Miniaturized standard Ames test for the evaluation of textile dyes

The Salmonella reverse mutation assay (Ames test, OECD 471) is a standard assay for the examination of mutagenic effects in chemicals, e.g. in the European REACh strategy. In the project 'Novel Sustainable Bioprocesses for European Color Industry' (Sophied, NMP2-CT-2004-505899) a fast mutagenicity test was needed for the screening of huge amounts of newly developed textile dye samples where only small sample volumes were available. The standard Ames test [1] was therefore compared with a miniaturized test version (mini Ames test). It could be shown, that the mini Ames test is a suitable test method for fast mutagenicity screening of colored samples. It needs less material and less time effort than the standard version and is therefore very cost effective.

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The traditional color industry was a major branch of industry in Europe until the end of the 20th century. Nowadays in Europe, a decline of business in favor of the developing world is taking place due to increasing environmental pollution control measures as well as high labor costs in Europe.

For the evaluation of the detoxification of wastewaters during the purification process, and of the toxicity of dye molecules and textile auxiliaries, several endpoints such as toxicity, ecotoxicity, and biodegradation/elimination have to be examined. One important factor in the evaluation process of textile dyes is the knowledge of mutagenic effects. Mutagenic dyes are a risk for the health of workers and consumers and for the environment. Especially workers can be exposed to high concentrations when preparing the recipes for the dyeing process. Mutagenicity of chemicals present on textiles can be seen as a major clue towards carcinogenic activities of these chemicals. Different researchers have identified mutagenic effects of textile dyes [2–5]. In a research project, 281 textile dye products were examined for available published and unpublished data [6–8]. 53 dye products not investigated so far for mutagenicity were selected for testing in the Ames test with *Salmonella typhimurium* following OECD 471. The results confirmed previous findings that there are dye products on the market which are not sufficiently tested and show mutagenic effects in in vitro tests. In addition, the new European Strategy of Chemistry, REACh [9], for Registration, Evaluation, and Authorization of Chemicals enhanced the constraints for the coloration industry. Moreover, during dyeing processes, approx. 10–40 % of the dyes are not bound on the substrate to which they are applied, and find their way into wastewaters.

The standard test version is performed with common Petri dishes. Previously, Flamand et al. [12] described the mini Ames test procedure as a screening test in the development of pharmaceuticals. This mini version uses 6-well micro-plates as shown in the picture, and a reduced test volume.

A reduction to one plate per strain and concentration is achieved instead of 6 Petri dishes in the standard plate incorporation assay. In the European Project developing 'Novel Sustainable Bioprocesses for European Color Industry' (Sophied, NMP2-CT-2004-505899) a fast mutagenicity test for the screening of huge amounts of colored samples with only small sample volumes was required. The standard Ames test [1] was compared with the mini Ames test version to investigate if this test system is also suitable to examine colored samples.

Results

Comparing investigations standard Ames test and mini Ames test

The mini Ames and the standard Ames test were performed in parallel with the two tester strains TA98 and TA 100 according to OECD 471 with three mutagenic control substances 2-aminoanthracene, nitrofluorene and sodium azide to determine if the mini Ames test is as sensitive as the standard version. The results achieved were quiet similar in both tests. In a few cases the mini Ames test shows lower sensitivity than the standard Ames (Table 1). Negative results should therefore be confirmed in the standard test version.

Mutagenicity testing of dyestuffs

12 textile dyes were tested in the mini Ames test with TA98 and TA100 with and without S9 activation in a concentration of 1,000 µg/plate (Table 2). Five dyes, Disperse Blue 1, Direct Blue 1, Direct Black 38, Acid Blue 62 and Acid Red 299, showed an effect which indicate that the dyes have to be classified as mutagenic in the Ames test. With some dyes

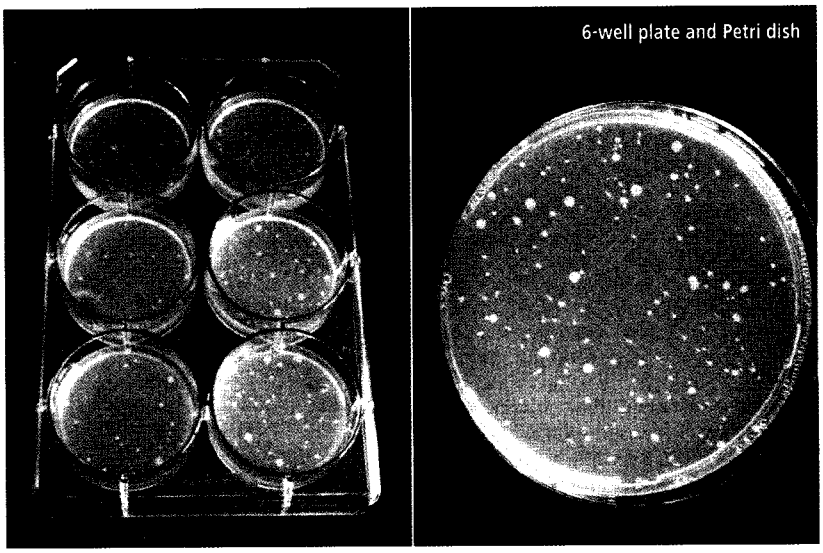

Table 1
Comparison of the dose-response relationship for positive control substances in the mini Ames test version and in the standard Ames test

