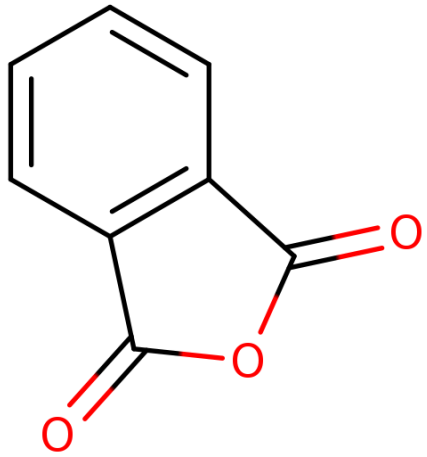
United States
Environmental Protection Agency

March 2026
Office of Chemical Safety and
Pollution Prevention

Draft Data Evaluation Record Information for *in chemico*, *in vitro*, and *in vivo* Assays for the Draft Human Health Hazard Assessment for Phthalic Anhydride

Systematic Review Support Document for the Draft Risk Evaluation

CASRN 85-44-9



March 2026

This supplemental file contains the data quality evaluation results for human health hazard data sources that met the PECO screening criteria and further filtering criteria for the *Draft Human Health Hazard Assessment for Phthalic Anhydride* (U.S. EPA, 2026a). EPA conducted data evaluation records based the adherence to OECD test guidelines of author-reported descriptions and results. Additional analyses (*e.g.*, statistical analyses performed during data integration into the risk evaluation) potentially conducted by EPA are not contained in this supplemental file. EPA used the TSCA systematic review process described in the *Draft Systematic Review Protocol Supporting TSCA Risk Evaluations for Chemical Substances (*U.S. EPA, 2021*)* (also referred to as the '2021 Draft Systematic Review Protocol') for all studies in this DER, which were considered mechanistic supplemental studies and therefore did not undergo the standard Data Evaluation and Extraction process. These updated steps in the systematic review process since the publication of the 2021 *Systematic Review Protocol Supporting TSCA Risk Evaluations for Chemical Substances* are reflected in the *Draft Systematic Review Protocol for Phthalic Anhydride* (U.S. EPA, 2026b).

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OECD 442C [Direct Peptide Reactivity Assay (DPRA)] (Bauch et al. 2012)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING

Product Manager: Not applicable
HERO ID: 8310407
Study Completion Date: Not reported. Peer-reviewed publication; publication year: 2012
Study No.: Not applicable. Peer-reviewed publication (DOI: 10.1016/j.yrtph.2012.05.013)
Testing Laboratory: Study authors associated with BASF SE (Ludwigshafen), SASF Perconsla Care and Nutrition GmbH (Dusseldorf, Germany), BASF Schweix AG (Basel Switzerland), and University of Manchester (Manchester, UK), however, exact laboratory in which testing was conducted is not stated.
Author: Caroline Bauch, Susanne N. Kolle, Tzutzuy Ramirez, Tobias Eltze, Eric Fabian, Annette Mehling, Wera Teubner, Bennard van Ravenzwaay, and Robert Landsiedel

Quality Assurance (40 CFR §160): GLP status not specified.

Test Material:
- Phthalic Anhydride (CASRN 85-44-9) purchased from Fluka (purity = 5%)
- Note: 54 chemicals of varying potency tested. Ten of the tested chemicals are described as proficiency substances in Appendix I, Annex 2 of OECD TG 442C for demonstrating technical proficiency with the DPRA (marked with asterisk '*' below).
    - 1-Chloro-2,4-dinitrobenzene*, Oxazolone*, Farnesal*, 2,3-Butanedione*, Formaldehyde > 36% (1% DMSO)*, Benzylidene acetone*, 6-Methylcoumarin*, DL-lactic acid*, n-Butanol*, 4-Methoxyacetophenone*, MCI/MI, p-Benzoquinone, 4-Phenylenediamine, Propylgallate, 2,4,6-Trinitrobenzenesulfonic acid, Phthalic anhydride, Methyldibromo glutaronitrile, Isoeugenol, Diethyl maleate, Ethylene diamine, Cobalt chloride, 2-Phenylpropionaldehyde, a-Hexyl-cinnamic aldehyde, Tartaric acid, 2-Mercaptobenzothiazole, Citral, Eugenol, Sodium lauryl sulfate, 4-Allylanisole, Hydroxycitronellal, Phenyl benzoate, Cinnamic alcohol, Imidazolidinyl urea, Undecylenic acid, Ethylene glycol dimethacrylate, Pyridin, Aniline, Methyl methacrylate, Xylene, Glycerol, 1.2-Propanediol, 4-Hydroxybenzoic acid, Fumaric acid, Glucose, Isopropanol, Methyl salicylate, n-Hexane, Nickel chloride, p-Aminobenzoic acid, Propyl 4-hydroxybenzoate, Salicylic acid, Sulfanilamide, Vanillin, Hexadecyltrimethylammonium bromide
        - Purity of these chemicals was reported to be equal to or greater than 95% except for the following: phthalic anhydride (5%), 2,3-butanedione (10.15%), glycerol (90%),

Concentration: Solutions of phthalic anhydride (and all other test chemicals) were prepared at a final concentration of 100 mM
Vehicle/Negative Control:
- "The test substances were preferably dissolved in acetonitrile (Sigma–Aldrich, Germany) to prepare a 100 mM solution. If the test substances were not soluble in acetonitrile, solutions were prepared in water, methanol, propanol, isopropanol, acetone or mixture of these solvents which is in accordance with the DPRA protocol used in the interlaboratory ring trials."
- Note: study authors do not explicitly state what phthalic anhydride was dissolved in.

Positive Control:

- No positive control stated. Cinnamic aldehyde [CASRN 104-55-2], the positive control recommended by OECD TG 442C, was not included. However, study authors included 10 proficiency substances recommended by OECD TG 442C, including 6 known sensitizers (1-Chloro-2,4-dinitrobenzene, Oxazolone, Farnesal, 2,3-Butanedione, Formaldehyde, Benzylidene acetone), which may serve as positive controls.

Concentration: 100 mM
Test System: Direct Peptide Reactivity Assay (DPRA)

Method: Pre-guideline study, but generally adheres to OECD TG 442C (In Chemico Skin Sensitisation)[1] with some deviations and reporting deficiencies noted below

Summary:

1. Phthalic anhydride was found to be positive for sensitization in the DRPA assay, with mean percent depletion of cysteine and lysine to be 16.7 and 31.3%, respectively.
2. **Classification:** Acceptable for qualitative use. The primary reasons for this classification are use of low purity test substance (reported to be 5%), and the fact that the ratios of test substance to peptide varied significantly from OECD TG 442C (*i.e.,* OECD TG 442C requires ratios of 1:10 (Cys) and 1:50 (Lys), while ratios used in the study were 1:15 (Cys) and 1:3 (Lys)). Additional minor deviations and limitations are documented below. However, the study still provides information that is useful as part of the weight of the scientific evidence.

Deviations from Guideline and other comments:

- Purity of phthalic anhydride was reported to be 5%, however, it is unclear if this was a typographical error, as the purity was equal to or greater than 95% for the majority of other evaluated test substances.
- The positive control (*i.e.,* cinnamic aldehyde) recommended by OECD TG 442C was not included in the DPRA assay in this study. However, study authors included all 10 substances recommended by OECD TG 442C for demonstrating technical proficiency with the DPRA, and DPRA predictions were accurate for all 10 substances (*i.e.,* 6 chemicals were accurately predicted to be positive and 4 to be negative). Therefore, lack of inclusion of cinnamic aldehyde is not anticipated to directly impact interpretation of study results.
- The HPLC standard calibration curve was not shown or discussed.
- The maximum standard deviation (SD) or Coefficient of variation CV) for the Reference Controls was not listed with the study.
- OECD TG 442C requires cysteine and lysine peptides to be incubated with the test chemical at 1:10 and 1:50 ratios, respectively. The peptides were run a different ratios than what is required under OECD TG 442c:
    - Cysteine model peptide (Ac-RFAACAA-COOH)
        - 1:4:15 (v/v/v) substance:acetonitrile:cysteine peptide
    - Lysine model peptide (Ac-RFAAKAA-COOH)
        - 1:3 (v/v) substance:lysine peptide

Procedure Highlights:

- A total of 54 chemicals were evaluated in the DPRA.
- All 10 of the OECD TG 442C recommended substances for demonstrating technical proficiency with the DPRA were included:

[1] https://www.oecd.org/en/publications/2023/07/test-no-442c-in-chemico-skin-sensitisation_g1g507cd.html

  - 1-Chloro-2,4-dinitrobenzene [CASRN 97-00-7]
  - Oxazolone [CASRN 15646-16-5]
  - Formaldehyde >36% (1% DMSO) [CASRN 50-00-0]
  - Benzylidene acetone [CASRN 122-57-6]
  - Farnesal [CASRN 19317-11-4]
  - 2,3-Butanedione [CASRN 431-03-8]
  - 4-Methoxyacetophenone [CASRN 100-06-1]
  - 6-Methylcoumarin [CASRN 92-48-8]
  - DL-Lactic acid [CASRN 50-21-5]
  - N-Butanol [CASRN 71-36-3]
- The methods used for DPRA were originally presented in Gerberick et al. (2004).
  - "Test chemical solutions at a concentration of 100mM were prepared in acetonitrile, or solubilized in DMSO and then diluted with an equal part acetonitrile"
  - "Test chemical solutions at a concentration of 100 mM were prepared in acetonitrile, or solubilized in DMSO and then diluted with an equal part acetonitrile"
  - "Triplicate reactivity samples were prepared containing 0.5 mM peptide, and either 5 mM or 25 mM test chemical for a peptide:test chemical ratio of 1:10 or 1:50. A Biomek 2000 automated workstation (Beckman Coulter, Fullerton, CA) was used to make additions of the peptide stock solution (400 ml), the appropriate buffer (350 ml), and the test chemical solutions (50 or 250 ml) into autosampler vial"
  - "Calibration standards were prepared manually from the peptide stock solution, diluted into the appropriate buffer for the peptide, and contained either 5 or 25% acetonitrile. The peptide concentrations were 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.50, 1.0 mM"
  - "Molecular weight confirmation was done by flow injection mass spectrometry with electrospray ionization in the positive mode"
  - "The column temperature was 30°C. The mobile phase consisted of 0.1% TFA in water (A) and 0.085% TFA in acetonitrile (B). A gradient of 90% (A) to 60% (A) over 25 min at a flow rate of 0.3 ml/min was used for the separation. The diode array detector scanned the wavelengths 210–400 nm."
- "… test substances were preferably dissolved in acetonitrile (Sigma–Aldrich, Germany) to prepare a 100 mM solution… If the test substances were not soluble in acetonitrile, solutions were prepared in water, methanol, propanol, isopropanol, acetone or mixture of these solvents… Substances were freshly prepared prior to use."
- Cysteine model peptide (Ac-RFAACAA-COOH)
  - Prepared as 0.667 mM stock solutions in 100 mM phosphate buffer (100 mM NaH2PO4, 100 mM Na2HPO4, both Sigma–Aldrich, Germany, pH 7.5; obtained from Synbiosci, Livermore CA, USA)
  - 1:4:15 (v/v/v) substance:acetonitrile:cysteine peptide
- Lysine model peptide (Ac-RFAAKAA-COOH)
  - Prepared as 0.667 mM stock solutions in 100 mM ammonium acetate buffer (1.542 g ammonium acetate in 200 mL H2O; pH 10.2; obtained from Synbiosci, Livermore CA, USA).
  - 1:3 (v/v) substance:lysine peptide
- The test substance and positive control were incubated with cysteine or lysine peptide for 24±2 hours at room temperature in the dark.

- All samples were run in triplicates

Results:

For phthalic anhydride, the mean percent peptide depletion for cysteine and lysine was 24% (lysine depletion: 31.3%, cysteine depletion: 16.7%). Under the conditions of the study, phthalic anhydride was shown to have a positive prediction in the DRPA assay (see Table below). For the 10 chemicals included in the OECD TG 442C proficiency set, 6 were accurately predicted to be positive, while 4 were accurately predicted to be negative under the conditions of the assay. Further, the percent peptide depletions for 9 out of 10 chemicals included in the OECD TG 442C proficiency set listed below were within the OECD stated range. For one chemical (1-Chloro-2,4-dinitrobenzene), depletion of depletion of Lys peptide was slightly outside of the OECD TG 442C range.

- **1-Chloro-2,4-dinitrobenzene:** Positive; Peptide depletion out of range for Lys, but not Cys (Cys & Lys depletion: 99.9% & 58.3%; OECD 442C range for Cys (90-100%) & Lys (15-45%)); Mean peptide depletion was 79.1%.
- **Oxazolone**: Positive, depletion in range (Cys & Lys depletion: 71.7% & 44.6%; OECD 442C range for Cys (60-80%) & Lys (10-55%)); Mean peptide depletion was 58.1%.
- **Formaldehyde >36% (1% DMSPO):** Positive, depletion in range (Cys & Lys depletion: 54.8% & 2.4%; OECD 442C range for Cys (30-60%) & Lys (≤24%)); Mean peptide depletion was 28.6%.
- **Benzylidene acetone**: Positive, depletion in range (Cys & Lys depletion: 92.4% & -1.4%; OECD 442C range for Cys (80-100%) & Lys (≤7%)); Mean peptide depletion was 45.5%.
- **2,3-Butanedione**: Positive, depletion in range (Cys & Lys depletion: 92.8% & 34.9%; OECD 442C range for Cys (60-100%) & Lys (10-45%)); Mean peptide depletion was 63.9%.
- **4-Methoxyacetophenone**: Negative, depletion in range (Cys & Lys depletion: -1.9% & -0.3%; OECD 442C range for Cys (≤7%) & Lys (≤5.5%)); Mean peptide depletion was -1.1%.
- **6-Methylcoumarin:** Negative, depletion in range (Cys & Lys depletion: -1.8% & -2.0%; OECD 442C range for Cys (≤7%) & Lys (≤5.5%)); Mean peptide depletion was -1.9%.
- **DL-Lactic acid:** Negative, depletion in range (Cys & Lys depletion: 3.0% & -1.2%; OECD 442C range for Cys (≤7%) & Lys (≤5.5%)); Mean peptide depletion was 0.9%.
- **N-Butanol**: Negative, depletion in range (Cys & Lys depletion: 0.7% & -1.3%; OECD 442C range for Cys (≤7%) & Lys (≤5.5%)); Mean peptide depletion was -0.3%.

Results of peptide reactivity assessments: OECD QSAR Toolbox v2.0 (*in silico*), the direct peptide reactivity assay (DPRA; *in chemico*), and the LuSens assay and KeratinoSens™ assay (both *in vitro*; ARE-reporter gene based assay).

