

|                                    | CEC (1991)   | Gettings et al. (1991)               | Bagley et al. (1992)   | Gettings et al. (1994)   | Vinardell and Macián (1994)   |
|------------------------------------|--|--------------------------------------|--|--|---|
| <b>Hen Strain</b>                  | Lohmann Selected Leghorn   | Lohmann's Selected White Leghorn LSL | White Leghorn  | White Leghorn  | Leghorn SA31  |
| <b>Egg Criteria for Use</b>        | Weight range of eggs between 50 g and 60 g   | Not Noted                            | Weight range of eggs between 50 g and 60 g                           | Not Noted  | Not Noted   |
| <b>Egg Storage (Prior to use)</b>  | Not Noted  | Not Noted                            | Not Noted  | Not Noted  | Not Noted   |
| <b>Incubation Temperature (°C)</b> | 37.5 ± 0.5   | Not Noted                            | 37.5 ± 0.5   | 38   | Not Noted   |
| <b>Relative Humidity (%)</b>       | 62.5 ± 7.5   | Not Noted                            | 62.5 ± 7.5   | 60   | Not Noted   |
| <b>Egg Rotation?</b>               | Yes  | Not Noted                            | Yes  | Yes  | Not Noted   |
| <b>Checking Egg Viability</b>      | Eggs candled on Day 5 and every day thereafter; non-viable embryos removed   | Not Noted                            | Not Noted  | Candled on Day 9 and non-viable embryos removed                              | Not Noted   |
| <b>Incubation Period</b>           | 10 Days  | 10 Days                              | 10 Days  | 9 Days   | 10 Days   |
| <b>Procedure for Opening Egg</b>   | Egg shell was scratched around the air chamber with a rotating dentist saw blade and then pared off. The inner shell was removed and the CAM was layed open. | Not Noted                            | Egg shell around the air pocket was removed with a dental rotary saw | Eggshell was scratched around the air cells and a small aperture was opened. | Egg shell was scratched around the air cell by a dentist's rotary saw and then pared off. After removal of the inner membrane, the CAM was exposed. |
| <b>Manipulation of CAM</b>         | Not Noted  | Not Noted                            | Not Noted  | Not Noted  | Not Noted   |

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|--|---|---|--|---|--|
| <b>Investigator Defined Test Substance Classes</b> | Chemicals from various chemical classes   | Hydro-alcoholic formulations  | Chemicals from various chemical classes, commercial products, and personal care products | Oil/water -based personal care formulations                               | Commercial disinfectants   |
| <b>Total Test Substances Evaluated</b>             | 21  | 10  | 32   | 18  | 6  |
| <b>Test Substance Quantity or Volume</b>           | 0.3 mL or 0.1 g   | Not Noted   | 0.3 mL or 0.1 g  | 0.3 mL  | 0.3 mL   |
| <b>Test Substance Concentrations Tested</b>        | 0.1% to 100%  | Not Noted   | Tested solutions at concentrations that were 10% of those tested <i>in vivo</i>          | 3 concentrations tested: threshold concentration, 10% solution, undiluted | Diluted or undiluted   |
| <b>Application of Solids to CAM</b>                | Placed directly on CAM  | Not Noted   | All tested substances appear to be solubilized   | All tested substances appear to be solubilized                            | All tested substances appear to be solubilized                         |
| <b>Preferred Solvent</b>                           | Not Noted   | Not Noted   | Not Noted  | Not Noted   | Distilled water  |
| <b>Rinse after Test Substance Application?</b>     | Yes, for solids. 20 seconds after application of test substance, rinsed with 5 mL warm water or saline solution | Not Noted   | 20 seconds after test substance applied rinsed with 5 mL warm water                      | Not Noted   | Not Noted  |
