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Journal of Chromatography B, 723 (1999) 81–94

JOURNAL OF CHROMATOGRAPHY B

Simultaneous determination of purine metabolites, creatinine and pseudouridine in ruminant urine by reversed-phase high-performance liquid chromatography

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Received 10 April 1998; received in revised form 26 November 1998; accepted 26 November 1998

Abstract

Determination of purine metabolites, pseudouridine and creatinine in both bovine and ovine urine using high-performance liquid chromatography (HPLC) is described. Following dilution and filtration, urine samples were analysed directly. Separation and quantification was achieved using a Spherisorb ODS II C_{18} column (250×4.6 mm I.D.) under isocratic conditions. The mobile phase contained 7.5 m*M* ammonium dihydrogen phosphate, 10 m*M* sodium 1-heptane sulphonic acid and 1.0 m*M* triethylamine at pH 3.0. Chromatography was achieved at a flow-rate of 1.0 ml/min and monitoring column effluent at 218 nm. Total analysis time was 60 min. Recovery of all compound standards added to urine was above 96%. In all cases, close spectral matches of compound standards and corresponding identified peaks in ovine and bovine urine were obtained. Lowest detectable concentrations of allantoin, uric acid, xanthine, hypoxanthine, creatinine and pseudouridine were 1.1, 1.0, 1.0, 1.0, 3.0 and 0.4 μmol/l, respectively. Advantages of simultaneous determination of purine metabolites, creatinine and pseudouridine in ruminant urine collected from both sheep and cattle exist over current methods.

Keywords: Purine metabolites; Allantoin; Pseudouridine; Uric acid; Xanthine; Hypoxanthine; Creatinine

1. Introduction

Microbial protein synthesised in the rumen contributes a significant proportion (between 0.42 and 0.93) of the total protein flux entering the small intestine in ruminants [1]. Quantification of microbial protein supply is fundamental to all ruminant protein evaluation systems, such as that adopted in the UK [2]. Protein evaluation systems are used to predict protein requirements to assist formulation of ruminant diets. Since information of microbial protein supply is central to accurate protein feeding, particularly in the dairy cow, prediction errors inherent in evaluation systems can lead to inefficient utilisation of dietary protein. Most estimates of microbial protein supply have been made using either internal e.g. (2,6-diaminopimelic acid, ribonucleic acid (RNA) or external (e.g. ^{15}N, ^{35}S) microbial markers. Use of such an approach is unsatisfactory due to the requirement for surgically modified ani-

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0378-4347/99/$ – see front matter
PII: S0378-4347(98)00549-0

mals while associated high costs are prohibitive to large-scale investigations.

Nucleic acid bases, nucleosides and nucleotides present in duodenal digesta are degraded during passage through the intestinal mucosa and generally absorbed as nucleosides [3,4]. Once absorbed, purine bases enter either cellular salvage or catabolic pathways. Purine catabolism proceeds via inosine and consists of a series of reactions leading to the formation of hypoxanthine, xanthine, uric acid and allantoin, all of which are excreted in the urine.

A number of studies [5–8], have demonstrated that measurements of urinary excretion of allantoin, hypoxanthine, uric acid and xanthine, collectively termed purine metabolites, could potentially be used as the basis of a non-invasive index of microbial protein supply in sheep and dairy cows. However, not all purine metabolites excreted in ruminant urine originate from metabolism of absorbed purines. During tissue nucleic acid turnover, a proportion of purine bases are not salvaged and re-utilised, but enter catabolic pathways, constituting an endogenous loss. Daily endogenous losses of purine metabolites are thought to account for between 136 and 217 [9,10] and 400 and 570 [8,10] $\mu mol/W^{0.75}$ in ovine and bovine ruminant species, respectively.

Pseudouridine, is one of the most important modified pyrimidine derivative components of transfer and ribosomal RNA [11]. Pseudouridine liberated during tissue RNA is not salvaged, but obligately excreted in the urine [12]. Assuming that RNA turnover and protein synthesis are directly related [13], urinary pseudouridine excretion could potentially be used as an indicator of ruminant nitrogen status [14].

Accurate assessment of urinary purine metabolite [15] or pseudouridine [16] excretion in dairy cows requires a total urine collection. In situations where this is not possible it has been suggested that use of creatinine as an internal marker of urinary output in sheep [17] and dairy cows [18] could allow collection of spot urine samples as a reliable alternative to total collection.

Allantoin, quantitatively the most important purine metabolite has traditionally been determined using a colorimetric analysis based on the Rimini-Schryver reaction [19], but is subject to criticism due to a lack of specificity [19].

Uric acid, xanthine and hypoxanthine are often quantified colorimetrically or as allantoin following enzymatic conversion [20,21]. Both approaches are problematic due to interference by compounds contained in biological fluids, while enzymatic conversion to allantoin is often incomplete [21].

Creatinine is routinely determined using the Jaffé alkaline picrate procedure [22]. In addition to being time consuming, this method has been reported to lead to overestimates due to interference by endogenous and exogenous pseudo-creatinine chromogens [23].

In recent years, a number of methods based on high-performance liquid chromatography (HPLC) have been reported for the quantification of creatinine [24,25], allantoin [25–32], purine metabolites [27,32] and pseudouridine [33,34] in biological fluids, but none allow simultaneous measurements. Furthermore, isocratic [25] or gradient [32] reversed-phase HPLC methods used to quantify purine metabolites have been developed using ovine urine while their application for the analysis of bovine urine has not been documented. Since the distribution of nitrogenous constituents [35] and the relative proportions of individual purine metabolites [10] are markedly different between ruminant species, a method sufficiently robust to allow analysis of both ruminant urinary sources would be advantageous. In order to conduct non-invasive studies of ruminant nitrogen metabolism there is often a requirement to quantify urinary concentrations of purine metabolites, pseudouridine and creatinine. This paper describes a simple and precise isocratic method based on a readily available C_{18} stationary phase allowing simultaneous quantitative determination of purine metabolites, creatinine and pseudouridine in bovine and ovine urine.

2. Experimental

2.1. Instrumentation

Analysis was performed using a Hewlett-Packard 1090 liquid chromatography system equipped with diode-array ultraviolet (UV) detector, autosampler and heated column compartment (Hewlett-Packard, Wilmington, DE, USA). Separation was achieved

using a 5 μm Spherisorb ODS II C_{18} reversed-phase column (250×4.6 mm I.D.; Waters, Milford, MA, USA) without the use of a precolumn. Column regeneration was performed by washing with the following solutions in sequence: distilled, deionised water, 50% (v/v) aqueous acetonitrile, acetonitrile, 50% (v/v) aqueous acetonitrile, distilled, deionised water.

