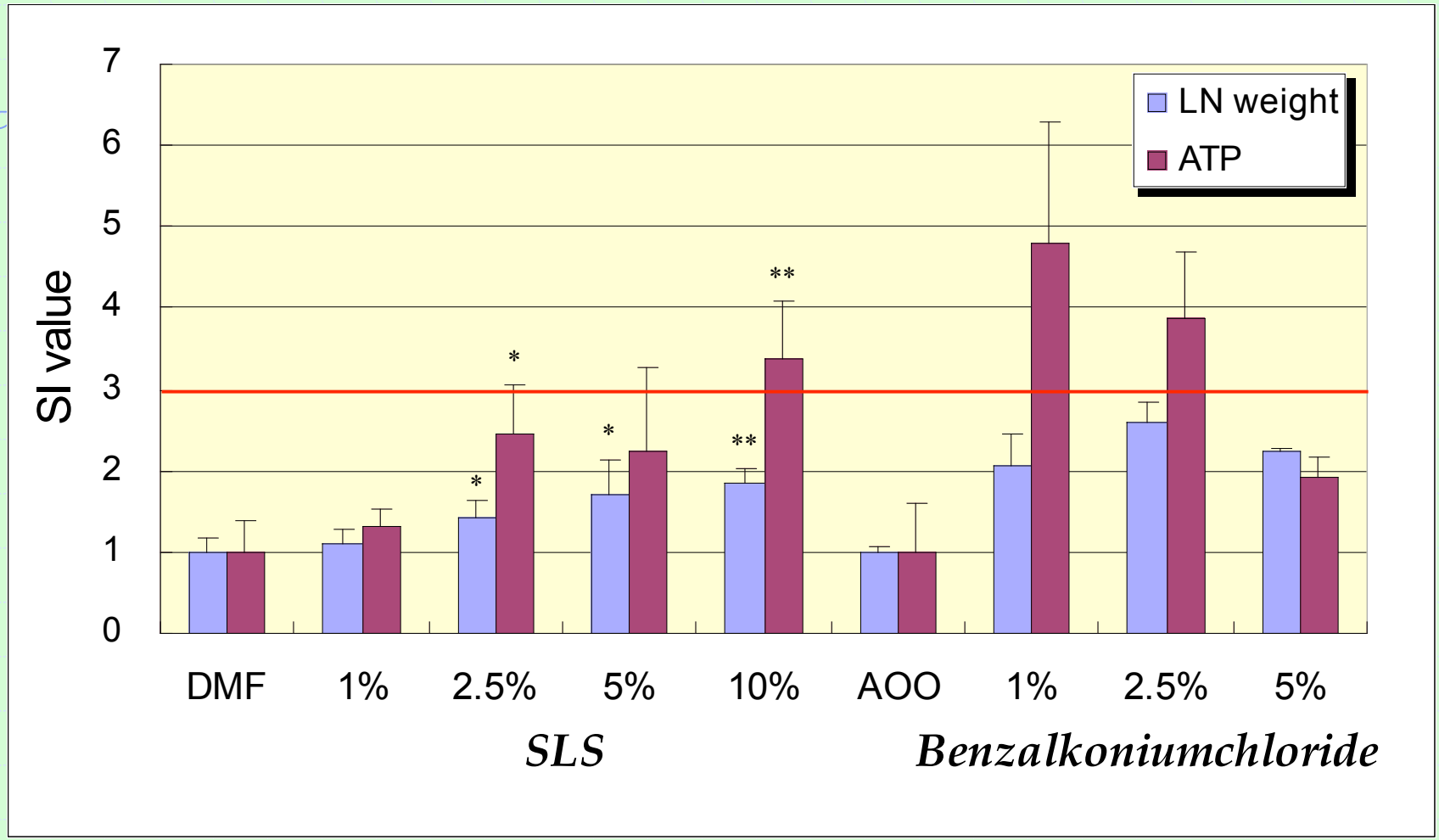


EC3 = 18.8%

Data presented as mean ± S.D., *$p < 0.05$, **$p < 0.01$.
DMF ; *N, N* –Dimethylformamide
MBT ; 2-Mercaptobenzothiazol

Reference information

False positive substances



Data presented as mean ± S.D., *$p < 0.05$, **$p < 0.01$.
DMF ; *N, N* -Dimethylformamide
AOO: acetone/olive oil (4:1, v/v)
SLS: Sodium lauryl sulfate

Reference information

The red bar ;

$$SI = \frac{\text{mean ATP content (RLU) of chemical treatment group}}{\text{mean ATP content (RLU) of vehicle treatment group}}$$

Cut-off point ;

positive, SI≥3 and negative, SI<3

Reference information

Historical data

Negative control: AOO
Positive control: 15% HCA

	Lymph Node Weight(mg)			ATP(RLU)		
	AOO	15%HCA/AOO	SI	AOO	15%HCA/AOO	SI
Average	4.08	9.81	2.42	4038	17349	4.45
S. D.	0.39	1.03	0.27	947	3398	1.05
CV(%)	9.6	10.5	11.3	23.4	19.6	23.5
N	42	42	42	42	42	42
max.	4.98	11.63	3.05	5877	23831	7.31
min.	3.03	7.54	1.73	2228	10801	2.73

Summary of results
31 well-known chemicals

- ICCVAM 20 (13 chemicals)
- ECVAM 14 (9 chemicals)

GPMT: guinea pig maximization test
BA: Buehler assay
HMT: human maximization test
HPTA: human patch test allergen

* K. E. Haneke, *et al.*, *Reg. Toxicol. Pharmacol.*, (2001) **34**, 274–286.