| <b>Evaluation Period</b>                           | Up to 300 seconds after test substance applied  | Not Noted   | At 0.5, 2, and 5 minutes after test substance applied                                    | Up to 300 seconds after test substance applied                            | Up to 300 seconds after test substance applied                         |
| <b>Controls and Test Standards</b>                 | Not Noted   | Not Noted   | Vehicle  | Not Noted   | 0.1 M NaOH, 1% SDS, 0.9% NaCl, distilled water                         |
| <b>Number of Control Eggs</b>                      | Not Noted   | Not Noted   | 2 eggs   | Not Noted   | 2 eggs per substance   |
| <b>Replicate Eggs</b>                              | Minimum 6 eggs  | Not Noted   | 4 eggs   | 3 eggs per concentration tested   | 6 eggs   |
| <b>Number of Replicate Experiments</b>             | Not Noted   | Not Noted   | Not Noted  | 2   | Not Noted  |
| <b>Endpoints Assessed</b>                          | Hemorrhage, lysis, and coagulation  | Hemorrhage, vascular lysis, coagulation                                       | Hyperemia, hemorrhage, coagulation   | Hemorrhage, coagulation, lysis  | Hemorrhage, vasoconstriction, coagulation                              |
| <b>Endpoint Evaluation</b>                         | The starting second that each of the three endpoints is observed is recorded.                                   | The starting second that each of the three endpoints is observed is recorded. | Numerical time-dependent scores for three endpoints.                                     | Seconds for the three endpoints recorded (see Kalweit, 1990)              | The starting second that each of the endpoints is observed is recorded |

|                              | CEC (1991)   | Gettings et al. (1991)  | Bagley et al. (1992)  | Gettings et al. (1994)   | Vinardell and Macián (1994)   |
|------------------------------|--|---|---|--|---|
| <b>Analysis Method</b>       | Irritation Index is calculated using the formula: $(301 - \text{sec H})/300 * 5 + (301 - \text{sec L})/300 * 7 = (301 - \text{sec C}) * 9$ ; where H=Hemorrhage, L=Lysis; C=Coagulation; sec=starting second | Two different analyses were used. Both calculated an irritation index using time (seconds) of appearance of hemorrhage vascular lysis, or coagulation. The differences between the two methods was in the particular calculations used. | Scores are totaled to give a single value (maximum of 21). Mean value of 4 eggs calculated for final value. | Calculation of an Irritation Score for each egg. Mean value of individual Irritation Scores calculated.  | Irritancy Potential calculated using the formula: $(301 - \text{sec H})/300 * 5 + (301 - \text{sec v})/300 * 7 = (301 - \text{sec C}) * 9$ ; where H=Hemorrhage, V=vasoconstriction; C=Coagulation of protein or blood; sec=starting second |
| <b>Classification Scheme</b> | Not Noted  | Not Noted   | Not Noted   | <b>Practically None:</b> 0-0.9; <b>Slight Irritation:</b> 1-4.9; <b>Moderate Irritation:</b> 5-9.9; <b>Strong Irritation:</b> 10-21                  | <b>Practically None:</b> 0-0.9; <b>Slight Irritation:</b> 1-4.9; <b>Moderate Irritation:</b> 5-8.9; <b>Strong Irritation:</b> 9-21  |
| <b>GLP Compliance?</b>       | Not Noted  | Not Noted   | Not Noted   | Not Noted  | Not Noted   |
| <b>Notes</b>                 |  | Cites Luepke (1985) as basis for protocol used  | Cites Luepke and Kemper (1986) as basis for protocol  | Cites Luepke (1985) as basis for scoring scheme  | Cites Ergatt/Frame Data Bank (1990) as basis for protocol and analysis method   |
|                              |  |   |   | To study the effects of slow acting materials, CAM scored 15 and 30 minutes after application on range from 0 (no reactions) to 3 (strong reactions) |   |

|                                    | Balls et al. (1995) (Non-Transparent Substances) | Balls et al. (1995) (Transparent Substances) | Kojima et al. (1995)  | Gettings et al. (1996) (HET-CAM I, II) | Gettings et al. (1996) (HET-CAM III)  |
