Determination of TCMTB and Other Fungicides in Leather

by

J. Font, M. Reyes,* S. Cuadros, A. Bacardit and A. Marsal[a]
Escola d'Enginyeria d'Igualada, Universitat Politècnica de Catalunya,
Plaça del Rei 15, 08700 Igualada, Spain
a IQAC-CSIC, Department of Chemical and Surfactants Technology,
J. Girona, 18-26, 08034 Barcelona, Spain

Abstract

The new ISO 13365:2011 develops a test method for the determination of the content of the preservative agents 2-(thiocyanomethylthio)-benzothiazole (TCMTB), 4-chloro-3-methylphenol (PCMC), 2-phenylphenol (OPP) and 2-Octyl-3(2H)-isothiazolone (OIT) in leather by liquid chromatography. The simultaneous determination of the fungicides TCMTB, PCMC, OPP, OIT, and also 2-mercaptobenzothiazol (MBT) and 3-iodo-2-propynyl-butylcarbamate (IPBC) in leather samples was carried out by liquid chromatography (HPLC) with diode array ultraviolet detection. The sample preparation and extraction step was performed following the new ISO 13365 Standard. 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 linear to 95% B in 9 min. The chromatographic detection introduced only a minor change with respect to the Standard: a photo diode array detector was used instead of a single wavelength ultraviolet one, thereby improving the reliability of the identifications and the sensitivity of the quantification. It has been ensured that 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) not interfere in the determination. The leather fortifications of 30 and 300 mg/kg yielded average TCMTB recoveries of 94% and 99%, respectively. The recoveries of the other fungicides were similar. The targeted fungicides were determined in 40 samples of commercial leather. Residues of TCMTB were found in 90% of samples.

In summary, the new ISO 13365:2011 Standard provides a quick and reliable method not only for the determination of the four molecules that are within the scope of the Standard but also for other fungicides such as IPBC and MBT.

Resumen

La nueva norma ISO 13365:2011 desarrolla un método analítico para la determinación del contenido de los agentes conservantes 2-(tiocianometiltio)-benzotiazol (TCMTB), 4-cloro-3-metilfenol (PCMC), 2-fenilfenol (OPP) y 2-octil-3(2H)-isotiazolona (OIT) en cuero por cromatografía líquida. La determinación simultanea de los fungicidas TCMTB, PCMC, OPP, OIT, y también de 2-mercaptobenzothiazol (MBT) y 3-yodo-2-propinil-butilo (IPBC) en muestras de piel se llevó a cabo mediante cromatografía líquida (HPLC) con detector ultravioleta de fotodiodos (PDA). La preparación de la muestra y el proceso de extracción se realizaron siguiendo la norma ISO 13365. La fase móvil consistió en agua con un 0.1% de ácido fórmico (A) y acetonitrilo con un 0.1% de ácido fórmico (B). La fase inicial tiene un 60% de B isocrático durante 6 minutos, entonces se inicia un gradiente lineal hasta el 95% de B en 9 min. En la detección cromatográfica se introdujo un pequeño cambio en relación con la norma. Se utilizó un detector de fotodiodos en lugar de un detector de longitud de onda fija para mejorar la fiabilidad de las identificaciones y la sensibilidad de la cuantificación.

Se ha comprobado que el 2,4,6-triclorofenol (TCP) y el pentaclorofenol (PCP) no interfieren en la determinación. Las fortificaciones de 30 y 300 mg/kg en cuero produjeron unas recuperaciones medias de TCMTB del 94% y 99%, respectivamente. Las recuperaciones de los otros fungicidas fueron similares. Los fungicidas investigados se determinaron en 40 muestras de cueros del mercado. En el 90% de las muestras se encontraron residuos de TCMTB.

En resumen, la nueva norma ISO 13365:2011 proporciona un método rápido y fiable, no sólo para la determinación de las cuatro moléculas incluidas en el ámbito de aplicación de la norma, sino también para otros fungicidas como el IPBC y el MBT.

