The Skin Compatibility of Distilled Tall Oils: Evaluation With the Bovine Udder Skin *In Vitro* Model System

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Summary — Distilled tall oil (DTO) is a natural product, often added as an emulsifying ingredient in cutting fluids used as lubricants and coolants in metal working. The *in vitro* model used to test the skin compatibility of these substances, was the isolated perfused *ex vivo* bovine udder skin (BUS) model. After three exposure periods (0.5, 1, and 5 hours), cytotoxic effects were determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and tissue levels of the pre-inflammatory mediator prostaglandin E_2 (PGE_2) in treated whole skin biopsies were assessed by using an enzyme immunoassay. The BUS standard study design, involving a single application, was previously developed to investigate the skin irritation potential of cosmetics and chemicals. In the current study, four different batches of undiluted DTO, and tall oil fatty acids as a reference compound, were applied both singly and repeatedly (three times), under open conditions which were in line with the potential usage conditions in the work place. Under the standardised single application conditions, no major differences in cytotoxic effects or PGE_2 levels between the samples were apparent, so no indication of a skin irritation potential could be concluded. This result is in accordance with prior *in vivo* studies for acute dermal toxicity. Under repeated application conditions, signs of cytotoxicity were observed after the application of one of the DTO samples, which was known to be derived from different raw materials. Therefore, it was concluded that, generally, the presence of DTO at a concentration of up to 10% in cutting fluids, is not expected to result in any DTO-related deterioration of the skin.

Key words: *BUS model, coolant, distilled tall oil, DTO,* in vitro*, MTT, PGE_2, skin compatibility, tall oil fatty acids*.

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Introduction

Cutting fluids are complex preparations that are used as process chemicals for the purpose of cooling, lubrication and swarf removal during metal cutting and forming. The main risk associated with repeated skin contact with cutting fluids, is the occurrence of industrial dermatitis among metalworkers (e.g. grinders and lathe, milling and drilling machine operators; 1, 2). Distilled tall oil (DTO) is a common component of cutting fluids, as it forms salts which are excellent natural emulsifiers, which reduce the need for synthetic surfactants. Tall oils, which are by-products of the wood pulp industry, are also used as ingredients in cleansing agents, emulsifiers, adhesives, paints and fungicides.

In recent years, workers' compensation funds, as well as the manufacturers, have taken the initiative and have searched intensively for ways of improving the compatibility of these cutting fluids with the respiratory passages and the skin. Numerous authors have published results concerning their skin compatibility, and have outlined details of the relevant methods used (3–5). Most of the existing data are derived from *in vivo* standard methods (i.e. studies with volunteers and animal testing). The variety of test methods does not usually allow any standardised comparisons of, for example, fresh and used products, or water-miscible and straight (i.e. non-emulsifiable) cutting fluids, nor of single ingredients, for possible skin irritation risks (5). An important feature of cutting fluids is the open (i.e. unprotected) exposure of workers to the substances during their routine use in the workplace (6). Therefore the study design should allow for the repeated open application of the test materials to the skin model.

In the current study, the skin compatibility of different types of undiluted DTO was tested by using the isolated perfused bovine udder skin (BUS) model. This model had been introduced previously as an *in vitro* model for use in studies of transdermal penetration and absorption, and of local tolerance of topically-administered compounds under open and occlusive exposure conditions (7–10). Fresh and used coolants (water miscible and non-water miscible) have been previously investigated with the same model, with regard to their potential skin incompatibility (11).

Materials and Methods

DTO

DTO (CAS No. 8002-26-4) is composed of fatty acids and resin acids, with some additional neutral materials. It is an intermediate cut in the distillation of crude tall oil, a by-product of wood pulp production. Typically, DTO comprises approximately 30% resin acids, 65% fatty acids and 5% neutrals. It is related to, but not chemically identical to, colophony (rosin), in which the resin acids are considerably different. The DTO distillation fraction contains a high content of resin acids, and thus is usually the lowest product in the final fatty acid fractionation column. Alternatively, it is taken as a side-stream between the fatty acid and rosin cuts. The utility of DTO fatty acids in cutting fluid is due to their long hydrocarbon chains (C18), the acid functionality of their carboxyl groups (–COOH), and the unsaturation of their double bonds. The presence of resin acids, among other substances, in the DTO is also an important characteristic in relation to its use in the cutting fluid. The resin acids add "cling" and binding properties that cannot be obtained with any other fatty acid type, making DTO a highly-suitable raw material for metal working fluids. The typical physical properties are: acid value = 185; viscosity (25°C) = 100mPa.s; and density (25°C) = 940kg/m^3. For this study, four different batches of DTO (Arizona Chemical, Sandarne, Sweden) were tested, and a tall oil fatty acids sample was used as a reference for comparison (Table 1). The tall oil fatty acids sample contained a higher level of fatty acids and a lower resin acid content than any of the DTO batches tested.