|------------------------------------|--|--|---|--|---|
| <b>Hen Strain</b>                  | Not Noted  | Not Noted                                    | Not Noted   | Lohmann's Selected White Leghorn       | White Leghorn   |
| <b>Egg Criteria for Use</b>        | Not Noted  | Not Noted                                    | Not Noted   | Not Noted                              | Not Noted   |
| <b>Egg Storage (Prior to use)</b>  | Not Noted  | Not Noted                                    | Not Noted   | Not Noted                              | Not Noted   |
| <b>Incubation Temperature (°C)</b> | Not Noted  | Not Noted                                    | 37.6  | Not Noted                              | 38 ± 0.5  |
| <b>Relative Humidity (%)</b>       | Not Noted  | Not Noted                                    | about 70  | Not Noted                              | 60 ± 5  |
| <b>Egg Rotation?</b>               | Not Noted  | Not Noted                                    | Yes, once per hour  | Not Noted                              | Yes   |
| <b>Checking Egg Viability</b>      | Not Noted  | Not Noted                                    | Not Noted   | Not Noted                              | Candled on Day 9 and returned to incubator in vertical position                     |
| <b>Incubation Period</b>           | 9 Days   | 9 Days                                       | 10 Days   | 10 Days                                | 10 Days   |
| <b>Procedure for Opening Egg</b>   | Not Noted  | Not Noted                                    | A portion of the egg shell was removed and a drop of water was placed onto the shell membrane to avoid bleeding. Then the CAM was exposed with forceps. | Not Noted                              | Shell and inner shell membrane were removed around the area defined by the air cell |
| <b>Manipulation of CAM</b>         | Not Noted  | Not Noted                                    | Not Noted   | Not Noted                              | Not Noted   |

|  | Balls et al. (1995) (Non-Transparent Substances)  | Balls et al. (1995) (Transparent Substances)  | Kojima et al. (1995)                                       | Gettings et al. (1996) (HET-CAM I, II)  | Gettings et al. (1996) (HET-CAM III)                 |
|--|---|---|--|---|--|
| <b>Investigator Defined Test Substance Classes</b> | Chemicals selected from the ECETOC database (acids, acyl halides, alcohols, aldehyde, alkalis, esters, heterocyclics, hydrocarbons, inorganic chemicals, ketones, organophosphates, pesticides, surfactants, misc.) | Chemicals selected from the ECETOC database (acids, acyl halides, alcohols, aldehyde, alkalis, esters, heterocyclics, hydrocarbons, inorganic chemicals, ketones, organophosphates, pesticides, surfactants, misc.) | Surfactants, solvents, formaldehyde                        | Surfactant-based personal care formulations   | Surfactant-based personal care formulations          |
| <b>Total Test Substances Evaluated</b>             | 59  | 59  | 24   | 25  | 25   |
| <b>Test Substance Quantity or Volume</b>           | Not Noted   | Not Noted   | 0.2 mL   | 0.3 mL  | 0.1 mL   |
| <b>Test Substance Concentrations Tested</b>        | Not Noted   | Not Noted   | 10% solution   | 3 concentrations tested: threshold concentration, 10% solution, undiluted                 | 10% solution   |
| <b>Application of Solids to CAM</b>                | Not Noted   | Not Noted   | All tested substances appear to be solubilized             | All test substances appear to be in liquid form   | All test substances appear to be in liquid form      |
| <b>Preferred Solvent</b>                           | Not Noted   | Not Noted   | Not Noted  | Not Noted   | Not Noted  |
| <b>Rinse after Test Substance Application?</b>     | After 3 minute exposure   | Not Noted   | Yes, after 20 seconds test substance was rinsed with water | Not Noted   | After 20 seconds, with a saline rinse                |
