An Improved Method for Accurately Determining Total Content of Four Fungicides (TCMTB, OIT, OPP And PCMC) in Leather

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Abstract

Determination of total contents of four fungicides (TCMTB, OIT, OPP and PCMC) in leather is required by Ecolabels due to health risks. Thus, an improved analytical procedure based on the current Standard ISO 13365-1-2020, was investigated to accurately test the total contents of the four fungicides in leather. It was verified that the ultrasonic assisted acetonitrile extraction described in this Standard is only effective for extracting TCMTB, but inefficient for PCMC, OPP and OIT. The extraction efficiency by acetonitrile was 93% for PCMC, 95% for OPP and 89% for OIT, respectively. Methanol was proven to be an optimal alternative of acetonitrile for sample extraction and presented satisfactory extraction efficiencies (~100%) for eluting the four fungicides at a wide content range (80-470 mg/kg). The detection limits were evaluated and well satisfy the requirement by the Ecolabels. Results of HPLC chromatograms from commercial leather samples demonstrated no obvious matrix interferences from methanol extraction. Series of leather samples from different origins were analyzed. TCMTB was found in 86% and OIT in 30% of the total tested samples, which were higher in content than the allowable limits by Ecolabels.

Introduction

Mold growth is a common problem for leather consumer products. It arises from the fact that leather is an organic material which has nutrient fats and proteins,[1] and is often exposed to mold under the daily storage and application conditions. Mold growth takes place particularly under humid conditions and at relatively high temperatures that enable mold to reproduce. Mold may hydrolytically degrade the collagen fibers and fat/oil components, thus impairing aesthetic, functional and other properties of leather. Researchers have stated that spue and some finishing defects in leather are closely related with mold growth.[2-3] Although precautions taken for leather can inhibit or slow down mold growth, the simple and precise solution is to use commercial preparations containing active ingredients called fungicides.[4]

Early fungicide products used in tanneries were chlorinated phenols (e.g. pentachlorophenol), or organo arsenic or mercury compounds (e.g. Phenyl mercuric acetate).[5] These products are effective for inhibiting the growth of mold, but they are also very toxic to human and environmental organisms. For example, pentachlorophenol has been proven to be a persistent toxic substance causing histopathological changes and mutations in aquatic life and is a probable human carcinogen.[6] Since the 1970s, the use of chlorophenols and organic compounds containing heavy metals as fungicides have been banned by legislation worldwide and have been gradually phased out.[7]

Organic pesticides are numerous and might be used as fungicides for leather. Their cost and anti-mold efficiency, as well as risk assessment by legislation, suggest that only a few pesticides are currently used in the leather industry.[8] In 2013, the European Biocidal Product Regulation (EU 528/2012) restricted the application of unregistered fungicides in leather manufacture. It led to four essential fungicides which are currently used. They are commonly known by their abbreviation PCMC, OPP, OIT and TCMTB, as listed in Table I. The four fungicides used in leather processing are also required to be controlled within a certain content range due to their toxicity and hazards, as indicated by the Ecolabels of OEKO-TEX®, BLUESIGN® and German BLUE ANGEL. The maximum limits of the four fungicides in leather recommended by BLUE ANGEL (DE-UZ 148, 2015) are 300, 500, 100, and 500 mg/kg for PCMC, OPP, OIT and TCMTB, respectively.

With this recognition of the allowable limits of the four fungicides in leather by brands, it is necessary to employ a robust procedure to detect their total content. The current standard method for analyzing the four fungicides in leather samples is ISO 13365-1-2020. It uses ultrasonic-assisted acetonitrile extraction followed by HPLC or LC-MS detection. This Standard has been in effect since 2011 due to its early edition ISO 13365-2011 and confirmed by the literature.[9] However, the full validation of this Standard has not yet been publicly reported, and the test results of the targets based on this procedure were found to be inconsistent with the desired contents in our laboratory. So, it is unsure whether the test results are real 'total contents' of these targets in leather, or not.