*Corresponding author, e-mail: m.reyes.reyes@eei.upc.edu.
Manuscript received March 29, 2011, accepted for publication May 24, 2011

INTRODUCTION

Fungicides are the substances used to inhibit the growth of fungi that cause the degradation of leather. 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). Figures 1 to 6 present the chemical structure and the UV spectrum of the fungicides studied in this paper. Before the decade of 1990's, pentachlorophenol (PCP) was the most employed fungicide in the leather industry. Since the withdrawal of PCP, TCMTB became the most widely used substance for controlling the fungi in the leather industry.

Determination of PCP and organochlorine pesticides in skins and leather is routinely carried out by gas chromatography (GC).[1,2] Determination of TCMTB is preferred by liquid chromatography (HPLC) rather than by GC for avoiding the possibility of thermal decomposition.[3,4] HPLC with careful calibration provides well reproducible results for quantitative analysis.[5] The first trials of TCMTB determination in leather were carried out in 1978 by subjecting treated wet blue to eight hours Soxhlet extraction with dichloromethane [6]. A poor recovery of 50% was obtained. Fowler et al. proposed a combined sonication and Soxhlet extraction with dichloromethane in 1987.[7] The efficiency of the extraction increased when the moisture of the sample was lowered. The determination stage was carried out with adsorption-HPLC eluting with non-polar solvents like hexane or dichloromethane. However, low recoveries were still obtained. Tomaselli et al, in 1991, introduced the chromatographic method of reversed phase HPLC eluting with acetonitrile/ water acidified with 0.1% H_3PO_4 for the analysis of fungicides.[8]

The ISO 13365:2011 Standard is based on the knowledge and experience of the *Lederinstitut Gerberschule* of Reutlingen on fungicide analysis. The ground leather sample is extracted with a mixture water/acetonitrile with the aid of ultrasonic waves for one hour. The filtered extract is analysed by reversed phase HPLC with UV detection.[9] With this method, the efficiency of the extraction of TCMTB from leather is considerably improved. This is mainly due to using samples of reduced humidity content. Wet blue and wet white leather are dried prior to grinding and extracting. The extraction of leather with a solvent miscible with water such as methanol or acetonitrile has the added advantage that the phase change for its injection in reversed phase chromatography is not necessary. Acetonitrile is preferred to methanol since acetonitrile allows the detection of molecules at a wavelength as short as 190 nm. This is convenient for molecules such as PCMC, OPP and IPBC that have the maximum absorptivity at short wavelengths.

Unlike the solvent proposed in the ISO Standard, a slightly acidified mobile phase was used in this work. Given that TCMTB is more stable in acidic conditions and given that phenolic preservatives are determined simultaneously, the use of acetonitrile and water at a slightly acidic condition is advisable. We used acetonitrile with 0.1% formic acid, and water with the same % of formic acid instead of acetonitrile and water. Unpublished work performed in our laboratory using acetonitrile and water acidified with trifluoroacetic acid gave results very similar to those obtained when using these eluents acidified with formic acid. However, formic acid has the advantage of being a suitable reagent also for HPLC - Mass Detection systems.

No method for simultaneous determination of more than four different fungicides in leather has been reported. In fact, very little is known about the residual contents of fungicides in hides and leather. Hauber and Germann have indicated that the TCMTB content of wet blue should be 250 ppm for minimum level of fungicidal protection, compared to 580/280 ppm for PCMC/OPP and 80 ppm for OIT.[10,11] A greater protection requires increased concentration of fungicide.

The aim of this paper is to describe a rapid and simple method for the simultaneous determination of TCMTB and seven other fungicides in hides and leather and to evaluate the parameters of its validation. This work deals with the application of easily available techniques such as HPLC with photo diode array (PDA) detection and ultrasounds assisted extraction.

