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Determination of aromatic metabolites in ruminant urine by high-performance liquid chromatography

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Abstract

A method based on reversed-phase HPLC is reported for the separation and quantification of various urinary aromatic metabolites: hippuric, phenylaceturic, salicyluric, benzoic, phenylacetic, salicylic, 3-phenylpropionic and cinnamic acids and several phenols in ruminant urine. In this method, a Nova-Pak C_{18} (4 μm) 150×3.9 mm I.D. column, two solvents [A: 15% methanol in 20 m*M* acetic acid (pH 3.3); B: methanol] in a gradient mode at a flow-rate of 0.8 ml/min, and UV detection at 210 nm were used. Quantification of the total (free and conjugated) benzoic, phenylacetic and salicylic acids present in urine was achieved by hydrolysis of the samples in 3 *M* HCl at 100°C for 24 h prior to HPLC analysis. The lowest detection concentration was 50 μmol/l. This method is useful for scanning the profile of aromatic metabolites in urine of ruminants, which provides information on the diets the animals receive.

Keywords: Benzoic acid; Phenylpropionic acid; Hippuric acid; *p*-Cresol; Phenylacetic acid; Phenylaceturic acid; Cinnamic acid; Salicylic acid; Salicyluric acid

1. Introduction

Ruminants excrete large quantities of aromatic metabolites in their urine due mainly to the ingestion of cellulosic plant materials. These metabolites are the excretory products of dietary phenolic acids, alicyclic acids and aromatic amino acids which are first metabolized by rumen microbes and further metabolized in body tissues after absorption [1–4]. Aromatic metabolites present in the urine of ruminants comprise aromatic acids (benzoic, phenylacetic, 3-phenylpropionic and cinnamic acid and their hydroxy derivatives) [2,5] and simple phenols (*p*-cresol, catechol and phenol) [4]. Most of these compounds are excreted in urine as conjugated forms, e.g. aromatic acids with glycine and glucuronic acid, and phenols with sulphuric and glucuronic acids. Hippuric acid (glycine conjugate of benzoic acid), phenylaceturic acid (glycine conjugate of phenylacetic acid) and *p*-cresol conjugates are the major aromatic metabolites in ruminant urine. Since hippuric acid is an important contributor to the energy content of urine and is a significant channel of glycine loss [6], earlier studies focused on the energy and nitrogen costs of its excretion. Interest has been renewed in the measurement of aromatic metabolites in ruminant urine because these metabolites could potentially be used as biological markers of feeding responses [7–9]. Studies have indicated that urinary benzoic acid is exclusively of dietary origin [3,10]

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and its excretion appears to be related to the amount of cellulosic feed ingested by the animals [11]. We are interested in studying the urinary outputs of aromatic metabolites in relation to the uptake of their precursors in feed and to the type of feeds.

There are a number of analytical procedures reported in the literature for the determination of urinary aromatic metabolites. For example, spectroscopic [12,13], gas–liquid chromatography (GLC) [14,15] and HPLC [16–18] methods are available for the determination of hippuric acid; GLC [19,20] and HPLC [21,22] methods for phenylacetic acid; HPLC methods [23,24] for salicylic acid and its glycine conjugate salicyluric acid; and GLC [25,26] and HPLC [27,28] methods for simple phenols. Most of the methods have been developed for human urine in clinical and toxicological studies. Methods for the determination of aromatic metabolites in ruminant urine using titrimetry [1–3,10], spectroscopy after thin-layer chromatography (TLC) [1,10] or low-pressure ion-exchange chromatography [1–3], GLC [1–5,29] and HPLC [30,31] have also been reported. The spectroscopic and titrimetric methods lack specificity. The GLC and HPLC methods can detect individual compounds, however the range of compounds is restricted since some of the conjugated forms of aromatic metabolites are not commercially available for standardization. In order to provide an estimate of the total amount of an aromatic acid or phenol (free and conjugated) in the urine, attempts have been made to hydrolyze the conjugates into their free forms either by heating the samples with alkali [1–3,5,10] or inorganic acid [4,19–23,28,29], or by incubation with β-glucuronidase (EC 3.2.1.31) and arylsulphatase (EC 3.1.6.1) enzymes [4,16,18,25–27,30].