2.2. Reagents

Allantoin, pseudouridine, sodium salts of uric acid and xanthine, hypoxanthine, (Sigma, St. Louis, MO, USA), creatinine (Merck, Darmstadt, Germany) were all assayed at 98% purity or above and used without further purification. Triethylamine, sodium 1-heptane sulphonic acid (Sigma) and ammonium dihydrogen phosphate (Tamro, Vantaa, Finland) were all HPLC grade. Distilled, deionized water was obtained from a Milli Q plus purification system (Millipore, Bedford, MA, USA).

2.3. Mobile phase preparation

Mobile phase was prepared by dissolving 2.02 g sodium 1-heptane sulphonic acid and 0.86 g ammonium dihydrogen phosphate in 1 l double deionised water. Following the addition of 0.14 ml of triethylamine, pH was adjusted to 3.0 with 10% (v/v) hydrochloric acid. Prior to elution through the column, the mobile phase was filtered through a polysulfane 0.45 μm filter (Millipore) and further degassed under a vigorous stream of helium for 10 min. The flow of helium was reduced to a steady stream during analysis.

2.3. Urine diluent

Urine diluent was prepared using the same procedure as described for the mobile phase, with the exception that triethylamine was omitted. Before filtration, urine diluent was further acidified to pH 2.1 with 4 ml/l of 10% (v/v) hydrochloric acid.

2.5. Sample preparation

Urine samples were centrifuged for 5 min at 2000 *g* (Eppendorf, Hamburg, Germany), diluted (1/50) with urine sample diluent and vortex mixed (Prolab, Espoo, Finland) for 20 s. A 2 ml aliquot of this mixture was passed through a 13 mm disposable syringe filter containing a 0.45 μm polysulfane membrane (Whatman International, Maidstone, England) and analysed directly. Urine samples were stable for seven days at 4°C, while storage at −20°C maintained sample integrity for several months.

2.6. Standard solutions, calibration and quantification

The stock standard solution was prepared by dissolving pure standards in 2 l of urine diluent. This resulted in allantoin and creatinine concentrations of 300 μmol/l, uric acid, xanthine and hypoxanthine concentrations of 100 μmol/l and a pseudouridine concentration of 25 μmol/l. Following filtration through a polysulfane 0.45 μm filter, the stock standard solution was stored at 4°C for one month. Additional working standards were prepared daily by diluting the stock standard 1/2 and 1/4 with urine diluent. Daily calibration was performed following duplicate injections of stock and working standard solutions. Calibration curves used for calibration were prepared over concentration ranges of 75–300 μmol/l, for allantoin and creatinine, 25–100 μmol/l for uric acid, hypoxanthine and xanthine, and 6.25–25 μmol/l for pseudouridine. Compound quantification was achieved by regression analysis of compound peak area against concentration.

2.7. Chromatographic conditions

Chromatography was achieved under isocratic conditions at a flow-rate of 1.0 ml/min. Separation was achieved at 20°C, with a total run time of 60 min. Eluted mobile phase was monitored at 218 nm. Sample injection volume was 20 μl.

Compound peaks were identified by their retention times and co-elution with authentic standards. Peak purity was assessed by comparison of compound UV spectra (range 190 to 600 nm, at 4 nm intervals) in standards with urine samples. Column dead time was assessed by eluting methanol through the column and determining elution time of acetonitrile, based on the assumption that it was not retained on the column.

3. Results and discussion

3.1. Chromatographic separation

Achieving satisfactory separation of allantoin is the major analytical problem in HPLC based methods, due to the presence of a number of compounds in urine with similar polarity and UV absorption [27,32]. During the initial stages of method development, chromatography using a mobile phase containing 5 m*M* phosphate buffer at pH 3.2 [28] was assessed. In order to improve chromatography, both sodium 1-heptane sulphonic acid (ion-pairing reagent) [31] and triethylamine [36] were included. The effects of mobile phase pH (3.00 and 3.20), ammonium phosphate, (5, 7.5 and 15 m*M*), triethylamine (30, 20, 10, 5 and 1 m*M*), and sodium 1-heptane sulphonic acid (10, 5 and 1 m*M*) concentrations were evaluated. Incorporation of sodium 1-heptane sulphonic acid improved the separation of allantoin and uric acid a finding consistent with earlier observations [31]. Inclusion of triethylamine improved all compound peak shapes and decreased the retention time of creatinine. Creatinine is a weak base and is therefore capable of binding to free or non-hydrogen bonded silanol groups present on the surface of the stationary phase which are highly acidic in nature. Since triethylamine is known to preferentially occupy silanol groups [36], decreases in creatinine retention time can be attributed to a reduction of creatinine–silanol group interactions.

Retention of allantoin was independent of changes in mobile phase composition which is consistent with other methods [25,32]. In a recent evaluation of three C_{18} stationary phases to separate allantoin, uric acid and parabanic acid, compound retention times were found to be column dependent [29]. Assessment of the relative concentration of free residual silanol groups by methyl red binding indicated that stationary phases which resulted in superior allantoin separation correspondingly bound more dye. Since mobile phase composition has little influence, variations in allantoin retention times appear to be due to characteristics of different stationary phases, indicating that column selection is critical in achieving an acceptable separation of allantoin. Recently it has been established that increases in the retention time and improvements in the separation of allantoin can be achieved by using a HPLC method based on an amino stationary phase eluted with an aqueous acetonitrile mobile phase [30]. Unfortunately this methodology does not provide a satisfactory separation of other purine metabolites, pseudouridine or creatinine (Shingfield, unpublished data). With the exception of allantoin, C_{18} materials remain the stationary phase of choice for the determination of purine metabolites, pseudouridine and creatinine in ruminant urine.

Separation of all compounds was best achieved using a mobile phase containing 7.5 m*M* ammonium dihydrogen phosphate, 10 m*M* sodium 1-heptane sulphonic acid and 1.0 m*M* triethylamine at pH 3.0. Chromatograms of the calibration standard and diluted urine samples collected from sheep and dairy cattle are shown in Fig. 1. Mean (±S.D.) retention times for allantoin, pseudouridine, uric acid, xanthine, hypoxanthine and creatinine were 2.8 (±0.1), 4.5 (±0.1), 7.9 (±0.1), 9.7 (±0.1), 12.1 (±0.2) and 37.6 (±2.6), respectively based on 50 calibration standard injections. Loss of resolution capacity indicated by peak tailing was most evident for the latest eluting analyte creatinine. The column maintained its resolution capacity for between 150 and 200 injections, and was subsequently regenerated. Chromatography was maintained following three successive column regenerations, representing a column lifetime equivalent to 800 injections. Despite sourcing stationary phases from the same manufacturer, variations in compound retention times were found to be column dependent. Coefficients of variation in compound retention times due to column were 3.4, 4.9, 4.2, 4.7, 2.4 and 6.9% for allantoin, pseudouridine, uric acid, xanthine, hypoxanthine and creatinine, respectively.