Substance	DPRA			OECD Toolbox v2.0		LuSens	KeratinoSens™
	Lys-peptide depletion	Cys-peptide depletion	Mean peptide depletion	Rationale by OECD toolbox	Prediction	EC1.5 in µM	EC1.5 in µM
Oxazolone	44.6	71.7	58.1	Nucleophilic acyl substitution	+	220	155
MCI/MI	4.3	90.9	47.6	Ring opening at the S–N bond followed by Nucleophilic addition.	+	3	2
p-Benzoquinone	95.9	99.7	97.8	Michael addition on Quinones/quinone imines	+	4	6
1-Chloro-2,4-dinitrobenzene	58.3	99.9	79.1	Nucleophilic substitution on activated aryl carbon atom.	+	2	3
4-Phenylenediamine	42.2	99.8	71.0	No binding	–	6	5
Propyl gallate	63.6	59.2	61.4	No binding	–	6	191
2,4,6-Trinitrobenzenesulfonic acid	22.6	99.7	61.1	No binding	–	23	41
Phthalic anhydride	31.3	16.7	24.0	Protein acylation by acid anhydride	+	Below threshold	Below threshold
Formaldehyde > 36% (1% DMSO)	2.4	54.8	28.6	Schiff base formation with aldehydes.	+	101	137
Methyldibromo glutaronitrile	28.4	75.3	51.8	Nucleophilic substitution on halogenated C sp^3 atom	+	7	8
Isoeugenol	21.8	99.1	60.4	No binding	–	3	6
Diethyl maleate	78.8	99.8	89.3	Michael addition on conjugated systems with electron withdrawing groups	+	4	2
Ethylene diamine	0.7	18.6	9.7	No binding	–	Below threshold	549
Benzylidene acetone	−1.4	92.4	45.5	Michael addition on conjugated systems with electron withdrawing groups	+	7	7
Cobalt chloride	35.0	30.3	32.6	No binding	–	75	106
2-Phenylpropionaldehyde	5.1	26.6	15.8	Schiff base formation with aldehydes	+	34	26
α-Hexyl-cinnamic aldehyde	−0.9	12.3	5.7	Michael addition on α,β-aldehydes, Schiff base formation with aldehydes.	+	13	23
Tartaric acid	−2.0	6.6	2.3	No binding	–	Below threshold	3
2-Mercaptobenzothiazole	3.2	99.9	51.5	Protein thiol-disulfide interchange	+	85	57
2,3-Butanedione	34.9	92.8	63.9	Nucleophilic cycloaddition to diketones	+	117	74
Citral	8.6	78.6	43.6	Michael addition on α,β-aldehydes, Schiff base formation with aldehydes.	+	6	17
Eugenol	4.2	38.3	21.3	No binding	–	79	64
Farnesal	−4.1	37.3	16.6	Michael addition on α,β-aldehydes, Schiff base formation with aldehydes.	+	6	11
Sodium lauryl sulfate	97.1	−0.1	48.5	No binding	–	Below threshold	Below threshold
4-Allylanisole	−2.2	98.5	48.1	No binding	–	274	39
Hydroxycitronellal	32.8	61.7	47.2	Schiff base formation with aldehydes	+	185	31
Phenyl benzoate	2.2	64.9	33.6	Diarylester aminolysis or thiolysis	+	Below threshold	Below threshold
Cinnamic alcohol	2.7	21.3	12.0	No binding	–	20	24
Imidazolidinyl urea	20.6	59.0	39.8	Protein acylation by *N*-acylamides	+	80	41
Undecylenic acid	−0.1	11.4	5.7	No binding	–	27	57
Ethylene glycol dimethacrylate	33.9	93.5	63.7	Michael addition on conjugated systems with electron withdrawing groups	+	18	14
Pyridin	−2.2	−0.1	−1.2	No binding	–	Below threshold	Below threshold
Aniline	2.4	−1.0	0.7	No binding	–	165	2
Methyl methacrylate	11.8	55.3	33.5	Michael addition on conjugated systems with electron withdrawing groups	+	Below threshold	Below threshold
Xylene	0.7	−0.7	0.0	No binding	–	Below threshold	Below threshold
Glycerol	1.3	−0.7	0.3	No binding	–	Below threshold	Below threshold
1.2-Propanediol	0.0	−3.0	−1.5	No binding	–	Below threshold	Below threshold
4-Hydroxybenzoic acid	0.7	3.0	1.9	No binding	–	Below threshold	Below threshold
4-Methoxyacetophenone	−0.3	−1.9	−1.1	No binding	–	168	56
6-Methylcoumarin	−2.0	−1.8	−1.9	No binding	–	14	6
α-lactic acid	−1.2	3.0	0.9	No binding	–	Below threshold	Below threshold
Fumaric acid	4.6	10.8	7.7	No binding	–	Below threshold	Below threshold
Glucose	15.3	0.1	7.7	Schiff base formation with aldehydes	–	Below threshold	Below threshold
Isopropanol	−1.7	−2.0	−1.9	No binding	–	Below threshold	Below threshold

Substance	DPRA			OECD Toolbox v2.0		LuSens	KeratinoSens™
	Lys-peptide depletion	Cys-peptide depletion	Mean peptide depletion	Rationale by OECD toolbox	Prediction	EC1.5 in µM	EC1.5 in µM
Methyl salicylate	−0.2	−2.4	−1.3	No binding	–	383	Below threshold
n-Butanol	−1.3	0.7	−0.3	No binding	–	Below threshold	Below threshold
n-Hexane	2.6	−0.9	0.9	No binding	–	Below threshold	562
Nickel chloride	0.3	43.1	21.7	No binding	–	355	Below threshold
p-Aminobenzoic acid	0.5	5.9	3.2	No binding	–	Below threshold	Below threshold
Propyl 4-hydroxybenzoate	−1.3	−1.5	−1.4	No binding	–	9	5
Salicylic acid	1.0	8.7	4.8	No binding	–	Below threshold	Below threshold
Sulfanilamide	−10.4	3.8	−3.3	No binding	–	Below threshold	Below threshold
Vanillin	19.8	−1.3	9.3	No binding	–	226	83
Hexadecyltrimethylammonium bromide	1.5	2.4	2.0	No binding	–	Below threshold	Below threshold

OECD 442C [Direct Peptide Reactivity Assay (DPRA)] (Gerberick et al. 2004)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING

Product Manager: Not applicable
HERO ID: 8356995
Study Completion Date: Not reported. Peer-reviewed publication, publication year: 2004
Study No.: Not applicable. Peer-reviewed publication (DOI: 10.1093/toxsci/kfh213)

Testing Laboratory: Study authors associated with The Procter & Gamble Company, Miami Valley Laboratories (Cincinnati, Ohio) and Universite´ Louis Pasteur, Laboratorie de Dermatochimie (UMR 7123, Strasbourg, France), however, exact laboratory in which testing was conducted is not stated.
Author: G. Frank Gerberick, Jeff D. Vassallo, Ruth E. Bailey, Joel G. Chaney, Steve W. Morrall, and Jean-Pierre Lepoittevin

Quality Assurance (40 CFR §160): GLP status not stated.

Test Material:

- Phthalic anhydride (CASRN 85-44-9) (purchased from Aldrich chemical company (Milwaukee, WI)) (purity ≥95%)
- Note: 38 chemicals of varying potency (based on Local Lymph Node Assay [LLNA] data) were evaluated as part of this study, including 4 extreme sensitizers, 5 strong sensitizers, 11 moderate sensitizers, 7 weak sensitizers, and 11 non-sensitizers. Five of the tested chemicals are described as proficiency substances in Appendix I, Annex 2 of OECD TG 442C for demonstrating technical proficiency with the DPRA (marked with asterisk '*' below).
- 38 chemicals tested:
    - 2,4 Dinitrochlorobenzene*, Oxazolone*, 1-Butanol*, 6-Methylcoumarin*, Lactic acid*, Diphenylcyclopropenone, p-Benzoquinone, Phthalic anhydride, 1,4-Hydroquinone, Glutaraldehyde, Lauryl gallate, CD3, 2-Hydroxyethyl acrylate, 3-Dimethylaminopropylamine, Cinnamic aldehyde, 3-Aminophenol, 3,4-Dihydrocoumarin, 1,2-Benzisothiazolin-3-one, Phenylacetaldehyde Squaric acid, Citral, 1-(4-Methoxyphenyl)-1-penten-3-one, Diethyl maleate, a-Hexylcinnamaldehyde, 5-Methyl-2,3-Hexandione, Hydroxycitronellal, Ethyleneglycol dimethacrylate, a-Amyl cinnamaldehyde, Benzyl benzoate, Lilial, Glycerol, Hexane, Diethyl phthalate, Octanoic acid, 2-Hydroxypropyl methacrylate, 4-Hydroxybenzoic acid, , Methyl salicylate, Chlorobenzene
        - Purity of these chemicals was equal to or greater than 95% except for the following: a-hexylcinnamaldehyde (85%), oxazolone (90%), glutaraldehyde (70%), phenylacetaldehyde (90%), and lactic acid (85%). Stock solutions that were prepared from chemicals with less than 95% purity were adjusted for purity."

Concentration: Solutions of phthalic anhydride (and all other test chemicals) were prepared at a final concentration of 100 mM
Vehicle/Negative Control:

- "Test chemical solutions at a concentration of 100 mM were prepared in acetonitrile, or solubilized in DMSO and then diluted with an equal part acetonitrile."
- "Samples without the test chemicals were also prepared in triplicate to function as controls."

- Note: study authors do not explicitly state what phthalic anhydride was dissolved in.

Positive Control: Cinnamic aldehyde (purity ≥95%)

- No positive controls explicitly stated. However, phthalic anhydride was evaluated alongside 26 other known sensitizers of varying potency, including cinnamic aldehyde [CASRN 104-55-2], which is the positive control recommended by OECD TG 442C
- **Concentration:** Cinnamic aldehyde evaluated at a concentration of 100 mM

Test System: Direct Peptide Reactivity Assay (DPRA)

Method: Pre-guideline study, but generally adheres to OECD TG 442C (In Chemico Skin Sensitisation)[2] with some deviations and reporting deficiencies noted below

Summary:

1. For phthalic anhydride the mean percent peptide depletions for cysteine and lysine were -1.9% and 75%, respectively. Based on these results, phthalic anhydride was considered positive under the conditions of the assay.
2. **Classification:** Acceptable for quantitative use

Deviations from Guideline and other comments:

- Stock solutions of lysine and cysteine peptides were prepared to final concentrations of 1.25 mM in 100 mM ammonium acetate buffer (pH 10.2) or 100 mM phosphate buffer (pH 7.5), respectively, while OECD TG 442C states that final stock solutions of both peptides should be 0.667 mM. However, this deviation is not expected to have an appreciable impact on study results, as the final ratios of cysteine and lysine to test substance were 1:10 and 1:50, as required by OECD TG 442C.
- The organization of the HPLC run was not listed:
    - Although the authors mentioned use of Reference Controls ("Samples without the test chemicals were also prepared in triplicate to function as controls") the data for these tests are not shown.
    - Details on the stability of reference controls (Reference Control B) and the ability of the test chemical's reaction buffer to react with free peptide (Reference Control C).
    - A co-elution control was not listed with the workflow.
- The r^2 value for the standard curve was not provided or commented on (OECD TG 442C states that the standard calibration curve should have an $r^2>0.99$).
- Organization of the HPLC run not listed:
    - The linear gradient from 10% to 25% acetonitrile over 10 minutes for the HPLC analysis was not mentioned. "A gradient of 90% (A) to 60% (A) over 25 min at a flow rate of 0.3 ml/min was used for the separation".
    - There was not mention about the total duration of HPLC analysis time being less than 30 hours, as required by OCED TG 442C.
- Storage conditions of test/control chemicals not stated.
- Replicate data was not shown in this reference.
- Reference control data was not presented.
- Co-variance was not listed. According to the Acceptance Criteria in the OECD TG 442C the, "… mean peptide concentration of reference controls A [used to verify the suitability of the HPLC system] should be 0.50±0.05 mM and the coefficient of variation (CV) of peptide peak areas for the nine reference controls B [verify stability of reference controls over the analysis time] and C [verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion] in acetonitrile should be <15.0%".

[2] https://www.oecd.org/en/publications/2023/07/test-no-442c-in-chemico-skin-sensitisation_g1g507cd.html

Procedure Highlights (as reported by Gerberick et al. 2004):

- "Test chemical solutions at a concentration of 100 mM were prepared in acetonitrile, or solubilized in DMSO and then diluted with an equal part acetonitrile"
- "Peptides, Ac-RFAAKAACOOH (lysine peptide, Fig. 1), Ac-RFAACAA-COOH (cysteine peptide), and Ac-RFAAHAA-COOH (histidine peptide), were made by the SynPep Corp. (Dublin, CA), and purified >90% by HPLC (Keough et al., 1997). Molecular weight confirmation was done by flow injection mass spectrometry with electrospray ionization in the positive mode."
- "Peptide stock solutions were prepared to a final concentration of 1.25 mM in either 100 mM ammonium acetate buffer, pH 10.2 (lysine peptide), or 100 mM phosphate buffer, pH 7.5 (histidine and cysteine peptides)"
    - "… the lysine peptide we used a 1:50 peptide to test chemical ratio with a 24 h reaction period"
    - "… a 1:10 peptide to test chemical ratio was used for the cysteine peptide"
- Samples were run at 3, 12, and 24 reactivity time and found to be stable in the amount of depleted peptide across all timeframes.
- "A gradient of 90% (A) to 60% (A) over 25 min at a flow rate of 0.3 ml/min was used for the separation"
- "For logistic reasons, the 24 h time point was chosen for analysis of the other chemicals in the dataset."
- "Triplicate reactivity samples were prepared containing 0.5 mM peptide, and either 5 mM or 25 mM test chemical for a peptide:test chemical ratio of 1:10 or 1:50. A Biomek 2000 automated workstation (Beckman Coulter, Fullerton, CA) was used to make additions of the peptide stock solution (400 ml), the appropriate buffer (350 ml), and the test chemical solutions (50 or 250 ml) into autosampler vial"
- "The autosampler vials were capped, gently vortexed, and incubated for 24h at room temperature in the autosampler (dark) prior to HPLC analysis."
- "Calibration standards were prepared manually from the peptide stock solution, diluted into the appropriate buffer for the peptide, and contained either 5 or 25% acetonitrile. The peptide concentrations were 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.50, 1.0 mM"
- "Ammonium acetate, ammonium hydroxide, sodium phosphate monobasic and sodium phosphate dibasic for the preparation of buffers were purchased from J.T. Baker. DMSO was obtained from Sigma. Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA, 99%) for the preparation of the HPLC mobile phase were purchased from EMD (Gibbstown, NJ) and Aldrich, respectively"
- "A Waters Alliance 2695 and 996 PDA detector comprised the chromatographic system. A 10 ml injection of the reactivity samples was made onto the column. The peptides were separated from the test chemicals and products on a Zorbax SB-C18 (2.1 3 100 mm) stationary phase (Agilent Technologies, Wilmington, DE) which was preceded by a SecurityGuard cartridge guard system (Phenomenex, Torrance, CA) containing a C18 cartridge (2.0 3 4.0 mm). The column temperature was 30°C. The mobile phase consisted of 0.1% TFA in water (A) and 0.085% TFA in acetonitrile (B). A gradient of 90% (A) to 60% (A) over 25 min at a flow rate of 0.3 ml/min was used for the separation. The diode array detector scanned the wavelengths 210–400 nm. Chromatograms were extracted at 220 nm. Quantitation was performed using either Millenium32 or Empower software packages. Peptide reactivity with the test chemicals was reported as percent peptide depletion, which was determined as the reduction of the peptide concentration in the samples relative to the average concentration of the controls."

