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Analysis of ergovaline in milk using high-performance liquid chromatography with fluorimetric detection

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Abstract

A high-performance liquid chromatographic method for the determination of the mycotoxin ergovaline in goat's milk is described here. Ergotamine was used as an internal standard. For a sample size of 5.0 ml, the cleanup method included precipitation of milk protein with acetone. Then, ergovaline was extracted twice with chloroform and purified by elution on an Ergosil® column. HPLC separation of the extract was accomplished on a C_{18} column: an isocratic elution, using acetonitrile–ammonium carbonate, was performed, and the analyte was detected by fluorimetry. The method was found to be linear between 0.7 and 8 ng ml^{-1}, a mean recovery rate of 99.8% was obtained, and the described assay appeared both repeatable and reproducible. The limit of detection and the limit of quantitation of ergovaline in milk were 0.2 ng ml^{-1} and 0.7 ng ml^{-1}, respectively. In order to apply the proposed method, four lactating goats were administered the toxin intravenously at a dose of 32 mg kg^{-1} body weight. The concentrations of the drug in plasma and milk were then determined at standardized intervals. Ergovaline (unequivocally identified by LC–MS–MS) could not be detected in the milk beyond eight hours post-dosing. Therefore, in goats, milk does not appear to be a major excretion route for the unmetabolized toxin.

Keywords: Ergovaline

1. Introduction

In mammals, milk is a classic route for mycotoxin elimination. The evaluation and the control of these compounds in dairy products are both of great importance for the prevention of harmful effects on human health [1]. Moreover, epidemiological investigations in cattle have demonstrated the interest of mycotoxin quantification in milk, when estimating feed contamination [2]. In addition, the assessment of mycotoxins in the mammary secretion could be of interest for studies in distribution, biotransformation and bioavailability.

HPLC is an extremely valuable method for mycotoxin analysis, making it possible in some cases to reach detection levels in the ppb (μg kg^{-1}) range [3]. This analytical tool has been widely used for many years for the determination of numerous mycotoxins in milk [4,5], such as aflatoxins [2], zearalenone [6], trichothecenes [7] or slaframine [8].

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Ergovaline [9,10], is the major ergopeptine alkaloid produced in some grasses (*Festuca arundinacea, Lolium perenne,...*) infected with the endophytic fungus *Neotyphodium sp*. This mycotoxin (dopamine D2 agonist and inhibitor of prolactine secretion [11]) is the main causative agent involved in fescue toxicosis, a severe syndrome which occurs in animals grazing endophyted tall fescue [12,13]. Although in steers, urine and bile were demonstrated to be the primary excretory routes for ergot alkaloids of the infected tall fescue [14], it seemed interesting to explore to what extent the endophyte toxins can be eliminated in milk. Some assays, based on HPLC, have already been described for ergovaline determination in plant material [15]. In animal matrices, HPLC determinations of the mycotoxin are scarce, but a few methods have been developed for: rumen fluid [12], bovine serum [16] and ovine plasma [17]. However, no analytical method for the identification and quantitation of ergovaline in milk has been published yet.

In the work presented here, we therefore intended to develop an HPLC assay adapted to ergovaline quantitation in milk. We used the proposed analytical method for a biological application: the detection of ergovaline in goat's milk, following an intravenous injection of the pure toxin to animals.

2. Experimental

2.1. Reagents

2.1.1. General reagents

Solvents used for extraction were analytical grade acetone, chloroform, methanol (Carlo Erba, RS-plus, Milan, Italy) ammonia (30% in water, Carlo Erba, RPE). Acetonitrile (far UV for HPLC gradient applications, Fisher Scientific, Loughborough, UK), ammonium carbonate (Carlo Erba, RPE for analysis), and HPLC grade water prepared using a Milli-Q plus system (Millipore, Molsheim, France) were used for the HPLC eluent preparation.

2.1.2. Standard preparations

A standard methanolic ergovaline solution (1 $\mu g\ ml^{-1}$ calculated as free base) was obtained from a pure crystallized tartaric salt (from Dr. F. Smith, Pharmacal Sciences Department, Auburn University, Auburn Alabama, USA).

