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Simultaneous determination of triclabendazole and its sulphoxide and sulphone metabolites in bovine milk by high-performance liquid chromatography

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Abstract

A simple method has been developed for the simultaneous determination of triclabendazole and its metabolites (sulphoxide and sulphone) in bovine milk by reversed-phase high-performance liquid chromatography (HPLC). A milk sample was homogenized with sodium sulfate anhydrous and acetonitrile, and centrifuged. The supernatant was isolated, rinsed with *n*-hexane saturated with acetonitrile, and evaporated. The residue was dissolved with 0.1 *M* potassium dihydrogenphosphate, and 0.1 *M* sodium hydrogencarbonate, and then cleaned up on a Bond Elut C_{18} cartridge. The three compounds were separated on a Capcell Pak C_{18} UG 120 (5 μm, 150×4.6 mm I.D.) column and determined by UV detection at 295 nm. The mobile phase was acetonitrile–0.05 *M* ammonium acetate (50:50), and the flow-rate was 0.8 ml/min at 40°C. The mean recoveries ($n=4$) were 89.1–95.0% with a relative standard deviation of 1.1–2.6%. The detection limits were 0.004–0.006 μg/g in milk. The proposed method was used to monitor raw milk samples for the market, and applied to the analysis of milk samples from 10 cows which had been administered with triclabendazole to control the liver fluke. The confirmation of the triclabendazole and its metabolites in the above milk sample has been achieved by electrospray LC–MS for the first time.

Keywords: Milk; Food analysis; Triclabendazole

1. Introduction

Triclabendazole is a benzimidazole anthelmintic widely used in the control of the liver fluke, *Fasciola hepatica*, in sheep and cattle. After oral administration, it has been found that triclabendazole is oxidized to sulphoxide and sulphone metabolites in animals, similarly to other benzimidazole sulphide compounds [1–3] (Fig. 1.)

FAO/WHO had reported the recommended maximum residue limits (MRLs) for triclabendazole in animal foods [4]. The MRLs of triclabendazole are scheduled to be adopted after June of 2000 in Japan. In order to monitor the residual triclabendazole and its metabolites in commercial milk samples, a simple, rapid and sensitive method is needed.

Methods have been published for the determination of triclabendazole and its metabolites in biological samples using HPLC with UV or fluorescence detection in pharmacokinetic studies [1,2,5–8].

A method has been published for the determination of eight benzimidazole anthelmintics includ-

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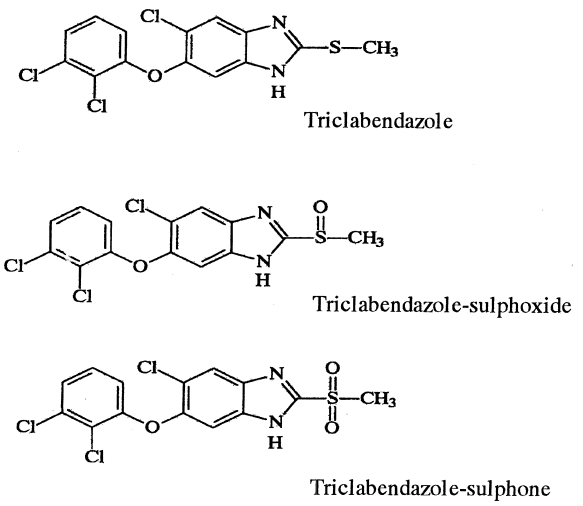

Fig. 1. Structures of triclabendazole and its metabolites.

ing triclabendazole in meat samples [9]. But, the metabolites of triclabendazole could not be simultaneously studied by this method. At present no method is available for the simultaneous determination of triclabendazole and its metabolites in milk.

The purpose of this study was to develop a simple method for the simultaneous determination of triclabendazole and its metabolites in milk.

2. Experimental

2.1. Materials and reagents

Triclabendazole and its metabolites (sulphoxide and sulphone) were kindly supplied by Ciba-Geigy (Munchwilen, Switzerland).