Chemicals	LLNA-DA	*LLNA	*GPMT/BA	*HMT/HPTA
2,4-Dinitrochlorobenzene	+	+	+	
p -Phenylenediamine	+	+	+	+
Toluene diisocyanate	+	+		
Glutaraldehyde	+	+		
$K_2Cr_2O_7$	+	+	+	+
Pthalic anhydride	+	+	+	
Trimellitic anhydride	+	+		
Formaldehyde	+	+	+	+
Cinnamic aldehyde	+	+	+	+
Isoeugenol	+	+	+	+
$CoCl_2$	+	+	+	+
Eugenol	+	+	+	+
Resorcinol	+	+	-	+
Benzocaine	+	+/-	+	+/-
Abietic acid	+	+	+	+
Hexyl cinnamic aldehyde	+	+	+	
Mercaptobenzothiazol	-	+	+	+
Citral	+	+	+	+
Hydroxycitronellal	+	+	+	+
Imidazolidinyl urea	+	+	+	+
SLS	+	+	-	-
$NiSO_4$	-	-	+	+
Benzalkonium chloride	+	-	-	+
Propyl paraben	-	-	-	+/-
Diethylphthalate	-	-		
1-Bromobutane	-	-		
Methysalicylate	-	-	-	-
Chlorobenzene	-	-	-	
Lactic acid	-	-	-	
Hexane	-	-		-
Isopropanol	-	-	-	

Reference information

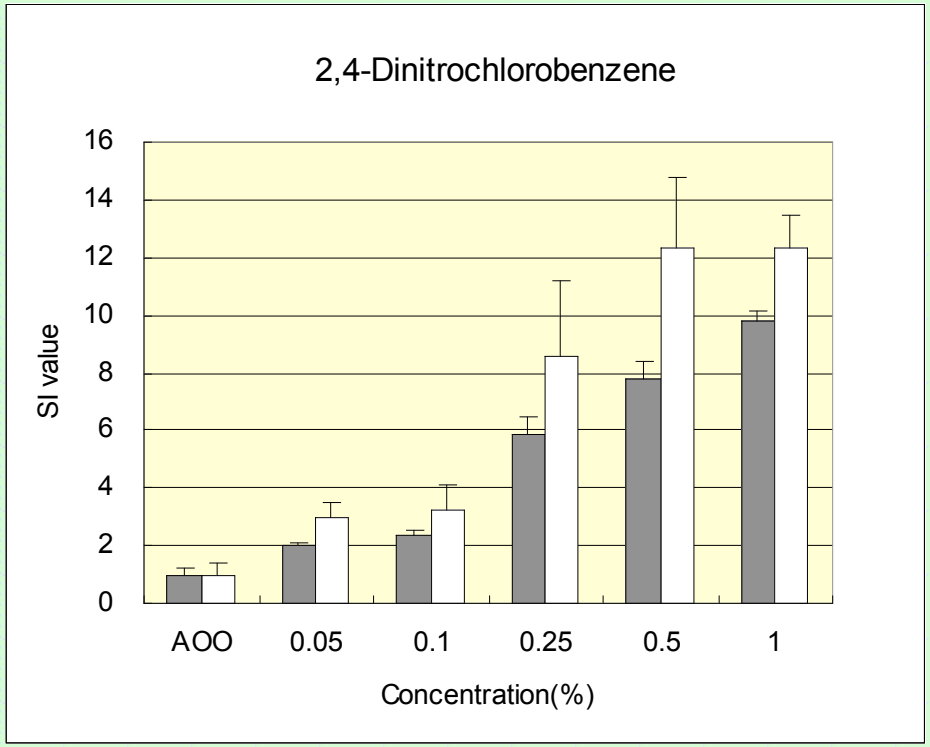
Performance of LLNA-DA against LLNA and other tests

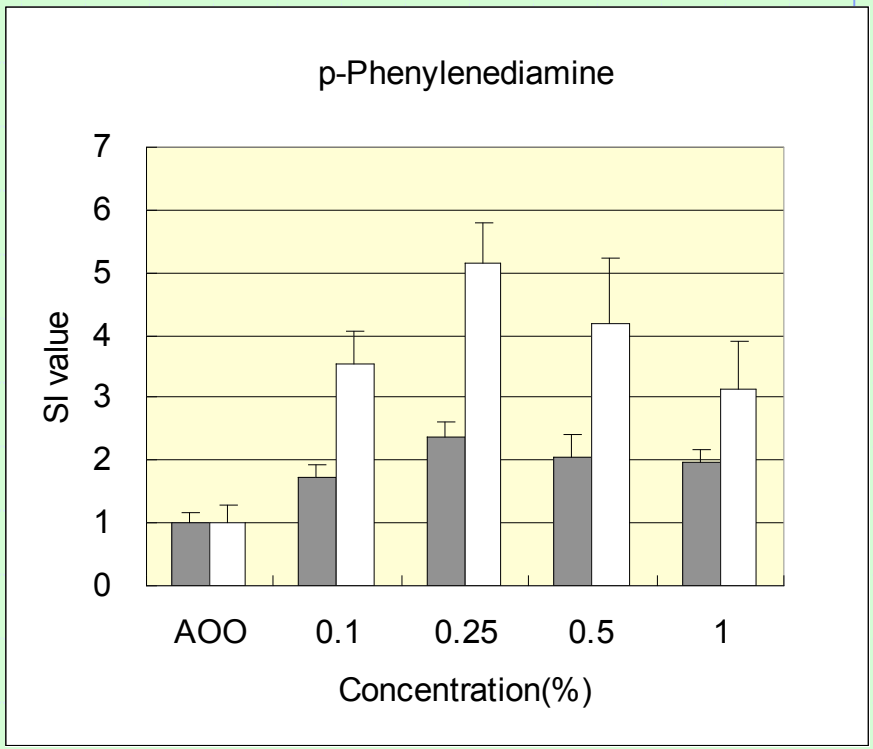
Comparison	No. of comparisons	Sensitivity	Specificity	Positive predictivity	Negative predictivity	Accuracy
LLNA-DA vs. LLNA	30	95% (19/20)	90% (9/10)	95% (19/20)	90% (9/10)	93% (28/30)
LLNA-DA vs. GPMT/	25	88% (15/17)	63% (5/8)	83% (15/18)	71% (5/7)	80% (20/25)
LLNA-DA vs. HMT/H	19	81% (13/16)	67% (2/3)	93% (13/14)	40% (2/5)	79% (15/19)
*LLNA vs. GPMT/B	97	91% (62/28)	83% (24/29)	93% (62/67)	80% (24/30)	89% (86/97)
*LLNA vs. HMT/HP	74	72% (49/68)	67% (4/6)	96% (49/51)	17% (4/23)	72% (53/74)