The substance used as the internal standard was ergotamine, the 5'α-benzyl analogue of ergovaline (analytical grade tartaric salt, 98% pure, E 4768, Sigma, St. Louis, MO, USA). A methanolic solution of ergotamine (200 $ng\ ml^{-1}$ calculated as free base) was prepared to spike milk pools or samples before they were analyzed.

To prepare the methanolic calibration curve, working standards in methanol, containing 400, 300, 200, 100 and 35 $ng\ ml^{-1}$ of ergovaline were all supplemented with the same amount of ergotamine (200 $ng\ ml^{-1}$).

The sample calibration curve was prepared by adding to 5 ml of a milk pool, 100 μl of each working standard solution. The concentrations in milk were then 8, 6, 4, 2 and 0.7 $ng\ ml^{-1}$ for ergovaline and 4 $ng\ ml^{-1}$ for ergotamine. The final levels were 400, 300, 200, 100 and 35 $ng\ ml^{-1}$ ergovaline, and 200 $ng\ ml^{-1}$ ergotamine, since the extraction procedure resulted in a fifty-fold concentration. Three series of spiked samples were prepared each on a different day.

2.2. Apparatus and chromatographic conditions

2.2.1. HPLC

The HPLC system, equipped with a Zorbax C_{18} column (150×4.6 mm I.D.; 3.5 μm particle size) and a guard column filled with the same phase, and the chromatographic conditions used, were those described in a previous paper [17], with the exception of the eluent phase. Here, an isocratic separation was performed, using acetonitrile–ammonium carbonate (2 m*M* in water) (36.5:63.5, v/v).

2.2.2. LC–MS–MS

The HPLC system (Spectra Physics) used was equipped with a P 1500 pump, a Nucleosil C_{18} column (150×4.6 mm I.D.; 3 μm particle size) and a Rheodyne injection valve fitted with a 20 μl-loop. Separations were performed with an isocratic methanol elution, at a flow-rate of 1 $ml\ min^{-1}$. The column effluent was continuously infused through a stainless capillary held at 4.5 kV into the atmospheric pressure ionization source with electrospray interface (Finnigan MAT) (sheath gas: 70 psi; auxiliary gas: 15

l min^{-1}). All positive ion electrospray ionization mass spectra were recorded on a TSQ 700 (Finnigan MAT) instrument. Daughter ion scan of m/z 534 was plotted using a collision energy of −23 eV and a collision gas pressure of 2.2 mTorr.

2.3. Sample extraction

A 5-ml milk sample was placed in a 30 ml glass tube, and spiked with 100 μl of the standard ergotamine (200 ng ml^{-1}) solution (to reach an ergotamine concentration of 4 ng ml^{-1}). Protein precipitation was carried out by adding 15 ml acetone. The tube was shaken for 10 min and centrifuged (800 *g*, 10 min). Then, the acetone was evaporated, and the aqueous residue was alkalinized (pH 9.0) with 20 μl of the 30% ammonia solution, and then twice extracted with 10 ml chloroform. The chloroform extract was partially evaporated to a 3 ml volume and was cleaned up as follows: 100 mg Ergosil® (Analtech, USA) were placed into a small column, and prewashed with 3 ml chloroform. The chloroform extract was applied on the column and the impurities were removed by washing the solid phase with 3 ml acetone–chloroform (75:25). Ergovaline was eluted with 1.5 ml of methanol, and the solvent was evaporated under a nitrogen stream (39°C). The dry residue was dissolved into 100 μl methanol, prior to HPLC analysis. Two 20 μl aliquots of the final extract were injected, the first into the HPLC system and the second into the LC–MS–MS system.

2.4. Validation procedure

2.4.1. Specificity and precision

In order to test the specificity of the analytical method, six blank milk samples, collected from four different adult goats (fed an endophyte-free diet), were extracted and analyzed as described previously.

