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Cytotoxicity of Leather Processing Effluents on the RTG-2 Fish Cell Line

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The leather tanning industry is characterized by the production of different kinds of effluents, generated in each step of leather processing. These effluents may have different chemical compounds present depending on their use in the leather tanning process, and these agents can cause toxicity (Adzet 2000; Konrad 2000). So, a comparative study of such effluent ecotoxicity is necessary in order to improve industrial processes.

In ecotoxicity assessment, acute fish bioassays are required but on the other hand, there is a social call for alternative/complementary toxicity tests in order to reduce or replace animal use (Castell 1992).

In aquatic toxicology, cytotoxicity tests with continuous fish cell lines have been used as a tool for screening and for toxicity ranking of anthropogenic chemicals, mixtures and environmental samples, in Toxicity Identification Evaluation protocols and in replacement or supplementation of in vivo animal tests (Babich et al. 1986; Babich and Borenfreund 1987; Castaño et al. 1989; 1994; 1996; Fent 2001; Bols et al. 1985; Vega et al. 1994). Thus the utilisation of fish cell lines has proven to be a valuable, rapid and cost-effective screening tool in ecotoxicological assessment of chemicals and environmental samples. This will probably become even more important in the future, as the new concepts of genomics and proteomics become incorporated into screening tests. Cellular test systems will provide the basis for application of automated and high-throughput technologies in ecotoxicological hazard assessment (for a review see Castaño et al. 2003).

The purpose of this work was to compare the acute toxicity of different wastewater baths from a leather tanning process on a fish cell line cytotoxicity test (Castaño 1992). Additionally, an activated charcoal adsorption treatment was performed on the samples and cytotoxicity was determined again in order to focus on analytical/toxicological studies for sample characterization and, if possible, to change or modify some leather tanning industrial processes to decrease their toxicity.

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MATERIALS AND METHODS

The wastewater was sampled from: soaking, scouring, liming, deliming, bating, pickling, tannage, retannage, fatliquoring and dyeing processes (Fig. 1), which are baths of the leather tanning process (Adzet 2000).

The acute cytotoxicity of each effluent was tested by means of the RTG-2 cytotoxicity test (Castaño 1992). Briefly, RTG-2 cells, a fibroblastic cell line (ATCC n°55) derived from rainbow trout gonads (*Oncorhychus mykiss*) were grown in tissue culture flasks, in Eagle's Minimum Essential Medium (MEM), with non-essential amino acids, 200 IU penicillin/ml, 100 μg streptomycin/mL and 0.85 g sodium bicarbonate/L, supplemented with 1.25 μg fungizone/L, 2 mM glutamine and 10 % foetal calf serum (FCS) at 20°C in a 5% CO_2 atmosphere.

Cells were seeded in 96-well culture microtiter plates, at 18000 cells/well and incubated for 48 hr at 20°C. After this acclimation period in which cells reached exponential growth-phase, the culture medium was removed and replaced by the test medium (samples + medium containing 1% instead 10% of foetal calf serum).

The effluents were filtered, under sterile conditions, with 0.45 μm and 0.22 μm syringe filters. Then, samples were mixed with MEM 10x (9:1) and the pH was adjusted to 7.3 with sterile $NaHCO_3$. Nine dilutions per sample were prepared in order to obtain a number of concentrations distributed in the range of 1 to 88%. Eight-well replicates were made for each dilution. Eight wells with cells non-exposed to wastewater were used as negative control.

Cytotoxicity was determined by measuring two endpoints, after 48 hr sample exposure: neutral red stain uptake (NRU) to evaluate cell viability (Borenfreund and Puerner 1984), and the kenacid blue protein assay (KBP) to evaluate cell detachment (Knox et al. 1986).

Furthermore, cytotoxicity of scouring, pickling, degreasing and fatliquoring baths was also measured after an adsorption during 1 hr with activated charcoal (UNE-EN 1485 1997) to check differences in cytotoxicity. Additionally, dissolved organic carbon (DOC) was measured with a Shimadzu model TOC-5050 total organic carbon analyzer to check differences due to the adsorption treatment.