*K. E. Haneke, *et al.*, *Reg. Toxicol. Pharmacol.*, (2001) **34**, 274–286.

GPMT: guinea pig maximization test
BA: Buehler assay
HMT: human maximization test
HPTA: human patch test allergen

Reference information



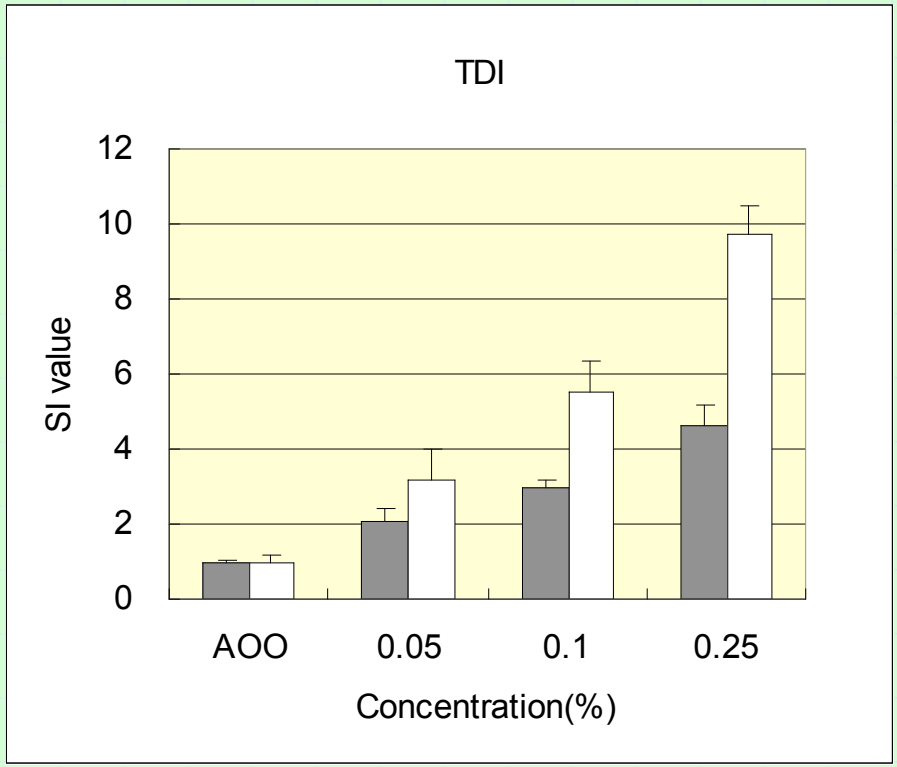


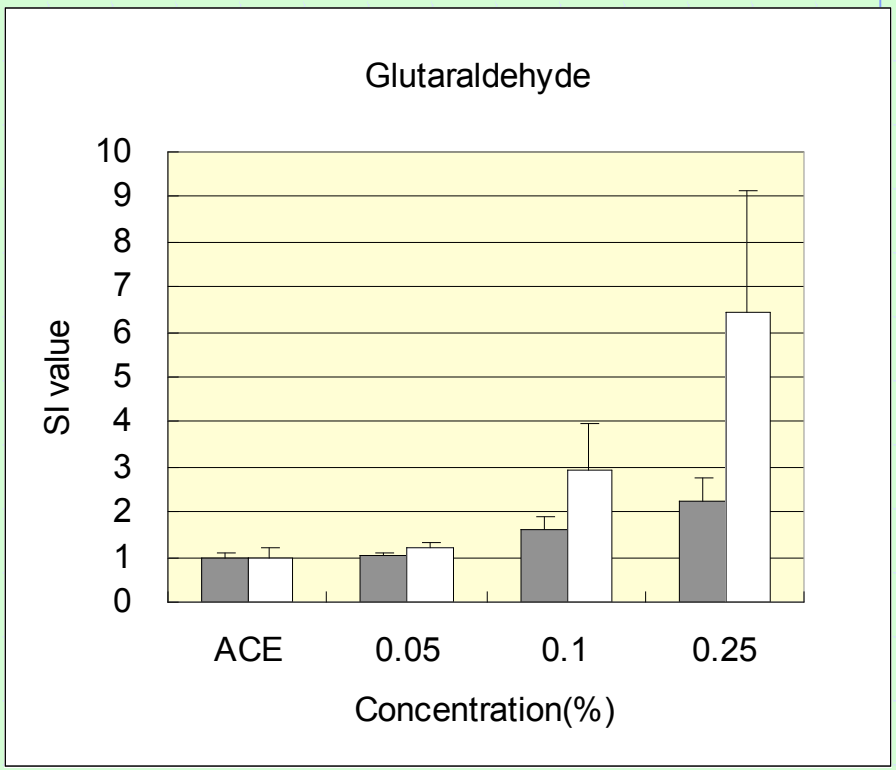
Data presented as mean ± S.E.

