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DETERMINATION OF MIMOSINE AND 3-HYDROXY-4(1*H*)-PYRIDONE IN *LEUCAENA*, AVIAN EXCRETA AND SERUM USING REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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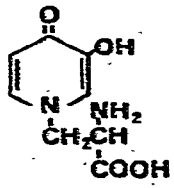
SUMMARY

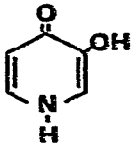
The estimation of mimosine and 3-hydroxy-4(1*H*)-pyridone in *Leucaena leucocephala*, *Leucaena* seeds, chick excreta and chick serum using reversed-phase ion-pair high-performance liquid chromatography was investigated. Isocratic elution of both compounds was achieved in 11 min using sodium octyl sulphate as the pairing agent in a pH 2.25 buffer. Good recoveries of both mimosine and 3-hydroxy-4(1*H*)-pyridone in all but serum samples were obtained.

INTRODUCTION

The legume *Leucaena leucocephala* is grown extensively in semi-arid tropical and sub-tropical areas of the world, and its wide variety of uses have been thoroughly discussed[1–4]. Its use as a protein source (224–344 g kg^{-1} on a dry matter basis)[5,6] for animals is limited because of a number of factors, one of the major constraints being its relatively high content (10–100 g kg^{-1} of dry matter)[6,7] of the unusual and toxic amino acid mimosine ((*S*)-*β*-[N-(3-hydroxy-4-pyridone)]-*α*-aminopropanoic acid; I). It is well documented that mimosine is a depilatory agent, and studies on the use of mimosine and its analogues as defleecing agents have been reported in detail[8–10]. The large variety of biochemical, biological and nutritional effects of mimosine, including inhibition of protein synthesis, is also well documented[10–15,22,23].



I



II

The major hydrolysis product of mimosine, 3-hydroxy-4(1*H*)-pyridone (DHP; II), has been found in dried *Leucaena*. It has also been found in ruminant and non-

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ruminant excreta when these animals have been fed diets containing *Leucaena* or mimosine[9,16–18]. DHP has been reported to be goitrogenic, a potent inhibitor of some enzymes[19,20] and a weak inhibitor of thymidine incorporation into mouse bone marrow cells *in vitro*[21].

The methods commonly used for the analysis of mimosine and/or DHP in *Leucaena* include colorimetry[24,25], paper chromatography[16], thin-layer chromatography[26], gas–liquid chromatography[27], ion-exchange chromatography (IEC)[28] and electrophoresis[29]. The use of IEC for the estimation of mimosine in ovine blood has also been reported[9,30]. All of these methods suffer from a variety of disadvantages, not least their inability to estimate mimosine and DHP simultaneously, rapidly, and in the case of colorimetry, specifically.

The use of high-performance liquid chromatography (HPLC) for the separation and estimation of amino acids from various sources is becoming popular[31]. Recently, however, the use of reversed-phase ion-pair HPLC (RP-IP-HPLC) for the separation and quantification of cation (and anion) forming compounds[32–34] has tended to predominate. A logical step in the estimation of both mimosine and DHP would, therefore, appear to be the use of RP-IP-HPLC. Two brief reports on the use of HPLC for the estimation of mimosine and DHP in *L. leucocephala*, ruminant urine, and chick excreta have appeared recently[35,36]. One of these methods involved the use of phosphoric acid as the ion-pairing agent[35], while the other utilised the ion-pairing effects of sodium octyl sulphate[36].

We report here the detailed methodology of RP-IP-HPLC, using the octyl sulphate anion as the pairing ion, for the estimation of mimosine and DHP simultaneously in *Leucaena* leaf meal (LLM), *Leucaena* seeds, chick excreta and serum.

EXPERIMENTAL

Sample preparation

LLM, Leucaena seeds and excreta. Finely ground samples of LLM, *Leucaena* seeds and freeze-dried excreta (or *ca.* 20 g of a homogenous mixture of fresh excreta) were prepared as described previously[28]. An aliquot of the resultant extract was forced through a Sep-Pak C_{18} cartridge (Waters Assoc., Stockport, Great Britain) to remove or reduce contaminants. Washing the Sep-Pak cartridges with 0.1 *M* HCl (2 × 5 cm^3), methanol (2 × 5 cm^3), distilled water (2 × 5 cm^3) followed by 2–3 cm^3 of extract, prior to collection of the eluate from the Sep-Pak produced a cleaner solution for chromatography. This procedure also allowed re-use of the cartridges.