Results:

For phthalic anhydride the mean percent peptide depletions for cysteine and lysine were -1.9% (SD = 1.0) and 75% (SD = 3.9), respectively (see Table below). The standard deviation (SD) for the phthalic anhydride replicates is less than 14.9% for cysteine depletion and 11.6% for lysine depletion, as required by OECD TG 442C. Therefore, the test substance, phthalic anhydride was predicted to be a sensitizer with high reactivity according to LLNA data and the corresponding DPRA results (see Table below). The positive control, cinnamic aldehyde, gave the expected result, causing 43.2% (SD = 4.1) and 70.6% (SD = 1.0) depletion of lysine and cysteine, respectively (see Table below). This is consistent with OECD TG 442C, which requires mean percent peptide depletion values between 60.8 and 100% for cysteine and between 40.2 and 69.0% for lysine for the positive control cinnamic aldehyde. Additionally, the five proficiency substances gave accurate DPRA predictions and depleted cysteine and lysine peptides within the expected ranges, as outlined in Table 1 in Appendix I, Annex 2 of OECD TG 442C.

Reactivity of Chemical Substances to Glutathione or Synthetic Peptides with Results Expressed as Percent Depletion

LLNA category	*(Conc. peptide:Conc. test substance)*	Glutathione (0.2 mM:20 mM)		Lysine (0.5 mM:25 mM)		Cysteine (0.5 mM: 5 mM)		Histidine (0.5 mM:25 mM)	
		Average	SD	Average	SD	Average	SD	Average	SD
Extreme	2,4 Dinitrochlorobenzene	43.6	2.6	14.7	4.2	100.0	0.0	0.3	4.9
	Diphenylcyclopropenone	22.0	7.5	0.7	3.8	98.8	2.0	−1.0	3.1
	Oxazolone	22.6	9.5	49.6	1.8	75.5	1.4	−4.9	11.6
	p-Benzoquinone	100.0	0.0	91.0	0.2	99.0	1.8	94.0	3.8
Strong	Phthalic anhydride	100.0	0.0	75.0	3.9	−1.9	1.0	−4.6	5.8
	1,4-Hydroquinone	76.5	6.1	51.1	6.5	83.3	0.9	30.0	0.9
	Glutaraldehyde	20.8	4.0	85.4	3.5	30.2	0.5	2.7	1.0
	Lauryl gallate	42.2	13.6	8.7	4.2	90.9	13.1	−0.1	1.0
	CD3	63.6	13.6	13.6	0.5	90.1	1.1	−8.9	4.0
Moderate	2-Hydroxyethyl acrylate	98.1	1.8	88.9	0.3	92.6	0.5	8.2	4.3
	3-Dimethylaminopropylamine	2.8	5.2	−1.8	1.9	10.2	3.4	1.9	2.5
	Cinnamic aldehyde	46.7	5.2	43.2	4.1	70.6	1.0	−3.8	7.8
	3-Aminophenol	2.0	2.4	1.2	1.7	6.6	1.5	2.0	2.4
	3,4-Dihydrocoumarin	2.3	2.6	7.5	1.0	ND	—	−1.8	2.8
	1,2-Benzisothiazolin-3-one	14.5	1.3	ND	—	97.7	0.1	ND	—
	Phenylacetaldehyde	−4.7	0.7	22.6	1.9	60.7	13.3	−3.1	0.8
	Squaric acid	16.5	4.0	4.8	4.9	46.9	8.7	3.1	0.4
	Citral	37.5	14.4	16.9	0.3	85.7	3.2	−7.9	1.0
	1-(4-Methoxyphenyl)-1-penten-3-one	−0.2	1.5	14.3	3.2	29.9	5.6	1.7	2.1
	Diethyl maleate	83.3	4.5	85.5	1.6	100.0	0.0	0.5	0.8
Weak	α-Hexylcinnamaldehyde	−2.6	3.2	−1.6	2.9	−0.3	1.2	−0.4	1.5
	5-Methyl-2,3-Hexandione	−2.6	9.9	7.5	1.1	25.8	4.0	23.1	3.9
	Hydroxycitronellal	−1.8	3.9	6.5	2.0	17.5	1.7	5.6	6.2
	Ethyleneglycol dimethacrylate	3.6	5.6	12.4	3.0	87.3	5.0	−1.2	1.8
	α-Amyl cinnamaldehyde	0.2	10.1	3.9	1.5	0.6	0.2	−1.1	1.7
	Benzyl benzoate	0.7	5.5	3.0	5.3	0.2	1.1	−2.5	0.8
	Lilial	7.7	0.8	0.7	0.2	14.0	6.4	−0.4	0.3
Non	Glycerol	1.2	4.2	2.1	0.9	−3.8	5.2	0.2	0.6
Sensitizers	Hexane	−0.8	4.1	−5.1	0.6	−0.4	0.8	−1.8	3.5
	Diethyl phthalate	10.9	13.3	−0.7	0.9	0.8	1.7	0.7	2.8
	Octanoic acid	−1.6	3.1	0.9	0.1	−1.0	0.7	0.7	0.3
	2-Hydroxypropyl methacrylate	5.5	4.8	ND	—	58.4	5.9	ND	—
	1-Butanol	6.1	7.5	1.2	0.8	−0.4	1.4	0.5	0.4
	4-Hydroxybenzoic acid	−1.0	5.8	2.2	2.1	−0.3	0.8	−0.4	0.2
	6-Methylcoumarin	−1.6	8.6	4.0	5.6	1.4	0.3	−1.6	2.5
	Methyl salicylate	4.2	3.5	1.6	0.3	0.3	0.8	0.5	1.1
	Chlorobenzene	3.2	2.3	1.3	0.2	0.4	0.2	−1.8	2.0
	Lactic acid	−1.1	11.1	0.8	0.5	−0.9	0.3	−0.8	1.3

OECD 442C [Kinetic Direct Peptide Reactivity Assay (DPRA)] (Wareing et al. 2017)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING

Product Manager: Not applicable
HERO: 6789397
Study Completion Date: Not reported. Peer-reviewed publication; publication year: 2017
Study No.: Not applicable. Peer-reviewed publication (DOI: 10.1016/j.tiv.2017.08.015)
Testing Laboratory: Study authors associated with BASF SE (Ludwigshafen, Germany), and BASF Personal Care and Nutrition GmbH (Dusseldorf Germany); however, exact laboratory in which testing was conducted is not stated.
Author: Britta Wareing, Daniel Urbisch, Susanne Noreen Kolle, Naveed Honarvar, Ursula G. Sauer, Annette Mehling, Robert Landsiedel

Quality Assurance (40 CFR §160): GLP status not specified.

Test Material:

- Phthalic anhydride (CASRN 85-44-9). Study authors purchased the test substance from Sigma Aldrich (Germany) and in a supplementary file provide the CASRN and SMILES structure, however the purity of each chemical is absent.
- Note: A total of 38 chemicals that were positive sensitizers in the local lymph node assay (LLNA) were evaluated. Five of the tested chemicals are described as proficiency substances in Appendix III, Annex 2 of OECD TG 442C for demonstrating technical proficiency with the kinetic DPRA (marked with asterisk '*' below).
    - 1-Chloro-2,4-dinitrobenzene (Dinitrochlorobenzene, DNCB)*, 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (Oxazolone)*, Isoeugenol*, 2,3-Butanedione*, Ethylene glycol dimethacrylate (EGDMA)*, Tetramethylthiuram disulfide, 1,4-Phenylenediamine, Formaldehyde, Phthalic anhydride, p-Benzoquinone, Phenyl benzoate, α-Hexyl cinnamic aldehyde, Diethyl maleate, 2-Mercaptobenzothiazole, Citral, Benzylidene acetone (4-Phenyl-3-buten-2-one), Eugenol, Cinnamyl Alcohol, Farnesal, Pentachlorophenol, Cobalt chloride, Dinitrofluorobenzene, Propyl gallate, Tosylchloramide sodium (Chloramine T), 1,2-Dibromo-2,4-dicyanobutane (MDGN, Methyldibromo glutaronitrile), 2,4-Dinitrobenzenesulfonic acid, sodium salt, 4-(Methylamino)phenol sulfate (Metol), MCI/MI, Ethylenediamine free base, Methylmethacrylate, Phenylacetaldehyde, Cinnamic aldehyde, Hydroxycitronellal, 3-Propylidenephthalide, Sodium lauryl sulfate / sodium dodecyl sulfate (SDS), Imidazolidinyl urea, Xylene, Nickel sulfate
        - Purity of these 38 substances was not stated.

Concentration: 5, 2.5, 1.25, 0.625 and 0.3125 mM
Vehicle/Negative Control:

- OECD guidelines state that 12 blank controls, 12 vehicle only controls, and the positive control should be used to calculate the relative peptide depletion in %.
    - The current protocol states that "Fluorescence intensities were normalized relative to the substance without the peptide [vehicle control], as well as the phosphate buffer and acetonitrile (background fluorescence) [blank control]. Peptide depletion was expressed as percent decrease in peptide concentration, which was calculated based on a calibration series…".

  - The number of replicates for controls was not stated.
- "Test substances were preferably dissolved in acetonitrile (Sigma–Aldrich, Germany) to prepare a 100 mM solution. If the test substances were not soluble in acetonitrile, solutions were prepared in water, methanol, propanol, isopropanol, acetone or mixture of these solvents which is in accordance with the DPRA protocol used in the interlaboratory ring trials."

Positive Control:

- No positive control stated. However, Cinnamic Aldehyde [CASRN 104-55-2] was evaluated, which is the positive control recommended by OECD TG 442C.

Concentration: 5, 2.5, 1.25, 0.625 and 0.3125 mM

Test System: Direct Peptide Reactivity Assay (DPRA)

Method: Pre-guideline study, but generally adheres to principles outlined in OECD TG 442C (In Chemico Skin Sensitisation)[3] with some deviations and reporting deficiencies noted below

Summary:

3. Sensitization was observed for phthalic anhydride. The log k of the highest reactivity timepoint (i.e., log k_{max}) was -067 $M^{-1}s^{-1}$. The threshold log k was determined to be -1.73 $M^{-1}s^{-1}$.
4. **Classification:** Acceptable for quantitative use

Deviations from Guideline and other comments:

- The OECD guideline (TG 442C) states to measure fluorescent intensity using an "excitation filter of 390 nm and an emission filter of 480 nm" however the fluorescent wavelength settings were not stated within the protocol.
- Under OECD TG 442C, reactions are to be measured at six time-points (10, 30, 90, 150, 210, 1440 minutes). Study authors state that reactions were measured at 5 or 10 minutes, 30, 60, 120, 240, and 1440 minutes. Although study authors state that reactions were measured after a 1440 minute incubation, results for this reaction were not presented by study authors.
- The Log K_{max} value for the positive control (cinnamic aldehyde) was -1.93. The OECD guidelines (TG 442C) stated that the log($k_{90\ min}$) of the positive control should be within the range of -1.75 to -1.40 $M^{-1}s^{-1}$.
  - There was not a 90 min sample recorded.
  - K values for 60 min and 120 min were -2.02 and -2.14 $M^{-1}s^{-1}$, respectively.
- The individual values of the vehicle controls was not reported nor was the coefficient of variance.
  - In the OECD guidelines, when a test chemical is not showing a linear relationship with reaction/concentration then the rates constances should be calculated based on individual depletion values according to this formula: k = = [ln (100/(100 − dp))]/($E \times t$).
  - There are no statements regarding the linearity of the concentration/response relationship.
- Proficiency testing was not stated, but 5 chemicals in the OECD guideline (TG 442C) that are listed for use in proficiency testing, were evaluated.

[3] https://www.oecd.org/en/publications/2023/07/test-no-442c-in-chemico-skin-sensitisation_g1g507cd.html

- Study authors selected a threshold value of log kmax = -1.73 to discriminate between GHS Cat 1A and 1B, whereas OECD TG 442C classifies chemicals as UN GHS subcategory 1A if the log k_{max} is ≥-2.0.
- The purity of each test chemical is not listed.
- Storage conditions of test/control chemicals is not listed.
- Replicate data was not shown in this reference.
- Reference control data was not presented.
- Co-variance was not listed.

Procedure Highlights:

- The following reference established the kinetic peptide depletion assay as a modified protocol from Roberts and Natsch 2009 (DOI: 10.1021/tx800431x).
- While other experiments were stated as following OECD guidelines for proper study execution, the kDPRA was not. It is listed as being performed as a modified assay from Roberts and Natsch 2009.
- Positive criterion was listed and from the original OECD 442C guideline. See Table 2 below for details on the "Cys-Only" criterion.
- "All substances were first dissolved in acetonitrile to yield stock solutions of 20 mM, and then dilution series of 20, 10, 5, 2.5 and 1.25 mM were prepared."
- The peptide used was diluted to "0.667 mM Cys-peptide solution in phosphate buffer (pH 7.5) were added to each well of a black 96-well plate." A final concentration of "0.667 mM Cys-peptide solution in phosphate buffer (pH 7.5) were added to each well of a black 96-well plate."
- The "diluted test substances were added leading to concentrations of 5, 2.5, 1.25, 0.625 and 0.3125 mM (final ratios of peptide and test substance = 1:10, 1:5, 2:5, 4:5, 8:5)."
- "All substances were tested in triplicate within the same run."
- "The plates were sealed with impermeable foil directly after application of the substance and incubated in the dark at 25 °C."
- "All substances were incubated for 30, 60, 120, 240 and 1440 min. In addition, ten substances of higher reactivity were incubated for shorter time periods, i.e. 5 min or 10 min in a second test run."
- Each reaction was stopped with "the addition of 3 mM of the fluorescence dye, i.e. monobromobimane solution (diluted in acetonitrile)."
- The fluorescence was measured with a "TriStar Multimode reader LB 942".
- "Fluorescence intensities were normalized relative to the substance without the peptide, as well as the phosphate buffer and acetonitrile (background fluorescence). Peptide depletion was expressed as percent decrease in peptide concentration, which was calculated based on a calibration series. For each incubation time, the remaining (non-reacted) amount of Cys-peptide was determined and plotted against the respective substance concentration. The obtained slope was divided by the incubation time to determine the second order reaction rate constants k".