□ : Lymph node weight

■ : ATP

Reference information



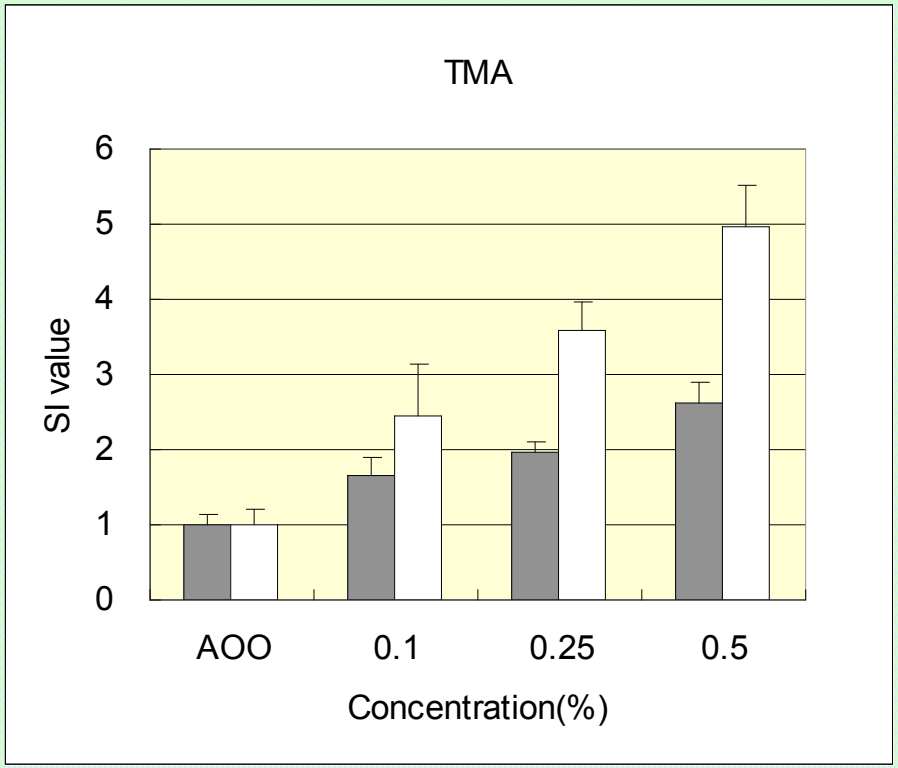


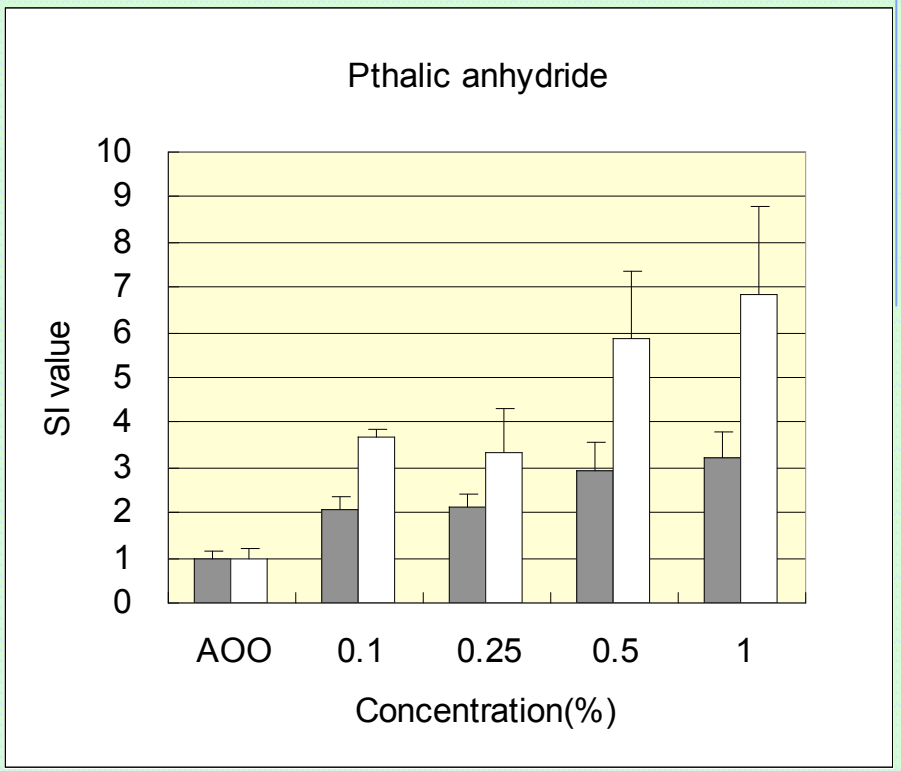
Data presented as mean ± S.E.

