



HUMANE SOCIETY
INTERNATIONAL

BRIEFING

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PRELIMINARY DRAFT COMMISSION REGULATION LAYING DOWN TEST METHODS PURSUANT TO REGULATION (EC) NO 1907/2006

For more than two decades, the EU has prohibited experiments on animals “if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available” (Article 7.2 of Council Directive 86/609/EEC).

Furthermore, Article 13 of REACH specifically directs that “in particular for human toxicity, information shall be generated whenever possible by means other than vertebrate animal tests, through the use of alternative methods...” and that “These methods shall be regularly reviewed and improved with a view to reducing experimentation on vertebrate animals and the number of animals involved. The Commission, following consultation with relevant stakeholders, shall, as soon as possible, make a proposal, if appropriate, to amend the test methods Regulation, and Annexes of this Regulation, if relevant, so as to replace, reduce or refine animal testing.”

With these considerations in mind, the following issues are raised in relation to the Annex (Parts B and C) to the draft Test Methods Regulation:

B.4 Acute Toxicity: Dermal Irritation/Corrosion

Action Requested: Delete Method B.4 and replace with a new test guideline for *in vitro* skin irritation based on the validated and ECVAM-endorsed EPISKIN™ Skin Irritation Test (EPISKIN-SIT).

Background: Corrosive agents cause irreversible tissue destruction, whereas irritants induce milder and reversible skin damage. Testing for skin corrosion/irritation is a first-tier requirement under EU regulations for industrial chemicals, plant protection products and biocides (i.e., Regulation (EC) No 1907/2006, Directive 91/414/EEC, and Directive 98/8/EC, respectively), which each apply to many thousands of substances. In addition, ingredients used in cosmetics and other preparations are also routinely tested to confirm their non-irritancy.

Several alternative methods have been accepted at EU-level and internationally as full replacements for animal tests for skin corrosivity. Among these is EPISKIN¹ – a multi-layered *in vitro* human skin model, in which cell viability over time is used as a measure of a chemical’s corrosive potential. Since April 2004, member countries of the Organisation for Economic Co-operation and Development (OECD) have accepted that positive results in EPISKIN™ are sufficient to classify a chemical as corrosive or severely irritating to the skin, and that negative results reflect that a chemical is not corrosive (i.e., OECD Test Guideline 431²). However, negative results are not generally accepted as evidence of non-*irritancy*; thus, negative results in EPISKIN™ are often subject to further testing in a rabbit skin irritation study.

Since the validation and endorsement of OECD TG 431, much effort has gone into the optimisation of EPISKIN™ to improve its accuracy in detecting milder irritants, and in distinguishing between mild and non-irritants. In 2003, ECVAM initiated an international, multi-laboratory validation study of the enhanced EPISKIN-Skin Irritation Test (EPISKIN-SIT), which determined that the new and improved test correctly predicted skin irritation potential for more than 90 per cent of chemicals tested.

¹ http://ecvam.jrc.it/publication/EPISKIN_statement.pdf

² <http://masetto.sourceoecd.org/vl=4395720/cl=13/nw=1/rpsv/ij/oecdjournals/1607310x/v1n4/s30/p1>

These validation results were subsequently forwarded to the ECVAM Scientific Advisory Committee (ESAC) for an independent peer review. Consequently, ESAC concluded in April 2007 that: *“the EPISKIN method showed evidence of being a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by MTT reduction, and for being used as a replacement ... for the Draize Skin Irritation Test (OECD TG 404 & Method B.4 of Annex V to Directive 67/548/EEC) for the purposes of distinguishing between R38 skin irritating and no-label (non-skin irritating) test substances.”*³

The reasonable and practicable availability of a full replacement method that has been validated and endorsed by ECVAM obviates the need for *in vivo* test guideline for skin irritation, and should be seen to render such testing unlawful in the EU. Accordingly, Method B.4 should be deleted from the Test Methods Regulation and replaced with a new test guideline for the EPISKIN-SIT.

B.42 Skin Sensitisation: Local Lymph Node Assay (LLNA)

Action Requested: Amend Method B.42 to incorporate the validated and ESAC-endorsed “reduced LLNA” methodology, which can reduce animal use by 50% relative to the conventional LLNA.