The BUS model

The BUS model was originally developed for use in pharmaceutical research, and is also used in the cosmetic and chemical industries for the testing of safety and efficacy (12–15). In the field of industrial occupational health, the model is used to demonstrate the skin compatibility of metal cutting fluids and the efficacy of skin protection products (11, 16). As no live animals are used in this *ex vivo* model, it is possible to test both fresh and used process chemicals, regardless of the degree of dilution and potential biocide content (11). In order to reflect the likely exposure to DTO-containing products in the workplace, the samples were applied directly to the skin under open exposure conditions. The cow udders were freshly obtained from a slaughterhouse, then cleaned and shaved in the laboratory. The test substances were applied after 1 hour of aerobic adaptation of the skin's metabolism by perfusion with oxygenated Tyrode's solution at 38.5°C. Under continuous perfusion, providing a skin surface temperature of 30–31°C, the udder (mammary gland), including the skin, was viable for more than 8 hours, during which the horny layer barrier (and reservoir) and the skin metabolism remained active. A detailed description of this methodology is given in Kietzmann *et al.* (7) and in Bäumer & Kietzmann (10). The hirsute skin on the udder side of the tissue has been proven to be histologically and functionally similar to human skin (18, 19).

The undiluted DTO samples (3–4ml/100cm^2) were applied to four udder samples, under open exposure conditions, either once or three times over a 20-minute period. Punch biopsies (6mm diameter; Stiefel, Offenbach, Germany) of all the skin layers were carried out after exposure times of 0.5, 1 and 5 hours, in order to evaluate the possible time-dependence of skin irritancy and cell damage. The skin samples were taken from treated and untreated areas and stored temporarily at –20°C, until required for further analysis.

Further details of the analytical methods, which are routinely used in *in vitro* methodology, are described in the literature (7–9, 11). An MTT assay (μg formazan/net weight) performed with skin biopsies was carried out, in order to evaluate any cytotoxic effects of the DTO samples on the tissue. Only undamaged, active mitochondria are able to reduce MTT, to form the water-insoluble formazan complex (8). The cell irritancy potential of the samples was determined by measurement of the eicosanoid concentration (ng PGE_2/net weight) in the tissue (17). In previous studies, it was shown that these assays could detect the tissue-irritating

Table 1: Composition of test materials

Distilled tall oil (DTO) product	Unsaponifiables (neutrals)	Fatty acids	Resin acids
DTO 1	~ 3%	~ 70%	~ 27%
DTO 2	~ 5%	~ 65%	~ 30%
DTO 3	~ 3%	~ 87%	~ 10%
DTO 4	~ 5%	~ 66%	~ 30%
Tall oil fatty acids	~ 1%	~ 96%	< 2%

potential of a number of known irritant compounds, e.g. sodium lauryl sulfate (SLS; 8, 12).

Evaluation

Depending on the strength of the irritant, a decreasing cell viability and an increased prostaglandin E_2 (PGE_2) concentration in the skin tissue could be observed. The irritation potential of a test substance was also defined as a comparable numerical quantity, by taking the mean measurement data and expressing the sample results as a percentage of the untreated skin result, according to the following equation:

$$100 - [(V_c - V_s) \times 100]$$

where V_c = the mean result obtained with the untreated skin; and V_s = the mean result obtained with the treated skin.

This calculated value was then used to obtain a weighted 'score' (see Table 2, column 2 and row 2). The scores were weighted to allow for the fact that total cytotoxic effects are determined by using the MTT assay, whereas the PGE_2 assay only recognises a small proportion of any inflammatory effect. PGE_2 is just one of the many mediators of the tissue inflammatory response. The weighted scores were obtained for the MTT assay (Table 2, column 2) and the PGE_2 determination data (Table 2, row 2) from each time-point, and a combined, weighted total score (MTT + PGE_2) for each exposure time was subsequently determined by reading across and down, into the body of the Table. A total score of approx. 3.0 was taken as the 'skin critical level' which indicated skin incompatibility.