□ : Lymph node weight

□ : ATP

Reference information



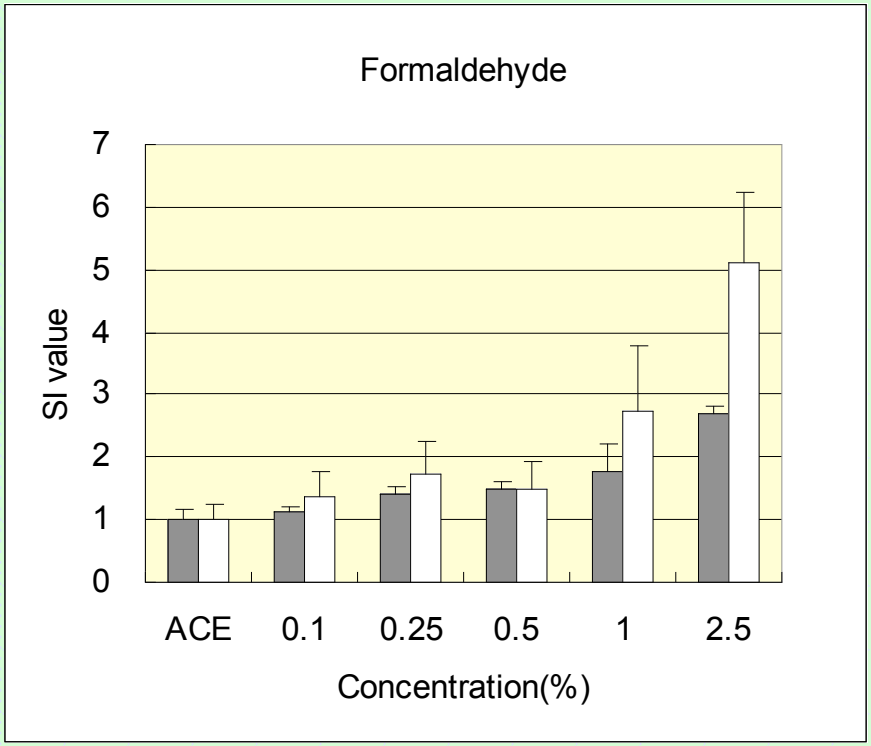


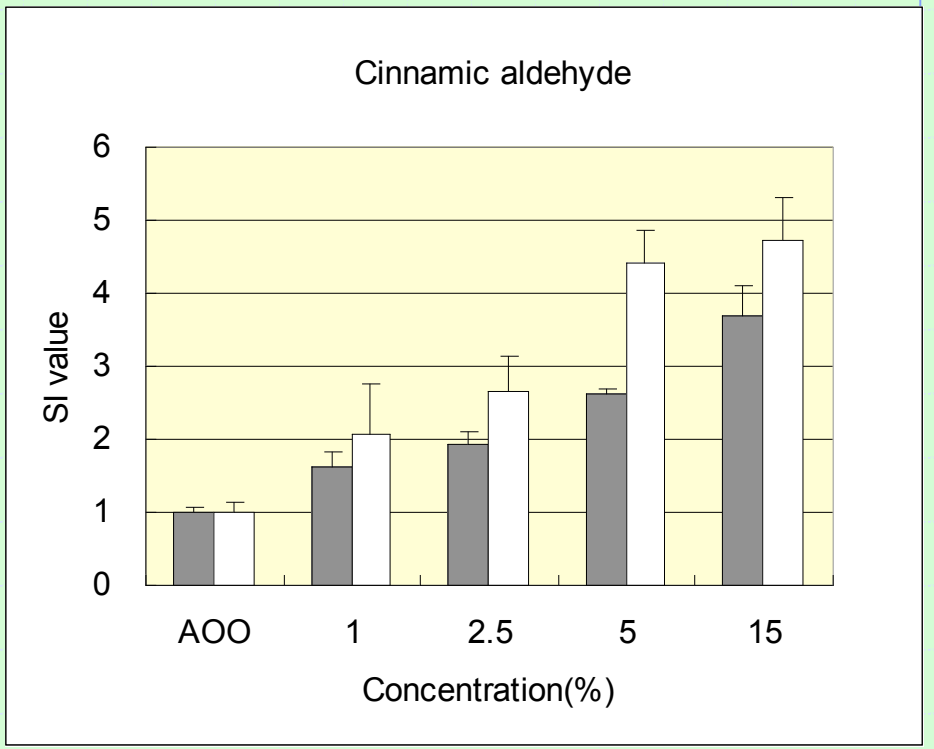
Data presented as mean ± S.E.

□ : Lymph node weight

□ : ATP

Reference information





Data presented as mean ± S.E.