Acetonitrile and *n*-hexane were of HPLC grade (Kanto, Tokyo, Japan). Sodium sulfate anhydrous, potassium phosphate monobasic, sodium hydrogencarbonate and ammonium acetate were of analytical-reagent grade (Wako, Osaka, Japan). Distilled water was of HPLC grade (Kanto).

Solid phase extraction C_{18} cartridge was of Bond Elut LRC, 500 mg (Varian, Harbor City, CA, USA). The cartridges were conditioned with 10 ml of acetonitrile followed by 10 ml of distilled water prior to use.

2.2. Preparation of standard solutions

Stock standard solutions (1 mg/ml) of triclabendazole and its metabolites were prepared by dissolving 10 mg of the compounds in 10 ml of acetonitrile. The working standard solutions were prepared by serial dilution of the stock solution with mobile phase. The stock and the working standard solutions were stored at 4°C.

2.3. Apparatus

The refrigerated centrifuge was a Model LX-130 (Tomy, Tokyo, Japan), the vacuum concentrator was a Speedvac Model AES 2010 (Savant Instruments, Holbrook, NY, USA), the homogenizer was a Ultra-Turrax Model T25 (Janke & Kunkel, Staufen, Germany), the sample extraction manifold was a Waters 20-position extraction manifold (Waters, Milford, MA, USA) and the ultrasonic bath was a Model 1210 (Branson, Danbury, CT, USA).

2.4. HPLC conditions

The HPLC system consisted of a Model LC-6AD (Shimadzu, Kyoto, Japan), UV–Vis spectrophotometric detector Model SPD-6A (Shimadzu) and autosampler Model AS-950 (Jasco, Tokyo, Japan). The data calculations were performed using a Chromatopac C-R3A (Shimadzu). Separation was performed on a Capcell Pak C_{18} UG 120, 5 μm, 150×4.6 mm I.D. column (Shiseido, Tokyo, Japan). The mobile phase consisted of acetonitrile–0.05 *M* ammonium acetate (50:50, v/v). The column oven was kept at 40°C, and the flow-rate was 0.8 ml/min. The wavelength of the detector was fixed at 295 nm.

2.5. LC–MS conditions

The LC–MS system comprised a Quattro II quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray (ES) ionization interface. This system was coupled to the outlet of the HPLC column at the split ratio of 1:4 using a length of polyether ether ketone (PEEK) tubing. The electrospray probe and desolvation temperatures were set at 120 and 350°C, respectively. The negative ES mass spectra were measured by

corn voltage of 45 V for triclabendazole, 35 V for triclabendazole sulphoxide, and 50 V for triclabendazole sulphone. The chromatographic conditions were as described in 2.4. HPLC conditions except for the flow-rate of 1.0 ml/min.

2.6. Sample preparation

Portions (5.0 g) of milk were weighed into 50-ml centrifuge tubes, and sodium sulfate anhydrous (5 g), and acetonitrile (20 ml) were added, and homogenized by a homogenizer. The tubes were centrifuged for 10 min at 3000 rpm, then the supernatant was transferred to a 200-ml separatory funnel. This extraction step was repeated with 10 ml of acetonitrile, then the supernatant was combined in the 200-ml separatory funnel. The acetonitrile extracts were rinsed with *n*-hexane (20 ml) saturated with acetonitrile. The lower layer was transferred into 50-ml centrifuge tubes and evaporated by a Speedvac vacuum concentrator. A 1-ml volume of 0.1 *M* potassium phosphate monobasic was added to the residues in the centrifuge tubes and sonicated for 1 min in an ultrasonic bath, then 5 ml of 0.1 *M* sodium hydrogencarbonate was added and sonicated for 1 min. Finally the extract solution was applied to Bond Elut LRC C_{18} cartridges using a manifold. The cartridges were rinsed with distilled water (2 ml) and dried by allowing air to pass through under vacuum for 3 min. Triclabendazole and its metabolites were eluted with acetonitrile (2 ml). Aliquots (20 μl) of the eluate and standard solutions were injected into the HPLC system for analysis.

