Paper D31

Alternative Fungicides for leather industry. Part I

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ABSTRACT

This work is focussed on the search of alternatives to the fungicides conventionally used in the tanning industry. These alternatives should have a high efficiency in front of a wide range of fungi and should be less toxic, more environmentally friendly and cost effective.

The main objective of this work is to evaluate the fungicidal capacity of the selected compounds (registered in the 98/8/EC Directive); diiodomethyl-p-tolylsulfone, DIMPTS, 3-iodoprop-2-ynyl-N-buthylcarbamate, IPBC and thiabendazole, TBZ, against different strains of fungi. The fungicidal capacity of the selected compounds has been compared with that of fungicides conventionally used in tannery such as TCMTB and a mixture of phenolics compounds.

The fungicidal capacity of the selected molecules was tested against strains of the following fungi, described in the literature as responsible for damage during the process of leather manufacture: *Aspergillus níger, Trichoderma harzianum, Alternaria alternata and Penicillium funiculosum.*

The Minimum Inhibitory Concentration (MIC) of the studied molecules against the selected fungi has been determined. Thereafter, a comparative study of the fungicidal capacity of the selected fungicides at different offers has been carried out with wet-blue leather.

The results obtained confirm that two of the three fungicides studied (diiodomethyl-p-tolylsulfone, DIMPTS, and 3-iodoprop-2-ynyl-N-buthylcarbamate, IPBC) are good candidates as alternative fungicides to be used in the leather industry. Their potential application against a wider spectrum of fungi especially those isolated in tannery constitutes the aim of the next study together with toxicity evaluation associated to such application and the determination of the fungicide that remain in the different layers of leather.

1. Introduction

After flaying, hides are attacked by bacteria, resulting in a total decomposition of the hide by the enzymes produced unless the hide is subjected to a curing process (salting) together with the addition of a bactericide. The raw hide must be protected from bacterial attack in operations that follow flaying, i.e. storage and soaking.

However, hides subjected to subsequent manufacturing steps i.e. pickling, tanning, dyeing and fatliquoring are susceptible to fungi attack. For these hides, the application of an effective fungicide

is necessary in order to avoid the development of fungi. Hide attacked by fungi is evidenced by the presence of permanent stains and impaired physical properties due to collagen degradation. [1]

Wet-blue leather provides suitable conditions for the growth of fungi: storage temperature, acid pH, presence of water, proteins and fats. A rise in temperature often leads to an increase in micro-organism growth. The optimum conditions for the growth of several fungi (*Penicillium* sp., *Aspergillus* sp., *Trichoderma* sp., *Rhizopus* sp. *and Mucor* sp.) are a pH range between neutral and slightly acid (3-6), a temperature of 25ºC and humidity between 12 and 15%. [2]

In accordance with Hauber [3-5] for a compound to be an effective fungicide, the requirements are as follows: optimal activity against fungi, compatibility with hide and with chemicals used for processing leather, effectiveness at acid pH, stability with respect to temperature and UV light, low solubility in water, low toxicity in humans and economically and environmentally acceptable. Although a large number of compounds meet these requirements to a greater or lesser extent [6-10], TCMTB possesses a wider range of application and has achieved the highest acceptance level in the tanning industry. However, its technical limitations and the considerable environmental impact of TCMTB [11, 12] reinforce the need for looking for new fungicides to replace those conventionally used [13].

The fungicides studied in this work have been selected in accordance with the 98/8/EC Directive [14, 15]. By means of this Directive, the European Union has established a normative framework for the commercialization of biocides so as to minimise risk to health and the environment.