In this paper, we report a method based on reversed-phase HPLC for the separation and quantification of a range of urinary aromatic metabolites: hippuric, phenylaceturic, salicyluric, benzoic, phenylacetic, salicylic, 3-phenylpropionic, cinnamic acids and several phenols, which are likely to be excreted by ruminants and other species of animals. When coupled with acid hydrolysis of the sample, the method also provides estimates of total (conjugated and free) content of each aromatic acid in urine.

2. Experimental

2.1. Reagents

Hippuric acid, phenylaceturic acid, phenylacetic acid, salicylic acid, 3-phenylpropionic acid, *trans*-cinnamic acid, catechol, guaiacol, resorcinol and β-glucuronidase (Sigma Cat. No G-0876) were purchased from Sigma (Poole, UK), salicyluric acid and *p*-cresol from Aldrich (Poole, UK), phenol from Fisons (Loughborough, UK), quinol from Fluka (Poole, UK); pyrogallol from May & Baker (Manchester, UK) and benzoic acid from BDH (Poole, UK)

2.2. Chromatographic condition

The HPLC equipment used consisted of Waters 625 LC system (including a controller and a pump), a tunable UV absorbance detector (Waters Model 484), Waters 712 WISP autosampler and Millenium 2100 data handling system (all equipment from Waters, Millipore, Bedford, MA, USA). The analytical column was a Nova-Pak C_{18} (4 μm) 150×3.9 mm I.D. (Waters, Millipore). A guard column (Upchurch Scientific, UK) of 25×2 mm I.D. packed with reversed-phase C_{18} (30-40 μm) pellicular packing material was used.

Two solvents were used: (A) 15% methanol in 20 m*M* acetic acid pH 3.3 (adjusted with a weak ammonium solution); (B) methanol. The glacial acetic acid and methanol used were of HPLC grade (HiPerSolv, BDH, UK). Both solvents were filtered through a 0.22-μm pore size cellulose membrane filter (Anachem, UK). An initial degassing of the solvents was made by ultrasonication and an on-line degassing was made by fluxing helium gas into the solvents at 30 ml/min. The gradient used was (% B): 0 at 0 min, 50 at 5–7 min, 0 at 9–14.5 min (the change was linear). The system pressure increased from ca. 11.72 to 15.17 MPa as the proportion of solvent B increased to 50%. The flow-rate was 0.8 ml/min. Injection volume was 20 μl. The UV detector was set at 210 nm. Total run time was 14.5 min. The separation was performed at room temperature.

2.3. Sample preparation

Urine was collected daily from animals into 1 *M* H_2SO_4 as a preservative. An appropriate amount of the acid was used so that the pH of the collected urine was below 3. A sample (about 20 ml) of the urine was stored at −20°C before analysis. For the determination of free aromatic acids and their glycine conjugates, the urine samples were diluted with 1 *M* sodium acetate buffer (pH 5.0, adjusted with glacial acetic acid). The dilution factor varied with the type of diets and the level of feed intake. The diluted urine was then centrifuged at ca. 40 000 *g* for 15 min, and the supernatant injected onto the HPLC system. For the determination of the sum of the free acids and their conjugates as the free acid equivalent, urine samples were subjected to acid hydrolysis using the following procedure. A 1-ml volume of diluted urine was mixed with 1 ml of 6 *M* HCl in a screw-sealed Pyrex glass tube and heated at 100°C for 24 h in an oil bath. After cooling, 1 ml of 6 *M* NaOH was added to neutralize the HCl and a further 1 ml of 1 *M* sodium acetate buffer (pH 5.0) was added. The mixture was then centrifuged at ca. 40 000 *g* for 15 min. The supernatant was injected onto the HPLC system within a day.

A mixture containing hippuric acid (HA), phenylaceturic acid (PUA), salicyluric acid (SUA), salicylic acid (SA), phenylacetic acid (PA), benzoic acid (BA), *p*-cresol (PC), 3-phenylpropionic acid (3PPA), and *trans*-cinnamic acid (CA) dissolved in distilled de-ionized water was used as the standard. Three to six solutions of the standard mixture at different concentrations were injected with every set of 45 urine samples to provide the calibration curve for the sample set. The concentration ranges of the standards were: 20–100 mg/l (ca. 0.20–0.66 mmol/l) for PUA, SUA, SA, PA, PC, 3PPA and CA, and 40–200 mg/l (ca. 0.30–1.60 mmol/l) for HA and BA. The standard solutions were stored in 1-ml vials at −20°C and fresh vials were used each day.