Capacity factors were calculated based on 50 calibration standard injections according to [36], based on the assumption that acetonitrile was not retained on the column (Table 1). Within-day and between-day variations in compound retention time assessed by analysing bovine and ovine urine samples four times daily over three consecutive days were less than 4% (data not presented). Dolan and Snyder [36] quote pH changes of ±0.01 can on average result in a ±1% change in compound retention times in reversed-phase based HPLC analytical systems. Since urine concentrations vary

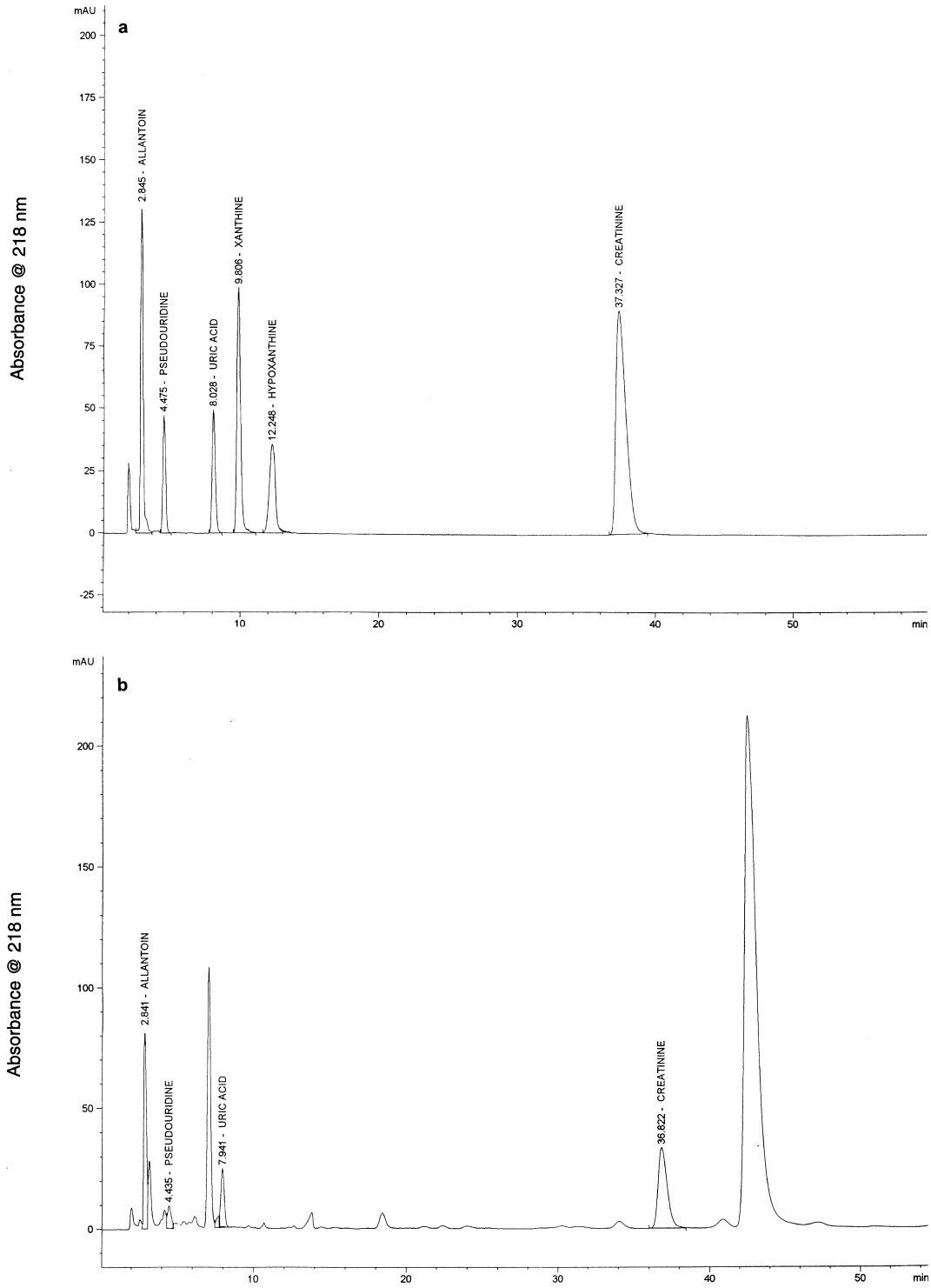


Fig. 1. (a) Chromatogram of calibration standard. (b) Chromatogram of bovine urine sample. (c) Chromatogram of ovine urine sample.

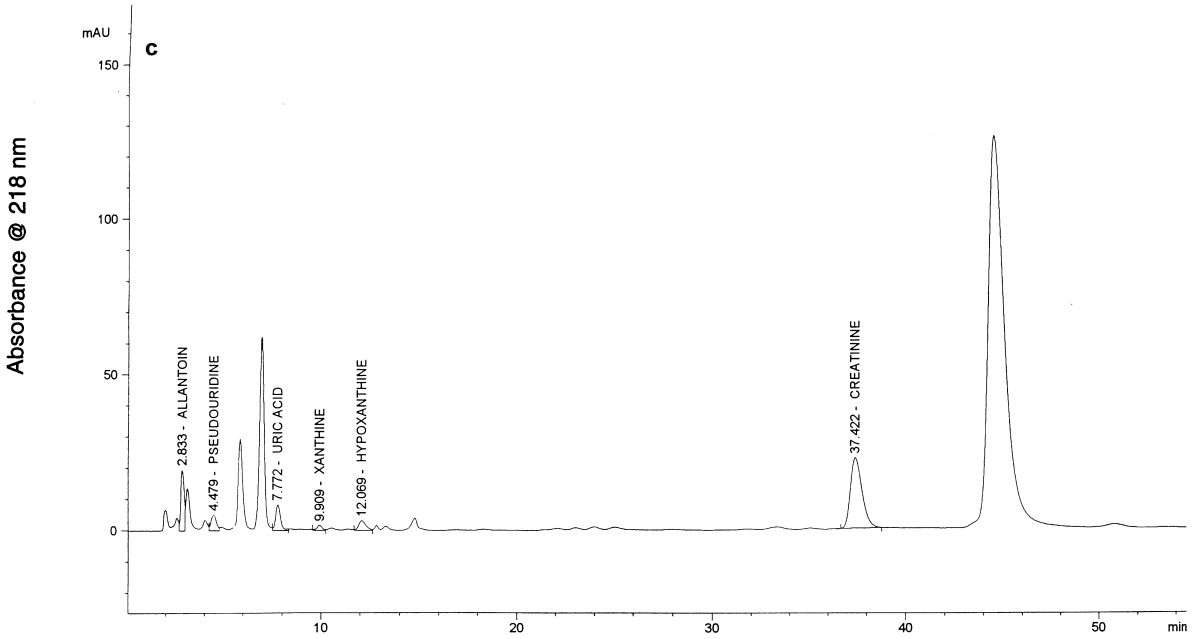


Fig. 1. (*continued*)