The total score figures obtained by using the standardised BUS test for skin compatibility (single application) were directly comparable to printed or online values (11, 15, 17). In contrast, repeated application provoked very complex conditions which were difficult to compare, due to evaporation effects of the topically-applied formulation (13).

Results

The perfusion of the four udders was technically straightforward, and was performed strictly according to the study plan. The comparison of the results from the MTT assay and the PGE_2 determination of the 'untreated' (control) skin with historical control data showed no major deviations, indicating that the viability of the skin used in this particular study remained within the historical range (Tables 3 and 4; 11, 15). Over the long perfusion period (5 hours), the number of viable cells (as determined by the MTT assay) slightly decreased (2–4%), whereas the tissue concentration of PGE_2 remained unchanged or, in fact, increased slightly (~ 4%).

The mean data used to obtain the MTT assay and PGE_2 determination 'score' values are presented in Table 3 (single application results) and Table 4 (repeated application results). Score values are compared between the single and repeated applications, after the three exposure times (0.5, 1,

Table 2: Calculation of the MTT, PGE_2, and (MTT + PGE_2) combined score values

	PGE_2	100	105	110	115	120	125	130	135	140	145
MTT	Score	0	0	0	1	2	3	4	5	6	7
100	0	0	0	0	1	2	3	4	5	6	7
95	0	0	0	0	1	2	3	4	5	6	7
90	1	1	1	1	2	3	4	5	6	7	8
85	2	2	2	2	3	4	5	6	7	8	9
80	3	3	3	3	4	5	6	7	8	9	10
75	4	4	4	4	5	6	7	8	9	10	11
70	5	5	5	5	6	7	8	9	10	11	12
65	6	6	6	6	7	8	9	10	11	12	13
60	7	7	7	7	8	9	10	11	12	13	14
55	8	8	8	8	9	10	11	12	13	14	15
50	9	9	9	9	10	11	12	13	14	15	16

From the mean measurement data, the sample results were expressed as a percentage of the untreated skin result. These results (column 1 for the MTT assay and row 1 for the PGE_2 determination) were correlated with a 'score' value (see column 2 for the MTT assay and row 2 for the PGE_2 determination data). A combined, weighted total score (MTT + PGE_2) for each exposure time was subsequently determined by reading across and down, into the body of the Table. A total score of 3.0 indicated the 'skin critical level' which indicated skin incompatibility.

Table 3: Results from the MTT assay and the PGE_2 determination for tissue treated with a single application of tall oils

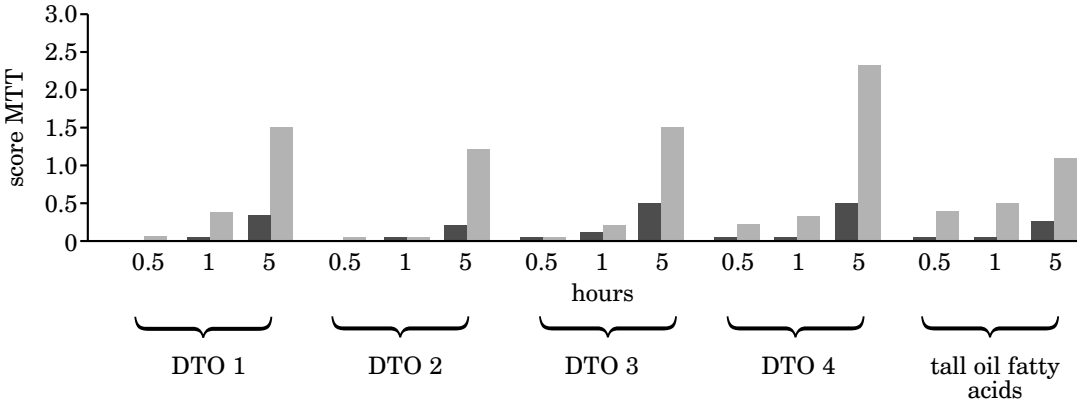
	MTT assay (μg formazan/net weight)	PGE_2 determination (ng PGE_2/net weight)
0.5-hour treatment		
Untreated (control)	1.07 ± 0.05	0.53 ± 0.03
Tall oil 1	1.05 ± 0.05	0.54 ± 0.01
Tall oil 2	1.04 ± 0.05	0.53 ± 0.02
Tall oil 3	1.02 ± 0.05	0.53 ± 0.02
Tall oil 4	1.06 ± 0.06	0.57 ± 0.02
Tall oil fatty acids	1.03 ± 0.05	0.53 ± 0.02
1-hour treatment		
Untreated (control)	1.05 ± 0.05	0.54 ± 0.04
Tall oil 1	1.02 ± 0.04	0.61 ± 0.05
Tall oil 2	1.01 ± 0.04	0.59 ± 0.01
Tall oil 3	1.00 ± 0.05	0.60 ± 0.02
Tall oil 4	1.02 ± 0.04	0.60 ± 0.01
Tall oil fatty acids	1.01 ± 0.05	0.57 ± 0.02
5-hour treatment		
Untreated (control)	1.04 ± 0.05	0.53 ± 0.03
Tall oil 1	0.97 ± 0.03	0.56 ± 0.02
Tall oil 2	0.98 ± 0.04	0.56 ± 0.02
Tall oil 3	0.96 ± 0.06	0.58 ± 0.01
Tall oil 4	0.96 ± 0.04	0.55 ± 0.02
Tall oil fatty acids	0.98 ± 0.05	0.54 ± 0.02

