Determination of fungicides in leather and residual tanning floats

M. Reyes Reyes, Joaquim Font, Anna Bacardit, Sara Cuadros*, Agustí Marsal*

Escola d'Enginyeria d'Igualada, Universitat Politècnica de Catalunya, Plaça del Rei 15, 08700 Igualada, España
* IQAC-CSIC, Departamento de Tecnología Química y de Tensoactivos, J. Girona, 18-26, 08034 Barcelona, España

Abstract

This article describes two methods of analysis to determine fungicides used in the leather industry. One of this method determines fungicides in skins and the other in tanning floats. The preservative agents are 2-(thiocyanomethylthio)-benzothiazole (TCMTB), 4-chloro-3-methylphenol (PCMC), 2-phenylphenol (OPP), 2-Octyl-3(2H)-isothiazolone (OIT), and also 2-mercaptobenzothiazol (MBT) and 3-iodo-2-propynyl-butylcarbamate (IPBC).

- In method of determination fungicides in leather sample preparation and extraction process has been conducted under the same conditions as described UNE-EN ISO 13365: 2011 Determination of the preservative (TCMTB, PCMC, OPP, OIT) leather by liquid chromatography. Some modifications have been introduced in the chromatographic method that expand the number of preservatives to be analyzed simultaneously, improve the reliability of the identification and quantitation sensitivity. It has also been found that 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) do not interfere with the determination.

- The method to determine fungicide in floats develops a method of extracting in aqueous samples fungicides using the technique SPME (solid phase microextraction). The identification and quantification is carried out by liquid chromatography with photodiode array detector (HPLC-PDA) using the chromatographic method described above.

This method enables us to determine quantitatively the fungicides contained in the residual floats. Consequently, it will constitute a very useful tool to improve the preservative uptake in leather manufacturing processes.

Toxicity is one of the parameters established in the canon of wastewater, and the presence of fungicides contributes, along with other products used.

Finally real samples from floats tannery processes as a tool for analysis of the effectiveness in the application of fungicides are analyzed.

Introduction

In the production and distribution of leather, two types of biodegradation may occur: due to bacteria and fungi. The first usually occurs in the initial stage of the process; the second usually appears after the tanning process.

Fungi are visually manifest as spots. The skin also suffers a decrease in the physical properties due to collagen degradation, reducing the quality of the skin.

Fungicides are substances used to inhibit the growth of fungi that cause the degradation of leather.

TCMTB, the more fungicide used to control fungal growth, not entirely effective against different strains that can grow and is usually combined with two or three molecules. Still, their antifungal capacity or vegetable tanned leather wet white is lower than in chrome tanned leathers. For this reason other molecules have been proposed as IPBC (3-iodo-2-propynyl butyl carbamate).

Cuadros et al. (2013) compared the effectiveness of TBZ (thiabendazole), DIMPTS (diiodomethyl p-tolyl sulphone) and IPBC in relation to TCMTB fungicides. The results showed that both DIMPTS as IPBC have very good antifungal capacity, compared with TCMTB. At equal quantity supplied, the DIMPTS and IPBC showed best response to 5 strains of fungi tested, while TBZ and TCMTB only protected against one of them. The toxicity of the tanning floats turned out to be,

in increasing order, IPBC <DIMPTS <TCMTB.

Fungicides used in the leather industry fall mainly into two broad chemical families: phenolics, (which include PCMC and OPP) and heterocyclics (which include TCMTB, OIT, and MBT). In Figure 1 the chemical structures and UV spectra of these molecules are presented.

Keywords: fungicides, biodegradation, floats.