markedly, it is essential that the pH of a diluted urine sample is not dissimilar to that of the mobile phase. Consistent compound retention times determined in both sources of ruminant urine suggest that in the current method, the pH of diluted samples approaches that of the mobile phase. Other methods have simply diluted urine samples with water [25,27,28] or a weak buffer [32] taking no account of variations in urine buffering capacity. Consistent compound retention times can be considered highly desirable, since acceptable separation, for allantoin in particular, is often difficult to achieve.

Table 1
Compound capacity factors

Compound	Capacity factor[a] (mean±S.D.[b])
Allantoin	0.3 (±0.01)
Pseudouridine	1.0 (±0.02)
Uric acid	2.7 (±0.05)
Xanthine	3.5 (±0.03)
Hypoxanthine	4.5 (±0.10)
Creatinine	16.3 (±1.17)

[a] Determined from 50 calibration standard injections.

[b] Capacity factor (k') calculated as: $k'=(t_r - t_o)/t_o$ [36] where: k' is the compound capacity factor; t_o is the column dead time (min)[c]; t_r is the compound retention time (min).

[c] Determined as 2.18 min, assuming that acetonitrile is not retained on the column.

3.2. Accuracy of the method

Peak identification was performed by the addition of authentic standards to ovine and bovine urine samples. Calibration curves were prepared to evaluate the relationships between peak area and compound concentrations. Curves were constructed by performing quadruplicate injections at five standard additions for each compound. Construction of each curve was based on the criteria that peak area responses were assessed over a range of compound concentrations typical of those present in diluted samples of ruminant urine. The standard addition method was also applied to diluted ovine and bovine urine samples. Peak area responses to each compound addition were highly linear with regression coefficients above 0.98 over the range of concen-

trations tested, i.e., 10–300 μmol/l (injected concentrations 10, 50, 100, 200 and 300 μmol/l) for allantoin and creatinine and 20–100 μmol/l (injected concentrations 20, 40, 60, 80 and 100 μmol/l) for hypoxanthine, pseudouridine, uric acid and xanthine. Regression equations of peak area (y) against compound concentration (x, μmol/l) obtained by calibration standard addition were $y=1.01x+0.55$ for allantoin, $y=3.14x-2.15$ for creatinine, $y=2.25x-3.07$ for hypoxanthine, $y=5.51x-2.75$ for pseudouridine, $y=3.95x+0.14$ for uric acid and $y=3.01x+19.3$ for xanthine. Corresponding peak area responses derived by standard addition to ovine urine were $y=1.04x+68.1$, $y=3.13x+346$, $y=2.17x+35.9$, $y=5.45x+22.2$, $y=4.01x+32.6$ and $y=3.04x+19.4$, respectively. Peak area responses derived by standard addition to bovine urine were $y=1.07x+755$ for allantoin, $y=3.02x+1052$ for creatinine, $y=5.55x+140$ for pseudouridine and $y=3.88x+216$ for uric acid.

Compound recoveries were calculated by comparison of peak area responses (regression gradient) determined for the calibration standard with those derived for diluted urine samples. Recoveries of allantoin, creatinine, hypoxanthine, pseudouridine, uric acid and xanthine added to ovine urine were 102.4, 99.7, 96.7, 98.9, 101.4 and 100.1%, respectively. Compound recoveries added to bovine urine were 105.2, 96.1, 100.7 and 98.1% for allantoin, creatinine, pseudouridine and uric acid, respectively.

Accuracy of the method was further assessed by determining the recovery of known quantities of allantoin, pseudouridine, uric acid, xanthine, hypoxanthine and creatinine added to ten diluted urine samples collected from sheep (Table 2) and ten diluted urine samples collected from dairy cows (Table 3). Mean recoveries determined over a wide range of compound concentrations were in excess of

Table 2
Recovery of compounds added to diluted samples of ovine urine[a]

Compound	Addition (μmol/l)	Mean ($n=10$) measured concentration (μmol/l)	Recovery of compound added (%) Ψ[b]
Allantoin	0	106.6	
	40	148.0	101.5 (±3.3)
	120	230.0	102.1 (±2.6)
	180	292.3	102.7 (±1.2)
Creatinine	0	79.9	
	40	121.7	100.7 (±2.2)
	120	203.4	101.8 (±2.1)
	180	264.3	102.4 (±0.8)
Hypoxanthine	0	8.8	
	20	28.4	97.7 (±3.4)
	60	68.6	98.5 (±3.6)
	90	98.6	98.9 (±2.8)
Pseudouridine	0	5.9	
	20	26.4	102.8 (±3.9)
	60	67.3	102.3 (±3.1)
	90	98.3	102.9 (±1.9)
Uric acid	0	18.2	
	20	38.6	101.0 (±2.5)
	60	79.0	101.5 (±1.7)
	90	109.7	101.9 (±1.4)
Xanthine	0	1.2	
	20	21.2	99.7 (±1.7)
	60	61.2	99.7 (±2.5)
	90	91.2	99.9 (±2.0)

[a] Compound recoveries (mean±S.D.) determined from standard additions to ten diluted samples.

[b] Ψ: Recovery calculated as recovery (%)=[{(C_1-C_0)×100}/A], where: C_0 and C_1 are determined compound concentrations before and after compound addition, and A is the quantity of compound added.