Serum. Whole chick blood was allowed to stand overnight at 4°C and was then centrifuged (2500*g*) for 8 min. The resultant serum was decanted off and stored at −20°C until required for analysis. Protein precipitation in the serum was accomplished using two precipitants: sulphosalicylic acid (SSA; 8 g in 100 cm^3 of distilled water) and phosphotungstic acid (PTA; $H_3PO_4 \cdot 12WO_3 \cdot xH_2O$; 6 g in 100 cm^3 of distilled water). (Both precipitants were obtained from BDH, Poole, Great Britain). Precipitation of protein was also attempted using both saturated $(NH_4)_2SO_4$ and ethanol, but these proved to be unsatisfactory because addition of SSA to the supernatant precipitated further amounts of protein. Precipitation of serum proteins using SSA and PTA was achieved by adding 0.25 cm^3 of either of the precipitants to 1 cm^3 of serum. The resultant mixture was then centrifuged (3000 g) for 5 min, the super-

natant was decanted off and subsequently forced through a C_{18} Sep-Pak cartridge, pretreated as described above, prior to chromatographic analysis. The Sep-Pak cartridges were then discarded.

Preparation of standards

Stock standard solutions of mimosine (0.25 m*M*) and DHP (0.5 m*M*) were prepared by dissolving the dried, crystalline materials in 0.1 *M* HCl. More dilute standards were prepared by dilution of appropriate volumes of the stock standards with 0.1 *M* HCl. Standard solutions with concentrations in the expected working ranges of 6.25 μM to 0.25 m*M* with respect to mimosine and 12.5 μM to 0.25 m*M* with respect to DHP were prepared. Mimosine (Sigma, Poole, Great Britain) was dried to constant weight in an oven prior to dissolution. DHP was prepared by a modification of the method of Hegarty *et al.*[16]. Mimosine (2 g) was refluxed in 0.1 *M* HCl (200 cm^3) for 24 h, and the resultant DHP separated from other impurities as described by Hegarty *et al.* DHP, however, could not be eluted from the ion-exchange resin using 0.1 *M* HCl (2.5 dm^3). Elution of DHP was accomplished by washing the resin with 1 *M* HCl (700 cm^3). Other preparative details are as described by Hegarty *et al.*[16], although vacuum sublimation was not performed. Repeated recrystallisation of DHP from ethanol followed by washing with diethyl ether yielded buff-coloured crystals (m.p. 240–243 dec; lit. 242–244 dec.[16,37]). Infrared spectroscopy produced a spectrum which was consistent with that expected for DHP, while chromatographic analysis did not reveal any impurities in the prepared DHP. The UV spectrum showed a λ_{max} of 269 nm and an extinction coefficient, in 0.1 *M* HCl, of 516 $m^2\ mol^{-1}$ at 269 nm.

Recoveries

Recovery of mimosine from LLM, estimated using IEC, has been previously reported[28]. Recovery of mimosine, added to excreta prior to extraction, also estimated using IEC, has been found to be 102.7% (± 3.0) (previously unreported results). A comparison of results obtained using IEC with those obtained using HPLC was regarded as yielding sufficient information on recovery of mimosine from excreta, LLM and *Leucaena* seeds. Recovery of DHP was measured by determining the DHP content of LLM and then adding crystalline DHP to the LLM at two levels. Recovery of DHP from excreta was ascertained by adding crystalline DHP to a freeze-dried DHP-free excreta sample. Extraction and subsequent preparation of the sample was as described in the Sample Preparation section of this report. An aliquot of a standard DHP solution was also added to the prepared LLM extract to measure chromatographic recovery, and give an indication of interference caused by any other components in the extract.

Recovery of both mimosine and DHP from chick serum was determined by the addition of 1 cm^3 of a standard solution (0.125 m*M* mimosine and 0.25 m*M* DHP) to 1 cm^3 of serum. Precipitation of protein was accomplished by the addition of 0.5 cm^3 of either PTA or SSA. Further treatment was as described earlier in this report.