□ : Lymph node weight

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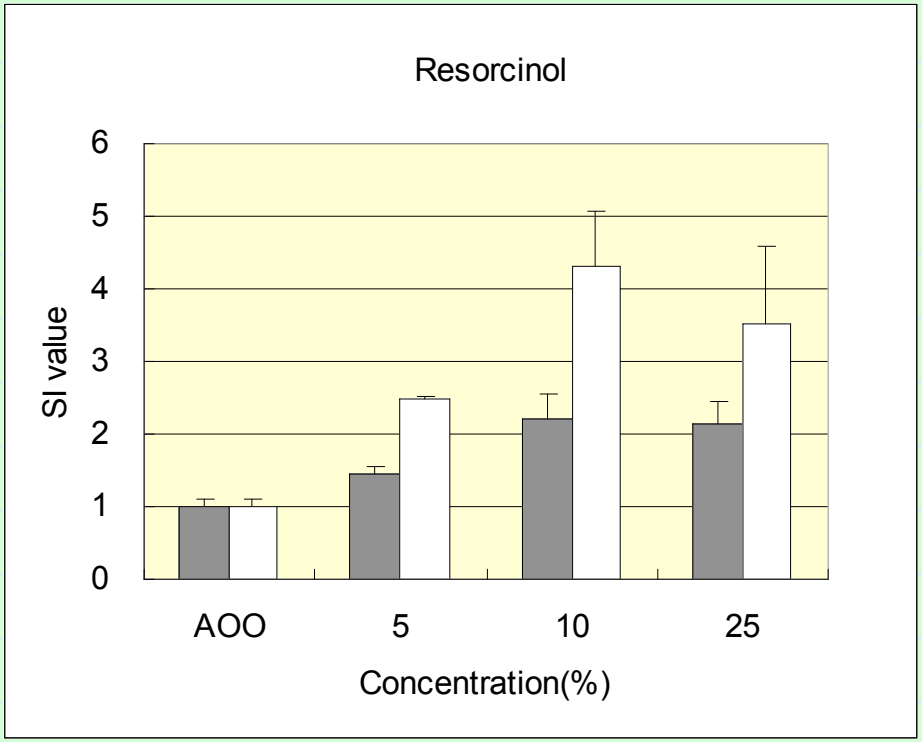
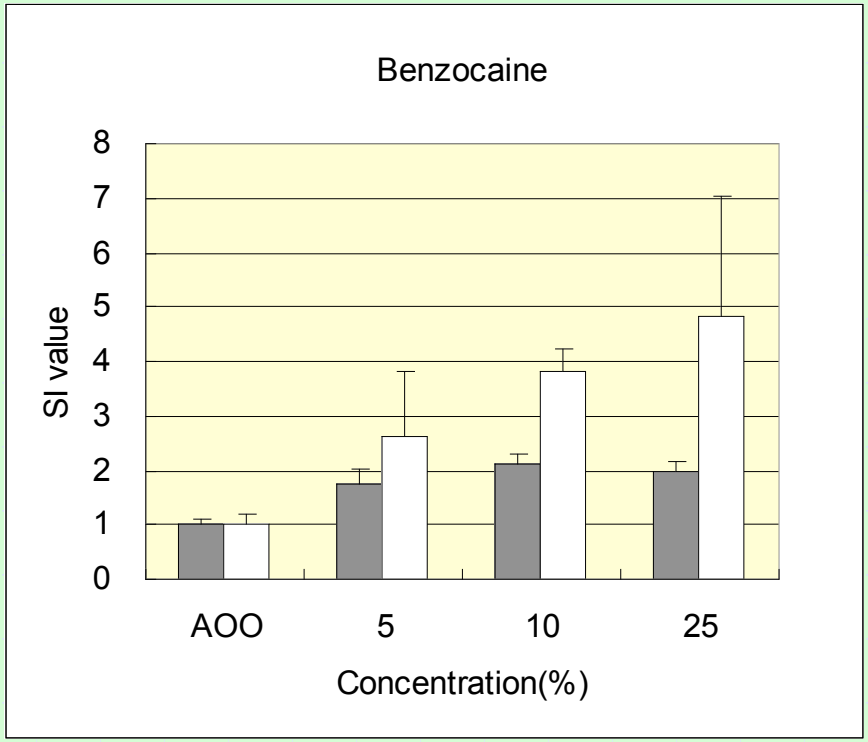
Reference information