Table 3
Recovery of compounds added to diluted samples of bovine urine[a]

Compound	Addition (μmol/l)	Mean (n=10) measured concentration (μmol/l)	Recovery of compound added (%) Ψ[b]
Allantoin	0	222.1	
	40	263.0	100.8 (±5.4)
	120	342.9	100.7 (±3.1)
	180	402.8	100.2 (±3.4)
Creatinine	0	72.1	
	40	112.8	100.8 (±5.8)
	120	188.1	97.6 (±2.7)
	180	246.8	97.9 (±2.9)
Pseudouridine	0	4.9	
	20	26.0	102.7 (±3.6)
	60	66.2	102.2 (±2.6)
	90	96.4	101.6 (±1.7)
Uric acid	0	36.8	
	20	55.4	97.4 (±3.1)
	60	93.9	96.9 (±3.0)
	90	122.9	96.4 (±2.6)

[a] Compound recoveries (mean±S.D.) determined from standard additions to ten diluted samples.
[b] Recovery calculated as recovery (%)=[{(C_1-C_0)×100}/A], where: C_0 and C_1 are determined compound concentrations before and after compound addition, and A is the quantity of compound added.

96%, and were consistent with values obtained by regression. Mean compound recoveries determined using the current method, are in good agreement with previously published methods. For example, mean recoveries of allantoin added to diluted ovine urine (102.1%) are consistent with previously reported values of 97.9% [25], 98% [28] and 96.7% [32].

Use of allopurinol as an internal standard [32] was evaluated despite no extraction procedure being used during sample preparation. Despite obtaining highly linear peak area responses using the standard addition method (data not presented), co-elution of allopurinol urine (retention time 13.6±0.05 min) precluded its use as an internal standard (refer to Fig. 2). Inability to use an internal standard was not considered a major disadvantage since recoveries of all identified compounds approached 100%. Detection limits were determined at a signal-to-noise ratio of 3:1 which corresponded to allantoin, uric acid, xanthine, hypoxanthine, creatinine and pseudouridine concentrations of 1.1, 1.0, 1.0, 1.0, 3.0 and 0.4 μmol/l, respectively.

3.3. Method specificity

Peak purity was assessed to check for chemical interference. UV spectra of compound standards were compared to UV spectra of corresponding identified peaks in ovine (Fig. 3) and bovine urine. Spectral comparisons for xanthine and hypoxanthine were unable to be performed in bovine urine, since urinary concentrations approached method detection limits. In all cases, close spectral matches were obtained, and all identified peaks were assumed to be pure and devoid of chemical interference from unidentified compounds.

Quantification of nucleic acid components (nucleotides, nucleosides and purine bases) based on HPLC techniques, typically monitor column effluent at 254 nm [28,32,34]. Balcells et al [32], determined purine metabolites based on UV detection at 205 nm, since maximum UV absorbance of purine compounds is within the range of 206–210 nm. Monitoring column effluent at 205 nm would however compromise specific and sensitive measurements of creatinine (refer to Fig. 3). Accurate and precise

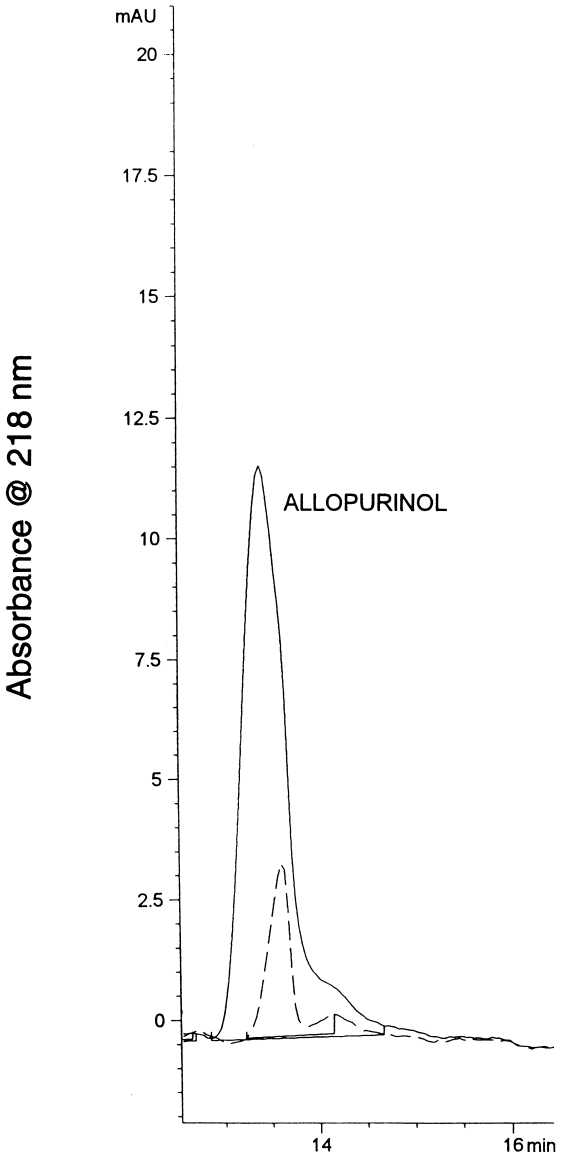


Fig. 2. Co-elution of allopurinol with unidentified peaks in ruminant urine.

quantification of creatinine in biological fluids has however been achieved using HPLC methods and UV detection at 215 [24] or 218 nm [25,27].

Creatinine is often used as an internal marker of urinary output [37,38], quantification of which is essential when attempting to estimate ruminant protein supply derived from rumen microbes by the collection of spot urine samples. In accordance with existing methods used to quantify urinary allantoin [25,28,30] and purine metabolite [27] concentrations, detection at 218 nm was adopted. Monitoring column effluent at this wavelength does however, compromise the specificity and sensitivity of uric acid, xanthine and hypoxanthine measurements (refer to Fig. 3). Compromised method specificity and sensitivity was considered acceptable and valid, since allantoin is quantitatively the most important purine metabolite excreted in ruminant urine [7–10,14,15,17,18,39]. Furthermore, capacity factors of uric acid, xanthine and hypoxanthine (Table 1) were within the range (1–10) considered to be ideal [36], and recoveries determined by standard addition to urine samples approached 100% (Tables 2 and 3).

Typical allantoin, pseudouridine, uric acid, xanthine, hypoxanthine and creatinine concentrations determined in samples obtained by total collection from dairy cows ($n=261$) and sheep ($n=24$) are presented in Table 4. Allantoin accounted for proportionately 0.86 (± 0.01; $n=261$) and 0.60 (± 0.05; $n=24$) of total urinary purine metabolite concentrations in bovine and ovine samples obtained by total collection, respectively. Purine metabolite proportions obtained in samples collected from dairy cows are highly consistent with values reported for cattle (0.85 [8], 0.82 [10] and 0.88 [18]) based on alternative analytical methods. Values obtained for sheep were within the range of between 0.49 and 0.82 reported in the literature [7,9,10,14]. Variation in the proportion of purine metabolites excreted in urine as allantoin in ovine species tend to be much larger than that in cattle due to a dependence on the amount of purine bases absorbed from the small intestine [7]. Xanthine and hypoxanthine were present in only trace amounts in bovine urine, a finding in agreement with other studies reporting negligible concentrations [5,10,13,30].