2.7. Calibration graphs

Standard levels of 0.05, 0.125, 0.25, 0.5, 1.0 and 1.5 μg/ml of triclabendazole and its metabolites were prepared from the stock standard solution. A 20-μl volume of these solutions was injected into the HPLC system. Calibration graphs were obtained by measurement of peak areas.

3. Results and discussion

3.1. Chromatographic conditions

To achieve an efficient separation of the triclabendazole, triclabendazole sulphoxide and triclabendazole sulphone, we investigated the retention factor (k) of these compounds in the mobile phase by changing the concentration (40–70%) of acetonitrile. The relationship between the retention factor (k) and concentration of acetonitrile in the mobile phase is shown in Fig. 2.

Because the k value of triclabendazole was remarkably increased when there was <50% acetonitrile in the mobile phase, it was difficult to simultaneously determine these compounds. To analyze these compounds within the range 1–10 of the k

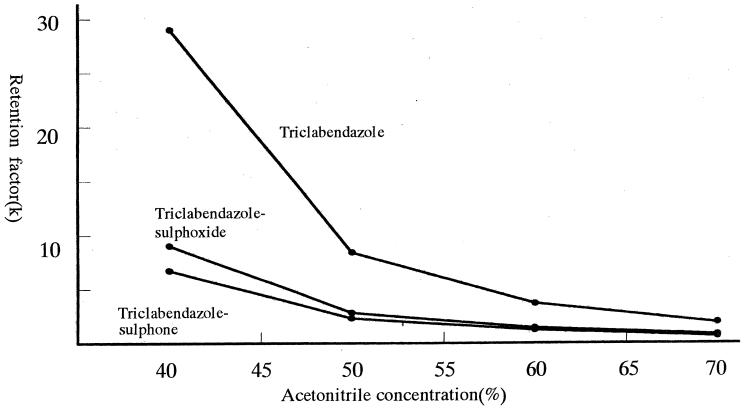

Fig. 2. Effect of acetonitrile concentration in mobile phase on the retention factors of triclabendazole and its metabolites; mobile phase: acetonitrile–0.05 *M* ammonium acetate.

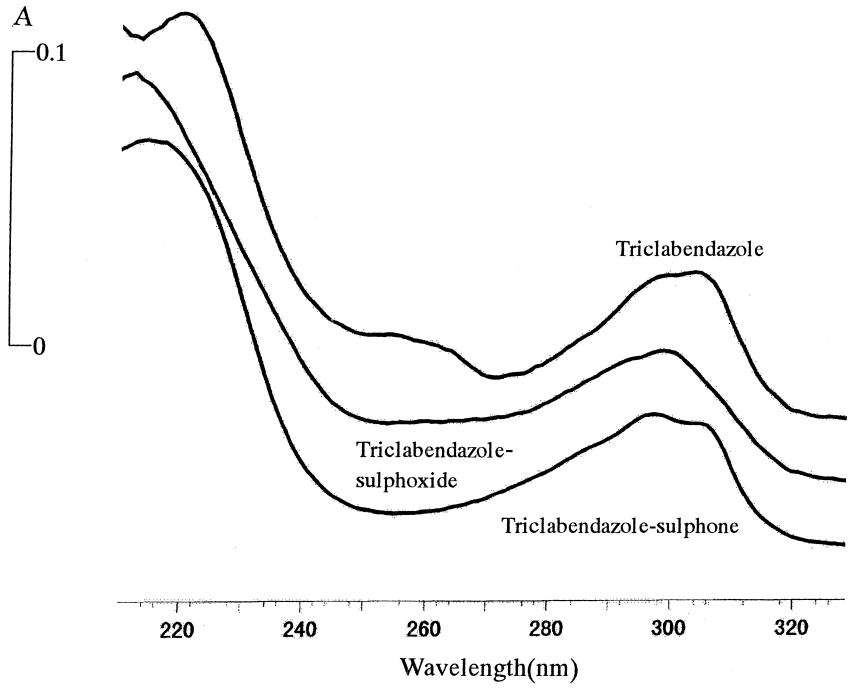

Fig. 3. UV spectra of triclabendazole and its metabolites in mobile phase.

value, the concentration of acetonitrile in the mobile phase needed to be >50%.