3. Results and discussion

3.1. Chromatographic separation

All nine compounds in the standard mixture were completely separated within 11 min as shown in Fig. 1. The chromatographic conditions have been used in our laboratory over the past two years for the analysis of more than 3000 samples and the method

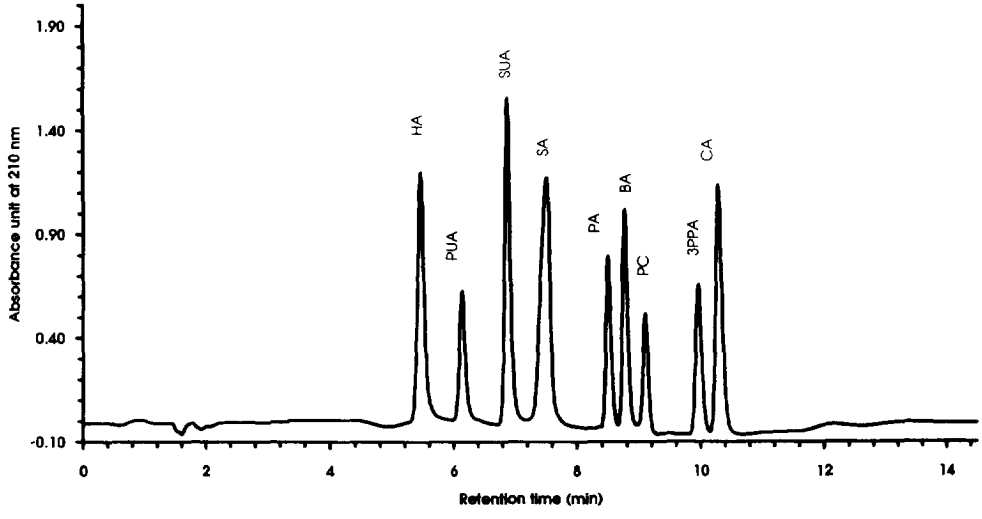

Fig. 1. Chromatographic separation of hippuric acid (HA), phenylaceturic acid (PUA), salicyluric acid (SUA), salicylic acid (SA), phenylacetic acid (PA), benzoic acid (BA), *p*-cresol (PC), 3-phenylpropionic acid (3PPA) and cinnamic acid (CA). These compounds were dissolved in water.

Table 1
Recovery of the aromatic metabolites added to six urine samples with or without acid hydrolysis

Compounds added to urine	Recovery (%)	
	Unhydrolyzed	Hydrolyzed
HA	100.4±2.1	0
PUA	99.7±3.0	0
SUA	100.5±0.9	0
SA	100.4±1.7	
PA	98.1±3.9	
BA	99.4±3.9	
PC	104.4±5.0[a]	45.7±6.0
3PPA	99.7±0.7	94.2±1.0
CA	97.8±0.3	70.8±1.9
HA+BA recovered as BA	99.7±1.9	96.5±5.1[b]
PUA+PAA recovered as PAA	98.9±2.0	95.8±3.7[b]
SUA+SA recovered as SA	100.5±1.0	96.2±5.0[b]

In the hydrolyzed samples, the glycine conjugates were recovered as their free acids (mean±S.D. of 18 measurements unless specified).
[a]Mean±S.D. of 4 measurements.
[b]Mean±S.D. of 36 measurements.