To determine the precision of the method, inter-day and intra-day assays were performed as follows: a pool of blank goat's milk was prepared by mixing four 60 ml milk samples, collected from four different adult goats. A 150-ml aliquot of the pool was then spiked with 180 μl of a 5 μg ml^{-1} ergovaline standard solution. The final ergovaline concentration in the pool was therefore 6.0 ng ml^{-1}. Six 5-ml aliquots were taken, each of them being extracted and chromatographed as described previously. This procedure was repeated three times, on three different days. The variances homogeneity was verified using the Cochran test, and to evaluate repeatability (within-day) and reproducibility (between-days), the coefficients of variation CV_r and CV_R were respectively determined [18].

2.4.2. LOD and LOQ

The noise level was estimated on six 5-ml samples obtained from the pool of blank goat's milk, which were extracted and chromatographed. The LOD was estimated by three times the mean value of the noise level. The LOQ was confirmed chromatographically by measuring the peaks obtained with standard ergovaline methanolic solutions of decreasing concentrations, after it had been calculated as ten times the mean noise level [18].

2.4.3. Linearity and accuracy

Five spiked samples (theoretical concentrations: 0.7, 2, 4, 6, 8 ng ml^{-1}) were freshly prepared by supplementing 5-ml aliquots of the pool of blank goat's milk with the working standards. Three series of spiked samples were prepared (each one on a different day), then extracted and analyzed as described previously. They were analyzed concurrently with the three series of methanolic standard solutions of ergovaline (see Section 2.1.2), to prepare five point-calibration curves. The peak area ratio of the analyte (ergovaline) on the internal standard (ergotamine) was represented as a function of ergovaline concentration.

The variances homogeneities were checked with the Cochran test, to evaluate both linearity and accuracy, and the linear regression equations of the spiked samples and standards were compared. The existence of a significant slope, and the validity of the regression line were demonstrated by means of adequate statistical tests [18].

Percent recovery of ergovaline (between 0.7 and 8 ng ml^{-1}) was determined by plotting the calibration curve of spiked samples, and comparing it with that obtained with standards [18].

2.5. Ergovaline administration and milk and plasma sampling

A dose of 32 $\mu g\,kg^{-1}$ was injected into the right jugular vein of four lactating goats (average weight of 48 kg) fed a non-endophyted diet. The drug was administered in the form of tartaric salt, dissolved in 1 ml of methanol and then diluted with 1 ml of sterile and apyrogen NaCl 9‰ in water at 9 a.m.

Blood samples were taken from the left jugular vein into heparinised tubes, at 0, 3, 6, 10, 15, 30, 60, 120 and 180 min after drug administration, to check the drug availability. Plasma was then separated and analyzed according to a previously described method [17]. The kinetic data analysis was performed using a programme for the residuals method (SIPHAR (v.4.0) software [19]).

Milk samples were collected using a milking machine. The mammary gland was completely emptied by stripping by hand, just before the toxin administration. Then, the goats were milked at 8, 24, 32 and 48 h post dosing. For each sampling time, a 60-ml aliquot was taken, and immediately frozen at − 20°C, until analysis. Five ml of each aliquot were then extracted and chromatographed as described previously.

3. Results and discussion

3.1. Validation of the analytical method

3.1.1. Specificity

Under the described chromatographic conditions, the capacity factor of ergovaline was 4.27, and that of internal standard 9.13 (Fig. 1). The HPLC chromatograms of the pool blank extracts (Fig.1b), compared with that of standard ergovaline (Fig.1a), exhibited a peak, of which the resolution factor (R_S) was 2.4. This last peak was not interfering with the analyte identification and quantitation. The chromatograms obtained for milk samples, either spiked with ergovaline (Fig. 1c), or collected after ergovaline administration in goats (Fig. 1d) exhibited the same specificity.

3.1.2. LOD and LOQ, linearity and accuracy

The LOD and LOQ were found to be, respectively 0.2 $ng\,ml^{-1}$ and 0.7 $ng\,ml^{-1}$ in milk. These values are similar to those obtained using HPLC assays to analyze certain mycotoxins in cow's milk, i.e. deoxynivalenol (LOD=5 $ng\,ml^{-1}$) [7] or zearalenone (LOQ=0.2 $ng\,ml^{-1}$) [6]. However in milk, aflatoxin M_1 could be detected with analogous methods at less than 0. 05 $ng\,ml^{-1}$ [20–22].