UV detection of triclabendazole and its metabolites was performed at 295 nm. The UV spectra showed two maxima at about 220 and 300 nm (Fig. 3). The detection at 220 nm was not suitable because of interference by the mobile phase. Under the described chromatographic conditions, a good separation of triclabendazole and its metabolites was achieved in 15–20 min (Fig. 4).

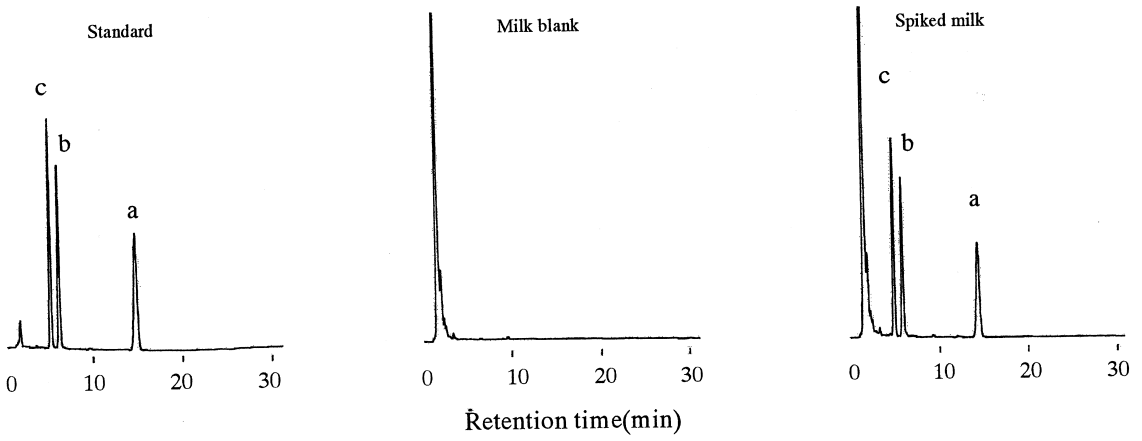

Fig. 4. Typical chromatograms: triclabendazole (a), triclabendazole sulphoxide (b) and triclabendazole sulphone (c). Column: Capcell pak C_{18} UG 120 (150×4.6 mm I.D.); mobile phase: acetonitrile–0.05 *M* ammonium acetate (50:50); flow-rate: 0.8 ml/min; detection: 295 nm.

3.2. Sample preparation

Since the regulatory monitoring of drugs in food requires the analysis of a large number of food samples in a short time period, a simple, rapid and cost effective preparation method is needed. Use of 0.1 *M* sodium hydrogencarbonate solution for sample preparation was effective in removing acidic substances from milk. With the proposed preparation method, no interfering peaks originating from the samples were observed in any milk samples. Typical chromatograms for the standards and milk samples are shown in Fig. 4.

3.3. Recovery

The linearity, accuracy and precision of this method were examined using the standard solutions. All calibration graphs obtained under the above-mentioned conditions were linear when the amount injected was 1.0–30 ng (Fig. 5).

The recovery of the compounds from milk are given in Table 1. The mean recoveries ($n=4$) of triclabendazole, triclabendazole sulphoxide and triclabendazole sulphone from milk spiked at levels of 0.2 μg/g were 89.1, 91.4 and 95.0%, respectively, and the RSD values were 1.6, 2.6 and 1.1%, respectively.