of separation was found to be robust. (Table 1 shows the results of an experiment to determine the accuracy of this chromatographic method for HA, BA, PUA, SUA, SA, PA, PC, 3PPA and CA). The relative retention times of the nine components were consistent and the absolute retention times showed only minor variations with different batches of solvent A prepared. The C.V. for the drift in retention time was between 1 and 4%, higher for the peaks with shorter retention times. Based on the data from 150 standard solutions injected over 1994 and 1995, the averaged C.V. of variation in the retention time was 1.0 (±0.4) and 1.3% (±2.2) for the two years respectively. The typical retention times (min) for the following compounds were: HA 5.5, PUA 6.2, SUA 6.9, SA 7.5, PA 8.6, BA 8.8, PC 9.1, 3PPA 10.1 and CA 10.4. The glycine conjugates of the aromatic acids were eluted earlier than their respective free acids. Within the concentration ranges used in the standard solutions, the calibration (standard) curves showed a slight quadratic trend for all components, and the model $y=a+bx+cx^2$ was therefore used to describe the calibration curves, where y is peak area, and x component concentration. The typical calibration equations for each component are listed in Table 2. Statistical analysis (t-test of the fitted values for a, b and c) showed that the quadratic trend was significant for all components except HA. However, at a concentration below about 0.3 mmol/l for PUA, SUA, SA, PA, PC, 3PPA and CA, and below 1.1 mmol/l for HA and BA, the calibration curves were linear. The variation in the peak response (peak area) of the same standard compounds injected repeatedly within a day averaged 0.5±0.5% (C.V.). The variation in the peak response of the same standard compounds injected for more than 150 runs over the last two years averaged 3.3±2.1% in 1994 and 1.9±2.6% in 1995. The low variation indicated a good reproducibility of the chromatographic procedure. For samples subjected to an additional step of acid hydrolysis, the C.V. for repeated measurements (hydrolysis+injection) within a day averaged 2.1±0.9%, and the between-day variability due to

Table 2
Calibration curves for the nine components detected in the HPLC method

Components	Concentration range (μmol/l)	Calibration curve	r^2	Linear	Quadratic
Benzoic acid	328–1638	$y=-79121(\pm14368)+6557(\pm33)x-0.32(\pm0.02)x^2$	0.999	$P<0.01$	$P<0.01$
Phenylpropionic acid	135–675	$y=-57\,811(\pm13\,731)+10\,809(\pm77)x-1.55(\pm0.09)x^2$	0.999	$P<0.01$	$P<0.01$
Hippuric acid	223–1116	$y=-153\,653(\pm37\,261)+11\,026(\pm124)x-0.09(\pm0.09)x^2$	0.999	$P<0.01$	$P>0.05$
p-Cresol	185–925	$y=-33\,290(\pm12\,873)+7885(\pm52)x-0.39(\pm0.05)x^2$	0.999	$P<0.01$	$P<0.01$
Phenylacetic acid	147–734	$y=-47\,710(\pm11\,285)+10\,029(\pm57)x-1.40(\pm0.06)x^2$	0.999	$P<0.01$	$P<0.01$
Phenylaceturic acid	103–517	$y=-487\,433(\pm173\,526)+16\,916(\pm1247)x-7.43(\pm1.97)x^2$	0.999	$P<0.01$	$P<0.01$
Cinnamic acid	135–675	$y=-547\,356(\pm172\,303)+24\,189(\pm949)x-7.32(\pm1.15)x^2$	0.999	$P<0.01$	$P<0.01$
Salicylic acid	145–724	$y=-316\,323(\pm103\,109)+25\,885(\pm529)x-8.85(\pm0.60)x^2$	0.999	$P<0.01$	$P<0.01$
Salicyluric acid	102–512	$y=-466\,125(\pm96\,502)+36\,432(\pm700)x-16.97(\pm1.12)x^2$	0.999	$P<0.01$	$P<0.01$

Each curve was constructed based on nine concentration levels. Peak area (y) was fitted into a quadratic function of the component concentration (x μmol/l): $y=a+bx+cx^2$. The t-test for the linear and quadratic trends is also presented. The S.E. values for the fitted constants are given within parentheses.

hydrolysis over a long time span has yet to be examined. The lowest concentration at which an accurate peak integration could be readily achieved was about 50 μmol/l for each compound.

The separation of some other compounds that may also be present in urine as reported by Martin [4], e.g. creatinine, quinol, pyrogallol, resorcinol, catechol, phenol and guaiacol, has also been tested, although some of these compounds are not normally present in significant quantities. Since glucuronic conjugates of aromatic acids and the sulphate and glucuronic conjugates of phenols are not available commercially, the separation of these compounds has not been tested. Except for phenol, these compounds were not co-eluted with any of the nine components in the standard mixture. The retention times are listed in Table 3. Phenol was eluted at the same time as SA. This chromatographic condition is therefore not suitable for the separation and the quantification of SA from phenol when both compounds are present. However, this restriction would only apply to urine samples after acid hydrolysis, since under physiological conditions, SA is mainly present as SUA [30] and phenol also as its conjugated forms [25,26] which can be determined separately. Catechol was not well separated from HA. Acid hydrolysis of the sample to convert HA to BA would give a more accurate measurement of HA and catechol even if catechol is present in the urine.