Salmonella typhimurium strain TA98 with S9 activation

	mini Ames test							standard Ames test					
2-AA		first study			second study		2-AA		first study			second study	
conc µg/well	NR	RSD	IR	NR	RSD	IR	conc µg/plate	NR	RSD	IR	NR	RSD	IR
NC	15,3	52,3		6,3	32,9		NC	17,0	20,4		25,5	13,9	
NC-solvent	6,3	18,2		4,0	50,0		NC-solvent	17,5	20,2		22,0	22,7	
0,4	43,7	19,2	6,9	77,0	15,8	19,3	2,0	176,7	8,5	10,1	298,7	5,0	13,6
0,28	31,0	14,1	4,9	51,0	2,0	12,8	1,4	121,7	8,8	7,0	199,7	6,7	9,1
0,16	18,0	14,7	2,8	25,0	13,9	6,3	0,8	62,0	11,4	3,5	118,3	7,8	5,4
0,08	10,3	27,9	1,6	11,3	22,2	2,8	0,4	38,0	11,2	2,2	63,7	10,5	2,9
0,04	6,0	28,9	0,9	6,3	9,1	1,6	0,2	26,0	5,4	1,5	38,7	18,2	1,8
0,02	4,7	81,1	0,7	7,0	51,5	1,8	0,1	22,0	19,3	1,3	27,5	48,9	1,3

Salmonella typhimurium strain TA98 without S9 activation

	mini Ames test							standard Ames test					
NF		first study			second study		NF		first study			second study	
conc µg/well	NR	RSD	IR	NR	RSD	IR	conc µg/plate	NR	RSD	IR	NR	RSD	IR
NC	12,0	16,7		2,7	21,7		NC	14,7	23,9		24,0	41,2	
NC-solvent	4,3	48,0		2,0	50,0		NC-solvent	16,5	30,0		17,0	23,5	
0,3	24,0	22,0	5,5	16,3	33,7	8,2	1,50	141,0	5,1	8,5	177,3	6,2	10,4
0,2	20,0	5,0	4,6	12,7	38,9	6,3	1,00	88,0	17,8	5,3	125,7	25,9	7,4
0,14	12,7	39,7	2,9	15,7	19,5	7,8	0,70	68,0	16,6	4,1	98,7	15,2	5,8
0,1	8,0	12,5	1,8	4,0	25,0	2,0	0,50	37,5	13,2	2,3	66,3	0,9	3,9
0,05	6,0	16,7	1,4	5,7	50,9	2,8	0,25	36,5	44,6	2,2	39,7	8,1	2,3
0,025	3,0	66,7	0,7	3,7	56,8	1,8	0,125	22,0	19,3	1,3	23,0	30,7	1,4

Salmonella typhimurium strain TA100 with S9 activation

	mini Ames test							standard Ames test					
2-AA		first study			second study		2-AA		first study			second study	
conc µg/well	NR	RSD	IR	NR	RSD	IR	conc µg/plate	NR	RSD	IR	NR	RSD	IR
NC	11,7	40,5		10,7	46,2		NC	67,7	9,4		65,5	11,9	
NC-solvent	15,0	11,5		14,7	7,9		NC-solvent	64,0	8,8		91,0	11,9	
0,5	86,7	18,3	5,8	112,7	6,5	7,7	2,5	374,7	14,0	5,9	654,0	8,0	7,2
0,4	59,3	6,8	4,0	92,0	4,7	6,3	2,0	264,3	10,8	4,1	480,7	4,0	5,3
0,28	51,7	12,6	3,4	65,3	0,9	4,5	1,4	187,5	12,4	2,9	349,0	4,8	3,8
0,16	31,7	28,5	2,1	36,7	12,6	2,5	0,8	126,5	18,4	2,0	204,0	14,1	2,2
0,08	20,0	32,8	1,3	19,3	18,2	1,3	0,4	108,0	0,0	1,7	127,0	8,5	1,4
0,04	16,3	12,7	1,1	15,0	40,0	1,0	0,2	95,0	7,4	1,5	114,5	16,7	1,3

Salmonella typhimurium strain TA100 without S9 activation

	mini Ames test							standard Ames test					
SA		first study			second study		SA		first study			second study	
conc µg/well	NR	RSD	IR	NR	RSD	IR	conc µg/plate	NR	RSD	IR	NR	RSD	IR
NC	5,7	27,0		15,3	18,8		NC	66,7	9,2		78,5	13,5	
NC-solvent	12,3	12,4		12,3	54,0		NC-solvent	59,5	3,6		74,0	13,7	
0,50	97,0	7,2	7,9	76,3	16,0	6,2	2,5	448,3	18,3	7,5	450,0	13,7	6,1
0,30	52,0	16,8	4,2	52,3	17,2	4,2	2,0	234,0	11,6	3,9	369,3	7,3	5,0
0,10	27,3	11,2	2,2	39,0	29,1	3,2	1,4	142,5	3,5	2,4	186,7	18,0	2,5
0,07	27,0	7,4	2,2	19,3	15,8	1,6	0,8	128,5	11,6	2,2	154,7	9,5	2,1
0,05	22,3	5,2	1,8	19,7	46,1	1,6	0,4	101,0	2,8	1,7	127,0	9,1	1,7
0,01	15,3	7,5	1,2	13,3	45,8	1,1	0,2	77,0	1,8	1,3	78,3	10,3	1,1