The aim of this study was to develop a reliable procedure for measuring the real 'total content' of the four targets based on ISO

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13365-1-2020. Firstly, leather samples containing known amounts of the four fungicides were carefully made according to leather-making processes.[10] These samples were then extracted according to the Standard method with acetonitrile, as well as with selected solvents as methanol, dimethylformide, ethanol, tetrahydrofuran and isopropanol. These results were finally analyzed and the extraction efficiencies were compared.

Experimental

Reagents and materials

Acetonitrile (ACN), methanol (MeOH), ethanol (EOH), tetrahydrofuran (THF), isopropanol (IPA) and N,N-dimethylformide (DMF) were used to dissolve standards, and to prepare the extracts. They were of HPLC-grade obtained from Aladdin (Shanghai, China). Reference standards of four fungicides (in Table I), were obtained from Sigma-Aldrich (Shanghai, China), and their stock solutions (1000 mg/L) were prepared by dissolving appropriate amounts of the commercial products in ACN and stored in glass-stoppered bottles at 4°. Appropriate volumes of the stock solutions were diluted to prepare solutions containing the four targets at 0.8-55 mg/L by the proper solvent. Table I lists the targets with numbers and abbreviations identifying the compounds.

The liquid commercial preparation containing the four fungicides was obtained from Kai-Mei Scientific Co. Ltd (China) with each concentration of ~8.5% (w/w). The preparation could well disperse and form an aqueous emulsion after being dropped into water. It was used for processing the leather crusts in drums to obtain samples containing the four targets.

Lime-split cattle-hides were obtained from a local tannery in Haining City (China). Commercial leather samples (50 pieces) were obtained from the market located in Haining China-Leather market. Leather samples with moisture content of 9-13% w/w, were cut into pieces (~3 mm × 3 mm) and sealed in bags lined with aluminum foil.

Apparatus

The following apparatuses were used: ultrasonic water-bath with frequency 40 kHz and power 200 W (Kunshan, China), PTFE membrane filter of 0.45 μm × 10 mm (Shanghai, China), and experimental drum with diameter 60 cm (Wuxi, China).

Sample preparation

Cattle-hide crusts were carefully prepared from limed hides at the leather laboratory (Jiaxing University), according to normal chrome-leather processing procedure.[10] After retanning and fatliquoring, the crusts were air dried to a moisture content of ~12% w/w and stored in sealed plastic bags at ~4°C. It was noted that during the processing, all the chemicals and auxiliaries were carefully selected and only those without the four fungicides were used, to ensure the crusts were not contaminated by those four targets. These crusts were used as negative controls.

Three positive samples (No. 1#, 2# and 3#) containing known amounts of the analytes were made by adding the preparation with 0.1%, 0.3% and 0.6% individually based on the negative crust weight in the experimental drums. The water amounts in the drum were 3-fold of the crust weight. The preparation used was first diluted with 10-fold water and then added gradually into the running drum within 3 min. After that the drum ran continuously for 120 min to allow the fungicides to penetrate into the crust thoroughly. The crusts were then taken out and lay on a porous mesh to dry under the room conditions for almost one week with a moisture content of ~12% w/w, and then stored in sealed plastic bags at 4°C. The distribution of the four targets in the three positive crusts was the same as in general leather processing.

The content of each fungicide in the positive samples was analyzed by also accounting for its residue in the float, as well as the amounts of the commercial fungicides used. For analyzing the float, this solution was first filtered with stainless meshes, and then directly analyzed with HPLC according to the procedure described in the following sample extraction. Then the real amounts of the four analytes in the three crusts were evaluated (as listed in Table III).

Prior to test, both the negative and positive crusts were cut into pieces (~ 3 mm × 3 mm) and conditioned for 24 h at standard atmospheric temperature of 20°C and relative humidity 65% (Temp. 20°C/R.H. 65%). The moisture contents of the samples were near to 12% w/w.

Sample extraction

Extraction of the four fungicides from leather samples was carried out by ultrasonic-assisted solvent extraction (single-cycle) according to ISO 13365-1-2020, but with the following modification. The operation was performed in a 50 mL screw-capped glass bottle charged with 2.0 g of accurately weighed sample pieces. After adding 40.0 mL aliquot of the selected solvent into the bottle, the contents were treated continuously in the ultrasonic water bath (40 kHz) at 25-35°C for 60 min. The bottles were then cooled to room temperature, and the extracts were filtered on a 0.45 μm PTFE filter, which were then ready for HPLC analysis. All tests were performed in triplicate. The calculations were all based on dry matter of leather sample.