| <b>Evaluation Period</b>                           | Within 30 seconds of rinsing  | Up to 300 seconds after test substance applied  | At 0.5, 2, and 5 minutes after test substance rinsed       | Up to 300 seconds after test substance applied  | At 0.5, 2, and 5 minutes after test substance rinsed |
| <b>Controls and Test Standards</b>                 | 5% Texapon AV (internal reference standard)   | 5% Texapon AV (internal reference standard)   | Not Noted  | Not Noted   | Not Noted  |
| <b>Number of Control Eggs</b>                      | Not Noted   | Not Noted   | Not Noted  | Not Noted   | Not Noted  |
| <b>Replicate Eggs</b>                              | 6 eggs  | 6 eggs  | 4 eggs   | 3 eggs per concentration tested   | Not Noted  |
| <b>Number of Replicate Experiments</b>             | Not Noted   | Not Noted   | Not Noted  | Not Noted   | Not Noted  |
| <b>Endpoints Assessed</b>                          | Hemorrhage, lysis, coagulation  | Hemorrhage, lysis, coagulation  | Hyperemia, hemorrhage, coagulation                         | Hemorrhage, lysis, coagulation  | Dilation, hemorrhage, coagulation                    |
| <b>Endpoint Evaluation</b>                         | Endpoints scored from 0 (no reaction) to 3 (strong reaction)  | The starting second that each of the endpoints is observed is recorded  | Not Noted  | The starting second that each of the endpoints is observed is recorded (see Kalweit 1990) | Numerical time-dependent scores for three endpoints. |

|                              | <b>Balls et al. (1995) (Non-Transparent Substances)</b>  | <b>Balls et al. (1995) (Transparent Substances)</b>  | <b>Kojima et al. (1995)</b>   | <b>Gettings et al. (1996) (HET-CAM I, II)</b>  | <b>Gettings et al. (1996) (HET-CAM III)</b>                                  |
|------------------------------|--|--|---|--|--|
| <b>Analysis Method</b>       | Calculation of "S Score". "S Score" is calculated using the most sensitive endpoint (endpoint can change from chemical to chemical). The scores recorded for the most sensitive endpoint are summarized for the 6 eggs. (Maximum for 6 eggs is 18) | Computer program calculates an Irritation Index. Irritation Index Is used to calculate a "Q Score". "Q Score" is a comparison of the Irritation Index of a test chemical with that of the reference chemical. If the effect of test chemical is identical to reference, Q is 1.0. If effect of test chemical is less irritating, Q is lower. If effect of test chemical is more irritating, Q is higher. | Score was calculated based on the time of onset for each endpoint. Mean value of 4 eggs calculated. | Mean Irritation Score (IS; determined at 10% concentration) of 3 eggs is calculated. The Irritation Threshold Concentration (ITC; lowest concentration producing a slight reaction during observation period) is calculated. | Time-dependent scores were used to calculate a single value (maximum of 21). |
| <b>Classification Scheme</b> | <b>Non Irritation:</b> $S < 6$ ; <b>Moderately Irritating:</b> $6 \leq S < 15$ ; <b>Severely Irritating:</b> $S \geq 15$   | <b>Non Irritating:</b> $Q < 1.5$ ; <b>Moderately Irritating:</b> $1.5 < Q < 2.0$ ; <b>Severely Irritating:</b> $Q < 2.0$   | Not Noted   | <b>Irritant (According to FHSA):</b> $IS \geq 5.1$ or $IS/ITC \geq 3.0$  | <b>Irritant (According to FHSA):</b> Score $\geq 4.83$                       |
| <b>GLP Compliance?</b>       | Yes  | Yes  | Not Noted   | Not Noted  | Not Noted  |
| <b>Notes</b>                 |  |  | Cites Luepke (1985) and Luepke and Wallat (1985) as basis for scoring scheme and analysis method.   |  |  |
|                              |  |  |   |  |  |

|                                    | Gilleron et al. (1996)   | Spielmann et al. (1996) | Gilleron et al. (1997)                                  | Hagino et al. (1999)  |
|------------------------------------|--|-------------------------|---|---|