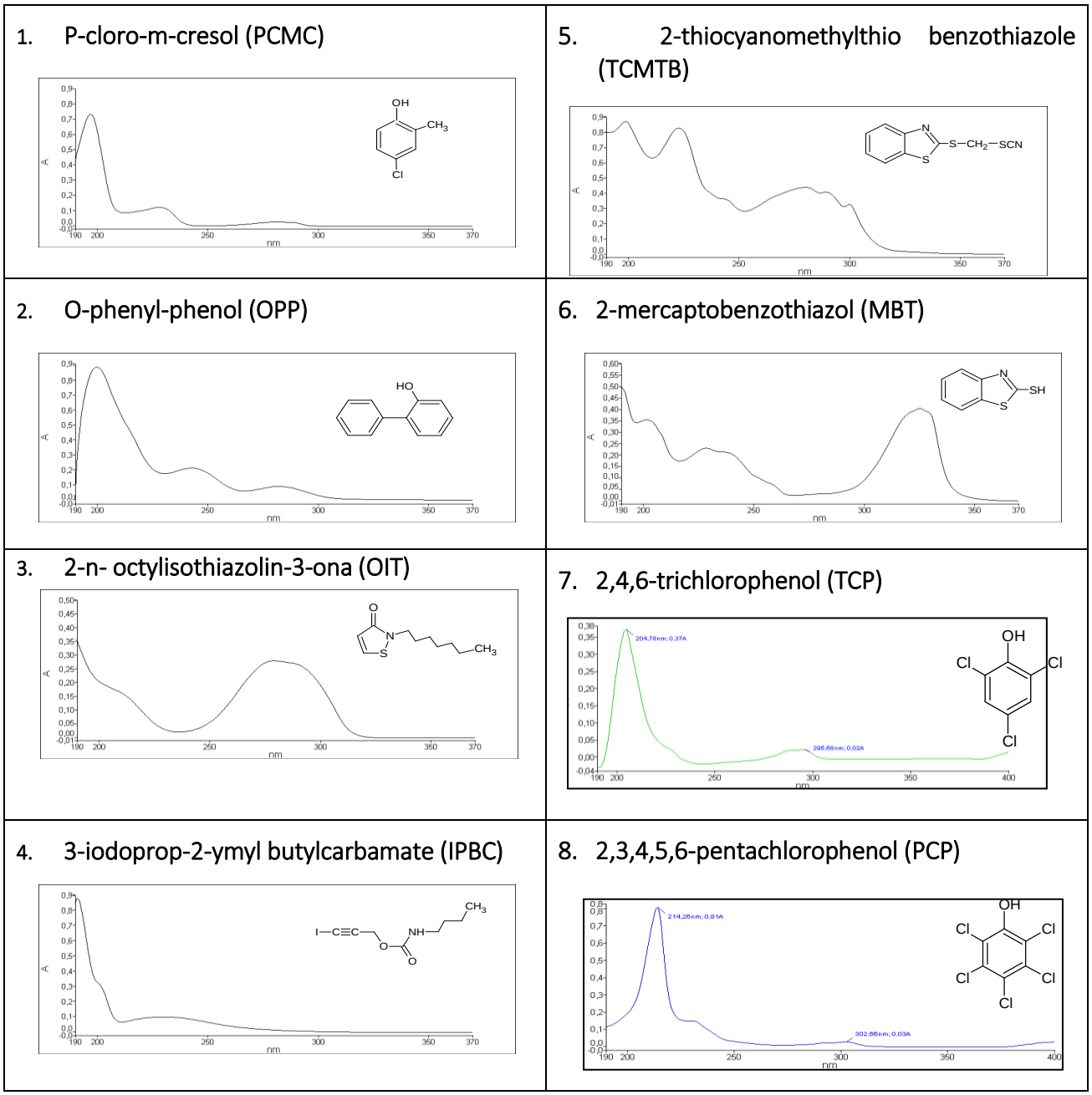

Figure 1. UV spectra and chemical structure of fungicides studied

1. Determination of fungicides in leather [5–7]

2.1. Procedure

2.1.1. Instrumentation

HPLC-PDA system: Alliance 2695 Separation Module (Waters Corporation, Milford, Massachusetts, USA) fitted with a 2998 PDA Detector. A Mediterranean Sea18 15 x 0.46 cm 3µm column (Teknokroma, Barcelona, Spain) packed with C18 reversed-phase was used. UV scanning detection was performed between 190 and 380 nm. The mobile phase was 0.1% formic acid in water (A) : 0.1% formic acid in

acetonitrile (B). Gradient: 60 % B, 6 min isocratic, then programmed linear to 95 % B in 9 min. Flow was 0.9 mL/min. The oven temperature was held at 30 oC. A 20-µL volume of analytical solution was injected. A smaller volume (10-µL or 15-µL) was chosen for the injection of the samples in which fungicides were detected at high concentrations.

2.1.2.Materials

Formic acid for mass spectroscopy, ~98% was obtained from Fluka. Analytical standards of fungicides were obtained from Supelco (TCMTB, PCMC), from Fluka (OIT, OPP, TCP), and from Aldrich (IPBC, PCP). 0.45 µm PVDF membrane filters were supplied by Micron Analítica (Madrid, Spain). The solvent acetonitrile was of HPLC-gradient grade from Panreac (Spain). Water used in the mobile phase was Milli-Q ultrapure water.

2.1.3.Samples

Forty commercial samples of leather from different countries were analyzed. Twenty-six were finished and 14 were semi-processed (wet-blue and wet white). Samples were collected within the period 2009-2011. Before the analyses, all the samples were conditioned in ISO 2419 standard atmosphere.

2.1.4.Procedure

1.000 ± 0.010 g of ground leather is weighed in a 50 mL screw top bottle. 20 mL of acetonitrile are transferred to the leather. The leather sample is extracted in an ultrasonic bath for 1 hour ± 5 min at room temperature. During extraction the temperature in the mixture increases to about 35 °C. Thereafter, a part of the extract is filtered through a 0.45 µm PVDF membrane filter into a suitable vial. The filtrate is analyzed by HPLC. Analyte peak identity is determined by matching the retention time with that obtained from the injection of analytical standards, and confirmed by diode array detection, which provides an UV spectrum for each compound peak for comparison with that obtained from the analytical standards of the fungicides.
Detected preservatives are quantified using the calibration plots prepared previously with known solutions of analytical standards of the fungicides.