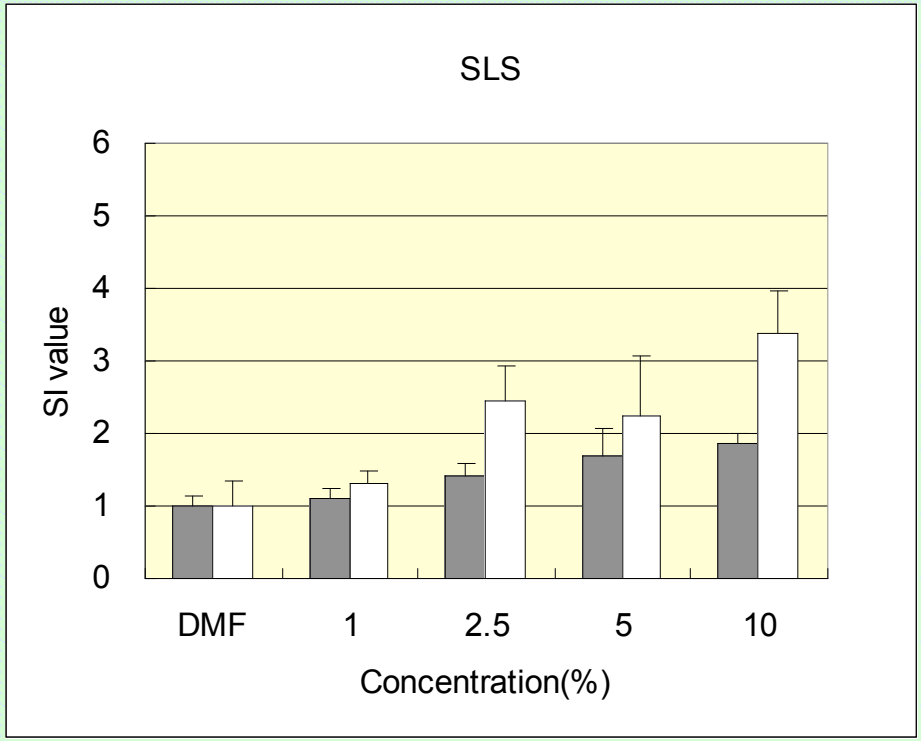


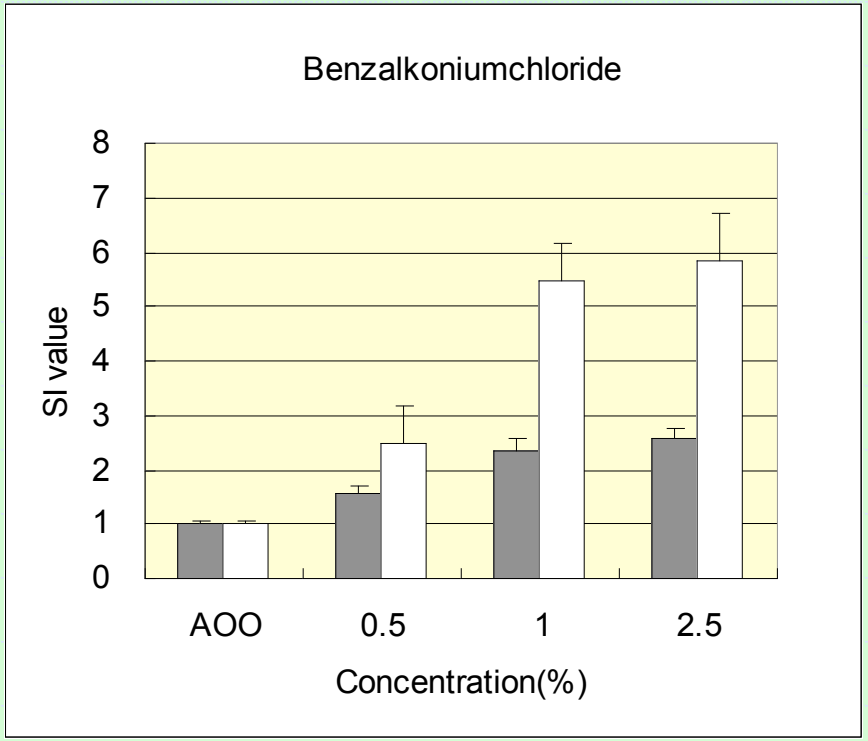
Data presented as mean ± S.E.

□ : Lymph node weight

■ : ATP

Reference information



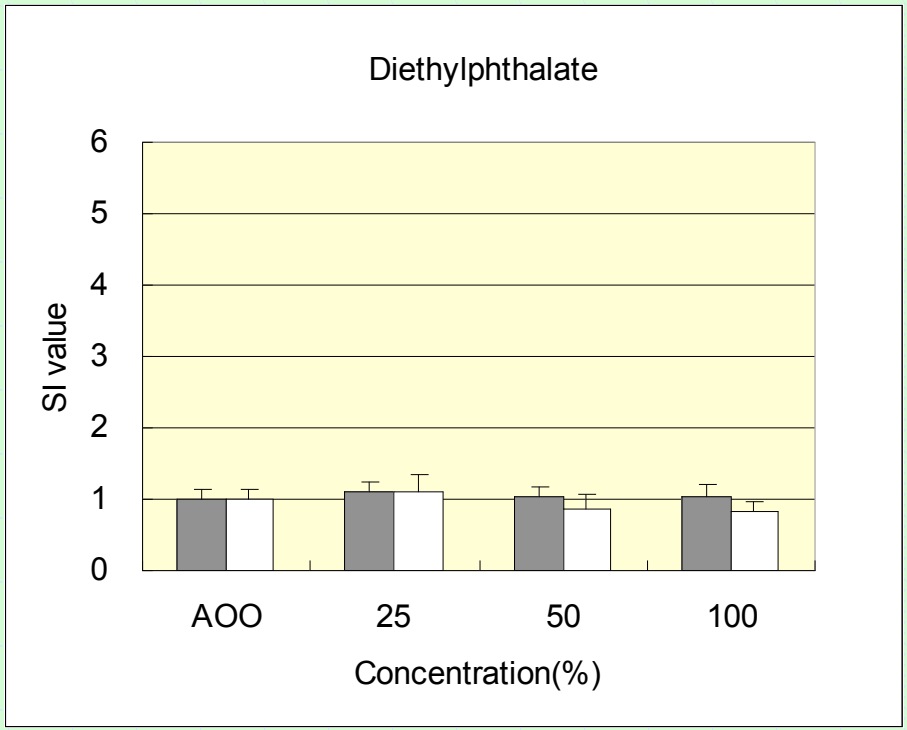


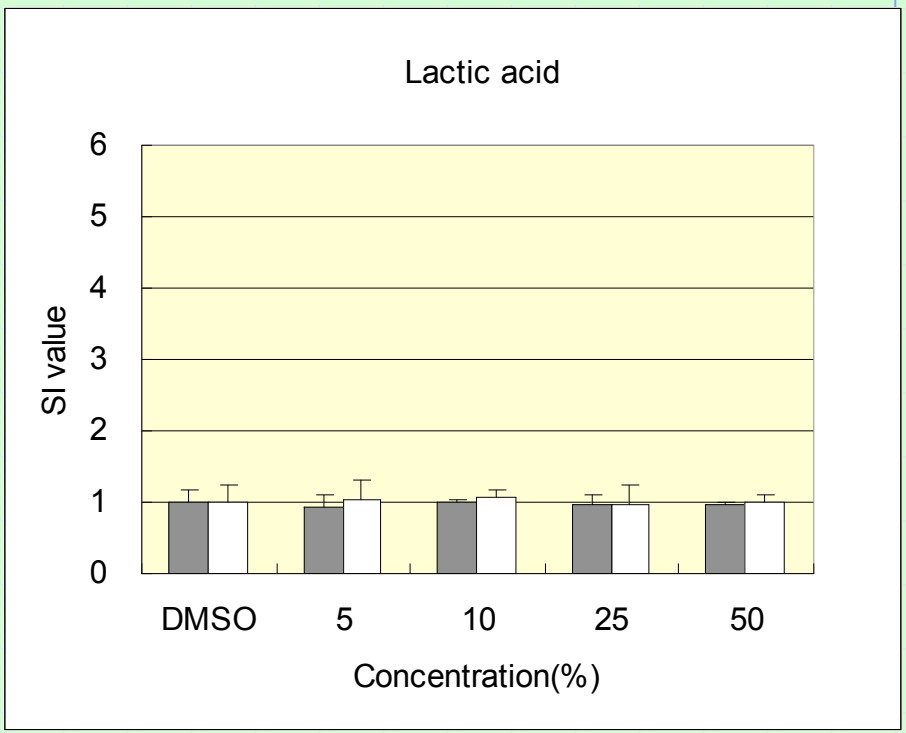
Data presented as mean ± S.E.