The variances homogeneity, for linearity and accuracy, was found to be verified, using the Cochran test at the limit rate of 5% (tabulated value: 0.68, calculated values: 0.32 for spiked samples, and 0.60 for standards). Moreover, the intercepts of the regression curves were not statistically different from zero.

The data obtained with the three series of both spiked samples and standards are shown in Table 1. They were shown to follow linear models between 0.7 and 8 $ng\,ml^{-1}$ (r^2=0.999 for the two fitted curves) (Table 1). Moreover, the slope and origin of the line of the spiked samples were found to be statistically equivalent to those obtained with standards.

The recovery rate values varied between 97.7 and 100.6%, with a mean value of 99.8% (Table 1). Such results are in accordance with those obtained for zearalenone (93%) [6], slaframine (91%) [8] or aflatoxin M_1 (90.8%) [21] in milk. On the other hand, the recovery rate value is higher for ergovaline than for deoxynivalenol [7].

3.1.3. Precision

Using the Cochran test at the limit rate of 5%, the variances homogeneity was found to be verified (tabulated value: 0.71, calculated value: 0.50). The CV_r and the CV_R were found to be of 6 and 9.6% respectively. The method appeared both repeatable and reproducible [23]. These values are almost identical to those obtained with ovine plasma samples [17].

3.2. Determination of ergovaline in plasma and goat's milk

3.2.1. Plasma profile

The arithmetic plot of the mean plasma ergovaline concentrations vs time, after intravenous administration, is shown in Fig. 2. A very rapid decrease of the mycotoxin plasma level occurs, since the LOQ (3.5 $ng\,ml^{-1}$) is reached between 1 h and 2 h postdosing. Each individual plasma profile could be fitted to a biexponential function, characterizing a two-com-