3.2. Recovery studies

The following experiment was carried out to examine the accuracy of this chromatographic method for HA, BA, PUA, SUA, SA, PA, PC, 3PPA and CA. Six urine samples, three from sheep and three from cattle, were each spiked with three levels of each of the nine compounds. The concentrations of each compound added were: 0.17–0.33 mmol/l for PUA, SUA, SA, PA, PC, 3PPA and CA, and 0.25–0.80 mmol/l for HA and BA. The mixtures were injected onto the HPLC system either directly or after hydrolysis in 3 *M* HCl. Results of the recovery of the added compounds with or without acid hydrolysis are shown in Table 1. For the unhydrolyzed samples, all compounds (free acids and glycine conjugates) were recovered almost completely (recovery 98–104%). For the hydrolyzed samples, all the glycine conjugates (HA, PUA, SUA) were removed but recovered as their free acids. The recovery for HA+BA, PUA+PA and SUA+SA was 96–97%, and that for 3PPA was 94%, slightly lower than the unhydrolyzed samples. The recovery of PC and CA was 46 and 71% respectively. The fraction

Table 3
Typical retention times of the nine compounds and other potential interfering compounds

Components	Retention time (min)	Remark
Main components		
Hippuric acid	5.5	
Phenylaceturic acid	6.2	
Salicyluric acid	6.9	
Salicylic acid	7.5	
Phenylacetic acid	8.6	
Benzoic acid	8.8	
p-Cresol	9.1	
3-Phenylpropionic acid	10.1	
Cinnamic acid	10.4	
Other components		
Quinol	2.8	
Pyrogallol	2.8	
Creatinine	3.5	
Resorcinol	4.4	
Catechol	5.3	Incomplete separation from hippuric acid
Phenol	7.5	No separation from salicylic acid
Guaiacol	8.0	

of loss was constant at a given hydrolysis time. No other peaks were detected as the possible derivatives from PC and CA to account for the incomplete recovery. There has been a report of a loss of phenols by volatilization during heating [18]. However, in the present work, the loss due to evaporation could not be the major factor as the tubes were completely sealed during hydrolysis.

The results show that under the hydrolysis condition used, BA, PA, SA and 3PPA are stable and the conversion of the glycine conjugates of BA, PA and SA to their free acids is complete. It has been reported in the literature that glucuronic acid conjugates of aromatic acids can be readily cleaved by acid hydrolysis [23,29]. Therefore, under the acid hydrolysis conditions used, glucuronic acid conjugates of the aromatic acids, if present in the sample, are probably also converted to their free acids. The stability of pyrogallol, quinol, catechol, resorcinol and guaiacol, and the hydrolysis of their conjugates, under the present acid hydrolysis conditions have not been tested.

3.3. Acid hydrolysis condition

Solutions of HA, PUA and SUA were mixed with equal volume of either 6 *M* HCl or water and heated at 100°C for 8, 16 and 24 h. When water was used, no hydrolysis of HA and SUA occurred, but 14.6% of the PUA was hydrolyzed to PA. Strong acidity was therefore essential for the hydrolysis of the aromatic acid conjugates. The hydrolysis of HA and SUA in 3 *M* HCl was complete by 24 h and that of PUA by 8 h.

3.4. Profiles of aromatic metabolites in urine of ruminants

The method has been used in our laboratory to examine the profiles of aromatic metabolites in urine of ruminants receiving a range of diets. In urine samples from animals receiving straw, hay or grass, HA and PUA were the predominant components. The concentration of HA in urine was normally 20–30 mmol/l and that of PUA 1–2 mmol/l. BA was present only when animals were given high levels of feed intake and PA was not normally present. Other components (SA, SUA and CA) were present only in very low concentrations (ca. 0.1 mmol/l) or not present (3PPA), and these compounds could be measured in undiluted urine. For measurement of HA and PUA, urine samples were analyzed twice using different dilution factors (typically fifty times for HA and five times for PUA). Fig.