Absolute values of each assay were transformed to control percentages. The results of EC50 values were expressed as dilution percentage (v/v) of the sample. The EC50 values and 95% confidence limits were calculated by computerized log-probit analysis using the SPSS© 10.0 package.

RESULTS AND DISCUSSION

Table 1 shows the EC50 values and the 95% confidence intervals for the two studied endpoints. Data show that cytotoxicity vary between the different baths of

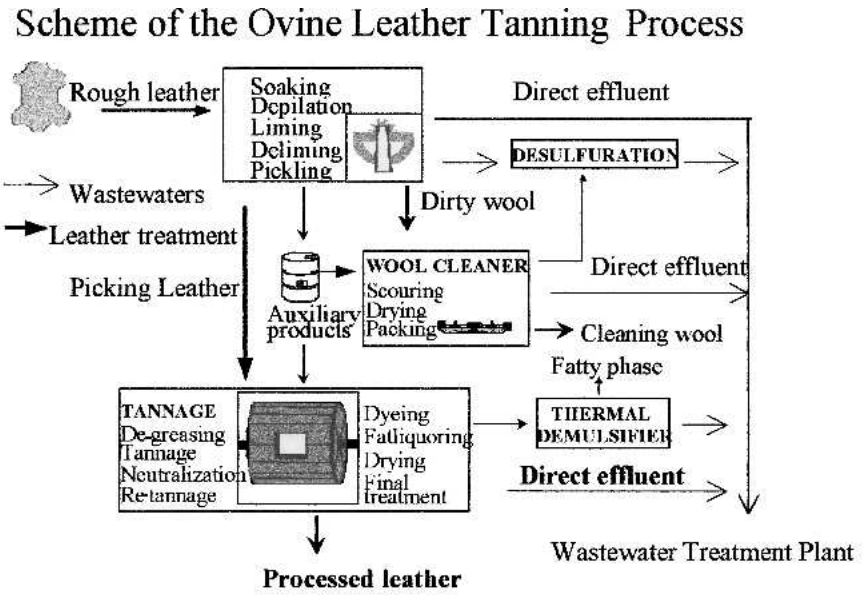

Figure 1. Scheme of the ovine leather tanning process.

Table 1. EC50 values obtained for each bath. Values are expressed as dilution percentage (v/v) of the sample. 95% confidence limits in parenthesis.

Bath	Cell Viability EC 50 (%)	Cell Detachment EC 50(%)
Soaking	19.51 (11.6-36.8)	>80
Scouring	29.35 (24.5-35.5)	>80
Liming	13.4 (9.5-22.3)	34.4 (27.06-48.9)
Deliming	>80	>80
Bating	46.13 (Nc)	-
Pickling	25.26(Nc)	>80
Degreasing	0.08 (0.03-0.13)	-
Tannage	Nc	Nc
Retannage	38.15 (23.4-92.9)	>
Fatliquoring	Nc	>80
Dyeing	8.18 (5.9-11.4)	-

(Nc) These 95% confidence limits have not been successfully calculated by SPSS
Nc The EC50 cannot be calculated because precipitates were observed.

the leather tanning process, with the most cytotoxic one being the degreasing effluent and the least the deliming effluent. It can be seen that the cell viability assay is more sensitive than the cell detachment assay because in the majority of the baths, the cell detachment assay cannot give EC50 values. In the case of the

soaking, scouring, deliming, pickling, retannage and fatliquoring baths, the percentage of cell detachment vs the control did not reach the 50% value of mortality in the range of the concentrations of sample tested (1-80%). We observed that there was a fixative effect: cells remained attached to the substratum, even after the extraction of the dye by sonication over four minutes in time.

The fixative effect on the cellular monolayer became evident by comparing the EC50 values for cell viability assay. Although the cells are non-viable, they remain attached to the growth substratum and the amino groups bind to dye maintaining similar absorbance values to these of control wells.