Aim of the work

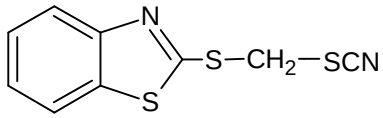
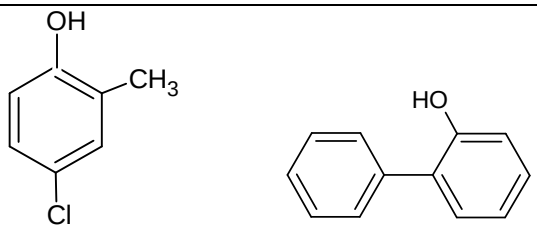
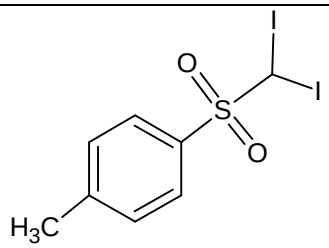
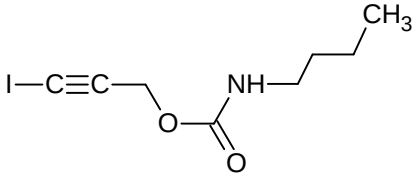
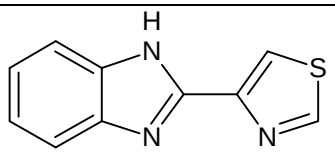
This work is focussed on the search of alternatives to the fungicides conventionally used in the tanning industry. These alternatives should have a high efficiency in front of a wide range of fungi and should be less toxic, more environmentally friendly and cost effective.
The main objective of this work is to evaluate the fungicidal capacity of the selected compounds (registered in the 98/8/EC Directive) against different strains of fungi. The fungicidal capacity of the selected compounds will be compared with that of fungicides conventionally used in tannery such as TCMTB and a mixture of phenolics compounds.

The Minimum Inhibitory Concentration (MIC) of the studied molecules against the selected fungi has been determined. Thereafter, a comparative study of the fungicidal capacity of the selected fungicides at different offers will be carried out with wet-blue leather.

2. Materials and methods

The following three compounds were selected to evaluate their potential application as fungicides in the leather sector: diiodomethyl-p-tolylsulfone, DIMPTS (provided by Dow Chemicals), 3-iodoprop-2-ynyl-N-buthylcarbamate, IPBC (provided by Lanxess) and thiabendazole, TBZ (provided by Tecnidex) Figure 1 shows the chemical structure of these compounds together with that of 2-(thiocyanatomethylthio)-1,3-benzothiazole, TCMTB (provided by Lamirsa) and a mixture of phenolic compounds (provided by Lanxess) used for comparison. The selection of fungicides was based on the following criteria: proved fungicidal capacity in other industrial sectors, compounds of different chemical family and compatibility with the substrate and with the conditions of application.

Figure 1. Molecular structure of fungicides used

FUNGICIDE	CHEMICAL STRUCTURE
2-(tiocianatometiltio)-1,3-benzotiazol TCMTB (~ 30% active ingredient)	
Mixture of phenolic compounds (PCMC+OPP) (clorometacresol + ortofenilfenol) (~ 40% active ingredient)	
Diiodometil-p-tolilsulfona DIMPTS (~ 40% active ingredient)	
3-iodoprop-2-in-N-butilcarbamato IPBC (~ 30% active ingredient)	
Thiabendazole TBZ (~ 60% active ingredient)	

The fungicidal capacity of the selected fungicides was carried out against strains of the following fungi, described in the literature as responsible for damage during the process of leather manufacture: *Aspergillus níger (CECT 2088), Trichoderma harzianum (CECT 2423), Alternaria alternata (CECT 2662) and Penicillium funiculosum (CECT 2914),* which were submitted by the Spanish Type Culture Collection (CECT) from Valencia University. Given that all the strains were received lyophilized, except *Alternaria alternata*, they were reconstituted in a suitable nutrient medium up to the production of viable spores. A suspension of spores of each strain was prepared with ringer solution. Thereafter, the counting of spores was carried out under the microscope and by the plate count method on sabouraud dextrose agar (Oxoid, Basingstocke, UK). Afterwards inoculum suspension was adjusted to 10^5spores/mL. Antifungal assay in leather samples were made using potato dextrose agar (Oxoid, Basingstocke, UK).