For the evaluation of the real contents of the four targets in the positive samples, repeated extraction (3-cycles) with MeOH under the same ultrasonic conditions was also performed as follows. Firstly, 2.0 g of sample pieces were extracted with 40 mL MeOH for 60 min. Then the leather pieces were filtered and retreated with 40 mL MeOH for another 60 min. Finally, the leather pieces were further treated with 20 mL MeOH for 30 min. All the extracts retrieved were merged into a 100 mL conical flask. After filled to the mark with MeOH, the solutions were filtered and analyzed with HPLC.

Extraction efficiency

Extraction efficiency (E, %) with different solvents was evaluated according to:

$$E = 100 \times w_e/w_r$$

where w_e refers to the test result with the used solvent, and w_r is the real content of the target in the sample.

HPLC analysis

The HPLC system was Agilent 1260 equipped with Diode Array Detection (DAD) and a thermostat. The separation was performed on a Diamond C_{18} reversed-phase column (250 mm × 4.6 mm I.D.; 5 µm particles (Dikma, China)) with an isocratic elution program. The condition was ACN/water solution 70:30 (v/v) for 10 min. The flow rate was 1.0 mL/min and the column was maintained at 40°C. Injection volume was 10 µL.

External calibration plots were built in the 0.8-55 mg/L concentration range for the four analytes. The curves were fitted by linear-ship and the correlation coefficient r^2 was calculated from the linear regression, which was expected to be greater than 0.995.

Results and Discussion

Analysis of fours targets with HPLC

According to ISO 13365-1-2020, HPLC parameters were further optimized to ensure the four fungicides were satisfactorily separated from one another. The chromatogram of the injected standard mixture exhibited complete resolution under the chosen HPLC operating conditions. The use of DAD allowed the acquisition of all analyte's spectra and the selection of the optimum detection wavelength for each compound. Besides, the spectrum was a means to verify the identity of analytes by comparison with the Standards. Table I reported the elution order, retention time of each analyte. Figure 1 presented all the spectra recorded at the peak retention time. A relative absorption maximum was evident at 225 nm and 280 nm for TCMTB, 246 nm and 285 nm for OPP, 229 nm and 282nm for PCMC, and 280 nm for OIT, respectively. In general, a long UV wavelength (as 280 nm) is clearly better than the short one (as 225 nm or 246 nm) for recovery and precision due to the noise and interferences from matrix and solvent (as DMF). In view of this consideration, 280 nm of UV detection was chosen for detecting the four targets.

Table I

The four fungicides with their names, CAS No. and retention times

No.	Target	Structure name	CAS. No	Retention time / min
1	PCMC	4-chloro-3-methylphenol	59-50-7	3.89
2	OPP	2-phenylphenol	90-43-7	4.56
3	OIT	2-octylisothiazol-3(2H)-one	26530-20-1	5.53
4	TCMTB	2-(thiocyanomethylthio)- benzothiazole	21564-17-0	5.86