Method specificity was also assessed by comparison of urinary allantoin, uric acid, hypoxanthine, xanthine, pseudouridine and creatinine excretion quantified using the current method with measurements reported in the literature. Measurements of urinary creatinine excretion quantified in ovine and bovine species were highly consistent with published values, determined using either colorimetric or

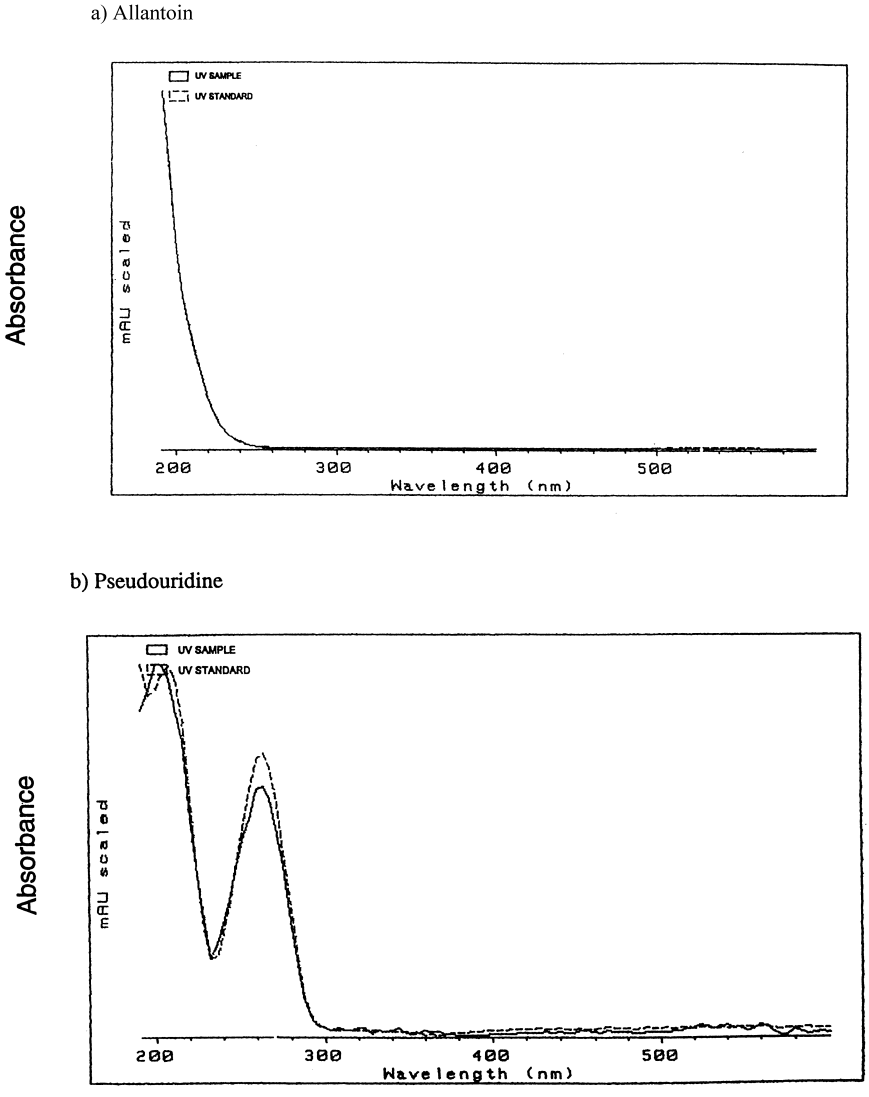
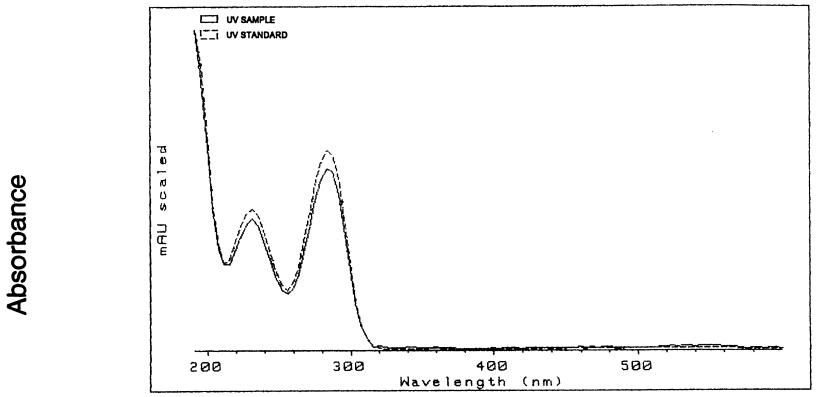


Fig. 3. Comparison of UV spectra of compounds in a calibration standard with corresponding compounds identified in ovine urine. (a) Allantoin, (b) pseudouridine, (c) uric acid, (d) xanthine, (e) hypoxanthine, (f) creatinine.

HPLC based techniques (Table 5). Urinary outputs of purine metabolites and pseudouridine were in general agreement with the range of values reported in the literature (Table 5). Direct comparisons of urinary purine metabolite excretion are less consistent due to variations in the quantity of microbial protein entering the small intestine, purine content of rumen microbes, dietary purines that escape rumen degradation and endogenous purine metabolite excretion [15]. Direct comparisons of urinary pseudouridine output may also be confounded by between-experiment variations, since pseudouridine excretion is dependent on animal factors, being proportionately much higher during periods of protein accretion