This HPLC method is adapted from the Standard ISO 13365. It is suitable for the four mostly used fungicides in the leather industry and could also be employed for other molecules that are currently being investigated. The sensitivity and selectivity of the method can be significantly improved compared to UV detection at a fixed wavelength by the selection of a specific optimal detection wavelength for each molecule. The use of a PDA detector enables us to obtain as many chromatograms (channels) as different fungicides under study for every injected sample. Each fungicide is detected at its own optimal wavelength.

Figures 1 to 6 show the UV spectra of the analytes. The different fungicides have absorption maximums at different wavelengths. The proposed detection wavelengths were the following: for IPBC, 193 nm; for MBT, 324 nm; for OIT, 279 nm; for TCP, 205 nm; for PCP, 214 nm; for OPP, 201 and 246 nm; for PCMC, 201 and 228 nm and for TCMTB 223 and 280 nm.

EXPERIMENTAL

Instrumentation

HPLC-PDA system: Alliance 2695 Separation Module (Waters Corporation, Milford, Massachusetts, USA) fitted with a 2998 PDA Detector. A Mediterranean Sea_{18} 15 x 0.46 cm 3μm column (Teknokroma, Barcelona, Spain) packed with C18 reversed-phase was used. UV scanning detection was

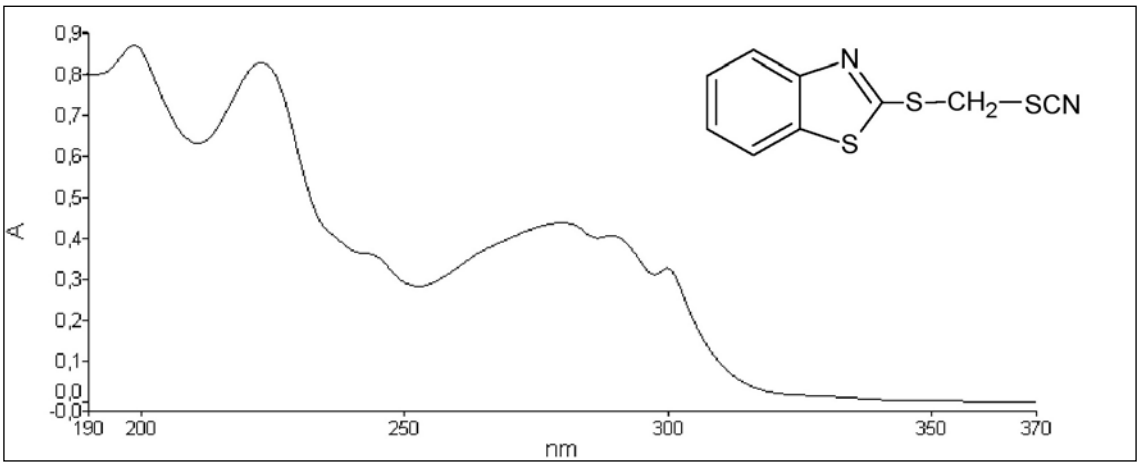

Figure 1. Structure and UV spectrum of TCMTB

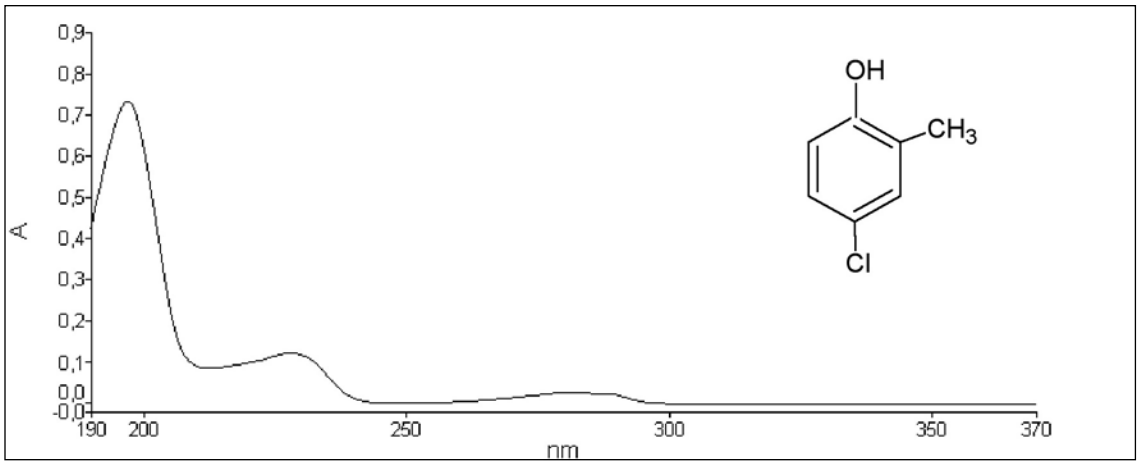

Figure 2. Structure and UV spectrum of PCMC

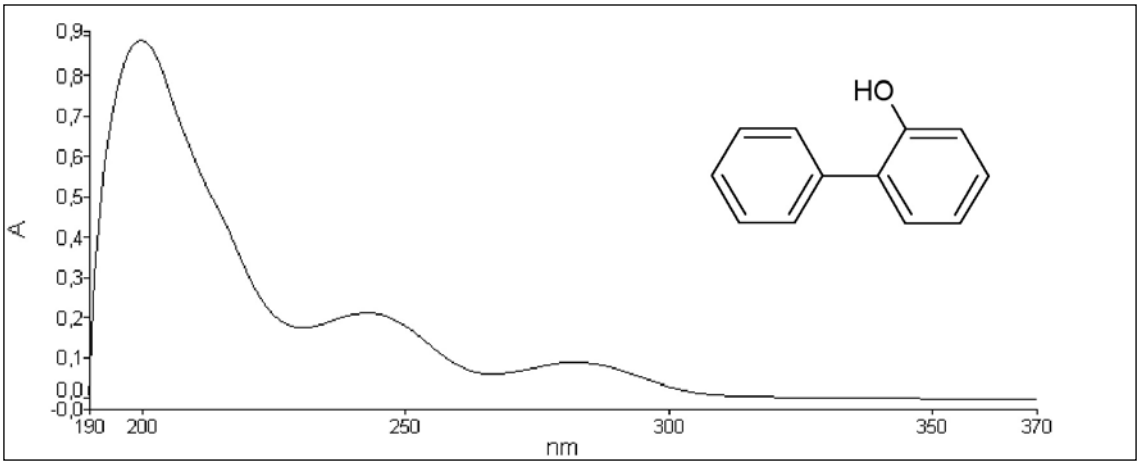

Figure 3. Structure and UV spectrum of OPP

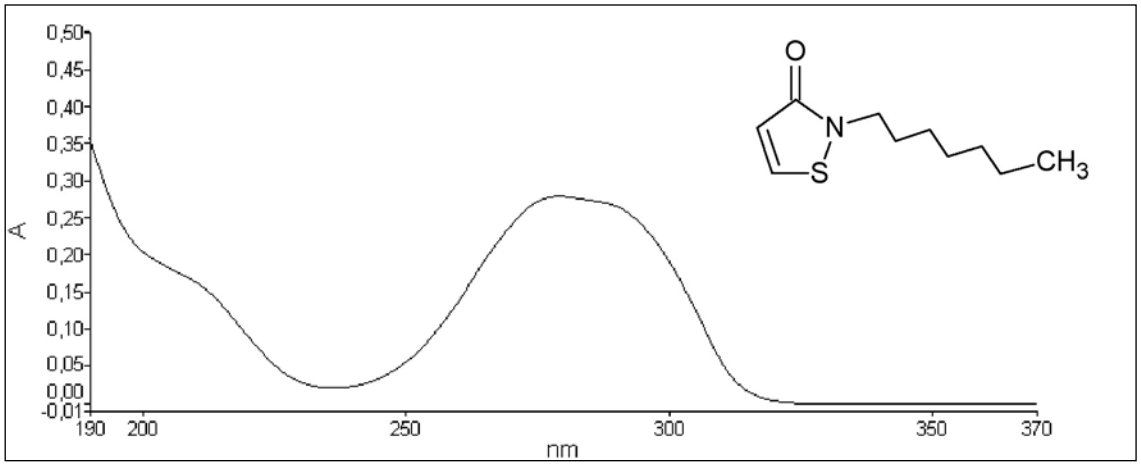

Figure 4. Structure and UV spectrum of OIT

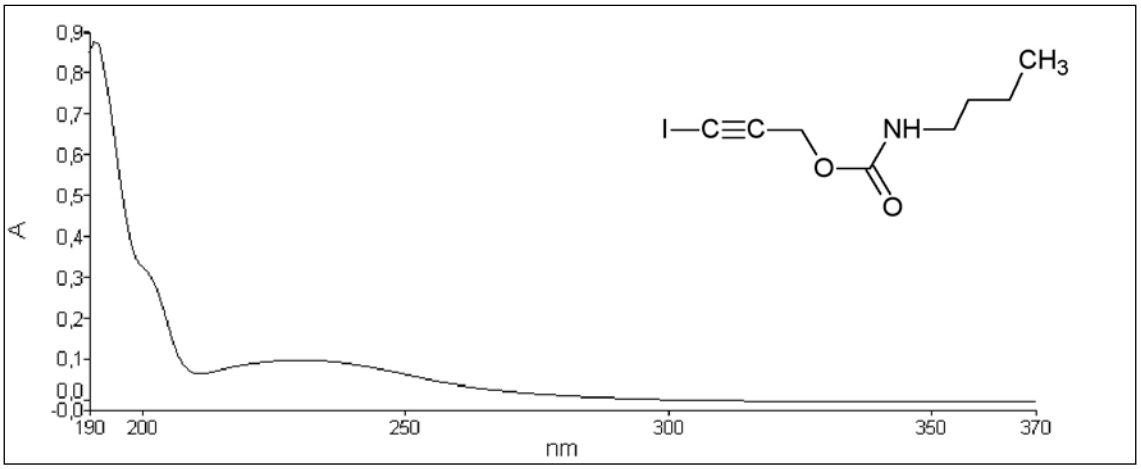

Figure 5. Structure and UV spectrum of IPBC

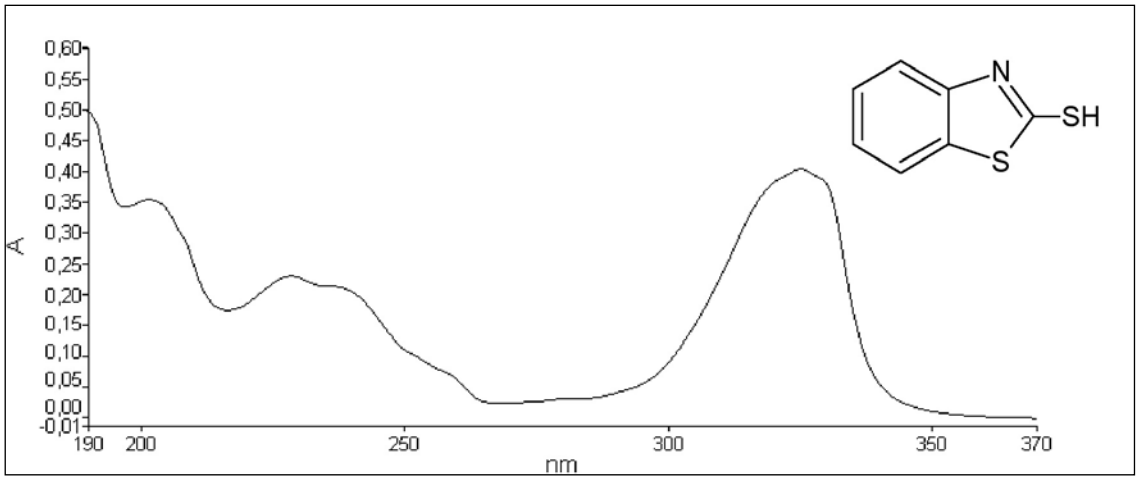