Results:

The highest reaction rate constant recorded for phthalic anhydride was at 5 min with a k 20 (reaction rate constant) of 0.2122 $M^{-1}s^{-1}$ (See table below). The log of k highest reactivity (i.e., log k_{max}) was -0.67 $M^{-1}s^{-1}$. The threshold value established from the kinetic DPRA prediction models was -1.73 $M^{-1}s^{-1}$. Under the conditions of the study, phthalic anhydride was considered positive,

with a log k_{max} value supporting a GHS category 1A classification for sensitization under both the threshold used by study authors (log $k_{max} \geq -1.73$) and OECD TG 442C (log $k_{max} \geq -2.0$).

Summary of the results of the 38 substances tested in the kinetic DPRA with calculated rate reaction constants for different time points. For further information on the substances see Supplementary Table S2.

Test substance	LLNA-EC3 [%]	CLP/GHS	Reaction rate constant [$s^{-1} M^{-1}$]							log *k* highest reactivity
			k 5	k 10	k 30	k 60	k 120	k 240	k highest reactivity	
Xylene	95.8	1B	n.d.	n.d.	0.0047	0.0019	0.0004	0.0001	0.0047	− 2.33
Methyl Methacrylate	90	1B	n.d.	n.d.	0.0025	0.0004	0.0007	0.0000	0.0025	− 2.60
EGDMA[a]	28	1B	n.d.	n.d.	0.0009	0.0011	0.0010	0.0008	0.0011	− 2.94
Imidazolidinyl urea	25	1B	n.d.	n.d.	0.0089	0.0006	0.0038	0.0008	0.0089	− 2.05
Hydroxycitronellal	23	1B	n.d.	n.d.	0.0031	0.0016	0.0001	0.0003	0.0031	− 2.51
Cinnamic alcohol[a]	21	1B	n.d.	n.d.	0.0016	n.d.	0.0009	n.d.	0.0016	− 2.79
Pentachlorphenol[a]	20	1B	n.d.	n.d.	0.0053	n.d.	0.0010	n.d.	0.0053	− 2.28
Phenyl benzoate[a]	17.1	1B	n.d.	n.d.	0.0007	0.0015	0.0005	0.0001	0.0015	− 2.82
Sodium dodecyl sulfate	14	1B	n.d.	n.d.	0.0009	0.0005	0.0005	0.0000	0.0009	− 3.03
Eugenol[a]	12.9	1B	n.d.	n.d.	0.0081	n.d.	0.0030	n.d.	0.0081	− 2.09
Farnesal[a]	12	1B	n.d.	n.d.	0.0028	n.d.	0.0012	n.d.	0.0028	− 2.55
α-Hexylcinnamicaldehyde[a]	12	1B	n.d.	n.d.	0.0072	0.0069	0.0086	0.0039	0.0086	− 2.07
2,3-Butanedione[a]	11.3	1B	0.0229	0.0173	0.0072	0.0028	0.0016	0.0010	0.0229	− 1.64
Citral[a]	7.3	1B	n.d.	n.d.	0.0067	n.d.	0.0040	n.d.	0.0067	− 2.17
Nickel sulfate	4.8	1B	n.d.	n.d.	0.0002	0.0000	0.0000	0.0000	0.0002	− 3.71
Benzylidenacetone[a]	3.7	1B	n.d.	n.d.	0.0042	n.d.	0.0077	n.d.	0.0077	− 2.12
3-Propylidenephthalide	3.7	1B	n.d.	n.d.	0.0019	0.0009	0.0000	0.0001	0.0019	− 2.71
Tetramethylthiuram disulfide[a]	3.1	1B	0.0061	n.d.	0.0006	n.d.	0.0071	n.d.	0.0061	− 2.22
Cinnamic aldehyde	3.1	1B	n.d.	n.d.	0.0118	0.0096	0.0073	0.0045	0.0118	− 1.93
Phenylacetaldehyde	3/4.7	1B	n.d.	n.d.	0.0007	0.0011	0.0007	0.0008	0.0011	− 2.96
Ethylenediamine	2.2	1B	n.d.	n.d.	0.0016	0.0006	0.0000	0.0000	0.0016	− 2.80
Diethylmaleate[a]	2.1	1B	n.d.	n.d.	0.0116	0.0121	0.0144	0.0172	0.0172	− 1.76
2,4-Dinitrobenzenesulfonic acid	2	1A	0.0310	0.0165	0.0090	0.0052	0.0043	0.0029	0.0310	− 1.51
Isoeugenol[a]	1.8	1A	0.0200	0.0147	0.0119	0.0078	0.0032	0.0005	0.0200	− 1.70
2-Mercaptobenzothiazole[a]	1.7	1A	n.d.	n.d.	0.1779	0.0878	0.0385	0.0190	0.1779	− 0.75
Methyldibromo glutaronitrile	0.9	1A	0.0164	0.0701	0.0012	0.0012	0.0004	0.0002	0.0701	− 1.15
4-Methylaminophenol sulfate	0.8	1A	0.0282	0.0286	0.0535	0.1295	0.1421	0.0437	0.1421	− 0.85
Formaldehyde[a]	0.7	1A	0.1225	0.0543	0.0227	0.0110	0.0038	0.0023	0.1225	− 0.91
Cobalt chloride	0.57	1A	n.d.	n.d.	0.0015	0.0006	0.0010	0.0002	0.0015	− 2.84
Chloramin T	0.4	1A	n.d.	n.d.	0.2872	0.0151	0.0082	0.0040	0.2872	− 0.54
Propyl gallate	0.32	1A	n.d.	n.d.	0.0027	0.0019	0.0011	0.0005	0.0027	− 2.56
Phthalic anhydride[a]	0.16	1A	0.2122	0.0906	0.0349	0.0120	0.0049	0.0015	0.2122	− 0.67
p-Phenylenediamine[a]	0.16	1A	0.0500	0.0313	0.0107	0.0055	0.0030	0.0016	0.0500	− 1.30
1-Chloro-2,4-Dinitrobenzene[a]	0.04	1A	n.d.	n.d.	0.0616	0.0512	0.0474	0.0423	0.0616	− 1.21
Dinitrofluorobenzene	0.03	1A	2.8300	0.4170	0.6742	0.0165	0.0074	0.0039	2.8300	0.45
p-Benzoquinone[a]	0.0099	1A	2.6725	1.3638	too reactive	too reactive	too reactive	too reactive	2.6725	0.43
Kathon CG	0.005	1A	n.d.	n.d.	0.0391	0.0656	0.1326	0.1899	0.1899	− 0.72
Oxazolone[a]	0.003	1A	n.d.	n.d.	0.1627	0.1102	0.0464	0.0173	0.1627	− 0.79

EGDMA = ethylene glycol dimethacrylate; *k* = Reaction rate constant [$s^{-1} M^{-1}$]; k 5, 30, 60, 120 or 240 = reaction rate constant at *t* = 5, 30, 60, 120 or 240 min; n.d. = not determined; too reactive = complete peptide depletion at any concentration.

[a] Data of substance used for ROC analysis.

OECD 442D [KeratinoSens™] (Natsch et al. 2013)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING

Product Manager: Not applicable.
HERO ID: 8315696
Study Completion Date: Not reported. Peer-reviewed publication; publication year: 2013
Study No.: Not applicable. Peer-reviewed publication (DOI: 10.1002/jat.2868)
Testing Laboratory: Study authors associated with the Procter & Gamble company (Cincinnati, Ohio) , Procter & Gamble company (Bever, Belgium), and Givaudan Schweiz AG (Duebendorf, Switzerland) however, exact laboratory in which testing was conducted is not stated.
Author: Andreas Natsch, Cindy A. Ryan, Leslie Foertsch, Roger Emter, Joanna Jaworska, Frank Gerberick, and Petra Kern

Quality Assurance (40 CFR §160): GLP status not specified.

Test Material:

- Phthalic Anhydride (CASRN 85-44-9). Study authors stated that "… all the test chemicals are commercially available…", and provide a supplementary file with CASRN and SMILES structures.
- Note: A total of 145 chemicals of varying potency (based on the Local Lymph Node Assay [[LLNA] were evaluated as part of this study, including all ten proficiency substances listed in Appendix IA - Annex 1 of the OECD Guideline (*i.e.*, Salicylic acid; Lactic acid; Glycerol; Isopropanol; Ethylene glycol dimethacrylate; Cinnamyl alcohol; 2-Mercaptobenzothiazole; 4-Methylaminophenol sulfate; Methyldibromo glutaronitrile; 2,4-Dinitrochlorobenzene). The full list of chemicals used in this report is provided at the end of this document.

Concentrations: Concentrations ranged from 0.98 to 2000 µM.

Vehicle/Negative Control:

- Dosing medium with 1% DMSO (DMEM containing Glutamax and supplemented with 1% fetal bovine serum); 6 wells/plate in 3 replicate plates.

Positive Control:

- No positive control stated. However, phthalic anhydride was evaluated alongside many other known sensitizers of varying potency based on predetermined LLNA results), including cinnamic aldehyde [CASN 104-55-2], which is a positive control recommended by OECD TG 442D

Concentration: Concentrations ranged from 0.98 to 2,000 µM.

Test System: KeratinoSens™ cell line

Number:

- 4 plates (3 parallel replicate plates for gene induction; 1 for cytotoxicity determinations) per repetition. 2-4 repetitions.

Method: OECD 442D (In Vitro Skin Sensitisation)[4]

Summary:

1. Phthalic anhydride: strong sensitizer
2. **Classification:** Acceptable for quantitative use

Deviations from Guideline and other comments:

- The study was not conducted in accordance with current GLP regulations (40 CFR §160).
- Confluency and cell passage number not reported; OECD No. 442D recommends use of 80-90% confluent cells be used and cells with 2 – 4 passages. The purity of all the test chemicals was not listed.
- Column statistics were not available for dataset presented in this report
    - Values from individual runs were not shown
    - Standard deviations for plates/groups was not provided
    - Coefficient of variation for groups is not presented
- The use of proficiency controls, including positive controls and negative controls) were complete, though they were not labeled as such.

Procedure Highlights:

- The procedure is described briefly in this study; the original procedure was described in Emter et al., 2010.
- Incubation of each of 12 concentrations of test substance (triplicates), negative/vehicle control (6 wells), and each concentration of positive control for 48 hour.
- Cell viability assessment: One plate of KeratinoSens™ cells was incubated with MTT for 4 hours at 37°C/5% CO_2, the cells were lysed with 10% SDS at 37°C/5% CO_2, and the absorbance of each well was read at 600 nm wavelength. These details were described in Emter et al., 2010.
- Sensitization potential assessment: Three plates were used to assess luciferase transcription/translation (used to detect activation of the second key event of the sensitization pathway). Following the 48-hour exposure, the supernatant was removed from each well, cells were incubated with lysis butter, and the luminescence of each well was determined using a GloMax® 96 Microplate Luminometer with automatic injection of 50 µl of the luciferase substrate to each well and integration of the luciferase activity for 2 s.
- Cytotoxicity, luciferase activity, I_{max}, and $EC_{1.5}$, EC_3, and IC_{50} were calculated.
- A chemical considered positive if it statistically significantly induced the luciferase activity more than 50% above control values at any of the tested concentrations in both independent repetitions. For chemicals with significant induction in only one repetition, two further repetitions were made. These chemicals were rated positive if the luciferase induction was statistically significant in at least 3 out of the total 4 independent repetitions. This is consistent with the acceptance criteria outlined in OECD 442D.

Results:

For phthalic anhydride, the mean $EC_{1.5}$ was >2000 µM, the EC_3 was >2000 µM, and the IC_{50} was >2000 µM. In comparison, cinnamic aldehyde (*i.e.*, positive control recommended by OECD TG

[4] https://www.oecd.org/content/dam/oecd/en/publications/reports/2022/06/test-no-442d-in-vitro-skin-sensitisation_g1g507d0/9789264229822-en.pdf

442D) had a mean $EC_{1.5}$ of 16.13 µM and a mean IC_{50} of 194.4 µM, indicating luciferase gene induction above 1.5-fold (threshold) without significant cell death.

The test article phthalic anhydride was predicted to be a skin sensitizer. The table below is from the Supplemental Excel file that accompanied the online version of this report. The list has been refined to only include phthalic anhydride, positive control, and the proficiency controls listed in Appendix IA - Annex 1.

				LLNA	U-937 Test	Direct Peptide Reactivity Assay		KeratinoSens Assay			Individual prediction models			
Name	**MW**	**logKow**	**Cas #**	**EC3 values %**	**EC150 for CD86 activation [μM]**	**Cysteine [% peptide remaining]**	**Lysine [% peptide remaining]**	**Concentration for 1.5-fold luciferase induction [μM]**	**Concentration for 3-fold luciferase induction [μM]**	**Concentration for 50% cytotoxicity [μM]**	**U-937 Test**	**DPRA**	**KeratinoSens Assay**	**Weight of evidence (2 or 3 tests)**
Glycerol	92.09	-1.76	56-81-5	NC	>7000	100.0	97.9	>2000	>2000	>2000	0	0	0	0
Isopropanol	60.1	0.05	67-63-0	NC	>7000	100.0	99.5	>2000	>2000	>2000	0	0	0	0
Lactic acid	90.08	-0.72	50-21-5	NC	>7000	100.0	99.2	>2000	>2000	>2000	0	0	0	0
Salicylic acid	138.12	2.26	69-72-7	NC	>7000	96.5	78.9	>2000	>2000	>2000	0	1	0	0
Ethylene glycol dimethacrylate	198.2	1.931	97-90-5	28	398.6	12.7	87.6	57.41	253.37	1655.76	1	1	1	1
Cinnamyl Alcohol	134.18	1.95	104-54-1	21	572.4	100.0	84.9	123.56	>2000	774.64	1	1	1	1
Cinnamic aldehyde	132.16	1.9	104-55-2	3.0	3.8	29.4	56.8	16.13	63.94	194.38	1	1	1	1
2-Mercaptobenzothiazole	167.25	2.42	149-30-4	1.7	37.4	2.5	100.0	48.12	340.15	1003.08	1	1	1	1
1,2-Dibromo-2,4-dicyanobutane (MDGN)	265.94	1.515	35691-65-7	0.9	>7000	0.0	71.4	7.79	18.14	25.59	0	1	1	1
4-(Methylamino)phenol sulfate (Metol)	344	0.85	55-55-0	0.8	>7000	0.0	55.3	9.40	2.68	11.70	0	1	1	1
Phthalic anhydride	**148.12**	**1.6**	**85-44-9**	**0.16**	**1080.2**	**98.1**	**25.0**	**>2000**	**>2000**	**>2000**	**1**	**1**	**0**	**1**
1-Chloro-2,4-dinitrobenzene (Dinitrochlorobenzene, DNCB)	202.56	2.17	97-00-7	0.05	1.5	0.0	85.3	2.50	3.89	8.20	1	1	1	1