2-AA: Aminoanthracene, NF: Nitrofluorene, SA: Sodium azide
NR: number of revertants (mean of n = 3)
RSD: relative standard deviation of the mean
IR: induction rate (number of revertants sample/number of revertants control)
NC: negative control, NC-solvent: solvent control DMSO
IR numerals in bold: IR ≥ 2, sample evaluated as mutagenic

such as Acid Blue 62 or Reactive Yellow 81 e.g. cytotoxic effects were observed. To confirm the results and to ensure that cytotoxicity does not mask mutagenic effects, all dyes were tested in a concentration range between 2,000 and 125 µg/plate.

By testing dose-response relationship in detail, comparable results were achieved as in the first test with 1,000 µg/plate. Acid Blue 62 showed a mutagenic effect in concentrations of 500 and 250 µg/plate, Reactive Yellow 81 was evaluated as not mutagenic.

Mutagenicity testing of waste water samples

Colored model waste water samples from different biotechnical purification procedures were tested before and after the purification process in the mini and standard Ames test version. It could be shown that the mini Ames test is also suitable to evaluate colored waste water samples.

Discussion

The Salmonella reverse mutation assay (Ames test, OECD 471) is a standard assay to detect mutagenic effects. During the development process of new chemicals a first screening on mutagenicity is essential to exclude substances from further research which do not meet the requirement of being non-mutagenic. In addition, there is often only a very little amount of the new chemical available or the substances are very expensive at this early stage. In recent years several attempts have been described to miniaturize the Ames test for a first high throughput screening on mutagenicity (e.g. Miniscreen test: [10], Ames II Mutagenicity Assay distributed by Xenometrics (www.xenometrics.ch, 2006), Ames Fluctuation Assay: [11–13]). Flamand et al. [12] describe a miniaturized Ames plate incorporation assay for a first screening for point mutagenesis assessment in the development of new chemicals. This protocol requires five times less reagents and is performed in six-well microwell plates. It is described as suitable for all tester strains, as well as those with low spontaneous mutation rates (e.g. TA1535, TA1537). Snyder (2004) [14] states that the concordance between standard Ames and mini Ames test is extremely high. The mini Ames test is routineously applied for pre-screening in drug development (DTC 2006) [15].

The mini Ames test system was checked whether it is suitable to evaluate mutagenicity of newly developed textile dyes.

In a comparative study with positive control substances in the standard Ames test and the mini Ames test using the *S. typhimurium* strains TA98 and TA100 it could be shown that the results were in line. The mini Ames version is a suitable tool to detect mutagenicity in textile dyes and it can be concluded to be applicable also for the testing of other chemicals and waste water samples. Substances which are mutagenic can be excluded at the beginning of the development process. Only if a substance does not show mutagenicity in the mini Ames it should additionally be tested in the standard Ames test.

The big advantage of the mini Ames test design in comparison to the standard version is the five-times lower sample amount which is necessary, the need of less consumable material and time and therefore the higher throughput which results in lower costs. In the standard Ames test for one substance tested in one strain 42 Petri dishes are required. In contrast for the mini Ames version only seven 6-well micro-plates are needed. In addition it is estimated that the test can be performed with a time reduction of 30 %, respectively also a big expense factor.

The mini Ames test proved to be a suitable tool to evaluate newly developed dyes and the efficiency of biotechnological waste water treatment processes in the textile industry. ■

Table 2
Dyes tested in the Ames test: Data for the stock solutions of 2,000 mg/l

Name	CAS-No.	Solubility in water	pH	cond [µS cm^{-1}]
Disperse Dyes				
Disperse Red 1	2872-52-8	partly soluble	5.4	3.6
Disperse Blue 1	2475-45-8	soluble	7.4	540
Disperse Yellow 1	2832-40-8	suspendable	6.6	364
Reactive Dyes				
Reactive Blue 19	2580-78-1	soluble	5.7	860
Reactive Black 5	17059-24-8	soluble	4.8	1513
Reactive Red 4	17681-50-4	soluble	7.2	818
Reactive Yellow 81	59112-78-6	soluble	4.4	1986
Direct Dyes				
Direct Red R	573-58-0	partly soluble	9.3	259
Direct Blue 1	2610-05-1	soluble	7.0	624
Direct Black 38	1937-37-7	soluble	8.4	1774
Acid Dyes				
Acid Blue 62	4368-56-3	soluble	5.6	540
Acid Red 299	57741-47-6	soluble	6.8	288

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