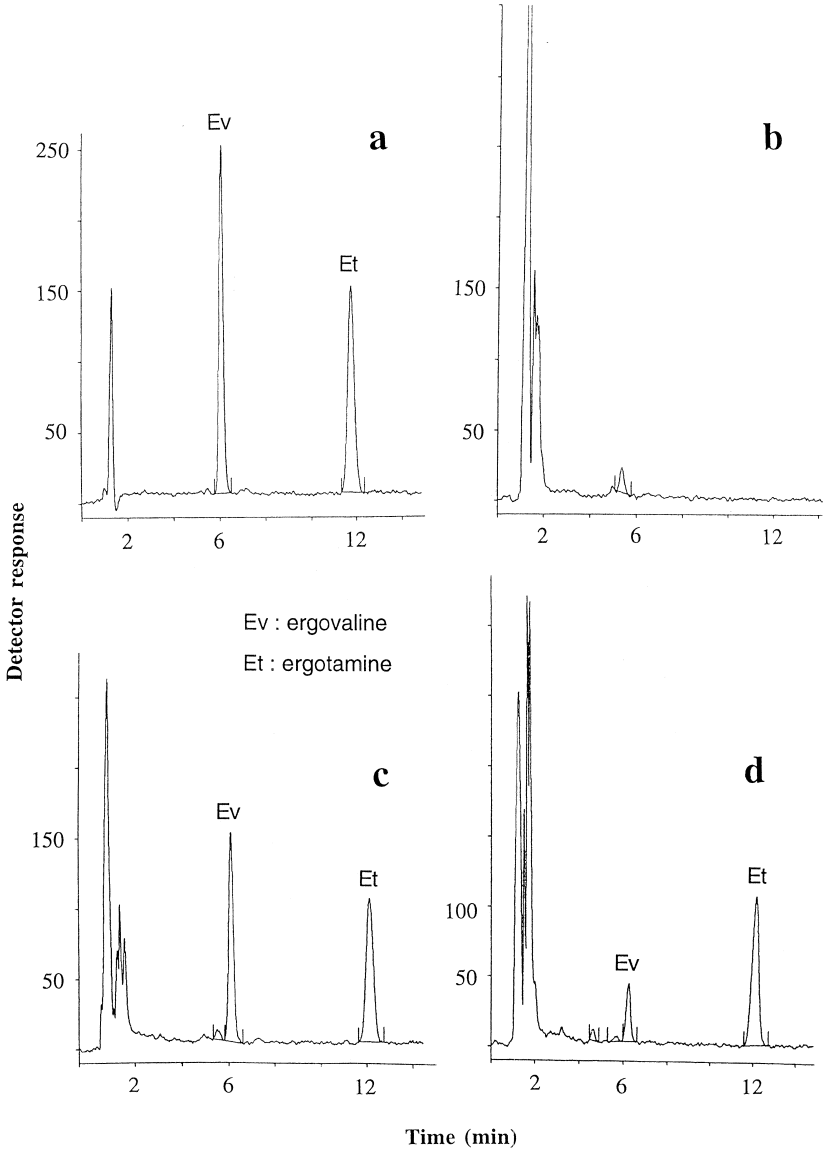

Fig. 1. HPLC chromatograms obtained with (a) a standard solution (200 ng ml^{-1} ergovaline, 200 ng ml^{-1} ergotamine) and with milk sample extracts from goat: (b) blank goat's milk, (c) goat's milk spiked with 4 ng ml^{-1} ergovaline and 4 ng ml^{-1} ergotamine, and (d) milk from a goat which had received intravenously a single dose of 32 μg kg^{-1} ergovaline body weight (sample collected 8 h post-administration, ergotamine added at the level of 4 ng ml^{-1} prior to extraction).

partment model, and a two-phase kinetic process. A comparison with the kinetic curve obtained after intravenous administration of a single dose of 17 μg kg^{-1} of ergovaline in sheep [17] is of interest. The two curves are quite similar, the LOQ being also reached after 1 h in sheep. Moreover, the plasma clearance and the elimination half-life of ergovaline during the second phase, were found to be of the same order in goats (0.034 l $min^{-1}kg^{-1}$ body mass and 32.41 min, respectively) and in sheep (0.020 l $min^{-1}kg^{-1}$ body mass and 23.6 min, respectively [17]).

Table 1
Recovery, accuracy and linearity derived from ergovaline standard solutions and milk samples spiked with the analyte

Concentration of ergovaline in spiked milk samples (ng ml^{-1})	Observed mean peak–area ratio	Standard mean peak–area ratio	Calculated ergovaline concentration (ng ml^{-1}) (percentage of theory)
0.7	0.19	0.20	0.70 (100.6)
2	0.56	0.57	2.04 (100.4)
4	1.08	1.11	3.93 (98.5)
6	1.63	1.63	5.86 (97.8)
8	2.19	2.74	7.99 (99.8)

3.2.2. Ergovaline identification in milk extract with LC–MS–MS

Under the described conditions, ergovaline shows the spectrum displayed in Fig. 3, which is in accordance with the fragmentation pathways previously proposed for the mycotoxin [24,25]. In this respect, the characteristic ions m/z 534 (parent ion) and 223, 268, 320 and 516 (daughter ions) have been selected. Single ion monitoring on these daughter ions was performed on the milk extract. Fig. 4 shows the two sets of ion chromatograms: (A) was obtained for the standard and (B) for the milk extract. Table 2 shows the ratios of the selected ions to the base ion. Most of the values obtained with the samples are in accordance with those of the standard. Apparent discrepancies for m/z 516 and 268 are probably related to the very low concentrations of the analyte in the milk samples. However retention times of ions recorded for the extract are consistent with those of the standard.