Chromatography

An Altex liquid chromatography system (Scotlab Instrument Sales, Lanark, Great Britain) consisting of an Altex Model 110A pump, a Rheodyne 7120 injection valve with 20-μl loop, and an Altex-Hitachi Model 100-10 variable wavelength detec-

tor was used. An Altex column (25 × 0.46 cm I.D.) packed with LiChrosorb RP-18 ($d_p \approx 10$ μm) was used for all chromatographic separations reported here. The column was packed in this laboratory using a Magnus P6050 column packer (Magnus Scientific, Cheshire, Great Britain). Column efficiency, determined using naphthalene eluted isocratically with aqueous methanol (70 cm^3 CH_3OH made to 100 cm^3 with distilled water) at a flow-rate of 1 cm^3 min^{-1}, was 4321 theoretical plates, (*i.e.* $N = 4321$). The buffer used for chromatography was prepared by mixing 200 cm^3 of 0.01 *M* sodium octyl sulphate in 2% v/v HPLC grade methanol (crystalline sodium octyl sulphate was obtained from Kodak, Liverpool, Great Britain and CH_3OH was obtained from Rathburn Chemicals, Peeblesshire, Great Britain) with 240 cm^3 of HPLC grade methanol. Analytical grade sodium nitrate (5.1 g; BDH) was added and the mixture made up to 2 dm^3 with "in glass" double distilled water. The buffer was filtered through a Whatman GF/F glass fibre filter under reduced pressure, and the pH adjusted to 2.25 with HNO_3 (≈ 7.9 *M*). It was then degassed ultrasonically prior to use.

The volume of extracts and standards loaded was restricted to 20 μl.

The methodology used for ion-exchange chromatographic analyses of samples has been previously reported[28].

RESULTS AND DISCUSSION

The excellent linear response of the system to both mimosine and DHP within their respective, expected working concentrations is shown in Fig. 1. Standards with concentrations ranging from 6.25 μ*M* to 0.25 m*M* for mimosine, and from 12.5 μ*M* to 0.25 m*M* for DHP were analysed in triplicate, and peak area was plotted against concentration. Correlation coefficients and standard errors for mimosine and DHP standards, measured at their λ_{max} wavelengths of 278 and 269 nm, respectively, and at different sensitivities, indicate an excellent linear response and precision of analysis for loaded amounts of mimosine from 0.125 nmol to 5 nmol. Loaded amounts from 0.25 nmol to 5 nmol of DHP produced similarly good results.

Typical chromatograms of a standard mixture of mimosine and DHP, those of a 6 *M* HCl extract of LLM and two deproteinised serum samples are shown in Fig. 2. Near-baseline resolution of mimosine and DHP was obtained. The mean resolution (R_S) obtained from nine samples was 1.62 (± 0.106). The relative standard deviation (R.S.D.; 6.54%) is fairly large presumably because the nine samples consisted of serum, LLM, excreta and standards analysed on different days. The resolution was calculated as follows: $R_S = 2\,(t_{RDHP} - t_{R\ mimosine})/(W_{DHP} + W_{mimosine})$ where $t_{R\ DHP}$ and $t_{R\ mimosine}$ are retention times for DHP and mimosine, respectively, and W_{DHP} and $W_{mimosine}$ are base widths of the DHP and mimosine peaks, respectively. The phase capacity ratios (k') of mimosine and DHP were 3.63 (± 0.33) and 5.56 (± 0.493), respectively. The k' values were calculated from the same nine chromatograms used to calculate the resolution.

A small shoulder appeared at the base of the mimosine peak in some excreta extracts, but had little or no effect on the estimation of recovery of mimosine or DHP (Tables I and II). Mimosine or DHP was not detected in the serum of chicks fed a diet containing LLM. An interesting feature of the chromatograms of serum (Figs. 2c and 2d), however, was the concentration of compounds which eluted prior to