Background: Skin sensitisation is another first-tier testing requirement for all agricultural, biocidal and industrial chemicals and preparations. Given that full replacement measures are not yet available for this toxicity end point, every effort should be made to promote the use of test methods and strategies that consume the smallest number of animals, while generating information required for classification and labelling purposes.

Classical tests for skin sensitisation (i.e., the Guinea Pig Maximisation and Buehler tests) consume at least 30 guinea pigs and involve multiple, stressful injections. In contrast, the mouse Local Lymph Node Assay (LLNA) reduces animal use to 16 per test, and is significantly less invasive. The developers of the LLNA have undertaken a retrospective analysis of published data and concluded that within the context of a tiered testing strategy, a reduced version of the LLNA – using only a negative control group and the equivalent of the high-dose group from the conventional LLNA – is sufficient to distinguish between sensitisers and non-sensitisers.

These data and conclusions have been independently peer reviewed and endorsed by ESAC, which concluded in April 2007 *“that the peer reviewed and published information is of a quality and nature to support the use of the rLLNA within tiered-testing strategies to reliably distinguish between chemicals that are skin sensitisers and non-sensitisers, and that animal use can be minimised....”*⁴ Accordingly, this strategy should be appropriately reflected in Method B.42.

B.44 Skin Absorption: *In Vivo* Method

Action Requested: Delete Method B.44.

Background: Information concerning the rate and degree of chemical absorption through the skin is not required under REACH, but is occasionally requested during the final stages of occupational risk assessments of agrochemicals. Method B.45 describes internationally accepted *in vitro* methods for evaluating this end point based on OECD Test Guideline 428 of April 2004.⁵ To the limited extent that skin absorption studies are carried out, *in vitro* methods have long been the preferred testing approach in the EU. Moreover, the reasonable and practicable availability of internationally accepted alternative methods obviates the need for *in vivo* test guideline for skin absorption, and should be seen to render such testing unlawful in the EU. Accordingly, Method B.44 should not be included in the Test Methods Regulation.

³ http://ecvam.jrc.it/ft_doc/ESAC26_statement_SkinIrritation_20070525_C.pdf

⁴ http://ecvam.jrc.it/ft_doc/ESAC26_statement_rLLNA_20070525-1.pdf

⁵ <http://puck.sourceoecd.org/vl=2472946/cl=15/nw=1/rpsv/ij/oecdjournals/1607310x/v1n4/s27/p1>

(NEW) *In Vitro* Eye Irritation/Corrosion: Bovine Corneal Opacity and Permeability (BCOP) and Isolated Chicken Eye (ICE) Tests

Action Requested: Promulgate two new test guidelines for *in vitro* eye irritation based on the validated, ECVAM-endorsed, and widely accepted Bovine Corneal Opacity and Permeability (BCOP) and Isolated Chicken Eye (ICE) tests.

Background: Eye irritation is a first- or second-tier testing requirement for agricultural, biocidal and industrial chemicals. Such testing is also routinely carried out to confirm the non-irritancy of ingredients used in cosmetics and other preparations. Given that full replacement measures are not yet available for this toxicity end point, every effort should be made to promote the use of test methods and strategies that consume the smallest number of animals, while generating information required for classification and labelling purposes.

Among the many tests proposed as alternatives to eye irritation tests in living rabbits are the Bovine Corneal Opacity and Permeability (BCOP)⁶ and Isolated Chicken Eye (ICE)⁷ tests, in which corneas isolated from the eyes of slaughtered cattle and chickens, respectively, are exposed to chemicals *in vitro* to quantify irritant effects. Since 2004, EU regulatory authorities have accepted that positive results from the BCOP, ICE, and two other *in vitro* tests can be used as a basis for classifying and labelling substances as severe eye irritants pursuant to Directive 67/548/EEC on Dangerous Substances. This conclusion has since been endorsed by ESAC in April 2007⁸ on the basis of a formal, retrospective validation study. Accordingly, new test guidelines for the BCOP and ICE tests should be promulgated as a matter of priority under the Test Methods Regulation.

(NEW) Mutagenicity: *In Vitro* Micronucleus Test

Action Requested: Promulgate a new test guideline for mutagenicity based on the validated, ECVAM-endorsed, and legislatively accepted *in vitro* micronucleus test.

