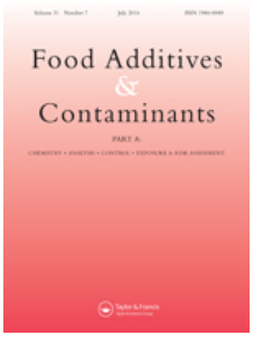

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Persistence of α-cypermethrin residues in milk of lactating donkeys (*Equus asinus*) using UHPLC-MS/MS

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The aim of this study was to measure the persistence of residues of the pyrethroid insecticide α-cypermethrin (ACYP) in the milk of lactating donkeys following pour-on treatment. Milk was collected from animals ($n = 7$) before the treatment and at 12, 24, 36, 48, 60, 72 and 84 h post-treatment. The last sampling was taken 7 days post-treatment (168 h). Milk samples were analysed by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The analytical method was validated following requirements of Commission Decision 2002/657/EC. All samples showed levels of ACYP below the maximum residue limit (MRL) of 20 μg kg^{-1} established for bovine milk (Commission Regulation (EU) No. 37/2010). The results demonstrate that there is minimal partitioning of ACYP into milk in lactating donkeys from pour-on treatment.

Keywords: alpha-cypermethrin; residues; depletion; milk; donkeys; HPLC-MS/MS

Introduction

The donkey (*Equus asinus*) plays a key role in subsistence agriculture and farm work, contributing to many social and economic sectors, especially in developing countries (Beja-Pereira et al. 2004; Yılmaz et al. 2012). Recently in some European countries, including Italy, there has been increasing interest in the donkey as a pet animal, in leisure activities, for onotherapy (a new instrument in animal-assisted therapy) and as a food-producing animal. Donkey milk is gaining popularity as a substitute for human milk, as its chemical composition (Vincenzetti et al. 2008) makes it suitable for feeding infants affected by cow's milk protein allergy and/or by multiple hypersensitivity (Iacono et al. 1992; Businco et al. 2000; Carroccio et al. 2000).

Therapeutics, such as anti-parasitic compounds, are often administered to donkeys; however, dosage and withdrawal intervals are usually based on recommendations for horses and cattle because very few veterinary medicines have donkey-specific label indications (Svendsen 1997; Grosenbaugh et al. 2011).

In fact classes of veterinary medicines used in horses and ruminants are commonly extrapolated for use in donkeys without the optimisation of dosing regimens and the determination of pharmacokinetic and pharmacodynamic properties. It has been reported by Lizarraga et al. (2004) that donkeys have a greater capacity to metabolise certain chemicals compared with the metabolic capacity of horses; thus, higher dosages or shorter intervals could be required to maintain effective drug concentrations for parasite control in donkeys.

The control of ectoparasites on donkeys, like horses, has traditionally relied on the application of insecticides. Currently, topically applied pyrethroids are the most widely used classes of insecticides for the control of lice in equids (Ellse et al. 2012).

Pyrethroids are manmade chemicals similar in structure to the naturally occurring pyrethrins. Natural pyrethrins rapidly knock down insects, have low mammalian toxicity and low persistence in the environment (Feo et al. 2010). The synthetic pyrethroids are more resistant to environmental degradation and highly active against a wide spectrum of ectoparasites. Their toxicity to mammals depends on their rate of bioavailability and route of administration (Kaneko 2011).

α-Cypermethrin (ACYP) belongs to the group of synthetic pyrethroid insecticides classified by the World Health Organization (WHO) (1992) as moderately harmful, class II. ACYP contains more than 90% of the most active enantiomer pair of the four *cis*-isomers of cypermethrin as a racemic mixture. It acts on insect axons in the peripheral and central nervous systems by interacting with sodium channels. This compound is effective against a wide range of pests of many crops and is also used in veterinary medicine to control ectoparasites such as ticks, lice and blowflies (EMEA 2001).

In Italy, ACYP is marketed as a pour-on formulation to treat louse infestation on cattle with a zero milk-

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withdrawal time (Dizionario del Medicinale Veterinario 2010). Veneziano et al. (2012) recently reported that ACYP applied as pour-on at a dose of 0.15 g per animal was highly effective, safe and user friendly for the treatment of chewing louse infestation on donkeys.