Figure 6. Structure and UV spectrum of MBT

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°C. 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.

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.

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.

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.

Results and Discussion

All the preservatives investigated were successfully separated under the detailed experimental conditions. The resolution between peaks was very good. The presence of 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) did not interfere in the determination. Figure 7 is an example of the chromatographic separation. The quantitative method was validated in terms of linearity, precision, sensitivity and recovery to determine the method quality and reliability.

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.

Linearity, recovery and precision

Five standards within the range 2 to 40 mg/L of OCP, OIT, PCMC, PCP and TCMTB were prepared. For MBT, the range of the standards was 0.5 – 20 mg/L and for IPBC was 5 – 50 mg/L. The peak areas were measured and calibration graphs were plotted on the linear regression analysis without forcing the curve through the zero. Linearity was verified over the entire working range. Correlation coefficients were all higher than 0.999.

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 25ºC, 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 23ºC 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.

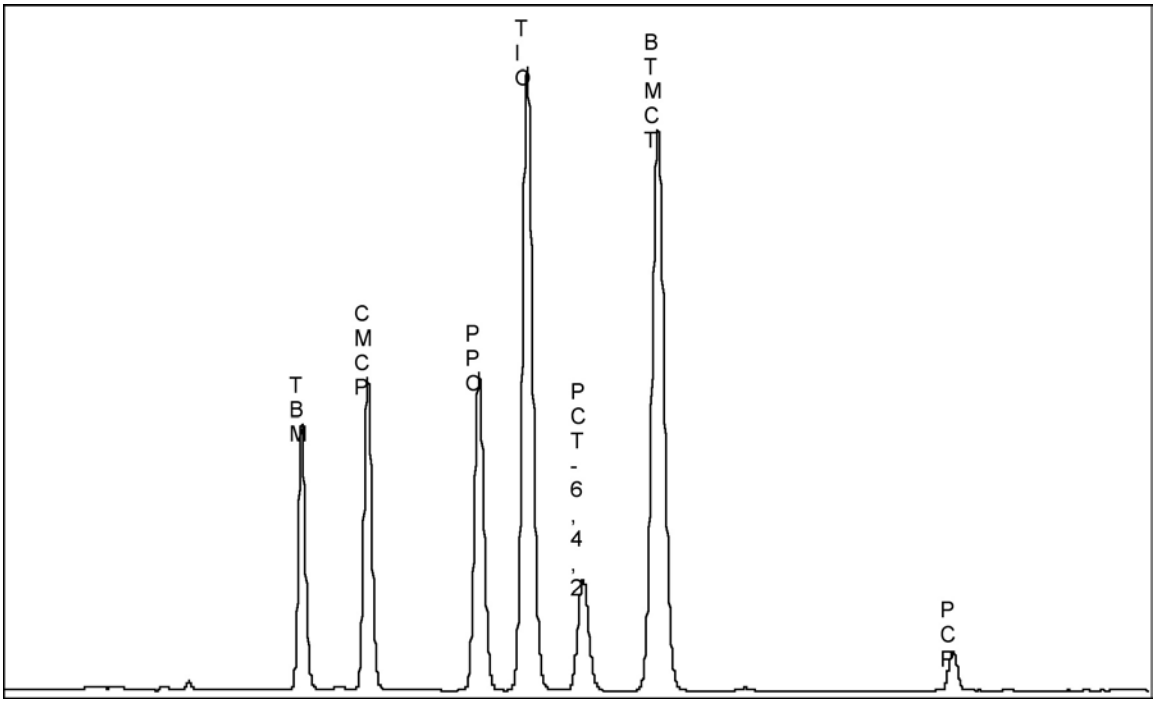

Figure 7. HPLC chromatogram of a standard of seven preservatives at concentrations ranging from 5 to 10 mg/L. See Experimental Section for chromatographic details. Wavelength of detection was 270 nm.