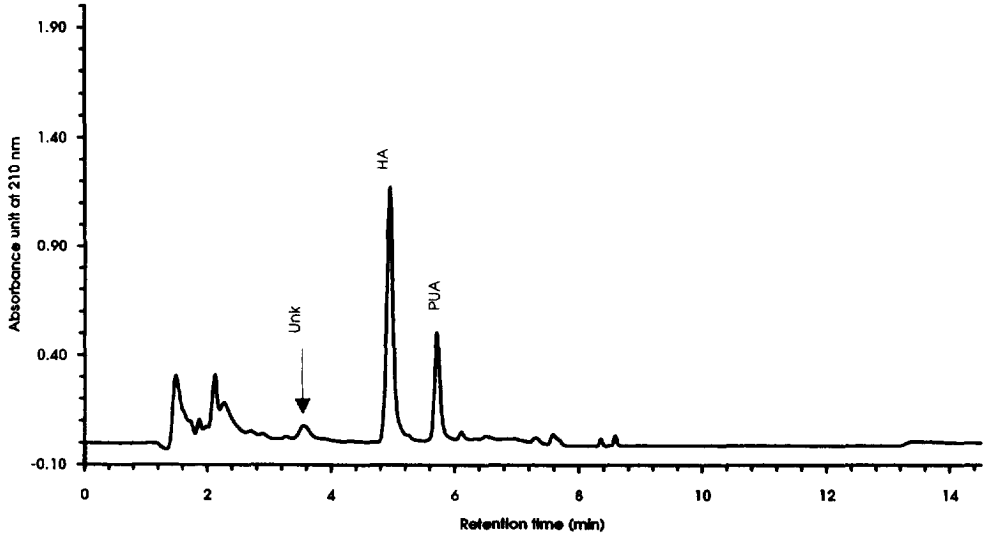

Fig. 2. Chromatographic separation of aromatic metabolites in a urine sample from a sheep receiving straw. The profile is typical for samples from animals receiving straw, hay and grass as the sole main diet. Urine samples from animals receiving silage usually contain an unknown peak (Unk) at 3.0 min.

2 shows a typical chromatogram from a sheep receiving straw. In the urine of animals receiving barley (and other non-cellulosic feeds), only trace amounts of HA and no BA were present. In order to examine the presence of glucuronic acid conjugates, urine samples were treated with β-glucuronidase as follows: 100 μl of urine was mixed with 800 μl of 0.2 *M* sodium acetate buffer (pH 5.0) and 100 μl of enzyme solution containing ca. 0.1 I.U. β-glucuronidase. The mixture was incubated at 37°C for up to 24 h. There was no increment in the amounts of BA, PA or PC, indicative of the absence of glucuronic acid conjugates of the aromatic metabolites. Therefore, we found that urine samples from animals receiving these feeds could be analyzed without acid hydrolysis. In urine samples receiving silage, an unknown peak appeared at retention time of ca. 3.0 min (see Fig. 2). This peak was not affected by β-glucuronidase treatment but was converted to PC after hydrolysis in 3 *M* HCl. It was possible that this peak was a sulphate conjugate of PC but there was no standard compound available to