Detailed ACYP pharmacokinetic studies were carried out for cattle (Bissacot & Vassilieff 1997; Rothwell et al. 2001; Sassine et al. 2004), but there is a lack of information for the asinine species. The absence of a specified withdrawal time and MRLs for ACYP content in donkey's milk provide the possibility for ambiguous interpretation by users when regarding its use in lactating animals.

Instead, studies about ACYP residues in donkey milk appear to be important considering its use in feeding children affected by hypersensitivity to cow, sheep and goat milk (Monti et al. 2008).

The aim of the present study was to investigate the persistence of ACYP in the milk of lactating donkeys after pour-on treatment. A simple analytical method that uses QuEChERS-based sample preparation and detection by UHPLC-MS/MS was developed and validated in-house to support this study.

Materials and methods

Chemicals and apparatus

α-Cypermethrin (ACYP) analytical standard (99.7%) and deltamethrin internal standard (99.8%) were purchased from Sigma Aldrich (Dublin, Ireland). Ultra-pure water (18.2 MΩ) was generated using a Milli-Q Plus water purification system. HPLC-grade methanol, acetonitrile (pesticide grade), ammonium acetate (p.a.) and anhydrous magnesium sulphate (p.a.) were from Sigma Aldrich. Sodium chloride was obtained from AppliChem GmbH (Darmstadt, Germany). A Dispensette® lll solvent dispenser (Brand GmbH + Co. KG, Wertheim Germany) was used for aliquoting acetonitrile; a Mistral 3000i centrifuge was from MSE (London, UK). PTFE syringe filters (13 mm, 0.22 µm) were obtained from Millipore (Cork, Ireland).

UHPLC-MS/MS conditions

Chromatographic separation was carried out on a Waters Acquity UHPLC system (Milford, MA, USA). The analytical column was an Acquity UHPLC® BEH C_8 (50 mm × 2.1 mm, 1.7 µm particle size) with a Vanguard pre-column C_8 (5 mm × 2.1 mm) and maintained at 60°C. A binary gradient separation was performed at a flow rate of 0.5 ml min^{-1}. Aqueous mobile phase (A) consisted of ammonium acetate solution (2 mM) while organic mobile phase (B) was MeOH. The gradient profile was as follows: (a) 0 → 0.1 min, 50% B; (b) 3 min, 95% B; (c) 3 → 4 min, 95% B; (d) 4.01 min, 50% B. The total run time was 6 min. UHPLC weak and strong washes consisted of H_2O:MeOH (90:10, v/v) and MeOH:H_2O (90:10, v/v), respectively. The sample injection volume was 5 µl.

ACYP residues were detected using a Waters Quattro Premier XE triple quadrupole instrument equipped with an electrospray ionisation probe operating in positive mode (Milford, MA, USA). Nitrogen was used for nebulisation, desolvation (1100 l h^{-1}) and as a cone gas (200 l h^{-1}). The source and desolvation temperatures were 140 and 400°C, respectively. Capillary voltage was 3.0 kV. Argon was used as a collision gas. The MS conditions were optimised by tuning the analyte-specific parameters, including cone voltage, collision energy and collision cell exit potential. This optimisation was carried out by infusion of a 1 µg ml^{-1} ACYP standard solution and monitoring the two most abundant fragment ions produced from the pseudo-molecular ion. The same routine was used for optimising MS conditions for internal standard. Dwell time was set to ensure 12–15 data points across the peak.

The UHPLC-MS/MS system was controlled by MassLynx™ software and data was processed using Target Lynx™ Software (both Waters).

Calibration

Primary stock standard solution was prepared in acetonitrile at concentrations of 1000 and 2000 µg ml^{-1} for ACYP and deltamethrin, respectively. Deltamethrin was used as internal standard (IS) as it is structurally similar to ACYP. Working standards were prepared in acetonitrile at 2 µg ml^{-1} for both ACYP and IS. Extracted matrix calibration curves were prepared by fortifying negative donkey milk samples with ACYP working standard (2 µg ml^{-1}) to give matrix concentrations of 1, 2, 4, 10, 20, 30 and 40 µg kg^{-1}. The recovery controls were used to monitor for loss of ACYP during extraction. Recovery was assessed by analysing milk samples extracts spiked post-extraction with ACYP at concentrations of 5 and 20 µg kg^{-1} in duplicate.