c) Uric acid



d) Xanthine

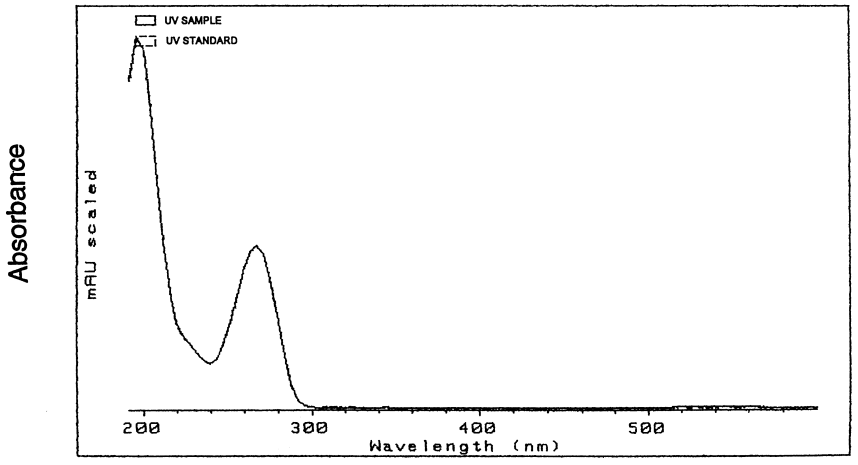
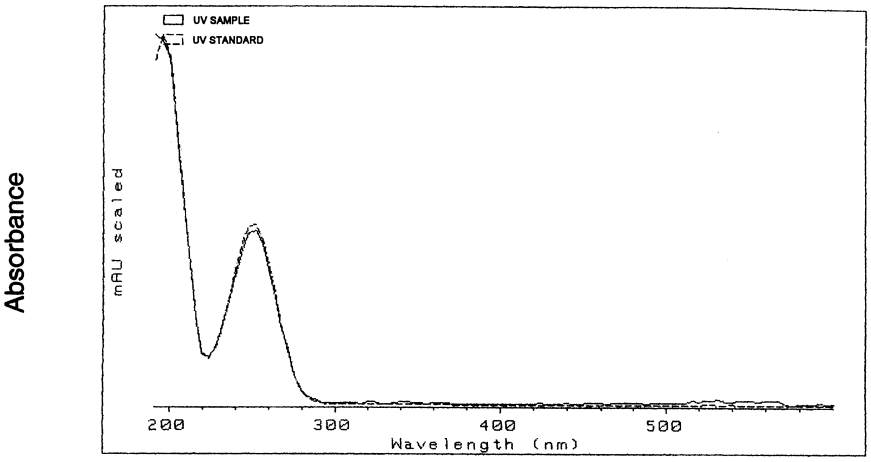


Fig. 3. (*continued*)

predominant in growing, pregnant or lactating animals [14,16].

Simultaneous determination of purine metabolites, pseudouridine and creatinine is particularly advantageous in ruminant nutritional studies in which a total urine collection is not possible. In such situations, use of creatinine as an internal marker of urinary output [32,33] has been suggested to allow the prediction of urinary excretion of purine metabolites and pseudouridine in sheep and cattle based on measurements of spot urine samples [12,13]. Analysis of spot urine samples collected from lactating dairy cows using the current method has demonstrated that expressing purine metabolite [15] and pseudouridine [16] concentrations as molar ratios to creatinine, reduces variations between-sampling in-

e) Hypoxanthine



f) Creatinine

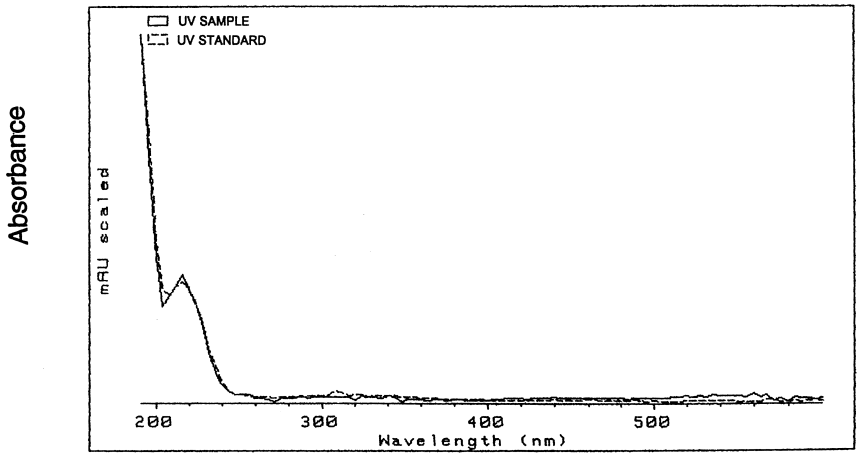


Fig. 3. (*continued*)

tervals and between-animals compared to measurements of absolute urinary concentration.

4. Conclusion

A robust and precise method has been developed for the simultaneous determination of purine metabolites, pseudouridine and creatinine in ruminant urine. Chromatographic conditions adopted were a compromise between the sensitivity and specificity of creatinine, uric acid, hypoxanthine and xanthine measurements, analysis time and resolution of purine metabolite, creatinine and pseudouridine peaks from interfering compounds. Compromises between specificity and sensitivity of the measurements of each analyte were considered acceptable since urinary excretion of creatinine, pseudouridine and purine metabolites in ovine and bovine species quantified using the current method were consistent with values reported in the literature based on alternative techniques. In conclusion, application of this method has

Table 4
Typical (mean±S.D.) purine metabolite, pseudouridine and creatinine concentrations in ruminant urine

Compound	Concentration (μmol/l)	
	Bovine[a]	Ovine[b]
Allantoin	12831 (±5546)	1736 (±564)
Creatinine	5690 (±1834)	3178 (±1347)
Hypoxanthine	ND[c]	541 (±194)
Pseudouridine	460 (±215)	185 (±78)
Uric acid	1894 (±1085)	593 (±299)
Xanthine	ND[c]	33 (±15)

[a] Determined in 261 samples obtained by total urine collection from lactating British Holstein/Friesian dairy cows (n=36).
[b] Determined in 24 samples obtained by total urine collection from Finnish Landrace sheep (n=4) fed at maintenance (35 g dry matter per kg $W^{0.75}$).
[c] ND: Not detected.

the potential to facilitate development and implementation of less invasive studies of ruminant nitrogen metabolism.

Acknowledgements

The authors would like to thank Kenny McIssac, Vesa Toivonen, Mervi Mikkola and Sari Siikjärvi for technical assistance during this work. SAC receives financial support from the Scottish Office Agriculture and Fisheries Department.