The detection limits of the compounds in milk were 0.006 μg/g for triclabendazole, 0.005 μg/g for triclabendazole sulphoxide and 0.004 μg/g for triclabendazole sulphone ($S/N=3$). The quantification limits were of the method were about five times as much as the individual detection limits.

Table 1
Recovery of triclabendazole and its metabolites from spiked milk samples, $n=4$

Compound	Spiked (μg/g)	Recovery (%, Mean±SD)	RSD (%)
Triclabendazole	0.2	89.1±1.4	1.6
Triclabendazole sulphoxide	0.2	91.4±2.4	2.6
Triclabendazole sulphone	0.2	95.0±1.1	1.1

This method was easy to perform, and the recovery and accuracy were good enough to make the procedure applicable to routine use in the analysis of residual levels of these compounds in milk.

3.4. Residual analysis of raw milk samples

To evaluate the suitability of the proposed method for the detection of the three compounds in milk, 40 raw milk samples collected in the Tokyo area were analysed. In no samples were the compounds detected.

Next, 20 raw milk samples (24-h milking and 48-h milking) from 10 cows to which Fasinex (the trademark name of triclabendazole) had been ad-

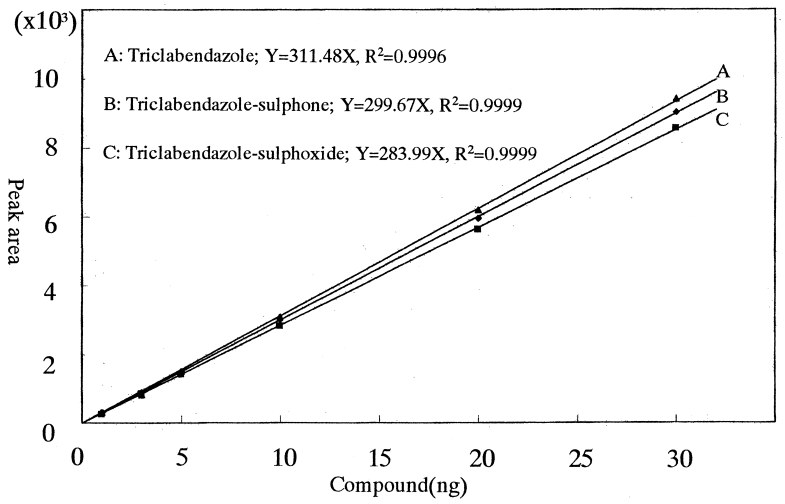

Fig. 5. Calibration graphs of triclabendazole and its metabolites.

Table 2
Residual concentrations of triclabendazole and its metabolites in the milk of cow following a single oral dose of triclabendazole (12 mg/kg body mass), $n=10$

Compound	Time after dosing (h)	Concentration (μg/g)	
		Mean	Range
Triclabendazole	24	0.024	0.008–0.072
	48	0.013	0.006–0.035
Triclabendazole sulphoxide	24	0.093	0.038–0.195
	48	0.044	0.017–0.114
Triclabendazole sulphone	24	0.168	0.038–0.395
	48	0.327	0.051–0.595

ministered orally at a single dose of 12 mg per kg body mass for the treatment of fascioliasis were analysed. The results of the analysis are given in Table 2, and the chromatograms are shown in Fig. 6.

The average levels of triclabendazole, triclabendazole sulphoxide and triclabendazole sulphone in the milk samples ($n=10$) from treated cows were 0.024, 0.093 and 0.168 μg/g at 24 h, and 0.013, 0.044 and 0.327 μg/g at 48 h, respectively. In several pharmacokinetic studies with sheep, goats and cows, the parent triclabendazole did not appear in plasma [1,2,10] or milk [6,7], but in the present study, it was detected in the milk samples analysed. This indicates that the proposed method is more sensitive than previously reported methods.

3.5. Confirmation of triclabendazole and its metabolites in raw milk samples

Triclabendazole and its metabolites in the milk samples from treated cows were concentrated and analysed by LC–ES-MS. The chromatograms and mass spectra of the standards and the milk extracts are shown in Figs. 7 and 8.