2.2.Results and discussion

Concentrations ranging between 440 and 540 mg / kg wet skins TCMTB blue in national export. In wet blue short retention periods, the concentrations of TCMTB ranged from 253 and 354 mg / kg. One of these samples also contained 154 mg / kg of MBT.
All values are expressed on conditioned sample weight in standard atmosphere.
Most samples containing PCMC also contained OPP. The found concentrations of the two fungicides ranged from 8 to 680 mg / kg and 8-480 mg / kg, respectively. The detected concentrations of MBT and OIT ranged from 4 to 154 mg / kg and 44 to 230 mg / kg, respectively.
This work has broadened the scope of the ISO 13365 standard: 2011 allowing the simultaneous determination of eight fungicides molecules. Including fungicides like IPBC and MBT are included. The presence of 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) did not interfere in the determination, as shown in the chromatogram of Figure 2. Their detection is possible simultaneously with the rest of preservatives.
Changes can improve the specificity of the method and the detection limits, setting a wavelength specific detection for each molecule, instead of a single standard as required. Furthermore, the UV spectrum obtained with PDA detector allows reliable confirmation of the identity of the analyte.

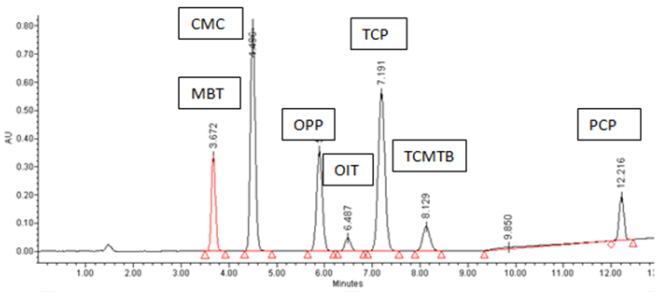

Figure 2. Chromatogram obtained at 279 nm

Limits of detection and quantification

Limits of detection (LOD), defined for a signal-to-noise ratio of 3 (S/N=3), were estimated for the different fungicides. The limits of quantification (LOQ), defined for a signal-to-noise ratio of 10 (S/N=10), were also estimated. The LODs of TCMTB, PCMC and OPP were measured at more than one wavelength. Results are given in Table 1. The largest analyte signal does not necessarily imply most sensitivity since a little baseline noise is also needed. The UV spectrum of TCMTB shows an absorption maximum at 223

nm. However, data in table 1 show that the most sensitive detection wavelength for TCMTB is 275 nm.
For OPP the most sensitive detection wavelength is 243 nm. For PCMC, the LOD at 197 nm resulted in an approximately 3 fold improvement in sensitivity when compared to the 228 nm detection. The lowest LOD obtained was for MBT and the highest was for IPBC, as expected.
These results show that the sensitivity of the method described in this paper allows the quantification of the fungicides in the range of concentrations used in the leather industry.

Fungicide	Wavelength (nm)	LOD (µg/g)	LOQ (µg/g)
OIT	279	0.46	1.5
OPP	201	2.2	7.3
	246	1.7	5.7
IPBC	193	17	57
MBT	324	0.06	0.2
PCMC	197	0.71	2.4
	201	1.3	4.3
	228	2.0	6.7
TCMTB	223	1.7	5.7
	275	1.2	4.0
TCP	203	1.7	5.7
PCP	214	0.39	1.3

Table 1. Límits of detection (LOD) and quantification (LOQ)

Linearity, recovery and precision
The recovery study was performed for each fungicide at two levels of concentration with three replicates per level. The levels were selected to reproduce the concentration ranges encountered in real samples according to Hauber and Germann data [10,11].

A sample of fresh Catalan calf hide was tanned to wet blue without using any preservative. Thereafter, it was analyzed to verify that it did not contain fungicides. This blank wet-blue leather sample was dried at 25 ºC, cut into small pieces, and ground in a cutter mill.
Portions of 1 g were transferred to 50 mL screw top bottles and were spiked with a standard solution of fungicide in acetonitrile. The solvent was allowed to evaporate at 23 ºC for 24 hours. Then, the concentration of fungicide was determined. This process was repeated three times for each fungicide. The average recoveries were greater than 84% in all cases, as shown in Table 2. For TCMTB, the recovery of the method was 99% at the level of concentration of 300 mg/kg.

The intraday precision of the method was evaluated by calculating the relative standard deviation (RSD) of replicated analysis (n=3) of the recovery study. RSD values were lower than ±5%. Results are included in Table 2.
Correlation coefficients were all higher than 0.999.

Fungicide	Wavelength	Level (µg/g)	Recovery (%)	% RSD (n=3)
OIT	279 nm	30	84	± 1.7
		350	91	± 4.6
OPP	201 nm	50	92	± 0.8
		450	96	± 0.8
OPP	246 nm	50	95	± 0.4
		450	96	± 0.3
IPBC	193 nm	130	98	± 5.0
		300	88	± 3.5
MBT	324 nm	20	88	± 1.8
		250	88	± 1.6
PCMC	201 nm	70	91	± 0.1
		600	98	± 1.1
PCMC	228 nm	70	93	± 0.5
		600	96	± 0.3
TCMTB	223 nm	30	94	± 5.0
		300	99	± 0.6

Table 2. Recoveries 6 fungicides at two levels of concentration

Selection of wavelength of detection
Three criteria must be borne in mind while choosing the wavelength detection: sensitivity, precision, and selectivity. The sensitivity is greater at shorter wavelengths, except for MBT and OIT. However, the precision of the analyses is better at longer wavelengths. For example, in the analysis of a commercial leather sample, the Relative Standard Deviation of eight determinations of OPP at 201 nm was ±3.8% while at 246 nm was only ±1.5%.