References

[1] M.D. Stern, in: Proceedings of the 1986 Cornell Nutrition Conference for Feed Manufacturers, Ithaca, NY, Oct. 1996, p. 10.
[2] Agricultural and Food Research Council, Nutr. Abstr. a. Rev. 62 (1992) 788
[3] H.C. Wilson, D.W. Wilson, J. Biol. Chem. 237 (1962) 1643.
[4] A.B. McAllan, Br. J. Nutr. 44 (1980) 99.
[5] R.H. Topps, R.C. Elliot, Nature 205 (1965) 498.
[6] A.B. McAllan, Proc. Nutr. Soc. 41 (1982) 309.
[7] X.B. Chen, F.D.DeB. Hovell, E.R. Ørskov, D.S. Brown, Br. J. Agric. Sci. (Camb.) 4 (1990) 243.
[8] J. Verbic, X.B. Chen, N.A. Macleod, D.S Brown, J. Agric. Sci(Camb.) 114 (1990) 131.
[9] J.E. Lindberg, K.G. Jacobsson, Br. J. Nutr. 64 (1990) 359.
[10] X.B. Chen, E.R. Ørskov, F.D.DeB. Hovell, Br. J. Nutr. 63 (1990) 121.
[11] G. Sander, H. Topp, G. Heller-Schöch, J. Wieland, G. Schöch, Clin. Sci. 71 (1986) 367.
[12] S. Weissman, A.Z. Eisen, M. Lewis, M. Karon, P. Clark, J. Lab. Clin. Med. 60 (1962) 40.
[13] G. Sander, J. Hülsemann, H. Topp, G. Heller-Schöch, G. Schöch, Ann. Nutr.a. Metab. 30 (1986) 137.
[14] R. Puchala, J.A. Shelford, W. Barej, G.W. Kulasek, H. Pior, M.V. Keyserlingk, N. Makoni, J. Anim. Physiol. a. Anim. Nutr. 69 (1993) 186.
[15] K.J. Shingfield, N.W. Offer, Anim. Sci. 66 (1998) 557.
[16] K.J. Shingfield, N.W. Offer, Agric. Fd. Sci. Finl. 7 (1998) 345.
[17] X.B. Chen, A.T. Mejia, D.J. Kyle, E.R. Ørskov, J.Agric. Sci. (Cambs.) 125 (1995) 137.
[18] R.J. Dewhurst, A.M. Mitton, N.W. Offer, C. Thomas, Anim. Sci. 62 (1996) 25.

Table 5
Comparison of urinary purine metabolite, pseudouridine and creatinine excretion in ruminants quantified using the current method with measurements reported in the literature

Compound	Excretion (μmol kg live weight $^{0.75}$/d)			
	Bovine		Ovine	
	Measured[a] (mean ±S.D.)	Published	Measured[b] (mean ±S.D.	Published
Allantoin	2354 (±988)	364–1988 [8], 1695–2196 [40], 1900–2875 [41][c]	291 (±50)	81–1073 [7], 287–1434 [14], 564–816 [42]
Hypoxanthine	ND[e]	4–6 [14][d]	90 (±12)	24–78 [7][d], 29–93 [14][d], 114–144 [42]
Xanthine	ND[e]	Not reported in the literature	5.7 (±2.4)	2–4 [42]
Uric acid	361 (±201)	55–289 [8], 122–193 [40], 182–388 [41][c]	99 (±36)	39–175 [7], 98–278 [14], 66–102 [42]
Creatinine	1054 (±288)	995–1048 [14], 891–983 [39], 856–1022 [40]	518 (±127)	405–502 [9], 407–525 [17], 576–612 [42]
Pseudouridine	85.7 (±40.2)	20–36 [14]	30 (±8)	19–36 [14]

[a] Determined from 261 samples obtained by total collection from lactating British Holstein/Friesian dairy cows (n=36).
[b] Determined from 24 samples obtained by total collection from Finnish Landrace sheep (n=4) fed at maintenance (35 g dry matter per kg $W^{0.75}$).
[c] Determined as ±S.D. of reported mean.
[d] Reported as the sum of hypoxanthine and xanthine.
[e] ND: Not detected.

[19] E.G. Young, C.F. Conway, J. Biol. Chem. 142 (1942) 839.
[20] M.A. Razzaque, J.H. Topps, J. Sci. Fd. Agric. 29 (1978) 935.
[21] T. Fujihara, E.R. Ørskov, P.J. Reeds, J. Kyle, J. Agric. Sci. (Cambs.) 109 (1987) 7.
[22] M.H. Kroll, R. Chesler, C. Hagengruber, D.W. Blank, J. Kestner, M. Rawe, Clin. Chem. 32 (1986) 446.
[23] S. Narayanan, H.D. Appleton, Clin. Chem. 26 (1980) 1119.
[24] G.P. Xue, R.C. Fishlock, A.M. Snoswell, Anal. Biochem. 171 (1988) 135.
[25] M.T. Díez, M.J. Arín, J.A. Resínes, J. Liq. Chromatogr. 15 (1992) 1337.
[26] X.B. Chen, D.J. Kyle, E.R. Ørskov, J. Chromatogr. 617 (1993) 241.
[27] J.A. Resínes, M.J. Arín, M.T. Díez, J. Chromatogr. 607 (1992) 199.
[28] W. Tiermeyer, D. Giesecke, Anal. Biochem. 123 (1982) 11.
[29] M. Hicks, L.S. Wong, R.O. Day, Anal. Biochem. 210 (1993) 428.
[30] K.J. Shingfield, N.W. Offer, J. Chromatogr. B 706 (1998) 342.
[31] O. Lux, D. Naidoo, C. Salonikas, Ann. Clin. Biochem. 29 (1992) 674.
[32] J. Balcells, J.A. Guada, J.M. Peiro, D.S. Parker, J. Chromatogr. 575 (1992) 153.
[33] W. Tiermeyer, M. Stohrer, D. Giesecke, J. Dairy Sci. 67 (1984) 723.
[34] R. Rosskopf, H. Rainer, D. Giesecke, Arch. Anim. Nutr. 41 (1991) 411.
[35] A.W. Bristow, D.C. Whitehead, J. E. Cockburn, J. Sci. Fd. Agric. 59 (1992) 387.
[36] J.W. Dolan, L.R. Synder, Troubleshooting LC Systems, Humana Press, Clifton, New Jersey, 1989.
[37] T.H. De Groot, J.H. Aafjes, Br. Vet. J. 116 (1960) 409.
[38] R.E. Erb, A.H. Surve, R.D. Randal, H.A. Carverick, J. Dairy Sci. 60 (1977) 825.
[39] P. Susmel, M. Spanghero, B. Stefanon, C.R. Mills, Lives. Prod. Sci. 44 (1995) 207.
[40] H.L. Gonda, J.E. Linberg, Acta Agric. Scand., Sect. A. Anim. Sci. 44 (1994) 236.
[41] D. Giesecke, L. Ehrentreich, M. Stangassinger, F. Ahrens, J. Dairy Sci. 77 (1994) 2376.
[42] J.C. Surra, J.A. Guada, J. Balcells, C. Castrillo, Anim Sci. 65 (1997) 83.