As shown in Fig. 7 (left), the peaks of standard triclabendazole and its metabolites were clearly observed on mass chromatograms monitored at individual deprotonated molecules (triclabendazole; m/z 357, triclabendazole sulphoxide; m/z 373 and triclabendazole sulphone; m/z 389). Their background subtracted mass spectra taken at the top of each peak on the mass chromatograms are shown in Fig. 7 (right). The deprotonated molecules, $[M-H]^-$ appeared strongly in the mass spectra of triclabendazole and its metabolites as the base peaks. Typical fragment ions, $[M-H-CH_3]^-$ for triclabendazole and triclabendazole sulphoxide, and $[M-CH_3-SO_2]^-$ for triclabendazole sulphone were clearly visible.

Fig. 8 (left) shows the mass chromatograms of the

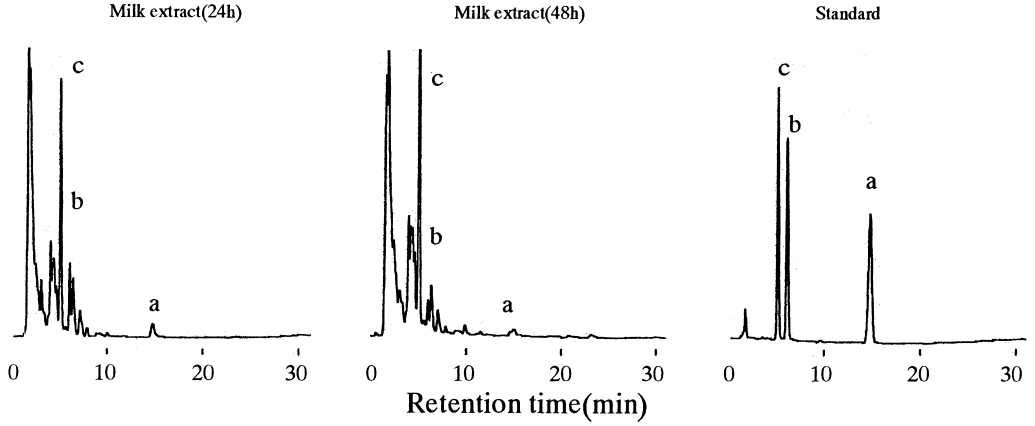

Fig. 6. Chromatograms of milk extract and standards. Triclabendazole (a), triclabendazole sulphoxide (b) and triclabendazole sulphone (c).