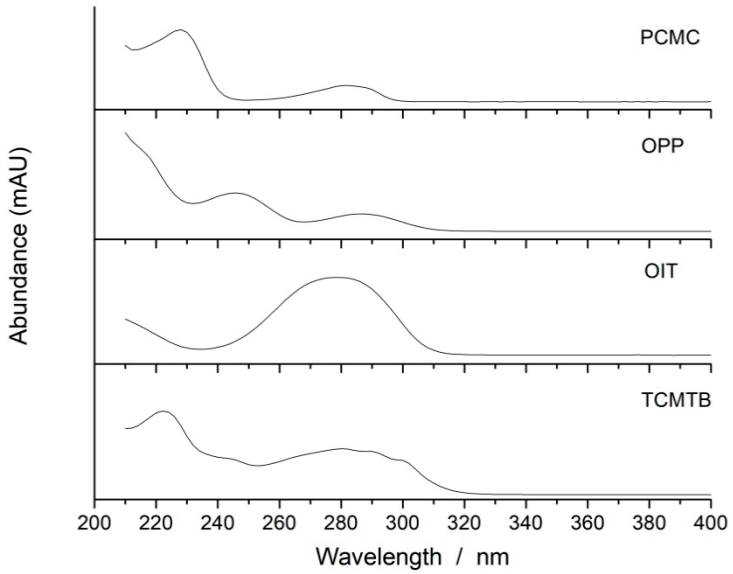

Figure 1. UV spectra of the four fungicides obtained with DAD

Calibration and detection limits

Linearity was tested based on the standard addition procedure by adding known amounts of the four fungicides to blank leather extracts. The negative leather sample was used as blank due to the absence of the four fungicides. External calibration curves (in terms of peak areas vs. concentration) were obtained in the range 0.8-55 mg/L at seven concentration levels. All calibrations were linear in the explored concentration range. Figure 2 presented the calibration plots relative to the four targets. It could be seen that the curves for the single target in ACN, MeOH, DMF, EOH, THF and IPA, almost completely coincided, indicating little interference of the six solvents for HPLC analysis. Thus, the calculation was based on one standard curve for one target even if different solvent was used in the following extraction experiment. The calibration parameters were reported in Table II.

Detection limits (LODs) were evaluated according to the instrumental detection limits, as well as weight of the sample and volume of the final extracts. LODs for targets determined by considering signal-to-noise of 3:1, were 20.0 mg/kg for PCMC, 10.0 mg/kg for OPP, 8.0 mg/kg for OIT and 4.5 mg/kg for TCMTB, respectively. These LODs can well satisfy the requirements by current Ecolabels.

Sample extraction

Repeated extraction (3-cycles) with MeOH was first carried out for measuring the total contents of the four targets. The results were compared with the real contents as described above. Three positive samples with different concentration levels were analyzed, and the data are listed in Table III. For any single target, the two results from one concentration level were close with each other, and their average deviation was less than 2%. Thus, it was believed that the repeated extraction (three times) with methanol could elute all of the four fungicides in leather. These results (in Table III) further verified the real contents of each target in the three positive samples and provided a basis for evaluating the extraction efficiencies of ACN, MeOH, DMF, EOH, THF and IPA in the followings investigation.

In contrast to repeated extraction, the single-cycle extraction operation as described in ISO 13365-1:2020 is obviously handy and efficient. However, the solvent used should be competitive for

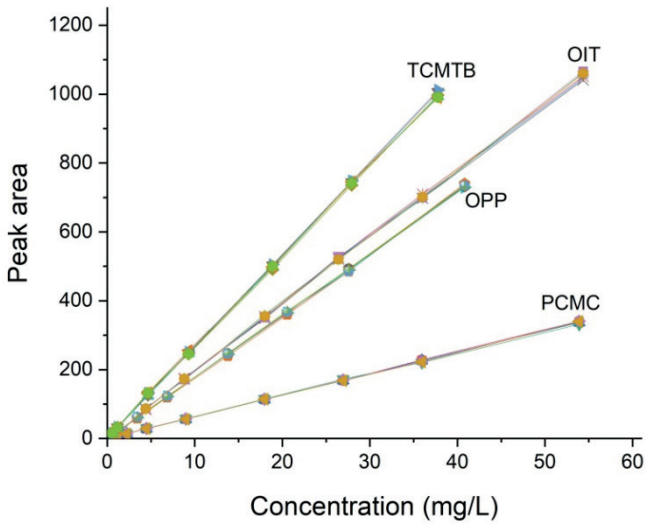

Figure 2. Calibration curves of the four fungicides in the six solvents: ACN, MeOH, DMF, EOH, THF and IPA, respectively (λ=280 nm)

Table II

Regression parameters and LODs for the four analytes

Analyte	Curve equation[a)]	r^2	LODs (mg/kg)
PCMC	$A_i = 6.33 \times C_i - 0.22$	0.9985	20.0
OPP	$A_i = 17.91 \times C_i - 1.01$	0.9991	10.0
OIT	$A_i = 19.49 \times C_i + 0.81$	0.9992	8.0
TCMTB	$A_i = 26.38 \times C_i + 0.44$	0.9989	4.5

[a)] A_i is the peak area, and C_i is the concentration, mg/L. The results were based on MeOH as solvent.