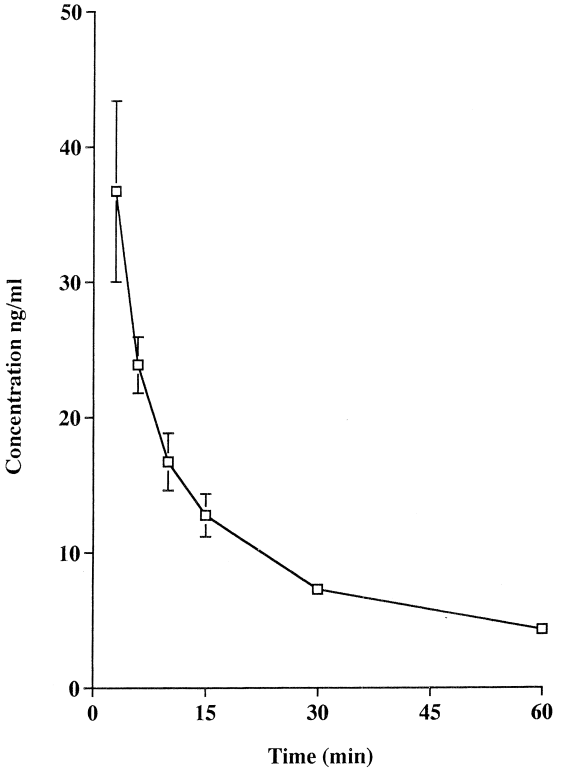

Fig. 2. Mean plasma ergovaline concentration–time profile in four goats, after intravenous administration of a single dose of 32 μg kg^{-1} body weight.

3.2.3. Ergovaline levels in milk

Under the described assay conditions, no peak interfering with ergovaline was found. The mycotoxin could not be detected later than 8 h post dosing. For this sampling time, ergovaline concentrations in goat's milk averaged 0.71 ± 0.17 ng ml^{-1}. Thus, the mycotoxin levels were in the range of the LOQ. These results show that a few hours after a single i.v. administration in goat, the mycotoxin is quite rapidly eliminated at low concentration in milk. The toxin dosage which was used reached the limit of toxicity, since some clinical signs were observed in the treated animals: difficulty in remaining standing, which occured some minutes after the mycotoxin administration, coolness of the ears, the nose and the udder, and a purplish-blue coloration of this last organ, observed about four hours after the toxin injection.

The present work shows that milk seems to be a minor excretion route for intact ergovaline, despite its slight molecular polarity [26]. In view of previous results obtained with ergot alkaloids [26], the low polarity together with the high molecular weight of