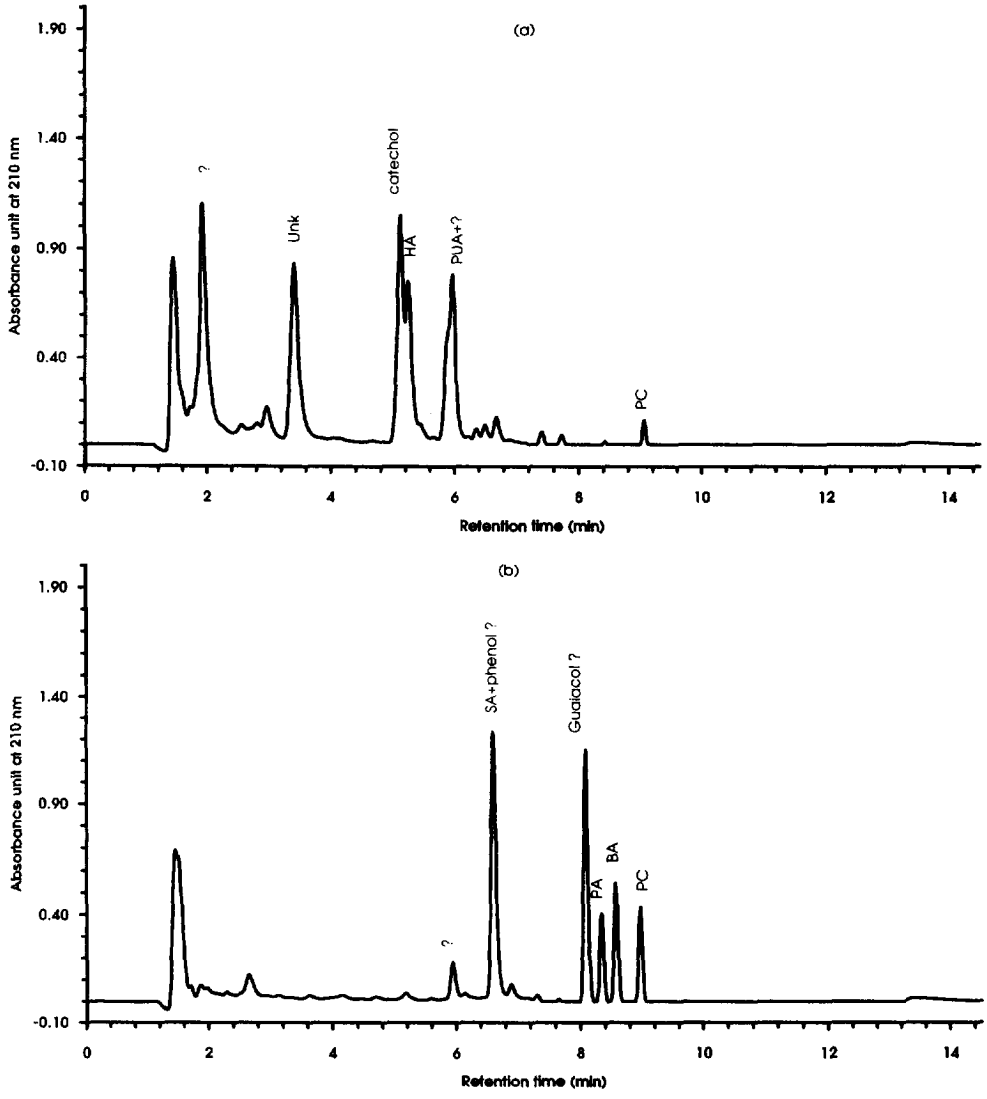

Fig. 3. Chromatographic separation of aromatic metabolites in a urine sample from a sheep receiving *Acacia spp.* leaves. (a) Without acid hydrolysis; (b) after acid hydrolysis. The identities of the peaks labeled with '?' are not certain.

confirm its identity. The concentrations of PC and PA (measured after acid hydrolysis) were substantial in urine of animals receiving silage, compared to those in urine of animals receiving hay or grass. The difference in the aromatic metabolite profiles in urine between grass/hay diet and silage is interesting. The higher urinary PC and PA associated with silage feeding could be an indication of a more extensive degradation of silage protein as a result of ensiling. With urine samples from animals receiving tree leaves (such as *Leucaena spp.*, *Acacia spp.* and *Ziziphus spp.*), the profile of aromatic metabolites were more complex containing several other unknown peaks (see Fig. 3a). Some of these peaks were probably compounds of phenolic nature [5] since these plant materials contain high levels of phenolics associated with tannins and lignin. However, after HCl hydrolysis, the profile became simpler (see Fig. 3b) indicating that those unknown compounds were conjugates of aromatic metabolites. Acid hydrolysis of the urine samples prior to HPLC separation is therefore required in order to estimate the total (free and conjugated) content of aromatic metabolites present in the urine.

In conclusion, the chromatographic procedure provides a rapid and accurate method for the separation and quantification of BA, SA and PA, and their glycine conjugates, and of 3PPA, PC and CA. By treating the sample in 3 *M* HCl at 100°C for 24 h followed by HPLC analysis, an estimate of the total contents (free and conjugated) of the aromatic metabolites can be achieved. The method is useful for the studies of the profile and the quantities of aromatic metabolites in the urine of ruminants that reflect the type and quantity of feed animals receive.

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