Table III

Contents of four fungicides in three positive samples

Sample No.	Contents based on float analysis (mg/kg)				Contents based on repeated extraction (mg/kg)			
	PCMC	OPP	OIT	TCMTB	PCMC	OPP	OIT	TCMTB
1#	78.0±4.5	80.7±5.2	80.2±4.3	81.1±4.4	80.1±3.1	81.4±3.8	81.8±3.0	82.3±2.2
2#	238.6±6.3	231.1±5.6	235.1±5.1	238.3±6.1	233.7±4.3	236.9±5.2	240.1±2.5	242.7±4.5
3#	462.1±5.1	457.8±5.7	464.0±4.7	463.1±5.6	470.4±5.0	450.2±6.3	457.3±4.1	468.1±5.1

solubilizing all the analytes to release them fully from the leather fiber. The solubility of target in solvent depends on their polarity, molecular structures, etc, as revealed by the Rule of the Like Dissolves Like in solubility. The four analytes and ACN are all polar compounds. Thus, other polar solvents as MeOH, EOH, THF, IPA and DMF should be comparative with ACN for dissolving the analytes. Especially for DMF, it is a Universal Solvent and possesses excellent solubility for the four analytes. So it is desired to fully elute the four targets from leather fibers.

The extraction efficiencies of MeOH, EOH, DMF, THF and IPA were tested and compared with ACN in one-cycle extraction. These results are reported in Figure 3. It can be seen that the three solvents (DMF, MeOH and THF) gave desired extraction efficiencies (~100%) for the four targets, indicating the total elution of the targets from the three positive samples. However, another three solvents (IPA, EOH and ACN) presented unsatisfactory results. For IPA, it only gave desired extraction efficiency (~100%) for OIT, but low results for PCMC (~90%), OPP (~95%) and TCMTB (~83%). For EOH, it presented relatively poor extraction efficiencies for PCMC (~90%), OPP (~88%), OIT (93%) and TCMTB (82%). For ACN, it gave a satisfied result (~100%) for TCMTB, but poor results for PCMC (93%), OPP (~95%) and OIT (~89%). These indicated that the three solvents (IPA, EOH and ACN) can't totally elute the four targets in one-cycle extraction. Thus, according to ACN extraction described in ISO 13365-1:2020, the test results of the three targets (PCMC, OPP and OIT) were not their 'total contents' in leather.