The results of the MTT assay (cytotoxicity determination) are expressed as μg formazan/net weight, and the tissue concentration of PGE_2 (measure of irritancy potential) expressed as ng PGE_2/net weight. The DTO samples were applied once and the exposure periods were 0.5, 1.0 and 5.0 hours. (Mean ± SD; n = 4).

and 5 hours), in Figure 1 (MTT assay), Figure 2 (PGE_2 determination), and Figure 3 (combined MTT + PGE_2 scores = total score).

The *cytotoxicity* parameter defines the loss of viable epidermal cells (basal and suprabasal cells), induced by the cytotoxic potential of the test sample after penetration of the horny layer (skin barrier/reservoir). A single application of the samples resulted in the penetration of very small proportion of the range of DTO components, causing slight cytotoxic effects after a 5-hour exposure (Figure 1). In comparison to the untreated site, there

Figure 1: Determination of the cytotoxicity of single and repeated applications of tall oils



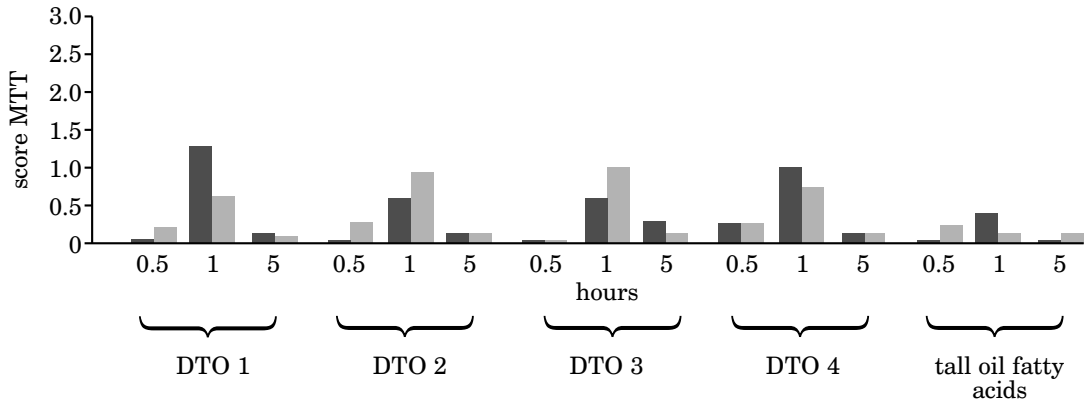
Cytotoxicity was determined by using the MTT assay. The DTO samples were applied to the tissue in a single application (■) or in repeated applications over a 20-minute period (■). Subsequent exposure times were 0.5, 1.0, or 5.0 hours, as indicated. The derivation of the MTT score values, is outlined in the text.