Finally, the proper selection of the wavelength improves the selectivity of the chromatography. The chromatograms of some finished leather samples are complex, richer in peaks than the chromatograms from wet blue samples. Peaks of unknown interfering substances in close proximity to retention time of the fungicide of interest may be present in some samples. The correct integration of the un-resolved peaks could be difficult. The selection of a wavelength where the difference in sensitivity between the fungicide and the interfering substance is maximal improves the quality of the peak integration in the chromatography.

2.3 Conclusions

The HPLC allows the rapid, sensitive and highly specific determination of fungicide preservatives in leather. Sample preparation of ISO 13365 Standard is as simple as 1-hour extraction, filtration, and injection. The UV spectrum from PDA detection allows the reliable confirmation of analyte identity.

The selection of wavelength detection is specific for each fungicide. The study shows that, in general, recovery and precision are better at long UV wavelengths (225-280 nm). Sensitivity is commonly greater at shorter wavelengths (193-225 nm), but noise and risk of interferences are enhanced.

The results of the analyses of real samples show that TCMTB is the most widely used molecule for protecting leather from fungi attack during storage.

3. Determination of fungicides in tannery floats

3.1. Introduction

Till the date, no method for the simultaneous determination of different commercial preservatives in leather manufacturing waste waters has been reported. Analyses of floats before and after leather processes are useful to give an indication of the effectiveness of the treatment as measured by the uptake.

Parbery et. al. determined methylene bis thiocyanate and TCMTB in leather process liquors by liquid-liquid extraction (LLE) with CH2Cl2 and HPLC separation and quantification5.

Two more papers have been published, but for only TCMTB determination. Meneses et al. proposed electroreduction of TCMTB and then electroanalytical determination 6 and Hinojosa et al. suggested indirect extraction-spectrophotometric determination after breakdown to 2- mercaptobenzothiazole7. Neither of these methods has been applied on a large scale, because the leather sector demands the simultaneous determination of different preservative compounds. In fact, very little is known about the residual content of fungicides in the effluent of a chrome tanning bath process. Parbery et al. found between 3 and 20 mg/L of TCMTB at the end of different industrial processes, and Hinojosa et al. found 42 to 116 mg/L in the industrial liquors analyzed in their research. Meneses et al. did not report any TCMTB concentration in real industrial waste waters.

The lack of data is due to the difficulty of analyzing such unclean and highly concentrated tanning waste waters. In leather manufacture, a portion of the trivalent chromium offer remains in the exhausted bath after the tanning process, with large amounts of substances such as sodium salts, proteins, aminoacids, carboxylic acids and fats among others. This constitutes a complex analytical matrix for the quantitative determination of the residual content of fungicide at the level of ppms.

The methods developed for the determination of TCMTB, MBT and other benzimidazoles in tannery industrial waste waters are also very limited. Fiehn et al.8 and Reemtsma9 used reversed phase HPLC coupled to UV detection and mass spectrometry, respectively. Although applied in the tanning process, TCMTB has not been detected in any of the analyzed tannery waste waters owing to the known hydrolysis of TCMTB under alkaline conditions, such as in tannery effluents.

Therefore, analyses for TCMTB uptake determination must be carried out in the residual tanning floats before mixing them with other waste waters.

The aim of this paper is to describe a rapid and simple method for the simultaneous determination of TCMTB and other fungicides in residual tanning floats and to evaluate the parameters of its validation. The waste bath sample is diluted, filtered, and the analytes are extracted by means of SPME technique. Determination is carried out by HPLC-PDA after desorption of the fiber. The extraction step of the developed method is absolutely innovative in the field of leather sector analysis.

The HPLC step of the method is the same as that previously validated in our earlier paper2. HPLC-PDA is the selected method because it is suitable for the simultaneous determination of the different fungicides used in the leather industry and also for other molecules that are currently being investigated. The sensitivity and selectivity of the method is enhanced by the selection of a specific optimal detection wavelength for each molecule. Moreover, the UV spectrum obtained from PDA detection allows the reliable confirmation of analyte identity. This is a robust method for the large number of variations that real samples may present i.e. variations that result from the origin of these samples (residual floats of chrome, wet-white or vegetable tanning process) and from the characteristics of the

final leather articles to be manufactured. Finally, given that it is the same technique used in the ISO Official Standard for the determination of preservatives in hides and leather2,4, the laboratories of leather companies already have the necessary equipment.

The classic LLE method involves three sequential extractions of the sample with dichloromethane. The layers are separated by centrifugation5. Other authors use ethyl acetate (twice) and toluene8. The remaining organic solvent must be removed by evaporation to be replaced by methanol, acetonitrile or another compatible solvent with the HPLC-reverse phase system. In the analyses of some real spent process liquors, emulsified layers are very often formed. These emulsions are difficult to break and, therefore, the quantitative recovery of the preservatives is not easy. LLE is time-consuming, uses hazardous organic solvents and is not cost-effective.
Very sensitive methods based on solid-phase extraction have been developed for determining some antifouling pesticides in water10, 11. However, of the fungicides used in the leather industry, only TCMTB has been included in such studies. In addition, these methods have been developed for environmental water samples with a matrix, which is much cleaner than that found in the residual floats of a tannery.