List of 145 Chemicals Tested in Natsch et al. 2013

1-Bromobutane	2-Ethylhexyl acrylate
1-Butanol	2-Methylundecanal
4-Hydroxybenzoic acid	1-(p-Methoxyphenyl)-1-penten-3-one
Diethyl phthalate	Citral
Glycerol	1,2,4-Benzenetricarboxylic anhydride (Trimellitic anhydride)
Hexane	Perillaldehyde
Methyl 4-hydroxybenzoate (methylparaben)	Methyl methanesulphonate
Octanoic acid (Caprylic acid)	1,1,3-Trimethyl-2-formylcyclohexa-2,4-diene (Safranal)
Propylene glycol	Dihydroeugenol (2-methoxy-4-propyl-phenol)
Sulphanilic acid	2-Phenylpropionaldehyde
Benzaldehyde	Diethyl maleate
Coumarin	2-methoxy-4-methylphenol
Propyl paraben	3.4-Dihydrocoumarin
Streptomycin sulfate	trans-2-Hexenal
Sulphanilamide	Resorcinol
4-Methoxyacetophenone	Tetramethylthiuram disulfide
6-Methylcoumarin	α-methyl-trans-cinnamaldehyde
Benzalkonium chloride	Squaric acid
Vanillin	2,4-Heptadienal
Ethyl vanillin	Benzylidene acetone (4-phenyl-3-buten-2-one)
Nonanoic acid	3-Propylidenephthalide
Tartaric acid	5-Amino-2-methylphenol
4-Carboxyphenylacetate	3-Aminophenol
Benzoic acid	Cinnamic aldehyde
3-Chloro-4-methoxybenzaldehyde (3-Chloro-p-anisaldehyde)	Phenylacetaldehyde
Octanenitrile	Methyl 2-nonynoate
3-Phenoxypropiononitrile	trans-2-decenal
Benzocaine	1,2-Benzisothiazolin-3-one (Proxel active)
2-Acetyl-cyclohexanone	1-Bromohexane
Clofibrate (Ethyl (2-(4-chlorophenoxy)-2-methylpropanoate)	3-Dimethylamino propylamine
Ethyl benzoylacetate	Ethylenediamine free base
Saccharin	2,4-Dinitrobenzenesulfonic acid, sodium salt
Vinylidene dichloride	2-Methyl-2H-Isothiazol-3-one
Isopropyl myristate[a]	2-Mercaptobenzothiazole
Ethanol-2-butoxy-, acetate	4-Vinyl pyridine
Sodium lauryl sulphate	Bisphenol A-diglycidyl ether
Chlorobenzene	4-Amino-m-cresol
Isopropanol	2-Hydroxyethyl acrylate
Lactic acid	Glyoxal
Methyl salicylate	1-Phenyl-1,2-propanedione
Salicylic acid	1-Naphthol

1-Iodohexane	Isoeugenol
2-Hydroxypropyl methacrylate	1,2-Dibromo-2,4-dicyanobutane (MDGN)
Aniline	Squaric acid diethyl ester
Diethyl acetaldehyde	1,2-cyclohexane dicarboxylic anhydride
Hydroxycitronellal	4-(Methylamino)phenol sulfate (Metol)
Butyl glycidyl ether	Formaldehyde
Penicillin G	4-(N-Ethyl-N-2-methan-sulphonamido-ethyl)-2-methyl-1,4-phenylenediamine (CD3)
(+/-) Linalool	N,N-dimethyl-4-nitrosoaniline
Ethyl acrylate	2-Aminophenol
Ethylene glycol dimethacrylate	2-Nitro-1,4-phenylendiamine
2,2,6,6-Tetramethyl-3,5-heptanedione	2,5-Diaminotoluene sulphate (PTD)
5-Methyl-2,3-hexanedione	Propyl gallate
Geraniol	Lauryl gallate
Imidazolidinyl urea	Benzyl bromide
cis-6-nonenal	**Phthalic anhydride**
Cyclamen aldehyde	Maleic anhydride
Cinnamyl Alcohol	1,4-Phenylenediamine
Methyl acrylate	Fluorescein-5-isothiocyanate
Pentachlorophenol	Hydroquinone
p-tert-Butyl-a-ethyl hydrocinnamal (Lilial)	Glutaraldehyde
1-Iodohexadecane	4-Nitrobenzyl bromide
4-Allylanisole	1-Chloro-2,4-dinitrobenzene (Dinitrochlorobenzene, DNCB)
3 and 4-(4-Hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde (Lyral)	Tetrachloro-salicylanilide
Benzyl benzoate	3-Methylcatechol
Abietic acid	Bandrowski's Base (N,N-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine)
Oxalic acid anhydrous	p-Benzoquinone
Eugenol	5-Chloro-2-methyl-4-isothiazolin-3-one (MCI)
Farnesal	7,12-Dimethylbenz[α]anthracene
2,3-Butanedione	Benzoyl peroxide
Butyl acrylate	4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone)
α-Amyl cinnamic aldehyde	Diphenylcyclopropenone
Hexyl cinnamic aldehyde	

OECD 442E [Human Cell Line Activation Test (h-CLAT)] (Nukada et al. 2012)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING

HEROID: 8238790
Study Completion Date: Not reported. Peer reviewed publication; publication year: 2012
Study No.: Not applicable. Peer reviewed publication (DOI: 10.1016/j.tiv.2012.07.001)
Testing Laboratory: Naohiro Nishiyama (Safety Science Research Laboratories, Kao Corporation, 2606 Akabane, Ichikai-machi, Haga-gun, Tochigi 321-3497, Japan)
Author: Yuko Nukada, Takao Ashikaga, Masaaki Miyazawa, Morihiko Hirota, Hitoshi Sakaguchi, Hitoshi Sasa, Naohiro Nishiyama

Quality Assurance (40 CFR §160): GLP status not specified.

Test Material:

- Phthalic anhydride (CASRN 85-44-9). Study authors purchased the test substance from Sigma Aldrich (St. Louis, MO), however the purity of each chemical is absent.
- Note: A total of 106 chemicals were tested that were previously evaluated in the LLNA and categorized as extreme, strong, moderate, or weak sensitizers, or non-sensitizers. Eight of the tested chemicals are described as proficiency substances in Appendix II of OECD TG 442E for demonstrating technical proficiency with the h-CLAT (marked with asterisk '*' below)
  - Diphenylcyclopropenone, Oxazolone, MCI/MI (act. 1.5%), Bandrowski's base, p-Benzoquinone, 1-Benzoylacetone, 2,4-Dinitrochlorobenzene *, 4-Nitrobenzyl bromide, Potassium dichromate, Glutaraldehyde (act. 50%), 1,4-Dihydroquinone, 1,4-Phenylenediamine*, Maleic anhydride, **Phthalic anhydride**, Benzyl bromide, Benzoyl peroxide, Lauryl gallate, Propyl gallate, Cobalt chloride, 2-aminophenol, Chloramine T, 2-Nitro-1,4-phenylenediamine, Formaldehyde (act. 37%), Iodopropynyl butylcarbamate, Methyldibromo glutaronitrile, Isoeugenol, 1-Naphthol, 1-Phenyl-1,2-propanedione, Glyoxal (act. 40%), 2-Hydroxyethyl acrylate, 2-Mercaptobenzothiazole*, Methylisothiazolinone (act.9.7%), 3-Dimethylaminopropylamine, Ethylene diamine, 1,2-Benzisothiazolin-3-one, Methyl-2-nonynoate, Cinnamic aldehyde, Phenylacetaldehyde, 3-Aminophenol, Diethyl sulfate, Benzylideneacetone, 3-Propylidenephthalide, a-Methyl cinnamic aldehyde, Nickel sulfate*, Tetramethylthiuramdisulfide, 3,4-Dihydrocoumarin, Resorcinol, Diethylenetriamine, Diethyl maleate, 2-Methoxy-4-methyl-phenol, 4-Chloroaniline, Trimellitic anhydride, 1-Bromohexane, Amyl cinnamic aldehyde, Hexyl cinnamic aldehyde, 2,3-Butanedion, Citral, Eugenol, Abietic acid, Oxalic acid, 4-Allylanisole, Lillial, Phenyl benzoate, Cinnamic alcohol, Benzocaine, Cyclamen aldehyde, Imidazolidinyl urea*, Geraniol, Ethyleneglycol dimethacrylate, Linalool, Penicillin G, Butyl glycidyl ether, Hydroxycitronellal, Pyridine, Aniline, Acetanisole, Benzalkonium chloride, Benzoic acid, 1-Bromobutane, 1-Butanol, Chlorobenzene, Dextran, Diethyl phthalate, Dimethyl formamide, Ethyl benzoylacetate, Ethyl vanillin, Furil, Glycerol*, 4-Hydroxybenzoic acid, 2-Hydroxypropyl methacrylate, Iodohexane, Isopropanol*, Kanamycin, Lactic acid*, 6-Methyl coumarin, Methyl salicylate, Octanoic acid, Propylene glycol, Propyl paraben, Saccharin, Salicylic acid, Streptomycin sulfate, Tween 80, Vanillin, Zinc sulphate, Sodium lauryl sulfate.

Concentration: A total of 8 doses were applied based on the concentration that results in 75% cell viability (CV75): 1.2 CV75 (480), 1 CV75 (400), 1/1.2 CV75 (0.83), 1/1.22 CV75, 1/1.23 CV75, 1/1.24 CV75, 1/1.25 CV75 and 1/1.26 CV75. For phthalic anhydride, the CV75 was the highest tested concentration (400 µg/mL). Therefore, concentrations were calculated to be: 134, 160, 193, 232, 278, 333, 400, and 480 µg/mL.

Vehicle/Negative control:

- No negative control was stated. However, Lactic Acid (CASRN 50-21-5) was evaluated, which is the negative control recommended by OECD TG 442E for CD86/CD54 expression measurement.

Positive Control:

- No positive control stated. However, 2,4-dinitrochlorobenzene (DNCB) [CASRN 97-00-7] in DMSO was evaluated, which is a positive control recommended by OECD TG 442E for CD86/CD54 expression measurement.

Test system: Human Cell Line Activation Test (h-CLAT)

Method: Pre-guideline study, but generally adheres to principles outlined in OECD 442E with some deviations and reporting deficiencies noted below. Study is cited in OECD 442E as an example of the use of h-CLAT data.

Summary:

1. Phthalic anhydride had a negative response. The maximum relative fluorescence intensity (RFI) for CD86 and CD54 were 115 and 160, respectively. No EC150 for CD86 expression or EC200 for CD54 expression could be calculated.
2. Classification: Acceptable for quantitative use

Deviations from Guideline and other comments:

- The OECD TG 442E describes the culture requiring 5% CO_2 and 37°C. In the study, some of the culture condition parameters were omitted (*i.e.*, CO_2 levels in incubator and temperature).
- Under OECD TG 442E, the EC150 and CV70 values are calculated based on the average variabilities. Study authors state that the median value of three experiments, not the average, was defined as EC150 (CD86) or EC200 (CD54) of the test chemical and was used as a threshold derived from the h-CLAT data.
- Authors indicate, “Final concentrations of the vehicle in h-CLAT were set at 1% for saline and 0.2% for DMSO.” However, it is not stated which chemical was dissolved in either vehicle.
- The purity of each test chemical is not listed.
- OECD TG 442E recommends a 15-minute incubation step with the globulin blocking solution for the cell staining process. The study reported a 10-minute incubation step.
- Details regarding the controls used (*i.e.*, isotype control, media/DMSO control, etc.) were not presented.
- Individual test results were not reported from each lab that conducted the experiment.
- Individual results from cell viability stain propidium iodide (PI) were not reported.

Procedure Highlights:

- In this human cell line activation test (h-CLAT) study, the skin sensitization potential of the test substance phthalic anhydride was assessed by monitoring the upregulation of the cell surface markers CD54 and CD86 using human acute monocytic leukemia cells (THP-1). The upregulation of these markers correlates to dendritic cell activation in the skin sensitization pathway.
- OECD 442E states that cytotoxicity measurement is conducted concurrently to assess whether upregulation of CD86 and/or CD54 expression occurs at sub-cytotoxic concentrations. Cell viability determined via staining with propidium iodide (PI, 0.625 lg/mL), which penetrates dead cells but not living cells.
- "The test concentration providing a cell viability of 75% (CV75) is derived from the dose response curve, and was calculated by loglinear interpolation."
- The CV75 value (test substance concentration that corresponds to 75% cell viability) was used to determine a range of eight serial test substance dilutions (CV75: 1.2 x CV75, 1 x CV75, 1/1.2 x CV75, 1/1.22 x CV75, 1/1.23 x CV75, 1/1.24 x CV75, 1/1.25 x CV75 and 1/1.26 x CV75) that were used in two independent repetitions to distinguish surface marker upregulation from a cytotoxic event.
- After 24-hour exposures to test substance or controls, the cells were rinsed and stained with fluorescently labeled anti-CD54 or anti-CD86 antibody. The upregulation of the surface markers was measured with flow cytometry to determine the mean fluorescence intensity (MFI) of the cell populations.
- A solvent control (DMSO), and a positive control (2,4-dinitrochlorobenzene [DNCB] in DMSO) were used. Additionally, an isotype control (*e.g.*, IgG-1 isotype) was performed for each test substance concentration to determine non-specific antibody binding (background) that was subtracted from the fluorescence values of the antibody-stained cells.
- The relative fluorescence intensity (RFI) was calculated from the mean fluorescent intensity (MFI) values as below:

$$RFI = \left(\frac{MFI\ of\ chemical\ treated\ cells\ -\ MFI\ of chemical\ treated\ isotype\ control\ cells}{MFI\ of\ solvent\ treated\ cells\ -\ MFI\ of\ solvent\ treated\ isotype\ control\ cells}\right) \times 100$$

- Consistent with OECD 442E (paragraph 26), the upregulation of either surface marker (CD54 or CD86) indicates a positive prediction, but the upregulation must be seen in the same surface marker for at least two valid definitive trials to make a positive prediction.
  - "All chemicals were tested in three independent experiments. If two of three independent experiments at any dose exceeded 150% of RFI for CD86, or exceeded 200% of RFI for CD54, the chemicals could be identified as a sensitizer. Otherwise, it is identified as a non-sensitizer. The prediction model was based on the historical data and several previous studies.
  - The Maximum RFI (MaxRFI) was defined as the highest values of RFI through the eight doses in three independent experiments for each chemical."
  - "In the h-CLAT, the test chemicals, which induce 1.5 times higher protein expression for CD86 or 2 times higher protein expression for CD54 against the vehicle control, are considered sensitizers."

Results:

As shown in Table 1, both the EC_{200} and the EC_{150} were unable to be calculated for phthalic anhydride. The MaxRFI is defined by "*the highest values of RFI through the eight doses in three independent experiments for each chemical*". This indicated that the RFIs for the CD54-stained

cells and the CD86-stained cells were <200 and <150, respectively, at all test substance concentrations tested. All other tables show correlation data between this test, LLNA, and MIT.