Method validation

The method was validated following guidelines detailed in Commission Decision 2002/657/EC concerning the performance of analytical methods and interpretation of results. Investigation included linearity, selectivity, within-laboratory repeatability (WLr), within-laboratory reproducibility (WLR) and recovery. CCα and CCβ were calculated according to calibration curve procedure (European Commission 2002). Negative control milk was fortified at 0.5, 1 and 1.5 times the MRL level of 20 µg kg^{-1}, which was established by Commission Regulation (EU) No. 37/2010 for ACYP residue in

bovine milk. Each validation run contained seven replicates at each fortification level (10, 20 and 30 μg kg^{-1}), seven point calibration curve (1, 2, 4, 10, 20, 30, 40 μg kg^{-1}), four recovery controls (two replicates at 5 and 20 μg kg^{-1}), and a negative control.

Sample preparation

Milk samples were thawed and weighed after gently end-over-end mixing at RT to ensure homogeneity prior to analyses. Milk samples (10 ± 0.1 g) were transferred to 50 ml centrifuge tubes. A 100 μl of working IS (2 μg ml^{-1}) was added to each sample. Extraction was performed by adding acetonitrile (10 ml), magnesium sulphate (4 g) and sodium chloride (1 g) to each sample and shaking vigorously by hand for 1 min. Samples were subsequently centrifuged (2842*g*, 10 min, 15°C), raw extract filtered through 0.2 μm PTFE syringe filters into 200 μl vials and injected by UHPLC-MS/MS system.

Animals studies

Seven cross-breed female lactating donkeys, with a mean age of 9 ± 3 years and a mean bodyweight of 240 ± 30 kg were selected for this study. Dairy donkeys were milked once a day and the milk production measured before and throughout the trial ranged from 1350 to 1500 ml day^{-1}. The study animals were tagged for identification and housed communally in an indoor pen from the day − 1 until the end of the trial (day 7) to avoid any impact of rainfall on insecticide persistence. The donkeys were fed hay and concentrated feed during the study. Water was provided *ad libitum* throughout the course of the study. The selected donkeys were not treated with any anti-parasitic drugs during the previous 6 months. On day 0 the study animals received ACYP pour-on (Renegade, 1.5%, Pfizer Animal Health s.r.l., Latina, Italy) at the manufacturer's recommended cattle dose of 10 ml per animal (15 g l^{-1}, 0.15 g/animal). The formulation was applied topically with a syringe along the midline of the back from the withers to the tailhead. The study animals were observed by a veterinarian periodically during the first day of the study (three times, every 4 h) for possible adverse reactions, then daily for 1 week until the end of the trial.

Milk samples were collected from each animal at 0 (before treatment), 12, 24, 36, 48, 60, 72, 84 and 168 h post-treatment. The total daily production was collected at each sampling. Aliquots of collected samples were stored at −20°C until analysis.

Results and discussion

Analytical method development and validation

In-house method validation was performed based on the guidelines detailed in Commission Decision 2002/657/EC (European Commission 2002). The identity of ACYP in matrix was confirmed by monitoring the retention time, the ion ratio of two product ions with acceptable tolerances, as described in Commission Decision 2002/657/EC, and a signal-to-noise ratio of the transitions better than 3. The mean ion ratio was obtained from extracted matrix calibrants. Individual transitions and respective collision energies and voltages for ACYP and IS are listed in Table 1. It has been previously reported by Fleet et al. (1996) that positive-ion electrospray ionisation of ACYP with ammonium acetate buffer produces predominantly ammoniated molecule ions $[M + NH_4]^+$ in their predicted isotopic abundances of 9:6:1 (433:435:437 *m/z*) for species that contain two chlorine atoms. This is in agreement with our selection of the most abundant transitions 433 > 190.8 and 433 > 415.9 *m/z* for ACYP. Deltamethrin formed ammonium adducts as well, and selected transition for quantification was 523.05 > 280.9 *m/z* (Martinez et al. 2006).

The selectivity of the method was evaluated through analyses and inspection of 20 negative donkey milk samples from different animals. ACYP MRM traces were found to be free of interference at the expected retention time (Figure 1). Different batches of blanks were used to prepare calibration curves during validation to increase the ruggedness of the study.