SPME has been used in the determination of both phenolic and benzimidazole fungicides, but not simultaneously. This SPME technique has two possible approaches: headspace exposure and direct immersion of the fiber in the aqueous sample. However, SPME extraction of fungicides from the headspace exposure would be limited to only volatile compounds, excluding molecules like OPP, TCMTB and OIT.

López et al. 12 indicated that SPME using a 75 µm carboxen–polydimethylsiloxane (CAR/PDMS) fiber is suitable for extraction of benzimidazole fungicides in environmental water samples. However, Hu et al.13 selected the 60 µm polydimethylsiloxane-divinylbenzene (PDMS/DVB) fiber because of its higher sensitivity in the determination of benzimidazole fungicides in aqueous apple extracts.

Phenolic preservatives have been widely extracted from environmental water samples by direct immersion SPME. Polar fibers, such as polyacrylate (PA), PDMS-DVB, and carbowax-templated resin (CW-TPR), have been successfully tested to extract phenolic compounds from water samples. However, when using PA fiber in SPME-HPLC, phenolic compounds are not totally desorbed from the fiber during the desorption step 14-17.

The novelty of this paper lies in the extraction step of the method. It is based on the SPME technique. It has clear advantages over LLE: a marked increase in sensitivity; the simplicity of performing the extraction in only one cycle of absorption and desorption, and a considerable saving in time, chemicals and laboratory wastes.
The method has been validated and applied in the analyses of ten residual tanning floats from four companies that produce different kinds of leather articles.

3.2. Experimental procedure

3.2.1. Instrumentation

HPLC-PDA system: Alliance 2695 Separation Module (Waters Corporation, USA) fitted with a 2998 PDA Detector. A Mediterranean Sea18 15 x 0.46 cm 3µm column (Teknokroma, Spain) packed with C18 reversed-phase was used. UV scanning detection was performed between 190 and 380 nm. The mobile phase was 0.1% formic acid in water (A): 0.1% formic acid in acetonitrile (B). Gradient: 60 % B, 6 min isocratic, then programmed linear to 95 % B in 9 min. Flow was 0.9 mL/min. The oven temperature was held at 30 ºC.

3.2.2. Materials and chemicals

Formic acid (~98%) for mass spectroscopy was obtained from Fluka. Analytical standards of fungicides were obtained from Supelco (TCMTB, PCMC), from Fluka (OIT, OPP, TCP), and from Aldrich (IPBC). Qualitative filter papers were supplied by Filtros Anoia (Spain). Acetonitrile as solvent was of HPLC-gradient grade from Panreac (Spain). Milli-Q ultrapure water (Millipore, USA) was used in the mobile phase and for reagents preparation. 2,4,6-Trichlorophenol from Fluka was used as Internal Standard (IS). IS stock solution was 6.25 mg/L in acetonitrile. Sodium chloride for ionic strength buffer solution (30 % w/v NaCl and 0.20 % v/v formic acid) was obtained from Panreac (Spain).

Portable manual samplers of SPME with an extraction fiber coated with polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65 µm were purchased from Supelco (USA) (Product number 57359-U).

3.2.3. Samples

Eleven samples of industrial residual floats were analyzed. Seven were from chromium tanning floats, two were from wet white tanning baths, one was a residual float of a retanning process, and the last one was a residual bath of the fatliquoring process of vegetable leather production.

3.2.4. Procedure

A sample of approximately 20 mL of the float is filtered through a qualitative filter paper. 1.00 mL of the filtered float is pipetted into a beaker which contains 10.0 mL of milli-Q water. From this solution, 1.00 mL (or 5.00 mL for low concentration floats) is transferred into a 50.0 mL volumetric flask, which contains 25.0 mL of ionic strength buffer and 1.00 mL of internal standard solution and is subsequently filled with milli-Q water to the mark.

8.0 mL of the former solution are then transferred to a 10mL vial. A magnetic stirring bar of 2 mm diameter and 8 mm long is added and the vial is capped.

The capped vial is preheated at 30ºC in a water bath for five minutes. The stirring speed is adjusted at 900 rpm. Then, the septum-piercing needle of the SPME portable sampler is introduced through the septum of the vial. The fiber is extended and immersed into the solution for 70 minutes at 30 ºC (Figure 3).

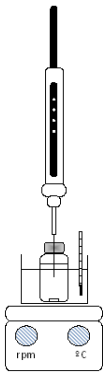

Figure 2. Fiber on extraction process

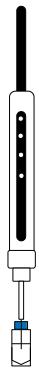

Figure 2. Fiber on desorption process

Once the extraction step is completed, the fiber is introduced into a glass screw neck vial that contains 0.400 mL of a solution of 60% v/v acetonitrile in water for the static desorption step. The vial is held at 45 ºC for 20 minutes (Figure 4). Subsequently, the fiber is retracted and the vial is homogenized. A 25-µL or 50-µL volume of this solution is injected into the HPLC system. After each analysis, the fiber is washed with Milli-Q water to remove the residues of NaCl.