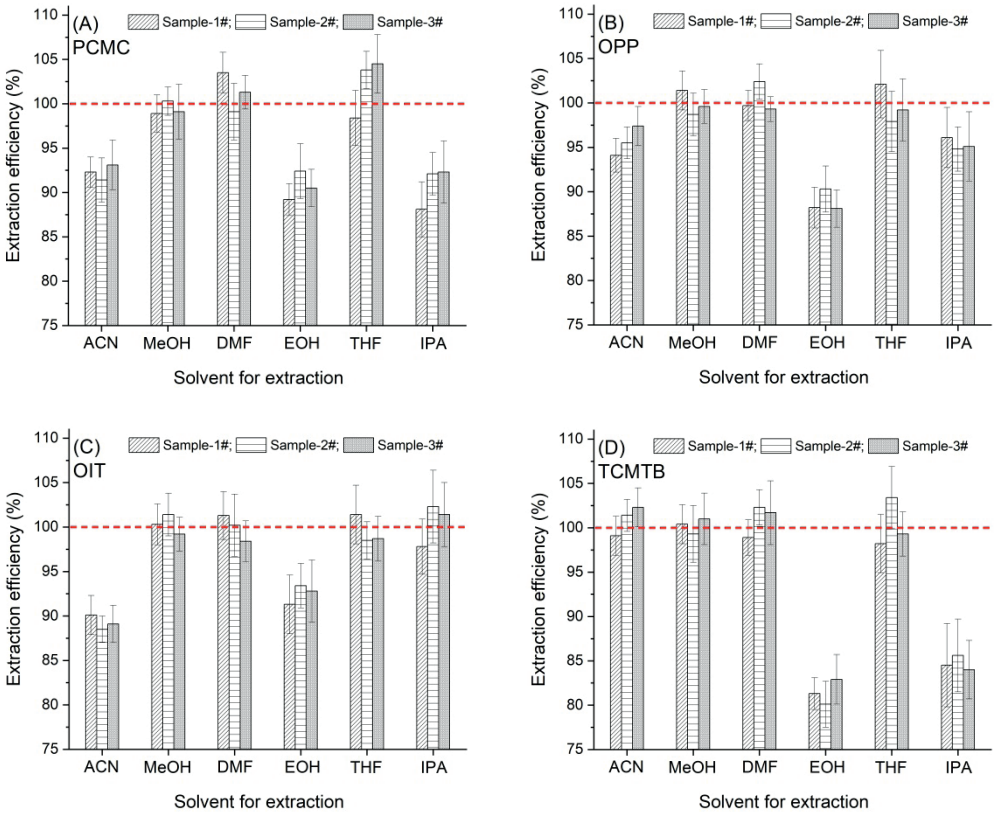

Figure 3. Extraction efficiencies of ACN, MeOH, DMF, EOH, THF and IPA for extracting **(A)** PCMC, **(B)** OPP, **(C)** OIT and **(D)** TCMTB from three positive samples in single-cycle extraction

The satisfied results determined by MeOH, DMF and THF rely on their polarity-based ability to dissolve the four targets. These data indicated the feasibility of using MeOH, DMF and THF to replace ACN for extraction. In comparison, MeOH should be given priority, because DMF and THF are very strong for solubilizing the leather fiber and leads to serious disturbance for HPLC analysis. Besides, MeOH has low toxicity, is readily available and a poor solvent of oils and polymers in the leather fibers. Thus, the new and improved procedure using ultrasonic assisted MeOH extraction was built and applied for testing real leather samples.

Analysis of real samples

Fifty commercial samples collected from garment, shoe and furniture leather, were tested by using the improved procedure, to verify its adaptability and to determine the contents of the four fungicides. All of the samples contained at least one of the four targets, and almost 40% of the samples contained two different compounds. Of all the tested samples, TCMTB was detected in 86%, OIT in 30%, OPP in 10% and PCMC in 2%. The concentrations ranged from 221 to 623 mg/kg for TCMTB, 79 to 211 mg/kg for OIT, 58 to 104 mg/kg for OPP, and 125 mg/kg for PCMC, particularly. These results demonstrate the popular application of TCMTB in current leather processing as well as its realistic levels. On the other hand, it was found that contents of TCMTB and OIT in 5 pieces were significantly higher than the allowable limits by Ecolabels from BLUE ANGEL (TCMTB < 500 mg/kg, OIT < 100 mg/kg). This may be a problem that should be addressed by leather tanneries.

Two samples each containing PCMC and OPP individually, and another sample containing both TCMTB and OIT, were further tested with ACN assisted ultrasonic extraction (single cycle, as described in ISO 13365-1-2020). These results were compared and listed in Table III. For PCMC, OPP and OIT, the contents based on ACN extraction were about 92.6%, 94.5% and 90.0% of that on MeOH extraction, respectively. This fact was quite similar with that obtained using positive samples above, and further verified the lower extraction efficiencies of ACN for the three targets.

Figure 4 showed the typical HPLC chromatogram with TCMTB and OIT. Their peaks were easily identified by their retention times

Table III

Comparison of ACN and MeOH extraction by testing commercial samples

Extraction solvent	Content of Targets[a] (mg/kg)			
	PCMC	OPP	OIT	TCMTB
MeOH	125.0±3.4	77.6±3.0	108.4±3.5	372.1±4.8
ACN	115.8±2.1	73.3±3.1	97.6±4.2	369.4±3.9

[a] PCMC and OPP were tested with each individual sample. OIT and TCMTB were tested together with another sample.