The assay results were conducted in two different labs (individual values not shown). Details regarding each experiment were not reported.

Subset of Data Table 1 from Reference

		LLNA						h-CLAT				
Chemical name	**CAS**	**Potency category**	**EC3(%)**	**Result**	**CD86**	**CD54**	**CV75 (µg/mL)**	**Max RFI (CD86)**	**Max RFI (CD54)**	**EC150 (CD86)**	**EC200 (CD54)**	**MIT**
2,4-Dinitrochlorobenzene:DNCB	97-00-7	Extreme	0.05	P	+	+	5	311	499	2.3	2.66	2.3
Phthalic anhydride	85-44-9	Strong	0.16	N	–	–	400.0*	115	160	–	–	–
Nickel sulfate	10101-97-0(7786-81-4)	Moderate	4.8	P	+	+	150	322	4146	42.2	45.3	42.2
Lactic acid	50-21-5	Non-sensitizer	NC	N	–	–	2800	Not determined				

Full Data Table 1 from Reference

Table 1
Test substances used (106 chemicals).

Chemical name	CAS	LLNA						h-CLAT				
		Potency category	EC3(%)	Result	CD86	CD54	CV75 (µg/mL)	Max RFI (CD86)	Max RFI (CD54)	EC150 (CD86)	EC200 (CD54)	MIT
Diphenylcyclopropenone	886-38-4	Extreme	0.003	P	–	+	6.0	121	349	–	3.92	3.92
Oxazolone	15646-46-5		0.003	P	+	–	166.6	249	183	2.71	–	2.71
MCI/MI (act. 1.5%)	Mixture/26172-55-4		0.005	P	+	–	3.2	156	127	2.21	–	2.21
Bandrowski's base	20048-27-5		0.008	P	+	+	2.3	215	5072	3.85	2.6	2.6
p-Benzoquinone	106-51-4		0.0099	P	+	+	4.3	170	140	2.68	2.25	2.25
1-Benzoylacetone	93-91-4		0.04	P	–	+	92.8	118	801	–	39.6	39.6
2,4-Dinitrochlorobenzene:DNCB	97-00-7		0.05	P	+	+	5.0	311	499	2.3	2.66	2.3
4-Nitrobenzyl bromide	100-11-8		0.05	P	+	+	3.6	504	468	0.95	0.91	0.91
Potassium dichromate	7778-50-9		0.08	P	+	+	3.2	161	300	2.09	1.06	1.06
Glutaraldehyde (act. 50%)	111-30-8	Strong	0.1	P	+	+	5.3	169	313	2.78	2.7	2.7
1,4-Dihydroquinone	123-31-9		0.11	P	+	–	5.0	208	145	2.13	–	2.13
1,4-Phenylenediamine	106-50-3		0.16	P	+	–	36.7	250	167	2.09	–	2.09
Maleic anhydride	108-31-6		0.16	P	–	+	658.0	56	217	–	298.4	298.4
Phthalic anhydride	85-44-9		0.16	N	–	–	400.0*	115	160	–	–	–
Benzyl bromide	100-39-0		0.2	P	+	+	7.5	230	624	3.2	2.86	2.86
Benzoyl peroxide	94-36-0		0.30	N	–	–	41.0	119	126	–	–	–
Lauryl gallate	1166-52-5		0.30	P	+	+	8.2	276	1042	2.02	2.91	2.02
Propyl gallate	121-79-9		0.32	P	–	+	125.0	110	1464	–	32.5	32.5

OECD 429 [Local Lymph Node Assay] (Arts et al. 2008)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING (OCSPP 870.2600)

Product Manager: Not applicable
HERO ID: 1222879
Study Completion Date: Not Reported. Publication year: 2008
Report No.: Not applicable. Peer-reviewed publication (DOI: 10.1093/toxsci/kfn199)

Testing Laboratory: National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands
Author: Josje H.E. Arts, Wim H. de Jong, Jos J. van Triel, Marcel A. Schijf, Arja de Klerk, Hen vn Loveren, C. Frieke Kuper

Quality Assurance (40 CFR §160): Non-GLP

Test Material: Phthalic anhydride (at least 99% pure, purchased from Fluka, Buchs, Switzerland)

Concentrations Tested: 25% phthalic anhydride (nominal) dissolved in 4:1 acetone:olive oil vehicle

Positive Control Item: No positive control stated. However, phthalic anhydride was tested in the LLNA alongside 9 other known sensitizers (*i.e.,*trimellitic anhydride, toluene 2,4-diisocyanate, hexamethylene 1,6-diisocyanate, isophorone diisocyanate, trimeric isophorone diisocyanate, 2,4-ditrochlorobenzene, oxazolone, methyl salicylate, formaldehydye).

Animals:	Mouse, BALB/c strain
Number/Sex:	Males: 3 in control group, 3 in phthalic anhydride treatment group
Age:	6-7 weeks
Weight:	Not reported
Source:	Obtained from Charles River Deutschland (Sulzfeld, Germany)

Method:

- No guideline stated
- Animals received 25 µl/day of a single concentration of each test substance (phthalic anhydride test concentration: 25%), dissolved in a 4:1 (vol/vol) mixture of acetone and olive oil (AOO) on the dorsum of both ears on three consecutive days, or AOO alone (negative skin control). The target concentrations of DNCB, OXA, PA, TDI, and TMA were based on dermal LLNAs, previously performed in BALB/c mice (Van Och et al., 2000).
- The animals were necropsied 3 days after the last exposure.
- The dermal sensitizing potency of the chemicals was investigated in a modified LLNA using *ex vivo* labeling of the proliferating LN cells (De Jong et al., 2002; Kimber and Weisenberger, 1989; Vandebriel et al., 2000; Van Och et al., 2002).
- The cells of left and right auricular LNs were pooled for each animal and suspended in 5 ml RPMI-1640 (Gibco, Life Technologies, Breda, the Netherlands) supplemented with 5% heat-inactivated fetal calf serum (FCS; Integro, Zaandam, the Netherlands), 100 U/ml penicillin, and 100 µg/ml streptomycin (referred to as supplemented medium). Single-cell

suspensions were prepared in supplemented medium under aseptic conditions by pressing the LNs through a 70-lm nylon cell strainer (Falcon, Franklin Lakes, NJ). Cells were washed twice (10 min, 300 g, 4°C), resuspended in 1 ml supplemented medium with 10% FCS, and counted using a Coulter Counter (Z2, Coulter Electronics, Mijdrecht, the Netherlands). After counting, the concentration of the cell suspensions was adjusted to 1×10^7 cells/ml. When necessary, cell suspensions of a few animals were pooled to obtain concentrations of 1×10^7 cells/ml, notably for vehicle-treated controls. To assess lymphocyte proliferation, cell suspensions (2×10^6 cells in 200 µl supplemented medium per well) were seeded in triplicate in round-bottomed 96-well microtiter plates (Greiner, Alphen aan de Rijn, the Netherlands). A 10-µl aliquot of [^{3}H]-methylthymidine ([^{3}H]-TdR; 37 kBq per well or 3.7 MBq/ml, specific activity 185 GBq/mmol; Amersham Int., Buckinghamshire, UK) was added to the wells immediately after the initiation of culture. Cultures were maintained at 37°C for 20–24 h in a humidified atmosphere of 5% CO_2 in air. The cellular DNA was harvested on glass fiber filters using an automatic cell harvester (Harvester 96, Tomtec, Orange, CT), scintillation liquid was added, and [^{3}H]-TdR incorporation was measured by liquid scintillation counting in a β-plate counter (1205 Betaplate, Wallac, Turku, Finland). Proliferation per animal was determined by calculating the incorporation of [^{3}H]-TdR for the total cell number harvested (left and right LNs combined). The mean [^{3}H]-TdR incorporation was calculated per experimental group. SIs were calculated by dividing the mean [^{3}H]-TdR incorporation per group by the mean [^{3}H]-TdR incorporation of the (vehicle-treated) control group.

Summary:

1. Phthalic Anhdyride: *Positive* for sensitization
2. **Classification:** Acceptable for qualitative use

Deviations from Guideline and other comments:

- No pre-screen test conducted to determine the maximum dose for use in the main LLNA and study authors do not report evaluating skin irritation or other signs of systemic toxicity (*e.g.*, body weight, clinical observations). Study authors note that the target concentration of phthalic anhydride (25%) was selected by on dermal LLNAs previously performed in BALB/c mice by Van Och et al. (2000). However, Van Och et al. (2000) do not report conduct of a pre-screen test to determine the maximum tested dose included in the study (*i.e.*, 25% phthalic anhydride).
- Tested concentrations of phthalic anhydride were not analytically verified.
- OECD 429 recommends young adult female mice of CBA/Ca or CBA/J strain to be used, but that other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist. This study was conducted prior to the establishment of OECD TG 429, and study authors use the BALB/c strain of mice. Previous comparative analyses of LLNA results for CBA versus BALB/c strains by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) have found that the pattern of LLNA responses seen in BALC/c mice are very similar to that seen in CBA mice (NICEATM, 2009). Therefore, this deviation is not anticipated to impact study results.
- Tested male mice 6-7 weeks of age. OECD TG 429 recommends use of female mice 8-12 weeks old.

- Included only 3 mice per treatment group. OECD TG 429 recommends use of at least 4 mice per treatment group.
- Study evaluated only a single concentration of phthalic anhydride (25%). OECD 429 recommends a minimum of 3 concentrations be evaluated.
- Single cell suspensions generated from auricular lymph nodes were cultured with [^{3}H]TdR *in vitro* to determine lymphocyte proliferation. OECD TG 429 requires *in vivo* [^{3}H]TdR incorporation.
- No statistical analyses for the dermal LLNA were conducted.

Results:

The results for phthalic anhydride (PA) are summarized in the table below. Using a modified LLNA, study authors report a mean stimulation index (SI) of 93 for phthalic anhydride. Under the conditions of the study, phthalic anhydride was considered positive for skin sensitization. Given the deviations from OECD TG 429 noted above, this study is considered **Acceptable for use qualitatively (*i.e.*, as part of the weight of the scientific evidence)**. The most significant deviation from OECD TG 429 that may impact study results and interpretation is the use of an *in vitro* protocol for [^{3}H]TdR incorporation and lymphocyte proliferation, rather than through *in vivo* [^{3}H]TdR incorporation as required by OECD TG 429.

SIs in Auricular LNs of Positive (dermal) Controls

Chemical	Concentration in % (wt/vol)	Number of animals tested	SI auricular LN[a]
TMA	50	3	130 ± 22
PA	25	3	93 ± 21
TDI	1	3	46 ± 6.3
HDI	1	6	285 ± 18
IPDI	1	6	415 ± 41
Trimeric IPDI[b]	12.5	3	1.0 ± 0.3
DNCB[c]	50	6	188 ± 30
OXA	0.1	3	52 ± 14
FA	10[d]	6	10 ± 1.7
MS[e]	25	3	2.3 ± 0.2

Note. 10% (vol/vol) FA was prepared by adding eight parts of AOO to three parts of FA (37%).
[a]Mean ± SEM.
[b]Three additional control animals were exposed to 1% DNCB (SI = 61 ± 3.5).
[c]In this experiment TMA (50% wt/vol) was used as the positive control.
[d]% (vol/vol).
[e]Three additional control animals were exposed to 1% DNCB (SI = 15 ± 3.9).

OECD 429 [Local Lymph Node Assay] (Basketter and Scholes, 1992)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING (OCSPP 870.2600)

Product Manager: Not applicable
HERO ID: 5353562
Study Completion Date: Not Reported. Publication year: 1992
Report No.: Not applicable. Peer-reviewed publication (DOI: 10.1016/0278-6915(92)90138-b)

Testing Laboratory: Unilever Environmental Safety Laboratory, Colworth House, UK
Author: D.A. Basketter, E.W. Scholes

Quality Assurance (40 CFR §160): Non-GLP

Test Material: Phthalic anhydride (Purity not reported, purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK)) (vehicle = 4:1 (v/v) acetone:olive oil)

Concentrations Tested: 2.5, 5.0, 10% phthalic anhydride (nominal) dissolved in 4:1 acetone:olive oil vehicle

Positive Control Item: No positive control stated. However, study conducted to investigate the correlation between the LLNA and GPMT for 40 chemicals covering a range of chemical types and levels of skin sensitization potential.

Animals:	Mouse, CBA/Ca strain
Number/Sex:	Males and females used across experiments for 40 chemicals, however, single experiments were limited to one sex. Study authors do not report what sex of mice was included in the phthalic anhydride study. Study authors state that the assay was carried out as described in Basketter et al. 1991 (DOI: 10.3109/15376519109036523). Basketter et al. (1991) indicates that this research group includes 4 mice per dose group in its LLNA study design.
Age:	8-12 weeks
Weight:	Not reported
Source:	Not reported

Method:

- No guideline stated, but generally conducted in a manner consistent with OECD TG 429 [Local Lymph Node Assay], with some deviations noted below.
- This assay was carried out as described elsewhere (Basketter *et al.,* 1991). CBA/Ca mice were used at the age of 8-12 wk. Animals of both sexes were used, but single experiments were limited to one sex. The test substance was assayed at three consecutive concentrations from the following range: 100, 50, 25, 10, 5, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05 and 0.01%. Groups of four mice were treated by a daily topical application of 25 µl of each concentration on the dorsal surface of each ear for 3 consecutive days. Control mice were treated with the vehicle alone. 4-5 days after the first topical application, all mice were injected intravenously through the tail vein with 250 µl phosphate buffered saline (PBS) containing [^{3}H]methyl thymidine (^{3}HTdR; 20/iCi). After 5 hr the mice were killed by carbon dioxide asphyxiation, and the draining auricular lymph nodes were excised and

pooled for each experimental group. A single-cell suspension of lymph node cells (LNC) was prepared by gentle mechanical disaggregation through a stainless-steel gauze (200-mesh size), using the plunger of a syringe. Pooled LNC were pelleted at 190g for 10 min, washed twice with 10 ml PBS and resuspended in 3 ml trichloroacetic acid (TCA; 5%) for the precipitation of macromolecules. After an overnight incubation with TCA at 4°C, the precipitate was recovered by centrifugation, resuspended in 1 ml TCA and transferred to 10 ml scintillation fluid. ^{3}HTdR incorporation was measured by β-scintillation counting. The proliferative response of LNC was expressed as radioactive disintegrations per min per lymph node (dpm/node), and as the ratio of ^{3}HTdR incorporation into LNC of test nodes relative to that recorded for control nodes (test/control ratio). A chemical was regarded as a sensitizer in the LLNA if at least one concentration of the chemical resulted in a three-fold or greater increase in ^{3}HTdR incorporation compared with control values. Also, the data had to be compatible with a biological dose response although an allowance was made, especially at high doses, for either local toxicity or immunological suppression.