| <b>Hen Strain</b>                  | White Essex  | Not Noted               | White Essex   | White Leghorn   |
| <b>Egg Criteria for Use</b>        | Eggs were 7 days old prior to start of incubation and weighed $60 \pm 5$ g   | Not Noted               | Eggs were 7 days old                                    | Not Noted   |
| <b>Egg Storage (Prior to use)</b>  | Not Noted  | Not Noted               | Not Noted   | Not Noted   |
| <b>Incubation Temperature (°C)</b> | $37.0 \pm 0.5$   | Not Noted               | $37 \pm 0.5$  | 37.6  |
| <b>Relative Humidity (%)</b>       | $62.5 \pm 1.5$   | Not Noted               | $52.5 \pm 2.5$  | about 70  |
| <b>Egg Rotation?</b>               | Yes, with large ends upward for 9 days   | Not Noted               | Not Noted   | Yes, once an hour   |
| <b>Checking Egg Viability</b>      | Not Noted  | Not Noted               | Not Noted   | Not Noted   |
| <b>Incubation Period</b>           | 10 Days  | Not Noted               | 10 Days   | 10 Days   |
| <b>Procedure for Opening Egg</b>   | Eggs were candled and non-viable eggs were discarded. The airspace delimited by the inner membrane at the large end of the egg was marked. The eggshell was removed using a dentist's rotating saw blade. The inner membrane was moistened with 1.5-2.0 mL of 0.9% NaCl and the egg was returned to the incubator (at 37) for a maximum of 20 mins. After incubation, the NaCl solution was removed, using a vacuum pump, and the inner membrane was removed with forceps. | Not Noted               | Not Noted   | Portion of the egg shell above the air space was removed.   |
| <b>Manipulation of CAM</b>         | A test substance applicator (TSA), which is comprised of a perlon mesh (pore diameter = 63 micron) locked between two Teflon rings, was placed on the CAM  | Not Noted               | A test substance applicator (TSA) was placed on the CAM | Drop of water is placed on the membrane (to avoid capillary bleeding). A silicone rubber ring is placed on the CAM. |

|  | Gilleron et al. (1996)   | Spielmann et al. (1996)   | Gilleron et al. (1997)   | Hagino et al. (1999)  |
|--|--|---|--|---|
| <b>Investigator Defined Test Substance Classes</b> | Chemicals from various chemical classes                                | Chemicals from various chemical classes   | Chemicals from various chemical classes                                | Surfactant, polyols, color additives, organic salts, PABA derivative, esters, inorganic chemicals, alcohols, amines, alkanolamines, carboxylic acids            |
| <b>Total Test Substances Evaluated</b>             | 46   | 200   | 60   | 39  |
| <b>Test Substance Quantity or Volume</b>           | 0.3 mL or 0.3 g of test substance placed inside the TSA                | Not Noted   | 0.3 mL or 0.3 g of test substance placed inside the TSA                | 0.2 mL (placed inside the rubber ring on the CAM). Solids (0.2 g) are reduced to a fine powder with a No. 200 sieve and placed inside a rubber ring on the CAM. |
| <b>Test Substance Concentrations Tested</b>        | Undiluted  | 1-100% solutions tested   | Not Noted  | 0.1, 1, 10, and 100%  |
| <b>Application of Solids to CAM</b>                | Placed inside TSA  | All tested substances appear to be solubilized  | Placed inside TSA  | Placed inside rubber ring   |
| <b>Preferred Solvent</b>                           | 0.9% NaCl  | 0.9% NaCl or olive oil  | Not Noted  | Not Noted   |
| <b>Rinse after Test Substance Application?</b>     | TSA (which contains the test substance) is removed after 20 seconds    | Yes, after 5 minutes (for substances that were insoluble in water or oil and were colored). | TSA (which contains the test substance) is removed after 20 seconds    | Yes, after 20 seconds with distilled water  |
| <b>Evaluation Period</b>                           | Up to 300 seconds after test substance applied                         | Up to 300 seconds after test substance applied  | Up to 300 seconds after test substance applied                         | At 0.5, 2, and 5 minutes after test substance applied   |