Table 4: Results from the MTT assay and the PGE_2 determination for tissue treated with repeated applications of tall oils

	MTT assay (µg formazan/net weight)	PGE_2 determination (ng PGE_2/net weight)
0.5-hour treatment		
Untreated (control)	1.04 ± 0.05	0.52 ± 0.03
Tall oil 1	1.02 ± 0.04	0.55 ± 0.01
Tall oil 2	1.01 ± 0.04	0.56 ± 0.01
Tall oil 3	1.00 ± 0.06	0.55 ± 0.02
Tall oil 4	0.98 ± 0.06	0.56 ± 0.01
Tall oil fatty acids	0.97 ± 0.05	0.55 ± 0.02
1-hour treatment		
Untreated (control)	1.02 ± 0.05	0.54 ± 0.01
Tall oil 1	0.94 ± 0.04	0.61 ± 0.02
Tall oil 2	0.98 ± 0.04	0.62 ± 0.01
Tall oil 3	0.97 ± 0.05	0.62 ± 0.02
Tall oil 4	0.96 ± 0.06	0.61 ± 0.03
Tall oil fatty acids	0.95 ± 0.06	0.58 ± 0.02
5-hour treatment		
Untreated (control)	1.00 ± 0.04	0.54 ± 0.02
Tall oil 1	0.88 ± 0.05	0.58 ± 0.02
Tall oil 2	0.89 ± 0.04	0.59 ± 0.01
Tall oil 3	0.88 ± 0.03	0.58 ± 0.01
Tall oil 4	0.83 ± 0.04	0.56 ± 0.03
Tall oil fatty acids	0.89 ± 0.05	0.56 ± 0.02

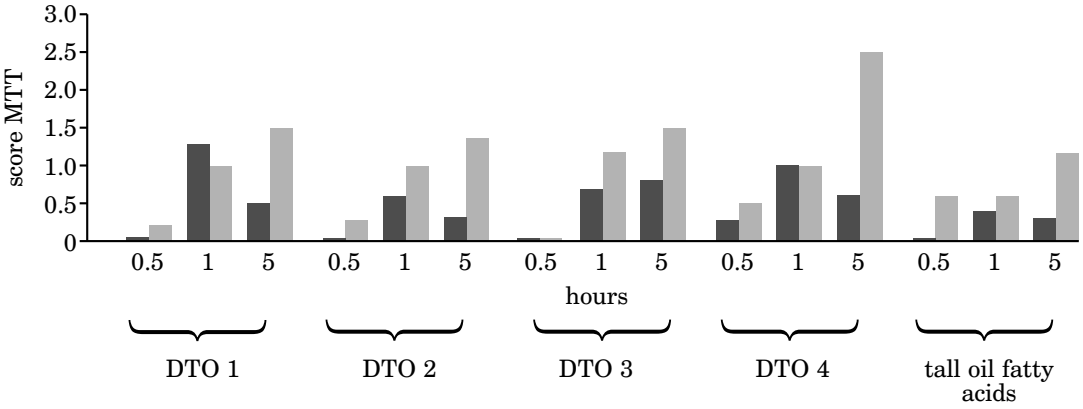
The results of the MTT assay (cytotoxicity determination) are expressed as µg formazan/net weight, and the tissue concentration of PGE_2 (measure of irritancy potential) expressed as ng PGE_2/net weight. The DTO samples were applied three times over a 20-minute period, then incubated for 0.5, 1.0 and 5.0 hours, as indicated. (Mean ± SD; n = 4).

Figure 2: Determination of the irritancy potential of single and repeated applications of tall oils



Irritancy potential was determined by measuring the tissue concentration of PGE_2. The DTO samples were applied to the tissue in a single application (■) or in repeated applications over a 20-minute period (■). Subsequent exposure times were 0.5, 1.0, or 5.0 hours, as indicated. The derivation of the PGE_2 score values, is outlined in the text.

Figure 3: The combined cytotoxicity and irritancy potential of single and repeated applications of tall oils



Cytotoxicity was determined by using the MTT test, and irritancy potential was determined by measuring the tissue concentration of PGE_2. The DTO samples were applied to the tissue in a single application (■) or in repeated applications over a 20-minute period (■). Subsequent exposure times were 0.5, 1.0, or 5.0 hours, as indicated. The derivation of the (MTT + PGE_2) score values, is outlined in the text.

was difference of about 3% or less. There were also no apparent differences in the effects of the five samples, following the single applications. After the repeated applications, the cytotoxicity results were slightly different. DTO samples 1–3 were cytotoxic to a very similar degree, resulting in a 12% change relative to untreated skin. The sample which had the greatest cytotoxic effect was DTO sample 4 (a 17% difference, relative to untreated control); the tall oil fatty acids were the least cytotoxic (Figure 1).