The calibration curve was designed to respond to the current MRL for ACYP in cow milk, which is set at 20 μg kg^{-1}. As there is no specific MRL determined for donkey milk, we set this value somewhere in the central region of the calibration curve. Therefore, seven-point calibration was chosen and negative donkey milk fortified for each set of samples at 1, 2, 4, 10, 20, 30 and 40 μg kg^{-1}. Calibration curve correlation coefficients

Table 1. MRM conditions for ACYP and deltamethrin (IS).

Compound	Parent ion (*m/z*)	Daughter ion (*m/z*)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
Alpha-cypermethrin (ACYP)	433.0	190.8	0.035	20	14	2.49
		415.9	0.035	20	9	2.49
Deltamethrin (IS)	523.1	280.9	0.035	17	15	2.50

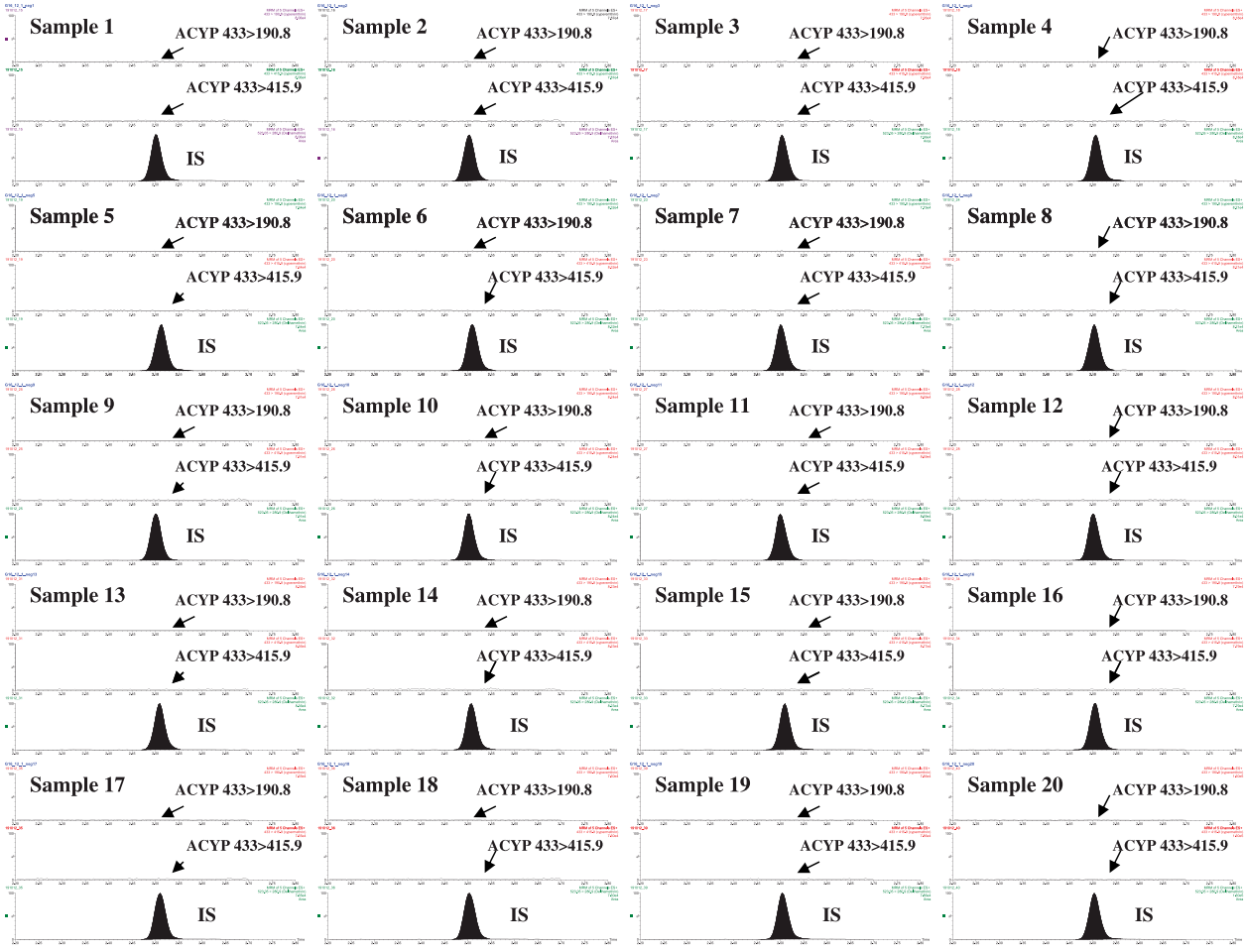

Figure 1. (colour online) MRM of 20 different negative donkey milk samples spiked with internal standard (deltamethrin).