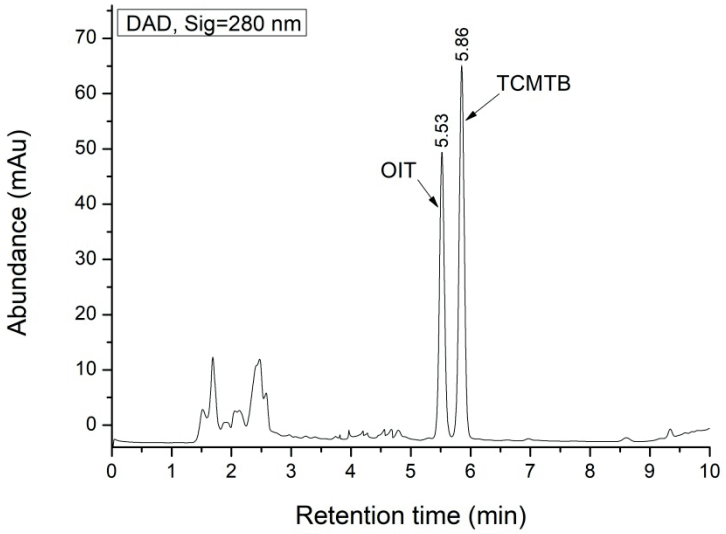

Figure 4. Chromatograms of OIT and TCMTB in MeOH extracts of real sample (concentrations of OIT and TCMTB were 181 and 176 mg/kg, respectively)

and UV spectra from DAD detection. The results demonstrate the absence of enhanced noise or interference from/in the MeOH extracts. These findings indicate the selectivity and reliability of this improved procedure.

Conclusion

The method of determining the total content of four fungicides (PCMC, OPP, OIT and TCMTB) in leather was investigated. The ultrasonic-assisted acetonitrile extraction method as described in ISO 13365-1:2020, was not effective for fully eluting the three fungicides (PCMC, OPP, and OIT) from leather. This problem is mainly due to the relative weak solubility of these fungicides in acetonitrile. By comparison, the alternative methanol extraction gave excellent results. This improved procedure was validated by testing samples with known contents of four fungicides and gave extraction efficiencies of almost 100% for the targets at wide concentration levels. The chromatogram of real samples indicated no obvious noise or interferences from methanol extracts. The detection limits were sufficient enough to satisfy the requirements of Ecolabels.

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References

1. Covington, A.D. Tanning Chemistry: The Science of Leather, Royal Society of Chemistry Press, 2011.
2. Tancous J.J. Skin, hide and leather defects. Tanners' Council Research Laboratory, Cincinnati, Ohio, 363, 1986.
3. Sharphouse, J.H. Leather technician's handbook. Leather Producers' Association, Northampton, 575, 1989.
4. Adminis, U., Huynh, C., Money C.A.; The need for improved fungicides for wet-blue. *J. Soc. Leather Technol. Chem*. **86**, 118-121, 2002.
5. Cuadros, S., Manresa, M., Font, J.; Alternative fungicides: Comparisons with conventional chemicals. *J. Soc. Leather Technol. Chem*. **95**, 263-269, 2011.
6. Igbinosa, E.O., Odjadjare, E.E., Chigor, V.N.; Toxicological profile of chlorophenols and their derivatives in the environment: the public health perspective. *Sci. World J.* 2013, 75-84, 2013.
7. Bayramoglu, E.E,, Gülümser, G., Karaboz, I.; Ecological and innovative fungicide for the leather industry: Essential oil of origanum minutiflorum. *JALCA*, **101**, 96-104, 2006.
8. Cuadros, S., Manresa, M., Font J.; Alternative fungicides for the leather industry: application in various processes. *J. Soc. Leather Technol. Chem*. **96**, 225-233, 2012.
9. Font, J., Cuadros, S., Bacardit, A.; Determination of TCMTB and other fungicides in leather. *JALCA*, **106**, 341-348, 2011.
10. John W. Stevens. Leather Manufacture, Kessinger Publishing, LLC, 2008.