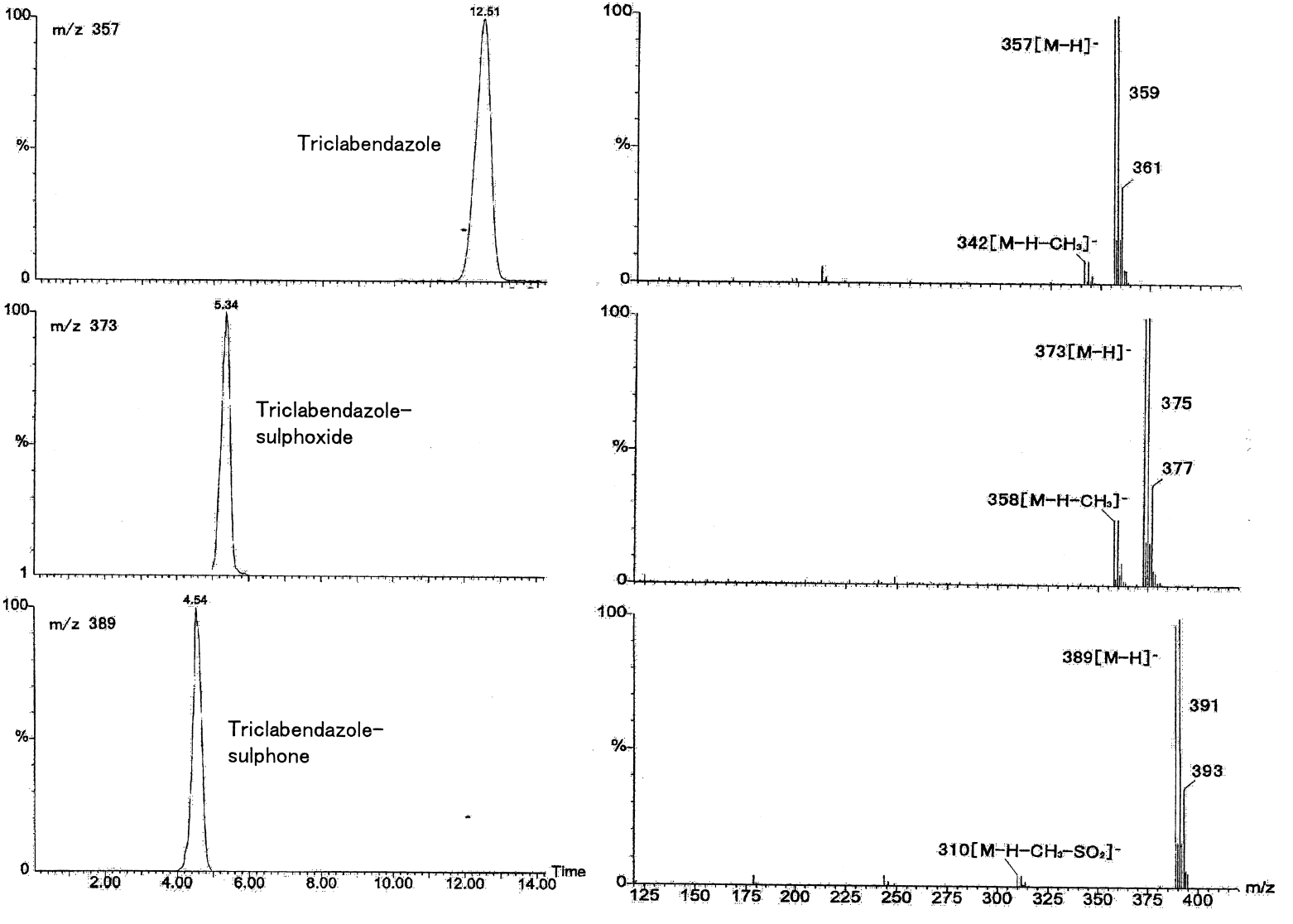

Fig. 7. Mass chromatograms and LC–MS spectra of standard triclabendazole and its metabolites.