r^2 higher than 0.995, residual plots and regression statistics confirmed linear correlation between response (y) and concentration (x). MRM chromatograms of donkey milk samples fortified with ACYP at 1, 2 and 4 µg kg^{-1} have a sufficient number of scans across the peak (12–15) and an acceptable signal-to-noise ratio for applied transitions (Figure 2). Stock standards and working standards were prepared in acetonitrile and stored in a fridge at 4–6°C during the study. Results of within-laboratory repeatability (WLr) and -reproducibility (WLR) study at three fortification levels (10, 20 and 30 µg kg^{-1}) are shown in Table 2. WLr was determined from three independent sets of analyses performed by the same operator. WLR was carried out by three different analysts on separate days. Overall accuracy for WLR ranged from 94% to 102%. Precision varied between 1.2% and 4.4%. Recovery was determined from samples fortified pre- and post-extraction with ACYP at 5 and 20 µg kg^{-1}. Mean recovery was 83% with an RSD of 10.8%. CCα was calculated to be 0.62 µg kg^{-1} in relation to the zero tolerance procedure from Commission Decision guidelines (European Commission 2002). If the MRL for bovine milk is applied to donkey milk (20 µg kg^{-1}), then the decision limit would be CCα = 20.44 µg kg^{-1}.

Persistence of ACYP in donkey milk following pour-on treatment

The donkeys were monitored by a veterinarian throughout the study. No adverse reactions were observed in any donkey treated with ACYP pour-on formulation.

The highest levels of ACYP in milk ranged from 1.0 to 5.5 µg kg^{-1} and were determined at the first milking, in samples taken 12 h post-treatment (Table 3). The results of this study showed that in all tested milk samples taken from seven treated lactating donkeys levels of ACYP were below the limit of 20 µg kg^{-1}, which is set by Commission Regulation 37/2010 for bovine milk. These findings might suggest that this pyrethroid has a rapid depletion in donkey species, which on the contrary, as