□ : Lymph node weight

■ : ATP

Reference information





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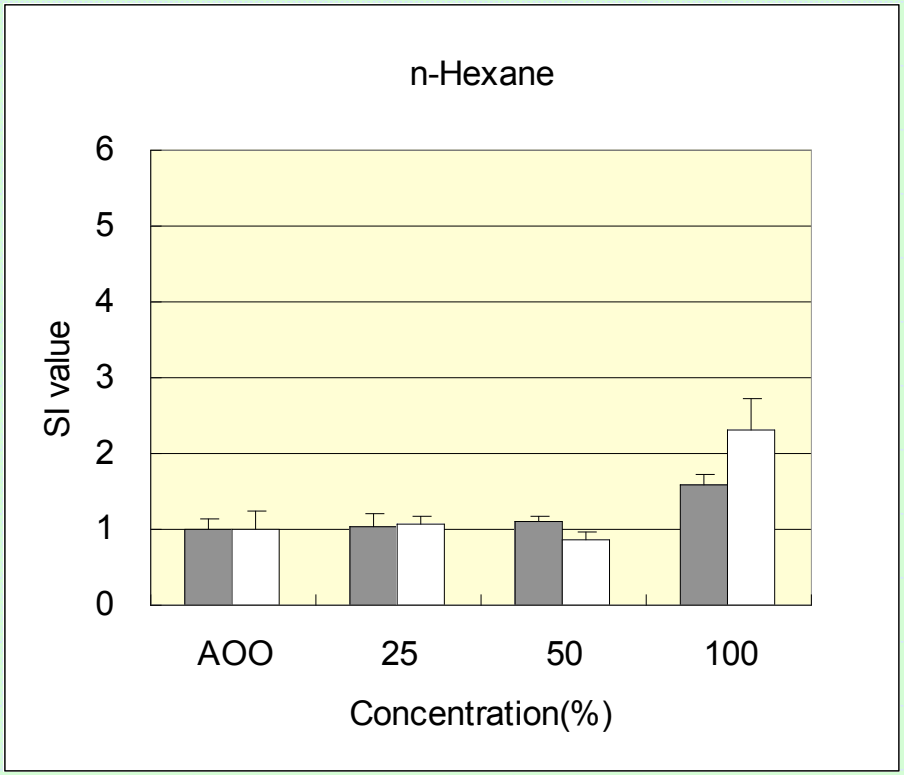
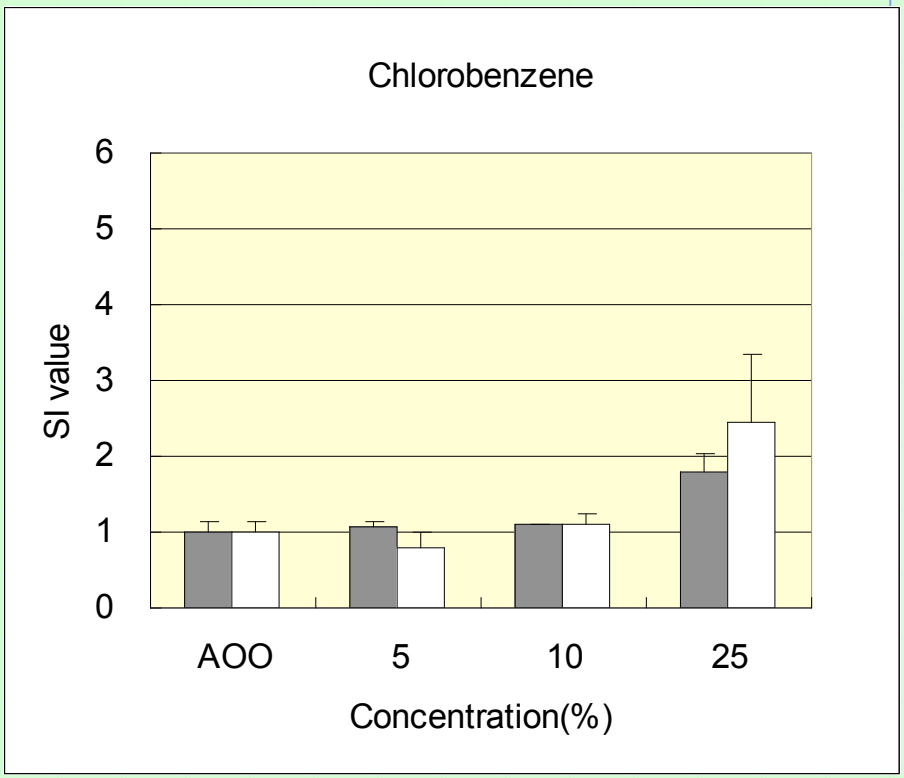
Reference information





Data presented as mean ± S.E.

□ : Lymph node weight

■ : ATP