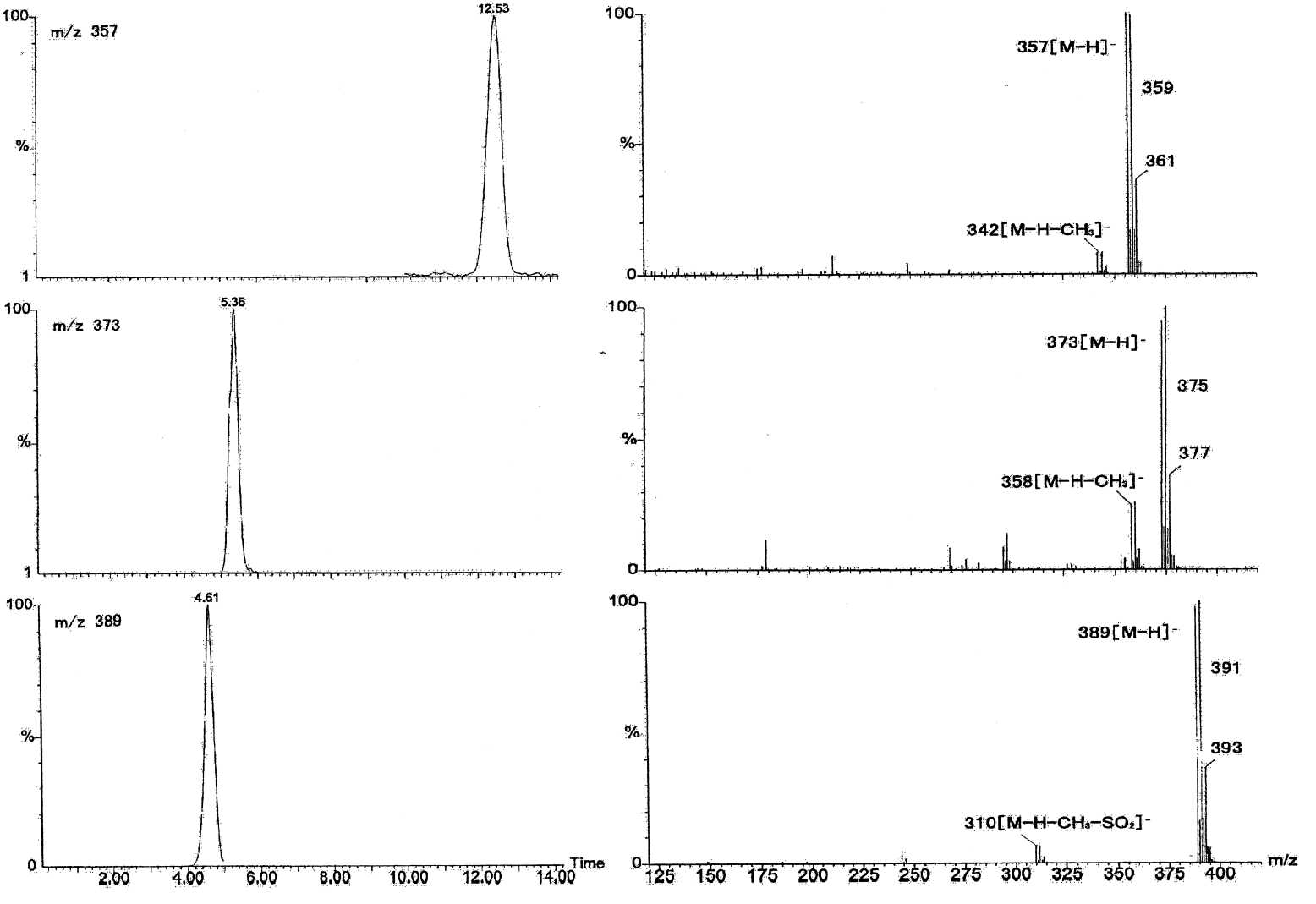

Fig. 8. Mass chromatograms and LC–MS spectra of milk extracts.

milk extract monitored at the individual deprotonated molecules of triclabendazole and its metabolites. As observed for the standards, peaks corresponding to triclabendazole and its metabolites were clearly observed on the mass chromatograms. Fig. 8 (right) shows background subtracted mass spectra recorded at the tops of each peak on the mass chromatograms. Although a few ions originating from co-extractive substances from milk appeared around m/z 290–300 in the spectrum of triclabendazole sulphoxide, the deprotonated molecules and the fragment ions were clearly observed in the spectra of the three compounds as the same as those of the standards. Thus, the three peaks in the chromatograms were confirmed as triclabendazole, triclabendazole sulphoxide and triclabendazole sulphone. The detection limits in the LC–ES-MS were the same as in the HPLC–UV method.

4. Conclusions

We described a rapid and sensitive HPLC method that allowed for the simultaneous determination of triclabendazole and its metabolites in milk using a simple sample preparation procedure and isocratic mobile phase with UV detection. The analysis time was ~15 min. The mean recoveries were $>$ 89% with RSD values within 3%. The detection limits were 0.004–0.006 μg/g. The proposed method may be applicable to the monitoring of triclabendazole and its metabolites (sulphoxide and sulphone) in milk.

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