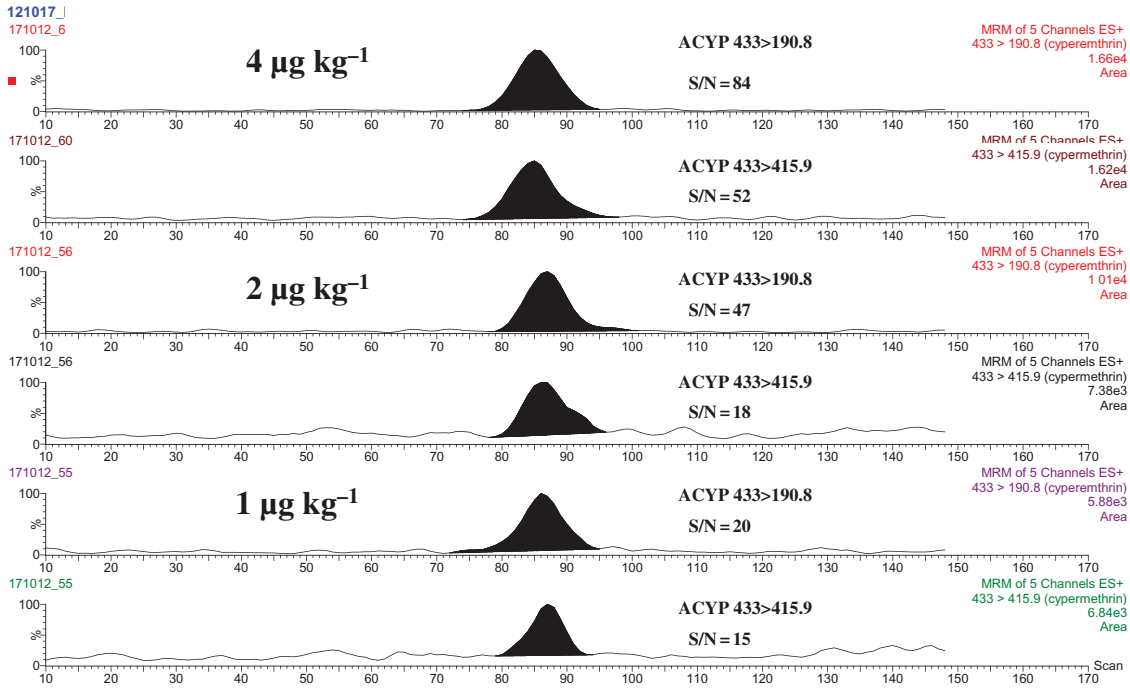

Figure 2. (colour online) MRM chromatograms of donkey milk samples fortified with ACYP at 1, 2 and 4 µg kg^{-1}.

Table 2. Accuracy and precision for fortified donkey milk under within-laboratory repeatability (WLr) and -reproducibility (WLR) conditions.

	ACYP (µg kg^{-1})					
	10	20	30	10	20	30
	Accuracy WLr (%)			RSD WLr (%)		
1	99	101	99	4.2	2.3	2.4
1	100	97	97	4.3	1.5	2.0
1	99	95	94	2.2	2.5	2.5
Mean	100	98	97	3.6	3.4	3.0
	Accuracy WLR (%)			RSD WLR (%)		
1	100	99	99	4.4	3.4	1.4
2	100	100	101	1.2	2.8	3.2
3	99	95	94	2.2	2.5	2.5
Mean	100	98	96	2.8	3.6	3.8

Note: CCα = 0.62 µg kg^{-1}.

demonstrated by many authors, persists in bovine milk (Bissacot & Vassilieff 1997; Rothwell et al. 2001; Sassine et al. 2004). Another possible explanation for low partitioning into milk would be poor dermal absorption of ACYP in donkey species. Although many studies (Wardhaugh 2005; Coman et al. 2013) have been performed to determine the pharmacokinetic behaviour of pyrethroids in farm animals, there is scarce information on the pharmacokinetics in asinine species. It is necessary that the veterinary medical community generates more pharmacological information specifically with, and for, donkeys because of the great importance of these species in developing countries, and especially because of food safety aspects, being milk-producing animals.

Large variations in pharmacokinetics are evident among animal species after topical administration of ACYP as a result of anatomical and physiological variation among animal species and irregular absorption of the drug from the site of application associated with differences in coat length and hair density (Gawler et al. 2005). Despite these differences in pharmacological assessment, ACYP has a broad spectrum of activity against ectoparasites in several large animal species and is often used in animal husbandry.

The results of the present study indicate that topical application of ACYP at the dosage recommended for cattle did not cause safety issues in donkeys. ACYP was tolerated well by all donkeys, with no adverse reactions following treatment.

Considering the pharmacokinetic disposition and efficacy of ACYP in donkey's louse infestations, it seems that topical administration of ACYP could be safely used in milking donkeys (Veneziano et al. 2012).

The level of ACYP excretion that has been found in this study may allow for the use of this pyrethroid in lactating donkeys, if applied within the investigated dose, without a withdrawal period. Further studies should be performed on the fate of ACYP's metabolites in

Table 3. ACYP residue in donkey milk from seven animals following pour-on treatment.

	Sampling time (h)								
	0	12	24	36	48	60	72	84	168
ID donkey	ACYP ($\mu g\,kg^{-1}$)								
1	n.d.	2.6	1.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	n.d.	1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	n.d.	n.d.	n.d.	1.6	n.d.	n.d.	n.d.	n.d.	n.d.
4	n.d.	5.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	n.d.	1.8	1.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	n.d.	2.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mean	n.d.	1.8	0.5	0.2	n.d.	n.d.	n.d.	n.d.	n.d.
SD	–	± 1.9	± 0.8	± 0.6	–	–	–	–	–

Note: n.d., Not detected.

milking donkeys, considering its important role as a substitute milk for a growing population of people with cow's milk protein allergies.

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