The *cell irritancy* parameter defines the increase in the production of a certain pre-inflammatory mediator by the tissue, as a result of the inflammation-inducing potential of the test sample after penetration of the horny layer (skin barrier/reservoir). In the standardised study design, as used here, the peak of the tissue concentration of PGE_2 is usually reached after an exposure period of 1 hour, and subsequently decreases toward the 5-hour exposure period (unpublished results, 11, 14, 15). These results were confirmed by an *in vivo* study, in which the initial increase in the concentration of tissue PGE_2 is followed by a decrease (20). This pattern was generally seen with both the types of application used in the current study (Figure 2). The PGE_2 score of 1.0 was assigned to a 15% difference in PGE_2 concentration, relative to the untreated control. For the single applications, the overall trend for the four DTO samples and the tall oil fatty acids reference sample was a peak in PGE_2 concentration at the 1-hour time-point, as outlined above. Following repeated applications, the induction patterns for the four DTO samples were very similar to those for the single applications, but a rather different induction pattern (and the lowest PGE_2 level) was obtained after the repeated application of the tall oil fatty acids reference sample (Figure 2).

The total score consists of the MTT score plus the PGE_2 score (Figure 3). In the standardised BUS test for skin compatibility (single application), any total score approaching the value of 3.0 after any exposure period, is taken to indicate a high probability of skin irritation after repeated skin contact during use. This conclusion is based on laboratory studies, comparisons of volunteer studies, and field experiences (11–16). In this particular study, none of the undiluted samples resulted in a combined score of more than 3.0 (skin critical level) after a single application, so excellent skin compatibility can be concluded for all the samples tested. In fact, the combined score level did not exceed 1.3 (DTO sample 1) in any case. The application of tall oil fatty acids did not induce any major cellular reactions (combined score, <0.5; untreated site combined score, 0.0).

Under repeated application conditions, DTO sample 4 gave a combined score value of 2.4, exceeding all the other results. This combined score was mainly the result of the increased cytotoxic properties of this sample (see Figure 1). DTO sample 4 was derived from a different source of raw material, which might explain the reason for this anomalous score value.

Discussion

The allergenic potentials and skin compatibilities of cutting fluid ingredients are important health

factors. The allergenic potential of DTO has been studied in the Guinea Pig Maximisation and Buehler skin sensitisation tests. The authors concluded that the risk of contact sensitivity induction by resin acids in workers exposed to tall oil-containing products like cutting fluids and cleansing agents was considered to be minimal (21–23).

To determine their skin compatibility, DTO products were previously tested in *in vivo* and *in vitro* studies performed in the authors' laboratories. In an acute dermal irritation study performed in 2000, the DTO products were considered to be non-irritant, as no adverse skin reactions were noted (24).

In the current study with the BUS test system, four further samples of different, defined batches of undiluted DTO samples and a tall oil fatty acids reference sample, were used to further study skin compatibility. The BUS model was used to determine the cytotoxic and irritant properties of the test samples after different exposure periods. In general, the *cytotoxicity* parameter dominates (albeit to a very low degree) the skin biology following application, and increases progressively throughout the exposure period. No signs of relevant irritant properties were obtained when considering this parameter.

Under the standardised single application conditions, it was concluded that all the samples showed excellent skin compatibility after repeated skin contact, as the total score values for the samples were similar to those of untreated areas (total score 0.0). These low total scores (maximum total score: 0.5–1.3) also indicated very good skin compatibility when compared with results for cutting fluids with known excellent skin compatibility, as judged from results obtained in previous systematic studies (11). The results obtained in this current *in vitro* study were also shown to correlate well with previously-obtained *in vivo* results (11). These authors also noted that the test results of this study were consistent with the results from the regulatory testing previously performed in the authors' laboratories, as outlined in Arizona Chemical internal report 2007-035 (23).

Following repeated application, DTO sample 4 gave a combined total score which exceeded the score values of the other samples. It is proposed that this difference was because this sample was derived from a different source of raw material. Nevertheless, in terms of skin compatibility evaluation, this marginal difference was deemed to be irrelevant.

The normal concentration of DTO in cutting fluids is approximately 5%. DTO has a very low solubility in water, so it is not possible to use the compound in cutting fluids at concentrations of greater than 10%. It is concluded that, when these low concentrations (5–10%) of DTOs are included in the formulation of cutting fluids, it is not expected that there will be any DTO-related deterioration of the skin after repeated contact.

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