| <b>Controls and Test Standards</b>                 | Positive Controls: benzalkonium chloride, dimethylformamide, imidazole | Not Noted   | Not Noted  | Not Noted   |
| <b>Number of Control Eggs</b>                      | Not Noted  | Not Noted   | Not Noted  | Not Noted   |
| <b>Replicate Eggs</b>                              | 3  | Not Noted   | Not Noted  | 4 eggs  |
| <b>Number of Replicate Experiments</b>             | 3  | Not Noted   | Not Noted  | Not Noted   |
| <b>Endpoints Assessed</b>                          | Hemorrhage, lysis, coagulation   | Hemorrhage, coagulation, lysis  | Hemorrhage, lysis, coagulation   | Hyperemia, hemorrhage, coagulation  |
| <b>Endpoint Evaluation</b>                         | The starting second that each of the endpoints is observed is recorded | The starting second that each of the endpoints is observed is recorded                      | The starting second that each of the endpoints is observed is recorded | Numerical time-dependent scores for three endpoints.  |

|                              | Gilleron et al. (1996)  | Spielmann et al. (1996)   | Gilleron et al. (1997)   | Hagino et al. (1999)                          |
|------------------------------|---|---|--|---|
| <b>Analysis Method</b>       | Irritation Index calculated using the formula: $(301\text{-sec H})/300*5+(301\text{-sec L})/300*7=(301\text{-sec C})^*9$ ; where H=Hemorrhage, L=Vessel Lysis; C=Coagulation; sec=starting second. Mean of 3 assays were calculated. Reproducibility also was assessed. | Irritation Score calculated ( $(301\text{-sec H})/300*5+(301\text{-sec L})/300*7=(301\text{-sec C})^*9$ ; where H=Hemorrhage, L=Lysis; C=Coagulation; sec=starting second) for 10% solution calculated (IS10). Irritation Threshold (ITC; lowest concentration of a test substance to induce an irritant reaction on the CAM) calculated.   | Irritation Index calculated using the formula: $(301\text{-sec H})/300*5+(301\text{-sec L})/300*7=(301\text{-sec C})^*9$ ; where H=Hemorrhage, L=Vessel Lysis; C=Coagulation; sec=starting second. Mean value and SEM of are calculated. | Not Noted                                     |
| <b>Classification Scheme</b> | <b>Non Irritant:</b> 0-4.9; <b>Irritant:</b> 5.0-21   | <b>BGA Classification Model: Non/Slight:</b> ITC > 10 and IS10 <16; <b>Moderate:</b> (1) ITC > 16 and IS10 > 16 or (2) ITC < 10 and IS10 < 16; <b>Irritant (R36):</b> (1) ITC < 10 and IS10 > 16 or (2) ITC < 2.5 and IS10 < 16; <b>Severe (R41):</b> (1) ITC ≤ 1% or (2) ITC between 1 and 2.5% and IS10 ≥ 16. <b>Proposed model using mtc10 (see notes below): R41:</b> mtc10 < 50 seconds. | <b>Non Irritant:</b> 0-4.9; <b>Irritant:</b> 5.0-21  | Not Noted                                     |
| <b>GLP Compliance?</b>       | Not Noted   | Yes   | Not Noted  | Not Noted                                     |
| <b>Notes</b>                 |   | Cites Luepke (1985) as basis for protocol. Cites Kalweit (1990) and Spielmann (1991) as publishing the protocol used. Cites the standard protocol for the test method as InVitox Protocol.  | Cites Gilleron 1996 as basis for protocol and analysis method  | Cites Luepke 1985 as basis for scoring method |
|                              |   | Nine additional endpoints were conducted using the raw data and the IS and IT scores in this analysis. Of these the endpoints evaluated, that best correlated with in vivo classification was mtc10 (mean detection time for appearance of coagulation when using a 10% solution).  |  |   |

Abbreviations: CAM = Chorioallantoic membrane, ECETOC = European Centre for Ecotoxicology and Toxicology of Chemicals, FHSA = Federal Hazardous Substances Act (1964), IS = Irritation score, IT = Irritation threshold concentration, NaCl = Sodium chloride, NaOH = Sodium hydroxide, SDS = Sodium dodecyl sulfate, Sec = seconds.