There were two baths, tannage and fatliquoring, where the EC50 values could not be calculated because precipitates were observed. These precipitates covered the cells making the microscopic inspection and absorbance readings impossible. In the case of the tannage bath, it could be due to the precipitation of Chromium^{3+} present at a neutral pH (Adzet 2000).

Table 2 shows the effect of the treatment of adsorption, during 1 hr with activated charcoal, on several of the baths. Data showed that the treatment was more effective in the case of the scouring bath, where there was a complete reduction of cytotoxicity after the adsorption process (Fig.2). In contrast, there were no differences in the EC50 values from the pickling bath. The previous results could mean that the inorganic dissolved compounds, that naturally cannot be adsorbed to the activated charcoal, produced the cytotoxicity. Indeed, in the pickling process inorganic compounds such as NaCl salt are present (Konrad et al. 2000) and this could cause the cytotoxicity (Fig.3).

Table 2. EC50 values (expressed as dilution percentage (v/v) of the sample) and dissolved organic carbon (DOC) values (expressed in ppm), of the baths before and after treatment for adsorption with activated charcoal for 1 hr. 95% confidence limits in parenthesis.

Bath	**Cell Viability EC 50(%)**		**Cell Detachment EC50(%)**		**D.O.C. (ppm)**	
	Before	**After**	**Before**	**After**	**Before**	**After**
Scouring	29.35 (24.4-35.5)	>80	>80	>80	92.97	40.18
Pickling	25.26 (Nc)	24.5 (18.1-52.8)	>80	>80	225.3	<1
Degreasing	0.08 (0.03-0.13)	1.4 (1.01-1.95)	-	-	8845	172.5
Fatliquoring	Nc	37.3 (19.7-160.6)	>80	>80	14058	1481

(Nc) These 95% confidence limits have not been successfully calculated by SPSS
Nc The EC50 cannot be calculated because precipitates were observed.
Detection limit of DOC is 1 ppm

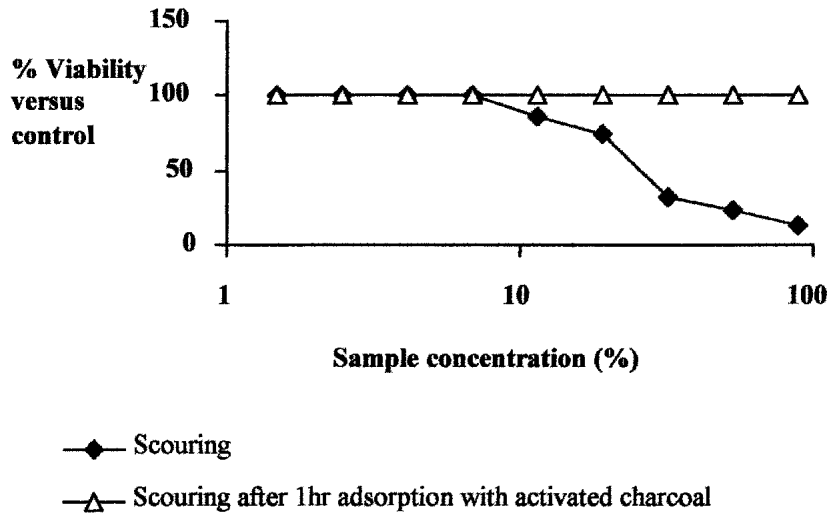

Figure 2. Differences in concentration-response curve for viability between scouring before and after adsorption with activated charcoal.