TABLE 1
Limits of detection (LOD) and quantification (LOQ) of 8 fungicides

Fungicide	Wavelength (nm)	LOD (µg/g)	LOQ (µg/g)
TCMTB	223	1.7	5.7
	275	1.2	4.0
MBT	324	0.06	0.2
PCMC	197	0.71	2.4
	201	1.3	4.3
	228	2.0	6.7
OPP	201	2.2	7.3
	243	1.7	5.7
OIT	279	0.46	1.5
TCP	203	1.7	5.7
PCP	214	0.39	1.3
IPBC	193	17	57

TABLE 2
Recoveries of 6 fungicides at two levels of concentration

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

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.

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.

Analysis of commercial samples

The six targeted fungicides were determined in 40 commercial samples of leather. All the samples contained residues of at least one of the determined fungicides. 40% of the samples contained two or more different molecules. Among fungicide residues identified, TCMTB was detected in 90% of the hides, PCMC in 35%, OPP in 25%, and MBT and OIT in 8%. As expected, IPBC was not detected in any sample. TCMTB was present in the ten Spanish wet-blue hides that were analyzed. One of these samples also contained 154 mg/kg of MBT. Concentrations ranging from 440 to 540 mg/kg of TCMTB were found in Spanish wet-blue hides for exportation. For short time conservation wet-blue, concentrations of TCMTB ranged from 253 to 354 mg/kg. All the values are expressed in weight basis of samples conditioned in the standard atmosphere.

Most samples that contained PCMC also contained OPP. The concentrations detected of the two fungicides varied from 8 to

680 mg/kg and from 8 to 480 mg/kg, respectively. The concentrations detected of MBT and OIT ranged from 4 to 154 mg/kg and from 44 to 230 mg/kg, respectively.

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.

Acknowledgements

The authors thank Ms. Núria Muñoz for her contribution in the analyses of real commercial samples and in the repeatability tests 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. J. Font, A. Marsal. *Journal of Chromatography A*, **811**, 256, 1998.
2. J.Font, E.Poch. *Bol. AQEIC*, **42**, 149 (1991).
3. N. Voulvoulis, M.D. Scrimshaw, J.N. Lester. *Chemosphere*, **38**, 3503, 1999.
4. S. T. Nawrocki, K. D. Drake, C. F. Watson, G. D. Foster, and K. J. Maier. *Arch. Environ. Contam. Toxicol.* **48,** 344, 2005.
5. L. Muthusubramanian, R.B. Mitra, V.S. Sundara Rao *J. Soc.Leath.Tech.Ch.* **82**, 22, 1998.
6. J. Van Deren, E.F.Weiss. *J.Am.Leather.Chem.As.* **73**, 498, 1978.
7. W. M. Fowler, A.E.Russell, I.H.Kruger, S.C.Pinchuck. *J. Soc. Leath. Tech. Ch.* **71**, 100, 1987.
8. M. Tommaselli, A. Cozzolino, *Cuoio Pelli Mat. Concianti*, **67**, 221,1991.
9. http://www.iso.org/iso/catalogue_detail.htm?csnumber=53718.
10. C.Hauber, H.P.Germann. *World Leather* **10**, 75, 1997.
11. C.Hauber. Microbicide applications in the leather industry in *Directory of Microbicides for the Protection of Materials*. W. Paulus. Springer, 317-324, 2004.