The fiber can be reused until we detect that the sensitivity of the method decreases, i.e., when the area of the IS peak decreases substantially. From our experience, the fiber can be reused 75 – 85 times.

Analyte peak identity is determined by matching up the retention time with that obtained from the injection of analytical standards, and confirmed by comparing the UV spectrum obtained from each compound peak with that obtained from the standards. Detected preservatives are quantified using the calibration plots previously prepared with solutions of a given concentration of the standard fungicides extracted by SPME in the same manner as the samples.

The sensitivity and selectivity of the method is enhanced by the selection of a specific optimal detection wavelength for each molecule. The use of a PDA detector enables us to determine each fungicide at its own optimal wavelength. In accordance with the results of our previous paper 2, the best detection wavelengths are the following: for IPBC, 193 nm; for MBT, 324 nm; for OIT, 279 nm; for TCP, 290 nm; for OPP, 246 nm; for PCMC, 228 nm and for TCMTB 223 nm.

3.3. Results and discussion

The development of a SPME method involves the selection of the fiber and the optimization of desorption and adsorption conditions.

Fiber choice and development of desorption conditions

The performance of three different commercial fibers was compared: PDMS-DVB (60 µm), CAR-PDMS (75 µm) and PDMS (100 µm). The mixture acetonitrile/water, which was recommended as mobile phase in the ISO: 13365 Standard, was used as desorption solution. The use of acetonitrile alone is not advisable since this shortens the fiber lifetime 15. The influence of temperature between 25 ºC and 45 ºC on fungicide recovery was investigated. A temperature of 45 ºC provided the best results. Moreover, the acidification of

the desorption solution with 0.1 % of formic acid impaired the efficiency of the process for all fibers under study.
Given that the PDMS fiber did not have sufficient affinity for the analytes, it was discarded. Comparative studies of desorption with the two other fibers at different times and temperatures were carried out. It was concluded that the PDMS-DVB fiber allowed a total desorption after 20 min at 45 ºC. This total recovery was not achieved with the CAR-PDMS fiber even though the desorption time was prolonged.
Consequently, the PDMS-DVB fiber was selected for the study.

Adsorption conditions
There are some variables that should be adjusted in SPME to achieve good extraction efficiencies. These parameters include time and temperature of adsorption, sample volume, stirring conditions, and ionic strength of the sample.

The volume of the sample was fixed at 8.0 mL so that the fiber was completely immersed but maintaining a safe distance with the stirring bar. A lower volume is not recommended to avoid the risk of breaking the fiber.
For many organic compounds, aqueous solubilities decrease in the presence of large amounts of salt due to salting-out effect 18. Two tests with different concentrations of NaCL (5 and 15 %), both with 0.1 % of formic acid, were carried out to evaluate the effect of the ionic strength.

Higher salt concentrations can damage the fiber 19. The results obtained showed that for all the fungicides, except for TCMTB, the recoveries substantially improved with the highest salt concentration (15% of NaCl). By contrast, TCMTB adsorption was independent of salt concentration.

The investigated temperatures were 30, 45, 60 and. 75ºC. For all the fungicides, the highest adsorption was achieved at 30 ºC.
The SPME technique is based on the equilibrium established between the aqueous medium and the fiber coating of the analyte concentration. The time necessary to reach this equilibrium may vary from a few minutes to several hours.

The studied interval was 30, 50, 70, 90 and 120 min for each analyte in the presence of all other target compounds. Shaking was kept constant at 900 rpm with the same type of magnetic bar. The results showed that 120 min were not sufficient to reach equilibrium.
Jiu Ai [24] demonstrated that if adsorption time and shaking conditions are kept constant, it is not necessary to reach equilibrium to carry out a quantitative analysis in reproducible conditions. He termed this as non-equilibrium conditions.
The robustness of the quantitative results under non-equilibrium conditions at 70 min and for three concentration levels, between 0.1 and 1 mg/L, was investigated. The best level for calibration corresponded to the lowest concentration.

Validation
Linearity
In order to compensate for the possible diminution in efficiency of the fiber during its lifetime, a calibration with 2,4,6-trichlorophenol as internal standard was preferred. Calibration was carried out by the same SPME treatment as real samples.
The calibration plots were obtained for each of the six fungicides from multi-component standard solutions that contain all the analytes. Four standards of different concentration and a blank were prepared. Table 3 shows the concentration range of the extraction solutions used as calibration standards. Internal standard concentration was 125µg/L in every solution. Each point on the calibration plot was the arithmetic mean from two independent chromatographic injections.

Fungicide	*Concentration range*	*Correlation coefficient (r)*
TCMTB	***48 - 244 µg/L***	***0.9992***
PCMC	***27 - 136 µg/L***	***0.9999***
OPP	***14 – 71 µg/L***	***0.9999***
IPBC	***199 - 995 µg/L***	***0.998***
MBT	***18 - 91 µg/L***	***0.998***
OIT	***21 - 108 µg/L***	***0.9999***

Table 1. Range and linearity of the calibration carried out for each analyte

Recovery and precision
Synthetic samples were prepared from a residual float of a tanning process free from fungicides and spiked with given amounts of the analytes. To measure the accuracy and the precision of the analytical method, these samples were analysed each day during one week. The results obtained (average of the

Recovery as well as the Relative Standard Deviation) are listed in Table 4.