Summary:

2. Phthalic Anhdyride: *Positive* for sensitization (EC3 <2.5%)
2. **Classification:** Acceptable for quantitative use

Deviations from Guideline and other comments:

- No pre-screen test conducted to determine the maximum dose for use in the main LLNA and study authors do not report evaluating skin irritation or other signs of systemic toxicity (*e.g.*, body weight, clinical observations). However, this deviation is unlikely to have a substantial impact on the study results, given the low concentrations (*i.e.,* 2.5, 5, and 10%) of phthalic anhydride tested.
- Tested concentrations of phthalic anhydride were not analytically verified.
- Study included only 1 rest day (OECD 429 calls for 2 rest days) between third application of test material and injection ^{3}H-TdR via the lateral tail vein.
- OECD 429 recommends young adult female mice of CBA/Ca or CBA/J strain to be used, but that other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist. Study authors used CBA/Ca mice and state that animals of both sexes were used, but single experiments were limited to one sex. However, the sex of animals included the study of phthalic anhydride was not stated.
- Study authors do not explicitly state how many mice were included per treatment group. However, study authors state that the assay was carried out as described in Basketter et al. 1991. Basketter et al. (1991) indicates that this research group includes 4 mice per dose group in its LLNA study design.
- A statistical analysis for presence and degree of dose-response relationship in the data was not conducted. However, under OECD TG 429 a statistical analysis is not required. Per OECD TG 429, an LLNA is regarded as positive when the stimulation index is ≥3. The strength of the dose-response and statistical significance may be used to determine whether a borderline result is positive. In the current study, stimulation indices were well above the threshold of 3 (see table of results below), and the result was not considered borderline. Therefore, lack of a statistical analysis is not expected to impact study results or interpretation.

Results:

Phthalic anhydride was evaluated at concentrations of 0 (vehicle control), 2.5, 5, and 10%. 4:1 (v/v) acetone:olive oil was used as the vehicle. Stimulation indices (SI) for each pooled treatment group were 26.0, 21.5, and 20.9 for the 2.5, 5, and 10% treatment groups, respectively. Under the conditions of the assay, phthalic anhydride was considered positive for dermal sensitization.

Chemical	Concentrations (%)	Exposure period	Vehicle	T/C ratio*			Classification
Abietic acid	5, 10, 25	4	AOO	3.3	4.1	6.4	+
m-Aminophenol	2.5, 5, 10	4	AOO	2.8	3.5	5.7	+
Ammonium tetrachloroplatinate	2.5, 5, 10	5	DMSO	16.0	15.4	18.1	+
Aniline	10, 25, 50	4	AOO	1.4	1.8	2.9	±
Benzocaine	10, 25, 50	5	AOO	1.7	2.0	0.9	–
p-Benzoquinone	0.5, 1, 2.5	4	AOO	36.4	42.3	52.3	+
Chloramine T	5, 10, 25	4	DMSO	7.7	7.5	10.7	+
p-Chloroaniline	2.5, 5, 10	4	AOO	1.0	1.5	1.8	–
Cinnamic aldehyde	5, 10, 25	5	AOO	12.5	18.4	15.4	+
Citral	5, 10, 25	4	AOO	2.1	5.0	9.3	+
Cobalt chloride	0.5, 1, 2.5	5	DMSO	3.2	3.7	2.8	+
Copper chloride	1, 2.5, 5	5	DMSO	8.1	13.8	13.6	+
Dextran	2.5, 5, 10	4	DMSO	0.9	1.5	1.5	–
Dimethyl isophthalate	5, 10, 25	5	AOO	1.0	0.9	1.0	–
Dinitrochlorobenzene	0.01, 0.05, 0.1	4	AOO	6.2	15.7	24.0	+
Eugenol	25, 50, 100	4	AOO	4.8	9.3	7.6	+
Formaldehyde	5, 10, 25	4	AOO	3.7	4.0	5.8	+
p-Hydroquinone	0.5, 1, 2.5	4	AOO	5.7	10.7	16.4	+
p-Hydroxybenzoic acid	5, 10, 25	5	DMSO	1.4	1.5	1.3	–
Hydroxycitronellal	25, 50, 100	4	AOO	3.6	5.9	8.5	+
2-Hydroxyethyl acrylate	10, 25, 50	5	AOO	9.0	8.2		+
2-Hydroxypropyl methacrylate	10, 25, 50	5	AOO	1.1	1.2	1.3	–
Imidazolidinyl urea	10, 25, 50	4	DMF	1.7	3.1	5.5	+
Isoeugenol	2.5, 5, 10	4	AOO	7.5	13.1	25.3	+
2-Mercaptobenzothiazole	10, 25, 50	5	DMF	4.5	4.6	5.5	+
Methyl dodecane sulphonate	1, 2.5, 5	5	AOO	21.6	39.9	48.6	+
Methyl salicylate	5, 10, 25	4	AOO	1.3	1.0	0.8	–
Metol	0.5, 1, 2.5	5	DMF	2.5	3.4	6.7	+
Nickel chloride	1, 2.5, 5	5	DMSO	1.5	2.2	2.4	–
Nickel sulphate	0.5, 1, 2.5	5	DMSO	1.1	1.5	1.5	–
Ovalbumin	5, 10, 25	4	0.9% NaCl	1.6	2.1	3.6	+
Penicillin G	10, 25, 50	5	DMSO	1.5	3.8	8.9	+
p-Phenylene diamine	2.5, 5, 10	4	AOO	12.8	16.5	23.3	+
Phthalic anhydride	2.5, 5, 10	4	AOO	26.0	21.5	20.9	+
Potassium dichromate	0.1, 0.25, 0.5	4	DMSO	3.5	10.2	10.4	+
Propyl gallate	5, 10, 25	5	AOO	22.3	18.3	33.6	+
Propyl paraben	5, 10, 25	4	AOO	1.3	1.6	1.3	–
Sulphanilic acid	2.5, 5, 10	4	DMSO	1.3	1.3	1.5	–
Toluene diamine bismaleimide	5, 10, 25	5	DMSO	11.9	12.2	11.8	+
Trimellitic anhydride	2.5, 5, 10	4	AOO	31.1	45.3	50.5	+

*Ratio of test to control lymphocyte proliferation (dpm/node, data not shown).
+ = positive; – = negative; ± = equivocal.
AOO = acetone–olive oil (4:1, v/v); DMSO = dimethylsulphoxide; DMF = dimethyl formamide.

OECD 429 [Local Lymph Node Assay] (Dearman et al. 2000)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING (OCSPP 870.2600)

Product Manager: Not applicable
HERO ID: 5178449
Study Completion Date: Not Reported. Publication year: 2000
Report No.: Not applicable. Peer-reviewed publication (PMID: 10797476)

Testing Laboratory: Zeneca Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK
Author: Rebecca Dearman, E. Vicky Warbrick, Ian R. Humphreys, Ian Kimber

Quality Assurance (40 CFR §160): Non-GLP

Test Material: Phthalic anhydride (99% pure, purchased from Sigma Chemical Co, St. Louis, MO, USA)

Concentrations Tested: 0.1, 0.25, 0.5, 1.0, 2.5% phthalic anhydride (nominal) dissolved in 4:1 acetone:olive oil vehicle

Positive Control Item: No positive control stated. However, phthalic anhydride was tested alongside 3 other known sensitizers (*i.e.,* trimellitic anhydride, maleic anhydride, hexahydrophthalic anhydride, all dissolved in 4:1 acetone:olive oil vehicle)

Animals:	Mouse, BALB/c strain
Number/Sex:	24 females: 4 in control group, 4 in each treatment group
Age:	6-12 weeks
Weight:	Not reported
Source:	Harlan Seralab, Bicester, Oxfordshire, UK

Method:

- No guideline stated, but generally conducted in a manner consistent with OECD TG 429 [Local Lymph Node Assay]
- Groups of mice (n = 4) were exposed topically on the dorsum of both ears to 25 µl of various concentrations of acid anhydrides or to vehicle (4:1 acetone:olive oil) alone, daily for three consecutive days. Five days after the initiation of exposure all mice were injected intravenously via the tail vein with 20 µCi of [3H]methyl thymidine (^{3}HTdR; specific activity 2 Ci mmol^{-1}) in 250 ml of phosphate-buffered saline (PBS). Five hours later, mice were killed and the draining auricular lymph nodes were excised and pooled for each experimental group. A single cell suspension of lymph node cells was prepared by gentle mechanical disaggregation through 200-mesh stainless-steel gauze. Cells were washed twice with PBS and precipitated in 5% trichloroacetic acid (TCA) at 4°C overnight. Pellets were then resuspended in 1 ml of 5% TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Hisafe 3', Wallac, Turku, Finland). Incorporation of ^{3}HTdR was measured by β-scintillation counting as disintegrations per min (dpm) per node for each experimental group. In each case a stimulation index (SI) relative to the concurrent vehicle-treated control was derived. The estimated concentration of chemical required to induce SI = 3 relative to concurrent vehicle-treated controls, i.e. the ec3 value, was derived by linear

interpolation as described previously. The EC3 value was calculated by interpolating between two points on the SI axis: one immediately above and the other immediately below SI = 3. The vehicle-treated control value (SI = 1) cannot be used for the latter. Where the data points lying immediately above and below SI = 3 have the coordinates (*a*,*b*) and (*c*,*d*), respectively, then the EC3 value may be calculated using the equation: EC3 = $c + [(3-d)/(b-d)] \times (a-c)$

Summary:

3. Phthalic Anhdyride: *Positive* for sensitization (EC3 = 0.16%)
2. **Classification:** Acceptable for quantitative use

Deviations from Guideline and other comments:

- No pre-screen test conducted to determine the maximum dose for use in the main LLNA and study authors do not report evaluating skin irritation or other signs of systemic toxicity (e.g., body weight, clinical observations). However, this deviation is unlikely to have a substantial impact on the study results, given the low concentrations (*i.e.,* 0.1–2.5%) of phthalic anhydride tested.
- Tested concentrations of phthalic anhydride were not analytically verified.
- OECD 429 recommends young adult female mice of CBA/Ca or CBA/J strain to be used, but that other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist. This study was conducted prior to the establishment of OECD TG 429, and study authors use the BALB/c strain of mice. Previous comparative analyses of LLNA results for CBA versus BALB/c strains by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) have found that the pattern of LLNA responses seen in BALC/c mice are very similar to that seen in CBA mice (NICEATM, 2009). Therefore, this deviation is not anticipated to impact study results.
- A statistical analysis for presence and degree of dose-response relationship in the data was not conducted. However, under OECD TG 429 a statistical analysis is not required. Per OECD TG 429, an LLNA is regarded as positive when the stimulation index is ≥3. The strength of the dose-response and statistical significance may be used to determine whether a borderline result is positive. In the current study, stimulation indices were well above the threshold of 3 (see table of results below), and the result was not considered borderline. Therefore, lack of a statistical analysis is not expected to impact study results or interpretation.

Results:

The results for phthalic anhydride (PA) are summarized in the table below. The stimulation index (SI) of the 0.1, 0.25, 0.5, 1.0, and 2.5% phthalic anhydride treatment groups were 1.98, 4.75, 4.81, 6.01, and 10.78, respectively. Treatment-related increases in the SI were also observed for other known sensitizers trimellitic anhydride, maleic anhydride, and hexahydrophthalic anhydride. These results indicate that phthalic anhydride is a dermal sensitizer and an EC3 value of 0.16% was calculated for phthalic anhydride by study authors.

Table 1. Local lymph node assay responses to the acid anhydrides TMA, HHPA, MA, PA and MTHPA

Acid anhydride concentration (%w/v)	TMA (dpm node^{-1})	SI	HHPA (dpm node^{-1})	SI	MA (dpm node^{-1})	SI	PA (dpm node^{-1})	SI	MTHPA (dpm node^{-1})	SI
0	341	1	306	1	245	1	240	1	333	1
0.1	287	0.84	ND		467	1.91	476	1.98	ND	
0.25	480	1.41	281	0.92	1191	4.86	1141	4.75	ND	
0.5	720	2.11	635	2.08	1548	6.32	1154	4.81	425	1.28
1	2190	6.42	1050	3.43	3431	14.00	1443	6.01	710	2.13
2.5	4405	12.92	2401	7.85	3915	15.98	2587	10.78	1881	5.65
5	ND		4040	13.20	ND		ND		3443	10.34
10	ND		ND		ND		ND		5701	17.12

Groups of mice received 25 μl of various concentrations of the acid anhydrides TMA, HHPA, MA, PA or MTHPA in AOO, or vehicle alone, on the dorsum of both ears daily for three consecutive days. Five days following the initiation of treatment, all mice were injected intravenously with 250 μl of 20 μCi ^{3}HTdR in PBS. Five hours later, draining auricular lymph nodes were excised and a single-cell suspension was prepared. The incorporation of ^{3}HTdR was measured by β-scintillation counting, and is displayed as dpm node^{-1} and stimulation index (SI). ND = not determined.

ICCVAM. 2009. Recommended Performance Standards: Murine Local Lymph Node Assay. NIH Publication Number 09-7357. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

OECD 429 [Local Lymph Node Assay] (Plitnick et al. 2003)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING (OCSPP 870.2600)

Product Manager: Not applicable
HERO ID: 117458
Study Completion Date: Not Reported. Publication year: 2003
Report No.: Not applicable. Peer-reviewed publication (DOI: 10.1016/s0300-483x(03)00264-6)

Testing Laboratory: The Dow Chemical Co., Midland, MI 48674, USA
Author: L.M. Plitnick, S.E. Loveless, G.S. Ladics, M.P. Holsapple, R.J. Smialowicz, M.R. Woolhiser, P.K. Anderson, C. Smith, M.J.K. Selgrade

Quality Assurance (40 CFR §160): Non-GLP

Test Material: Phthalic anhydride (99% pure, purchased from Sigma Chemical Co, Milwaukee, WI, USA)

Concentrations Tested: 0.15, 1.5, 15% phthalic anhydride (nominal) dissolved in 4:1 acetone:olive oil vehicle

Positive Control Item: No positive control stated. However, Phthalic anhydride was tested alongside 3 other known sensitizers (*i.e.,* trimellitic anhydride (TMA), maleic anhydride (MA), hexahydrophthalic anhydride (HHPA), all dissolved in 4:1 acetone:olive oil vehicle)

Animals:	Mouse, BALB/c strain
Number/Sex:	20 females: 5 in control group, 5 in each phthalic anhydride group
Age:	8-12 weeks
Weight:	Not reported
Source:	Charles River Laboratories (Kingston, NY, USA)

Method:

- No guideline stated, but generally conducted in a manner consistent with OECD TG 429 [Local Lymph Node Assay], with some deviations noted below.
- Female BALB/c mice (8–12 weeks old) (Charles River Laboratories, Kingston, NY) were housed in stainless steel, wire-mesh cages suspended above cage boards. Mice were fed Certified Rodent LabDiet® Diet 5002 (PMI Nutrition International, Inc., St. Louis,
- 12.5 µl of each test substance or control (vehicle = 4:1 acetone:olive oil) was administered on both sides of each ear (equaling 25 µl total per ear) of 8–12 weeks old BALB/c mice (n =5) for 3 consecutive days (days 0, 1 and 2). A 1 day rest was followed by an i.v. injection of 20 µCi ^{3}H-TdR (Amersham) in 0.25 ml PBS (Sigma) which was delivered to each mouse via the lateral tail vein on the morning of test day 4. Approximately 5 h following the initial injection of ^{3}H-TdR, auricular LN were harvested and pooled for each mouse. Single cell suspensions were prepared in PBS by gentle mechanical disaggregation using a Stomacher 80 Lab System® (Seward Ltd., London, UK). Cell suspensions were washed twice in PBS and placed in 3ml 5% TCA (Sigma) for approximately 65 h at 4◦C. Cells were centrifuged and resuspended in 1ml 5% TCA and transferred to glass scintillation vials (Kimble Glass, Inc., Vineland, NJ) containing 10 ml Aquasol® scintillation cocktail

(Packard Instrument Co., Meriden, CT). 3H-TdR incorporation was measured on a Beckman LS6000SC β-scintillation counter (Beckman Coulter, Fullerton, CA) as dpm per mouse.