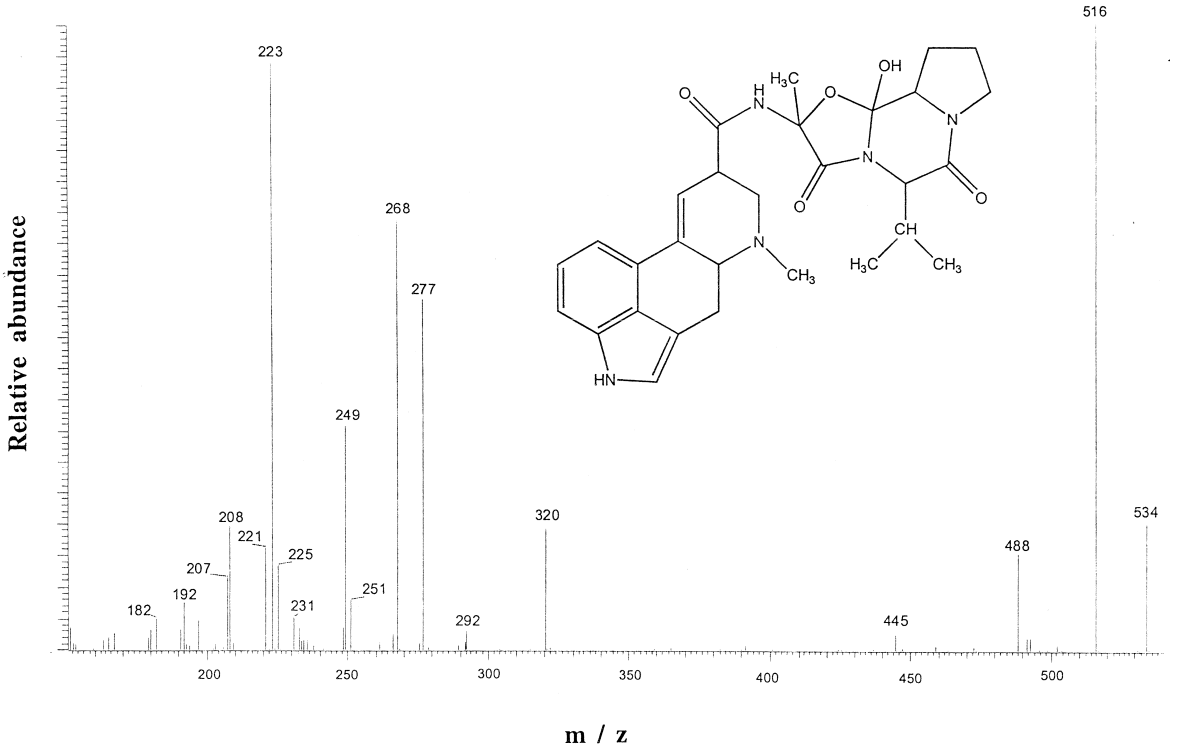

Fig. 3. Positive fixed precursor ion scan of MH^+ (m/z 534) of ergovaline obtained by LC–MS–MS.

ergovaline seems to indicate that this toxin might be preferentially excreted in bile.

4. Conclusion

In view of its characteristic parameters (LOD=0.2 ng ml^{-1}, LOQ=0.7 ng ml^{-1}, CV_r and CV_R respectively 6 and 9.6%), the tested bioanalytical method can be considered an acceptable procedure to evaluate ergovaline levels in goat's milk. Its relative simplicity makes its routine use feasable when there are a large number of samples to be tested. Of course, the recovery of very low amounts of ergovaline in milk (under the LOQ) should make an immunological bioassay or an HPLC–MS method a basic requirement. The use of HPLC–MS for an unequivocal identification of the mycotoxin has been shown to be of great utility. In addition, these methods could allow for the detection of biotransformation products (e.g. lysergic acid amide) [14] which could be excreted in milk.

Table 2
Comparison of the relative abundance of each selected ion of ergovaline for the standard and the milk sample

m/z	Standard	Milk sample
534	40	40
516	99	50
320	24	16
238	60	44
223	100	100

The preliminary application of the described HPLC method showed a quite rapid decrease of ergovaline levels in the mammary secretion, after intravenous administration at a subtoxic dosage in goats. A more complete kinetic study, performed during the first eight hours post dosing, should be of interest to obtain more precise results. Nevertheless, the present work indicates that ingestion by goats of forage endophyte-infected with *Neotyphodium sp.,*

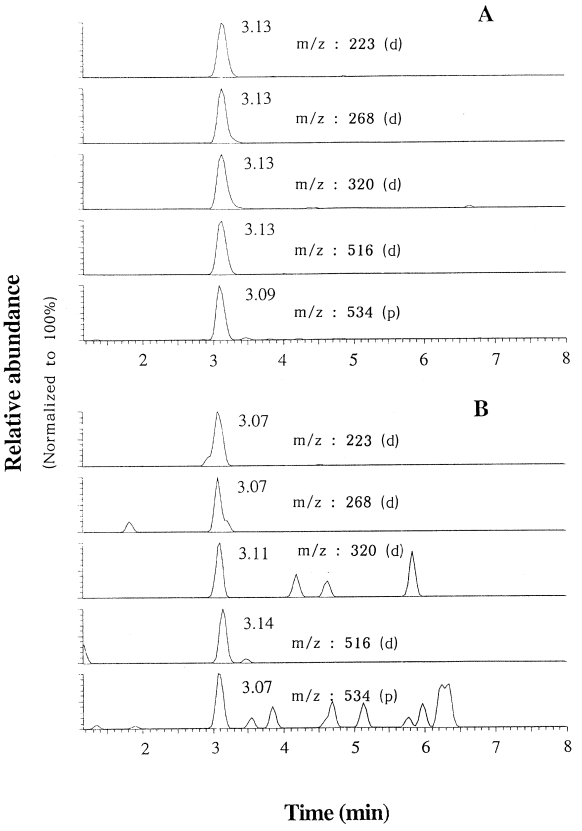

Fig. 4. Reconstituted chromatograms of characteristic ions of ergovaline, obtained by LC–MS–MS for: (A) standard solution, (B) milk extract. From bottom to top are displayed the ions of m/z 534 (parent ion: p), 223, 268, 320, 516 (daughter ions: d). From left to right: retention time and m/z.

should entail no toxic consequences in humans who drink milk obtained from contaminated animals, provided that no intact lysergic metabolites were excreted in milk after oral ingestion.

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