Fungicide	Concentration	Recovery (%, n=5)	RSD (%, n=5)
TCMTB	2.0 mg/L	95.0	± 8.6
PCMC	2.0 mg/L	116	± 12
OPP	1.7 mg/L	88.0	± 7.7
IPBC	14 mg/L	84.8	± 9.1
MBT	1.4 mg/L	81.9	± 12
OIT	1.9 mg/L	99.4	± 12

Table 2. Recovery and precision

Limits of detection and limits of quantification

Detection limits (LOD) were calculated for a signal-to-noise ratio of 3. Limits of quantification (LOQ) defined for a signal-to-noise ratio of 10 were also estimated. These values are calculated for 50-µL injection volumes and are shown in Table 5.

Fungicide	LOD (mg/L)	LOQ (mg/L)
TCMTB	0.25	0.83
PCMC	0.14	0.47
OPP	0.06	0.20
IPBC	6	17
MBT	0.05	0.17
OIT	0.07	0.25

Table 3. Límits of detection and quantification

Robustness of calibration

Four months later, a new calibration was carried out. New standard solutions were prepared but employing the same fiber, which had been used in approximately 70 absorption-desorption processes during this period. The Student's t-test was used to compare the differences between the results of the same samples calculated with both calibrations. No significant difference was observed in spite of the ageing of the fiber and the possible dispersion introduced as a consequence of the preparation of new solutions of standards or reactants. Moreover, small deviations in the shaking speed or in the control of temperature and absorption-desorption time made no difference.

Analyses of real samples

The method was applied in the analyses of eleven residual tanning floats from four companies. The results are presented in table 6.

As can be seen, residual floats 1 to 6 have higher concentrations than floats 7 to 11. This can be explained because samples 1 to 6 are residual floats from two tanneries that produce wet blue and wet white for storage purposes. By contrast, samples 7 to 11 are residual floats from two tanneries that employ a lower amount of fungicide because leather is produced in a follow-on process.

Some floats contained more than one fungicide molecule. In the recipe of sample number eleven, PCMC and OPP were applied in the tanning process and TCMTB in the fatliquoring one. The fatliquoring residual float contained TCMTB and the remains of PCMC and OPP released from the tanned leather to the waste bath.

There was no fiber damage or poor chromatographic resolution or any problem in the analyses of such real samples from diverse sources containing complex matrixes.

Sample	Fungicide detected	Concentration found
Residual float of wet blue production. Company 1- wb1.	TCMTB	78.5 mg/L
Residual float of wet blue production. Company 1- wb2.	TCMTB	77.7 mg/L
Residual float of wet blue production. Company 1- wb3.	TCMTB	47.2 mg/L
Residual float of wet white production. Company 1- ww4.	TCMTB	18.7 mg/L
Residual float of wet white production. Company 1- ww5.	TCMTB	20.2 mg/L
Residual float of wet blue production. Company 2- wb6.	TCMTB	28.2 mg/L
Residual float of tanning process. Company 3- tan7.	TCMTB	6.0 mg/L
Residual float of tanning process. Company 3- tan8.	TCMTB	5.7 mg/L
Residual float of tanning process. Company 3- tan9.	TCMTB	5.3 mg/L
Residual float of retanning process. Company 3- ret10.	TCMTB PCMC OIT	4.7 mg/L 2.4 mg/L 2.1 mg/L
Residual float of fatliquoring process in vegetable leather production. Company 4- veg11.	TCMTB PCMC OPP	2.7 mg/L 7.0 mg/L 2.2 mg/L

Table 4. Results obtained in the analyses of eleven residual floats from different sources

3.4. Conclusions

A very sensitive and highly specific method based on solid-phase microextraction and liquid chromatography-photo diodes array detection was developed and validated to determine residual amounts of preservatives in wasted floats. This method avoids the use of hazardous materials like solvents and does not generate significant laboratory wastes.

Sample preparation consists of a 2-hour extraction, filtration, and injection. The UV spectrum from PDA detection allows us to reliably confirm analyte identity. The wavelength used for quantitative determination is specific for each fungicide.

The analyses of real samples showed residual amounts of TCMTB ranging from 28 to 80 mg/L in wasted tanning floats for wet blue for storage production and from 5 to 6 mg/L for wet blue to be shaved and retanned in a short period of time. Wet white residual floats contained a lower amount of TCMTB. This was due to a better yield of the process, because the amount of TCMTB, the length of the process and the volume of the float were the same as in wet blue production. Furthermore, the residual amounts of TCMTB in the wet white leathers were higher than in the wet blue ones.

Acknowledgements

The authors thank Ms. Núria Muñoz for her contribution in part of the experimental trials and Mr. George von Knorring for reviewing the English version of the manuscript. The authors would also like to acknowledge the Spanish Ministry of Science and Innovation for the funding obtained through the CTQ 2009-08347 Project.