Background: Testing for genetic toxicity and mutagenicity is required for all agricultural, biocidal and industrial chemicals, as well as food and feed additives (including sweeteners and colours),⁹ flavourings,¹⁰ food contact materials,¹¹ and food enzymes. Such testing is also routinely carried out to confirm that ingredients used in cosmetics and other preparations do not possess mutagenic or genotoxic properties.

Regulatory confidence in the results of classical *in vitro* tests for mutagenicity and genotoxicity has been limited due to the implausible number of positive results they generate. Consequently, much attention has been paid to the creation of more accurate and human-relevant tests. In November 2006, ESAC endorsed the validity of such a method – the *in vitro* micronucleus test – for use in lieu of a more costly and technically demanding *in vitro* test.¹² Almost immediately, the test achieved legislative acceptance through its inclusion in the final REACH regulation, and is currently being developed into an OECD Test Guideline.¹³ However, despite being specifically listed in the REACH annexes, the *in vitro* micronucleus test does not yet appear in the Test Methods Regulation.

C.1 Acute Toxicity for Fish

Action Requested: Amend Method C.1 to incorporate ECVAM-endorsed “upper threshold concentration step-down approach,” which can reduce animal use by up to 73% relative to the conventional fish acute lethality study.

Background: Acute lethality testing on fish is required under REACH for all substances manufactured or imported in quantities of 10 metric tonnes or more per annum, consuming at least 42 animals per chemical tested. Yet despite the severity of the end point and the number of

⁶ http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_brd_bcop.htm

⁷ http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_brd_ice.htm

⁸ http://ecvam.jrc.it/ft_doc/ESAC26_statement_Organotypic_20070510_C.pdf

⁹ http://ec.europa.eu/food/fs/sc/scf/out98_en.pdf

¹⁰ http://www.efsa.europa.eu/EFSA/Scientific_Document/guidancedocument1.0.pdf

¹¹ http://www.efsa.europa.eu/EFSA/Scientific_Document/afc_noteforguidancecfcm_en1.0.pdf

¹² http://ecvam.jrc.it/publication/ESAC25_statement_MNT_20061128_C.pdf

¹³ <http://www.oecd.org/dataoecd/33/22/37865944.pdf>

animals consumed, fish data are seldom used as a basis for EU hazard classifications for acute aquatic toxicity. This is because classifications are based only on the *lowest* toxic concentration seen in aquatic plants (algae), single-celled water fleas (daphnia), or fish—and fish are usually the *least* sensitive of these species.¹⁴

Given the limited value of fish data and the desire to minimise vertebrate testing, scientists have proposed a tiered testing strategy for acute aquatic toxicity, whereby testing is first carried out on algae and daphnia to determine the lowest concentration of a chemical that is harmful to these species (called the “upper threshold concentration” or “UTC”). Then, instead of conducting a standard fish acute study (6 groups of 7 fish), only one group of fish is exposed to the UTC. If this concentration results no lethal effects on fish, testing is terminated and the chemical is classified based on the algae or daphnia results. Although this testing strategy does not fully replace testing of fish, a reduction of up to 73% is possible for chemicals of low toxicity.

The feasibility of the UTC strategy has been confirmed by ECVAM and the European Chemicals Bureau¹⁵ using a large database of industrial and agrochemicals. The strategy was then subjected to an independent scientific peer review by an *ad hoc* panel of the ECVAM Scientific Advisory Committee (ESAC), which concluded in March 2006: “*The Committee therefore recommends that the UTC approach should be implemented as a valid strategy to significantly reduce the number of fish used in the assessment of acute aquatic toxicity for hazard classification.*”¹⁶ Accordingly, this strategy should be appropriately reflected in Method C.1.

¹⁴ <http://cat.inist.fr/?aModele=afficheN&cpsidt=15369374>

¹⁵ http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WPT-4GC1RM9-1&_user=10&_coverDate=07%2F31%2F2005&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_acct=C000050221&_version=1&_urlVersion=0&_userid=10&md5=bd186a2114bcff9c13737cec7280cd

¹⁶ http://ecvam.jrc.it/ft_doc/ESAC_statement UTC_step_down_approach_20060515.pdf