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is defined as the lowest concentration (expressed in µg/mL) of an antimicrobial that will inhibit the visible growth of a microorganism after an incubation period. The antimicrobials are dissolved at different concentrations together with a standardized amount of the isolated microorganism. Thereafter, the solutions are incubated at 26 to 30ºC for 18 – 24 hours and allowed to set until microorganism growing is observed. The minimum

amount of antimicrobial which is necessary to inhibit the fungal growth provides the minimum inhibitory concentration. Each antimicrobial has a specific MIC for each different microorganism. [16]

To determine the minimum inhibitory concentration, several dilutions at concentrations ranging from 10240µg/mL to 0.078µg/mL of the fungicides under study were prepared. One mL of each dilution was mixed with 20mL of melted sabouraud dextrose agar medium and poured into a plate to which one drop of each inoculum suspension was platted onto the medium, each test was carried out by duplicate. Plates were incubated at 25ºC for 96 hours. Plates without fungicides were used as positive control. The plate with the lowest concentration where fungal growth was not observed provided the minimum concentration that inhibited growth, i.e., the minimum inhibitory concentration of the fungicide. [17]

Preparation of a wet-blue skin free of bactericides and fungicides

The study of fungi growth was carried out on a wet-blue skin. In order to avoid possible interferences with the fungicides under evaluation, a wet-blue skin was prepared ensuring that no bactericides and fungicides were added in the soaking process.

The tanning process was carried out in accordance with the recipe shown in Table 1.

Table 1. Tanning recipe for pickled skin free from bactericides and fungicides

Offers on pelt weight	
60 % H_2O	
4 % NaCl	
4 % chromium salt 33 % basicity	Drum 1/2 hours
4 % chromium salt 33 % basicity	Drum 3 hours
1 % sodium formate.	Drum 3 hours
Rest overnight	
pH (2.8 – 3.0)	
2 % sodium hydrogen carbonate	Drum 3 hours
pH (3.5 – 4.0)	
Ts > 100 ºC Drain off	

Control of fungal growth on wet-blue skin

Samples of 250mm x 250mm were cut from the wet-blue skin. In order to simulate real application conditions, the wet-blue samples were placed in a closed recipient and shaken with a solution of the corresponding amount of fungicide and 100% of water (on wet-blue weight) for three hours. The mould growth resistance of the treated samples was tested in accordance with the ASTM D4576-01 Standard [18] against two different strains (*Aspergillus niger* and *Trichoderma harzianum*), which are the most invasive strains of those previously mentioned. A wet-blue sample without fungicide was used as control for comparative purposes.

After shaking, the fungicide treated samples were placed in sterile plates surrounded by potato dextrose agar culture medium. After solidification of the agar, one drop of the spore suspension ($1x10^6$ spores per mL) of each mould was deposited directly on the sample and other one on the culture medium as shown in Figure 2.

Each test was carried out for triplicate. The plates were stored in humid atmosphere at 26ºC and the control of fungal growth was performed weekly against the control sample. The percentage (from 0% to 100%) of wet-blue sample surface overgrown by mould was recorded. This control was carried out during a period of 90 days. After three months, the wet-blue samples without mould growth were stored in hermetic plastic bags to check the mould growth resistance after a long period.

Two different growth controls were carried out:

- **Offers of 0.1% - 0.5% - 1% of fungicide**

Three different offers (0.1%, 0.5%, and 1% on wet-blue weight) of each of the five selected fungicides in 100% of water were studied.

- **Comparative study with 0.2% of fungicide**

The results obtained in the tests described above revealed that an offer of 0.2 % on wet-blue weight in 100% of water could provide satisfactory mould growth resistance. Consequently, a comparative study with 0.2% of each of the selected fungicides was performed.

3. Results and discussion

Determination of Minimum Inhibitory Concentrations (MIC)

Table 2 shows the results of the MICs of the five selected fungicides against the strains of the fungi considered.