References

1. Hauber, C. & Germann, H.-P. The addition of fungicides and their Penetration. *World Leather* 75–82 (1997).
2. Landau, L. *Biocides in leather*. *Zhurnal Eksperimental'noi i Teoreticheskoi Fiziki* (1937).
3. Orlita, A. Microbial biodeterioration of leather and its control: a review. *Int. Biodeterior. Biodegradation* **53,** 157–163 (2004).
4. Ramadori, L. New generation of fungicides for leather preservation. *Cuoio, Pelli, Matiere Concia*. **86,** 19–23 (2010).
5. Planas, X. Determinació de TCMTB i altres fungicides en pells. (Treball Final de Carrera, UPC, Escola d'Enginyeria d'Igualada, 2004).
6. Vázquez, A. M. Implantació de noves aplicacions cromatogràfiques al laboratori de l'EUETII. (Treball Final de Carrera, UPC, Escola d'Enginyeria d'Igualada, 2004).
7. Bautista, S. Desenvolupament de Metodologia pel Control Analític de Fitosanitaris. (Treball Final de Carrera, UPC, Escola d'Enginyeria d'Igualada, 2006).
8. Parbery, C. & Taylor, C. D. Determination of methylene bis(thiocyanate) and 2-(thiocyanatomethylthio)benzo[d]thiazole in leather process liquors by high-performance liquid chromatography. *Analyst* **114,** 361–363 (1989).
9. Hinojosa Reyes, L., Wróbel, K. & Wróbel, K. Indirect extraction-spectrophotometric determination of 2-(thiocyanomethylthiol)benzothiazole in chrome tanning liquors after its breakdown to 2-mercaptobenzothiazole. *Talanta* **56,** 515—521 (2002).
10. Meneses, E. S., Arguelho, M. L. P. M. & Alves, J. P. H. Electroreduction of the antifouling agent TCMTB and its electroanalytical determination in tannery wastewaters. *Talanta* **67,** 682–5 (2005).
11. Fiehn, O., Reemtsma, T. & Jekel, M. Extraction and analysis of various benzothiazoles from industrial wastewater. *Anal. Chim. Acta* **295,** 297–305 (1994).
12. Reemtsma, T. Determination of 2-substituted benzothiazoles of industrial use from water by liquid chromatography / electrospray ionization tandem mass spectrometry. **1618,** 1612–1618 (2000).
13. Font, J., Reyes, M., Cuadros, S., Bacardit, A. & Marsal, A. Determination of TCMTB and Other Fungicides in L eather. *J. Am. Leather Chem. Assoc.* **106,** 341–348 (2011).
14. AENOR (2011). Cuero. Ensayos químicos. Determinación del contenido en conservantes (TCMTB, PCMC, OPP, OIT) del cuero mediante cromatografía de líquidos. (ISO 13365:2011
15. Martínez, K., Ferrer, I. & Barceló, D. Part-per-trillion level determination of antifouling pesticides and their byproducts in seawater samples by off-line solid-phase extraction followed by

high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr. A* **879,** 27–37 (2000).
16. Agüera, A., Piedra, L., Hernando, M. D. & Fernández-Alba, A. R. Multiresidue method for the analysis of five antifouling agents in marine and coastal waters by gas chromatography–mass spectrometry with large-volume injection. *J. Chromatogr. A* **889,** 261–269 (2000).
17. López Monzón, a, Vega Moreno, D., Torres Padrón, M. E., Sosa Ferrera, Z. & Santana Rodríguez, J. J. Solid-phase microextraction of benzimidazole fungicides in environmental liquid samples and HPLC-fluorescence determination. *Anal. Bioanal. Chem.* **387,** 1957–63 (2007).
18. Hu, Y. *et al.* A sensitive determination method for carbendazim and thiabendazole in apples by solid-phase microextraction-high performance liquid chromatography with fluorescence detection. *Food Addit. Contam. Part A. Chem. Anal. Control. Expo. Risk Assess.* **25,** 314–9 (2008).
19. Möder, M., Schrader, S., Franck, U. & Popp, P. Determination of phenolic compounds in waste water by solid-phase micro extraction. *Fresenius J. Anal. Chem.* **357,** 326–332 (1997).
20. González-Toledo, E., Prat, M. D. & Alpendurada, M. F. Solid-phase microextraction coupled to liquid chromatography for the analysis of phenolic compounds in water. *J. Chromatogr. A* **923,** 45–52 (2001).
21. Sarrión, M. ., Santos, F. . & Galceran, M. . Determination of chlorophenols by solid-phase microextraction and liquid chromatography with electrochemical detection. *J. Chromatogr. A* **947,** 155–165 (2002).
22. Peñalver, a, Pocurull, E., Borrull, F. & Marcé, R. M. Solid-phase microextraction coupled to high-performance liquid chromatography to determine phenolic compounds in water samples. *J. Chromatogr. A* **953,** 79–87 (2002).
23. Pawliszyn, J. *Handbook of Solid Phase Microextraction*. (Chemical Industry Press, 2009).
24. Ai, J. Solid Phase Microextraction for Quantitative Analysis in Nonequilibrium Situations. *Anal. Chem.* **69,** 1230–1236 (1997).