- The mean dpm values for each group were determined using the individual dpm values with blank mean subtracted. A stimulation index (SI) was then derived for each animal by dividing the dpm of each mouse by the mean dpm of the AOO vehicle group. A mean SI ± S.E. was then calculated for each experimental group. A SI of ≥3.0 indicated a positive response (NIEHS, 1999). Mean SI and the SI of the highest dose of each chemical were analyzed by ANOVA followed by an all pairwise multiple comparison procedure (Tukey Test) using SigmaStat. Significance was judged at $P < 0.05$.

Summary:

4. Phthalic Anhdyride: *Positive* for sensitization (0.15% < EC3 <1.5%)
2. Classification: Acceptable for quantitative use

Deviations from Guideline and other comments:

- No pre-screen test conducted to determine the maximum dose for use in the main LLNA and study authors do not report evaluating skin irritation or other signs of systemic toxicity (e.g., body weight, clinical observations). However, this deviation is unlikely to have a substantial impact on the study results, given the low concentrations (*i.e.,* 0.15 and 1.5 %) of phthalic anhydride tested.
- Study included only 1 rest day (OECD 429 calls for 2 rest days) between third application of test material and injection ^{3}H-TdR via the lateral tail vein.
- DNA precipitated in 5% TCA at 4◦C for approximately 65 hours (OECD TG calls for 18 hour precipitation at in 5% TCA at 4◦C).
- Tested concentrations of phthalic anhydride were not analytically verified.
- OECD 429 recommends young adult female mice of CBA/Ca or CBA/J strain to be used, but that other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist. This study was conducted prior to the establishment of OECD TG 429, and study authors use the BALB/c strain of mice. Previous comparative analyses of LLNA results for CBA versus BALB/c strains by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) have found that the pattern of LLNA responses seen in BALC/c mice are very similar to that seen in CBA mice (NICEATM, 2009). Therefore, this deviation is not anticipated to impact study results.

Results:

The results for phthalic anhydride (PA) are summarized in the figure below. Mean stimulation indices (SI) ± SEM were provided by study authors graphically only. For phthalic anhydride, dose-dependent increases in the SI were observed. The mean SI in the 0.15% treatment group (low dose) was less than 3, while the mean SI was greater than 3 in both the 1.5% and 15% treatment groups. The SI was statistically significantly higher than the vehicle control in both the 1.5% and 15% treatment groups. These results indicate that phthalic anhydride is a dermal sensitizer. No EC3 value was estimated, however, from results provided in the figure below the EC3 value can be inferred to be greater than 0.15%, but less than 1.5%. Treatment-related increases in the SI

were also observed for other known sensitizers trimellitic anhydride, maleic anhydride, and hexahydrophthalic anhydride.

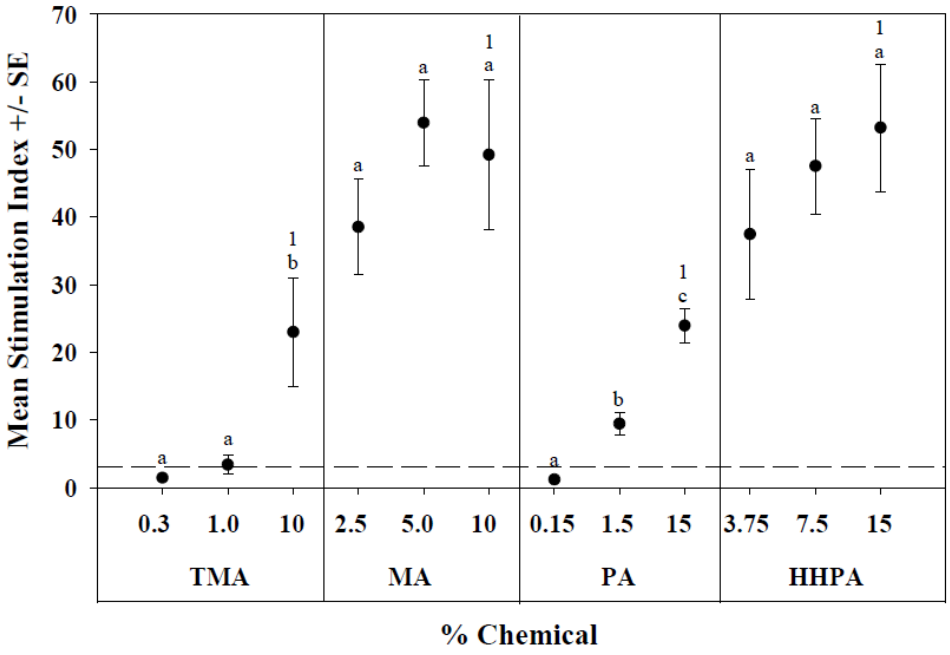

Fig. 2. Proliferation in local lymph nodes in response to TMA, MA PA or HHPA as measured by LLNA. Mice were exposed on both ears on days 0, 1 and 2 with 0.3, 1.0 or 10% TMA, 2.5, 5.0 or 10% MA, 0.15, 1.5 or 15% PA, or 3.75, 7.5 or 15% HHPA. ^{3}H-Thymidine uptake in draining LN was assessed and data are expressed as mean SI ± S.E. Dashed line indicates SI = 3. TMA and PA were tested at The Dow Chemical Co. (Midland, MI) and MA and HHPA were tested at The DuPont Haskell Laboratory (Newark, DE). Shared letters within each chemical indicate values are not different based on $P < 0.05$ using an ANOVA analysis. Shared numbers within highest dose group of each chemical indicate values are not different based on $P < 0.05$ using an ANOVA analysis.

ICCVAM. 2009. Recommended Performance Standards: Murine Local Lymph Node Assay. NIH Publication Number 09-7357. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

OECD 429 [Local Lymph Node Assay] (van Och et al. 2000)

DATA REVIEW FOR DERMAL SENSITIZATION TESTING (OCSPP 870.2600)

Product Manager: Not applicable
HERO ID: 1943046
Study Completion Date: Not Reported. Publication year: 2000
Report No.: Not applicable. Peer-reviewed publication (DOI: 10.1016/s0300-483x(00)00165-7)

Testing Laboratory: National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands
Author: Francoi M. M. van Och, Wout Slob, Wim H. de Jong, Rob J. Vandebriel, Henk can Lovren

Quality Assurance (40 CFR §160): Non-GLP

Test Material: Phthalic anhydride (99% pure, purchased from Sigma- Aldrich)

Concentrations Tested: 0.25, 1.0, 2.5, 10, 25% phthalic anhydride (nominal) dissolved in 4:1 acetone:olive oil vehicle

Positive Control Item: No positive control stated. However, phthalic anhydride was tested in the LLNA alongside 9 other known sensitizers (*i.e.,* benzocaine, diethylamine, 2,4-ditrochlorobenzene, 2-mercaptobenzothiazole, 4-ethoxymethylene, oxazolone, toluene diisocyanate, trimellitic anhydride, tetramethylthiuramdisulfide).

Animals:	Mouse, BALB/c strain
Number/Sex:	Males/females: 3 in control group, 3 in each treatment group (sex of animals used in the phthalic anhydride experiment not reported)
Age:	6-8 weeks
Weight:	Not reported
Source:	Obtained from RIVM's own breeding colony

Method:

- No guideline stated, but generally conducted in a manner consistent with OECD TG 429 [Local Lymph Node Assay]
- Groups of mice (*n*=3, 4, or 6) were pretreated with 1% SDS (w:v) one hour before exposing the animals to 25 ml of test solution in vehicle or vehicle alone on both ears daily for three consecutive days. A positive response is not seen at the SDS concentration that we used (1%). However, application of 1% SDS and the test chemical generally resulted in an increased response compared to the test chemical alone (data not shown).
- The concentrations of phthalic anhydride tested were 0 (vehicle control, 4:1 acetone/olive oil), 0.25, 1, 2.5, 10, 25%. Three days following the last topical application, the auricular lymph nodes were excised. The lymph nodes were weighed and pooled for each animal and suspended in 5 ml RPMI- 1640 (Gibco, Breda, NL) supplemented with 5% heat inactivated Fetal Calf Serum (PAA, Linz, Austria), 100 U/ml penicillin and 100 µg/ml streptomycin (standard medium). Single cell suspensions were prepared under aseptic conditions by pressing the lymph node through a sterile 70 mm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed twice in standard medium (10 min, 311 g,

4°C) and resuspended in 1 ml standard medium with 10% FCS. The cells were counted using a Coulter Counter (Coulter Electronics, Mijdrecht, NL) and cultured at a concentration of $1x10^7$ cells/ml. When necessary, cell suspensions of several animals were pooled to obtain the concentration required. The cell suspensions (200 ml) were seeded in triplicate into round-bottomed 96-well microtitre plates (Greiner, Alphen a:d Rijn, NL). The cells were cultured with 10 ml of [^{3}H]TdR (Amersham, Buckinghamshire, UK; 37 kBq:ml) for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The [^{3}H]TdR incorporation was determined by liquid scintillation counting in a β plate counter (1205 Betaplate™ Wallac, Turku, Finland). The [^{3}H]TdR incorporation is expressed per animal, i.e. the [^{3}H]TdR incorporation is multiplied by the cell number of the two lymph nodes and divided by the cell number in culture.

- The dose-response data were analysed by nonlinear regression analysis, using the following family of models:

  model 1: y_a
  model 2: $y\_a \exp(bx)$
  model 3: $y\_a \exp(bxd)$
  model 4: $y\_a(c-(c-1) \exp(bx)$
  model 5: $y\_a(c-(c-1) \exp(bxd))$.

  where y represents the response ([3H]TdR incorporation) and x the applied concentration. In these models the parameter a represents the background [3H]TdR incorporation of the particular assay. The parameter b reflects the 'slope' or the 'strength' of the response with increasing dose. The selection of the model to be used for a particular data set follows from a procedure of successively fitting the above models, and applying likelihood ratio tests to establish if an increase in the number of parameters leads to a significantly better fit to the data. A model with more parameters is considered better only if this leads to a significantly better fit (Slob, 1999). Then the selected model is used to derive the concentration (EC3) associated with a stimulation index of 3. The uncertainty in the estimate of the EC3 is assessed by a bootstrap method (Slob and Pieters, 1998), resulting in an uncertainty distribution from which any desired confidence interval can be derived. In this paper the 5% and 95% confidence limits are reported (i.e. 90%-confidence intervals).

Summary:

5. Phthalic Anhdyride: *Positive* for sensitization (EC3 = 0.357%)
2. **Classification:** Acceptable for qualitative use

Deviations from Guideline and other comments:

- No pre-screen test conducted to determine the maximum dose for use in the main LLNA and study authors do not report evaluating skin irritation or other signs of systemic toxicity (e.g., body weight, clinical observations). However, this deviation is unlikely to have a substantial impact on the study results, given the low concentrations (*i.e.,* 0.25, 1, 2.5, 10, 25%) of phthalic anhydride tested.
- Tested concentrations of phthalic anhydride were nominal and were not analytically verified.
- Tested mice 6-8 weeks of age. OECD TG 429 recommends use of mice 8-12 weeks old.

- OECD 429 recommends young adult female mice of CBA/Ca or CBA/J strain to be used, but that other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist. This study was conducted prior to the establishment of OECD TG 429, and study authors use the BALB/c strain of mice. Previous comparative analyses of LLNA results for CBA versus BALB/c strains by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) have found that the pattern of LLNA responses seen in BALC/c mice are very similar to that seen in CBA mice (NICEATM, 2009). Therefore, this deviation is not anticipated to impact study results.
- Authors report use of male and female mice, however, the sex of animals used in the phthalic anhydride experiment were not reported. OECD TG 419 recommends use of young adult female mice.
- Included only 3 mice per treatment group. OECD TG 429 recommends use of at least 4 mice per treatment group.
- Animals were pretreated with 1% SDS. Study authors stated that a positive response was not seen at the SDS concentration that was used (1%). However, application of 1% SDS and the test chemical generally resulted in an increased response compared to the test chemical alone (data not shown).
- Single cell suspensions generated from auricular lymph nodes were cultured with [^{3}H]TdR *in vitro* to determine lymphocyte proliferation. OECD TG 429 requires *in vivo* [^{3}H]TdR incorporation.

Results:

The results for phthalic anhydride (PA) are summarized in the table below. Study authors did not report the mean stimulation index (SI) per dose group. However, using the modified LLNA, an EC3 value of 0.357 (95% confidence interval: 0.226–0.560) was estimated. Under the conditions of the study, phthalic anhydride was considered positive for skin sensitization. Given the deviations from OECD TG 429 noted above, this study is considered **Not Acceptable**. The most significant deviation from OECD TG 429 that may impact study results and interpretation, including the EC3 estimate, is the use of an *in vitro* protocol for [^{3}H]TdR incorporation and lymphocyte proliferation, rather than through *in vivo* [^{3}H]TdR incorporation as required by OECD TG 429.

Lymph node weights and cell counts after epicutaneous treatment of BALB/c mice

Chemical	% Concentration	Lymph node weight/animal (mean ± SD, in mg)	Cell counts/animal (mean ± SD × 10^6)
PA	0	2.53 ± 0.46	5.42 ± 0.91
	0.25	4.10 ± 0.58	9.53 ± 2.00
	1	5.83 ± 0.90	13.19 ± 3.61
	2.5	6.12 ± 1.55	16.28 ± 4.12
	10	10.77 ± 1.43	25.01 ± 8.36
	25	11.10 ± 2.56	29.44 ± 2.10

References

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