Table 2. MICs, in μg/mL, of each of the studied fungicides against the fungi assayed.

Fungicide	Aspergilius niger	Penicilium funiculosum	Trichoderma harzianum	Alternaria alternata
TCMTB	7.6	15.3	7.6	0.95
Mix of phenolics	62	31	124	62
DIMPTS	3.8	1.9	7.6	1.1
IPBC	0.8	1.9	3.9	0.8
TBZ	3.9	0.95	1.9	---

The results shown in table 2 confirm the good fungicidal capacity of the chemicals conventionally used in the leather industry. Likewise, the three selected alternative compounds show potential applicability as fungicides in the tannery sector. In general, the alternative compounds have a lower minimum inhibitory concentration than the chemicals conventionally used.

Control of fungal growth on wet-blue skin

The results obtained of fungal growth resistance on wet-blue skin are shown next:

- **Offers of 0.1% - 0.5% - 1% of fungicide**

The easiest way to evaluate the results obtained in this study is to observe the images of fungal growth in the treated wet-blue samples placed in the plates. However, in accordance with the ASTM Standard [18], the report of results of mould growth is rated as a percentage of wet blue surface covered by mould. We found that wet-blue samples with 0% of mould growth in their surface had

mould growing around the samples, therefore in order to be more precise in the report of the results, we have introduced another parameter called “inhibition zone” (Figure 2), which can be defined as the area on an agar plate where growth of the mould is prevented by the fungicide placed on the wet-blue leather sample.

Figure 2. Sample with inoculum locations (X).
Inhibition zone provided by the fungicide impregnated wet-blue sample

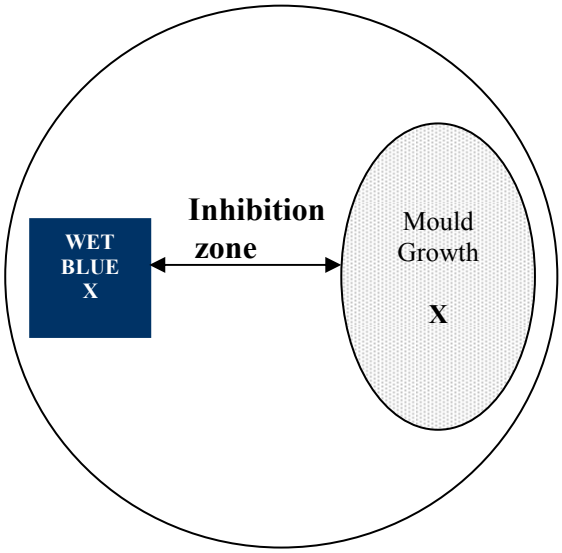


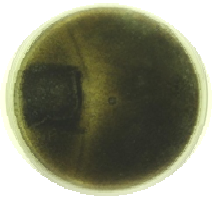
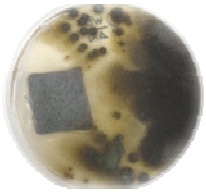
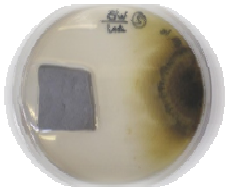
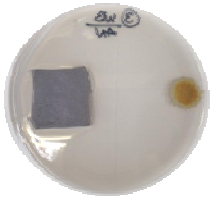
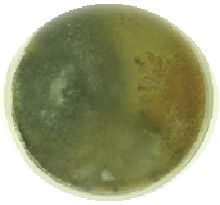
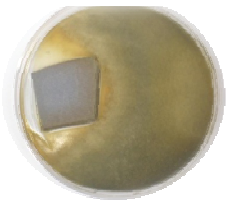
Table 3. Results of surface growth and the inhibition zone observed in the different samples after 90 days of testing.