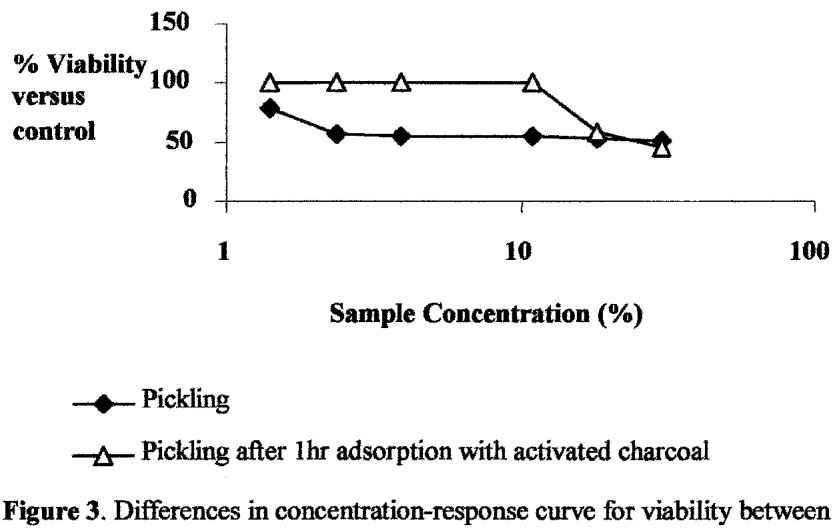

Figure 3. Differences in concentration-response curve for viability between pickling before and after adsorption with activated charcoal.

In the case of the degreasing bath, there was an important decrease in the initial dissolved total organic compound (DOC) values (Table 2), and there was also a high reduction (almost 20 times less) in the cytotoxicity after the adsorption to activated charcoal (Fig.4). Nevertheless the effluent still remained highly cytotoxic. The high cytotoxicity may be due to the fact that during the degreasing process, tensoactives and organic solvents were used and probably, these substances were present at high concentrations in the bath and could cause a disturbance of the cellular lipidic membrane and produce the high cytotoxic effect.

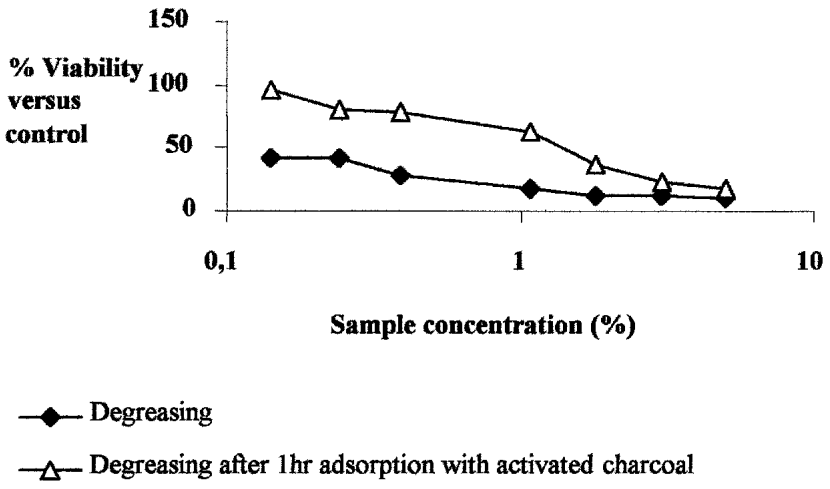

Figure 4. Differences in concentration-response curve for viability between degreasing before and after adsorption with activated charcoal.

In the fatliquoring bath, the EC50 value could not be estimated because precipitates were observed when fatliquoring concentration was high. However, after the adsorption with activated charcoal these precipitates disappeared, hence allowing for EC50 determination.

According to these results and the results obtained in previous studies (Alañón 2001) it can be seen that the use of activated charcoal reduces cytotoxicity when organic compounds, that can be adsorbed to activated charcoal, cause it. That is the case of the scouring bath shown in Table 2 and Figure 2, thus suggesting that activated charcoal could be used as an effective system to reduce toxicity of industrial effluents.

These results suggest also the usefulness of applying an in vitro cytotoxicity test using fish cell lines in sewage characterization and in combined chemical and biological tools such as Toxicity Identification Evaluation (TIE) protocols to rank sample toxicities and to lead the toxicological characterisation process. In vitro

cytotoxicity tests represent a cost-effective and versatile tool for acute toxicity testing, thus they may be a useful surrogate to laboratory animals toxicity test.

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