		Aspergillius niger		*Trichoderma harzianum*	
% of fungicide		**Surface growth (%)**	**Inhibition zone (mm)**	**Surface growth (%)**	**Inhibition zone (mm)**
0.1 %	**TCMTB**	0	45	4	0
	Mix of phenolics	77	0	98	0
	DIMTPS	0	15	0.5	2
	IPBC	0	26	0	15
	TBZ	53	0	100	0
0.5%	**TCMTB**	0	45	0	5
	Mix of phenolics	0	18	0	19
	DIMTPS	0	16	0	8
	IPBC	0	36	0	30
	TBZ	0	22	100	0
1 %	**TCMTB**	0	45	0	8
	Mix of phenolics	0	35	0	30
	DIMTPS	0	45	0	10
	IPBC	0	45	0	32
	TBZ	0	38	0	32
CONTROL		100	0	100	0

In Table 3, an inhibition zone of 45mm means that no mould growth on the agar plate was observed. As expected, all control samples (without fungicide) had a 100% of surface growth

after one or two weeks of testing and the inhibition zone was 0mm. For TCMTB, the sample surface growth was not observed for any of the three offers considered (0.1, 0.5 and 1.0%) nor for any of the fungi studied. In the case of *Aspergilius niger*, the inhibition zone was maximum (45mm) even for the lowest offer, i.e, the lowest offer was sufficient to impede the growth of mould in the plate. On the contrary, the growth of *Trichoderma harzianum* in the plates was observed for any of three offers of fungicide considered so that the highest offer applied resulted in the lowest fungal growth. In summary, a 0.1% of TCMTB on wet-blue weight is sufficient to protect against *Aspergilius niger* and barely sufficient against *Trichoderma harzianum*. When the mixture of phenolic compounds is considered, an offer of 0.1 % was not sufficient for any of the two fungi studied since after 90 days of testing the percentage of sample surface growth was between 70% and 100%. An offer of 0.5% on wet-blue weight of the mixture of phenolic compounds was sufficient to protect the leather samples against both fungi, whereas the protection provided by a 1% offer was much higher. All of this can be observed in Figure 3.

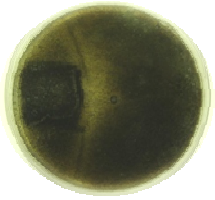
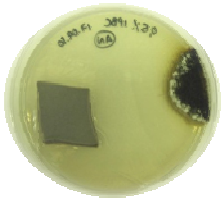
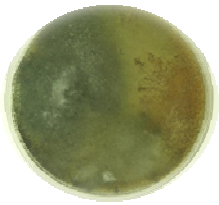
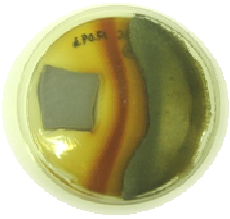
Figure 3. Results of the fungicidal capacity of different offers of the phenolic compound mixture after 90 days of testing

90 days	**CONTROL**	**0.1% offer**	**0.5% offer**	**1% offer**
Aspergilius niger				
Trichoderma harzianum				

With the lowest offer of DIMPTS (0.1% on wet-blue weight), the sample surface growth was inhibited. Mould growth was observed in the agar culture medium. However, the diffusion caused by the treated sample was sufficient to impede the progress of the mould. A higher offer would confer a higher protection but it would be completely unnecessary.

For IPBC, no mould growth was observed in the wet-blue sample surface for any of the three selected offers. Therefore, an offer of 0.1% on wet-blue weight of IPBC was sufficient to control mould growth in sample surface. An offer ten folds higher (1%) avoided almost totally the mould growth also in plates thanks to the diffusing capacity of the treated wet-blue sample. Figure 4 shows the results obtained with IPBC.

Figure 4. Results of the fungicidal capacity of different offers of IPBC after 90 days of testing

90 days	CONTROL	0.1% IPBC	0.5% IPBC	1% IPBC
Aspergilius niger				
Trichoderma harzianum				

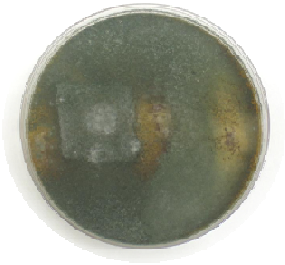
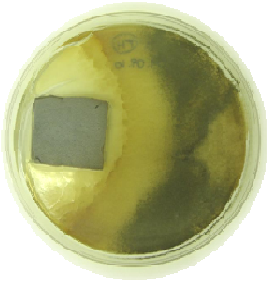
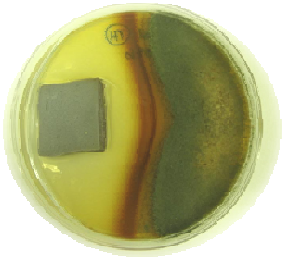
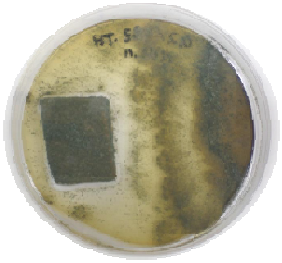
In accordance with the results obtained for TBZ, it can be confirmed that the application of this chemical in tannery was not very efficient against the selected fungi, at least, the commercial formulation tested. For an efficient attack against mould, a very high amount of product would be required and this is not economical or environmental advisable. TBZ against *Trichoderma harzianum* was only effective with an offer of 1% on wet-blue weight whereas an offer of 0.5% was necessary to be efficient against *Aspergilius niger*.

- **Comparative study with 0.2% of fungicide**

In accordance with the results obtained in the previous section, a comparative study with 0.2% on wet-blue weight in 100% of water of each of the selected fungicides was performed to check if this offer was sufficient to avoid mould growth not only on the wet-blue sample surface but also in plates.

In the case of *Aspergillius niger*, the offer of 0.2% of fungicide was sufficient to avoid mould growth both in leather samples and in plates for all fungicides except for the mixture of phenolic compounds. When the study was carried out against *Trichoderma harzianum*, the mould growth in the wet-blue samples was avoided by the alternative fungicides, which caused a wider inhibition zone in plates, except for TBZ. However, the 0.2% of the conventional fungicides was not sufficient to impede the progress of the hyphae towards the wet-blue samples, which were partially covered by mould. Figure 5 shows the results obtained in the comparative study with this type of mould.

Figure 5. Results of the fungicidal capacity of 0.2 % of the five selected chemicals against trichoderma harzianum after 90 days of testing

Trichoderma harzianum (90 days)		
Control	**0.2 % TCMTB**	**0.2 % mix of phenolic compounds**
		
0.2 % DIMPTS	**0.2 % IPBC**	**0.2 % TBZ**
		

3. Conclusions

From the fungicides conventionally used in tannery and studied in this work, the 2-(thiocyanatomethylthio)-1,3-benzothiazole (TCMTB) shows the highest antifungal capacity against the tested fungi (*Aspergillus níger* and *Trichoderma harzianum*), mainly against *Aspergillus níger*. The mixture of phenolics compounds is not adequate against *Trichoderma harzianum* at normal application offers and *Aspergillus niger* is also resistant to the action of the mixture when this is applied at low offers.

Low offers of two of the three alternative fungicides studied (diiodomethyl-p-tolylsulfone, DIMPTS and 3-iodoprop-2-ynyl-N-buthylcarbamate, IPBC) confer satisfactory mould growth resistance to the treated wet-blue leather samples as shown by the values of sample surface growth and inhibition zone obtained. As far as TBZ is concerned, the commercial formulation tested resulted inefficient against the selected fungi, at least at the lowest offers applied in the tests.

The results obtained confirm that diiodomethyl-p-tolylsulfone (DIMPTS) and 3-iodoprop-2-ynyl-N-buthylcarbamate (IPBC) are good candidates as alternative fungicides to be used in the leather industry. However, their potential application should be checked against a wider spectrum of fungi especially those isolated in tannery. This constitutes the aim of the next study together with toxicity evaluation associated to such application.

5. Acknowledgements

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6. References

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