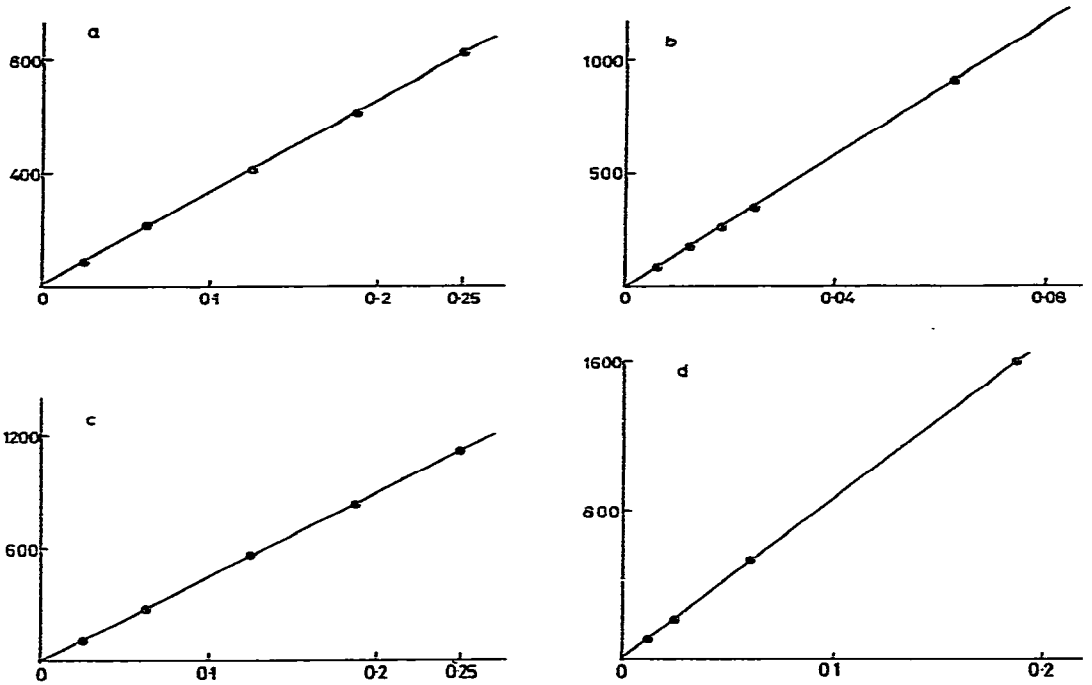

Fig. 1. HPLC response curves for mimosine (a, b) and 3-hydroxy-4(1*H*)-pyridone (c, d) at 278 and 269 nm, respectively. Concentration (m*M*) plotted on abscissa, peak area (mm^2) on ordinate. (a) 0.05 a.u.f.s.: $y = 3271.0x + 6.374$; standard error (S.E.) = 10.3; correlation coeff. (R) = 0.999. (b) 0.01 a.u.f.s.; $y = 14{,}738x - 15.208$; S.E. = 11.2; $R = 0.999$. (c) 0.02 a.u.f.s.: $y = 4486.6x - 0.77$; S.E. = 19.3; $R = 0.999$. (d) 0.01 a.u.f.s.: $y = 8513.5x + 3.57$; S.E. = 10.0; $R = 1.000$.

mimosine. Serum from chicks fed LLM had a higher concentration of these components than had serum from chicks fed LLM-free diets. The identities and significance of these compounds are not known but further study is warranted.

Mimosine values obtained in samples of LLM, chick excreta and *Leucaena* seeds, using IEC, agree well with those obtained using HPLC (correlation coefficient = 1.000) although slightly lower values were obtained for the two excreta samples when determined using HPLC. The percentage R.S.D. values for the seven samples vary somewhat, becoming fairly large when different extracts of the sample were analysed. The R.S.D. values for replicate analyses of the same extract, however, are fairly low even when mimosine was estimated at two different wavelengths. All R.S.D. values are within the limits expected for this type of analysis[38]. The precision, and good agreement with IEC values, obtained for the mimosine content of the LLM sample, analysed at 278 and 269 nm, indicate that no interfering compounds eluted simultaneously with mimosine.

The recovery of DHP (Table II), when added in crystalline form to LLM and excreta, and in solution to extracts of LLM, averaged 98.9%. The excellent recovery of DHP when added to extracts of LLM indicates that no interference from other compounds in the extract had occurred. Losses during clean-up of the sample were also negligible. Recovery of DHP, added in the crystalline form to LLM prior to extraction (Table II), is slightly low being least for the lowest level of addition. The

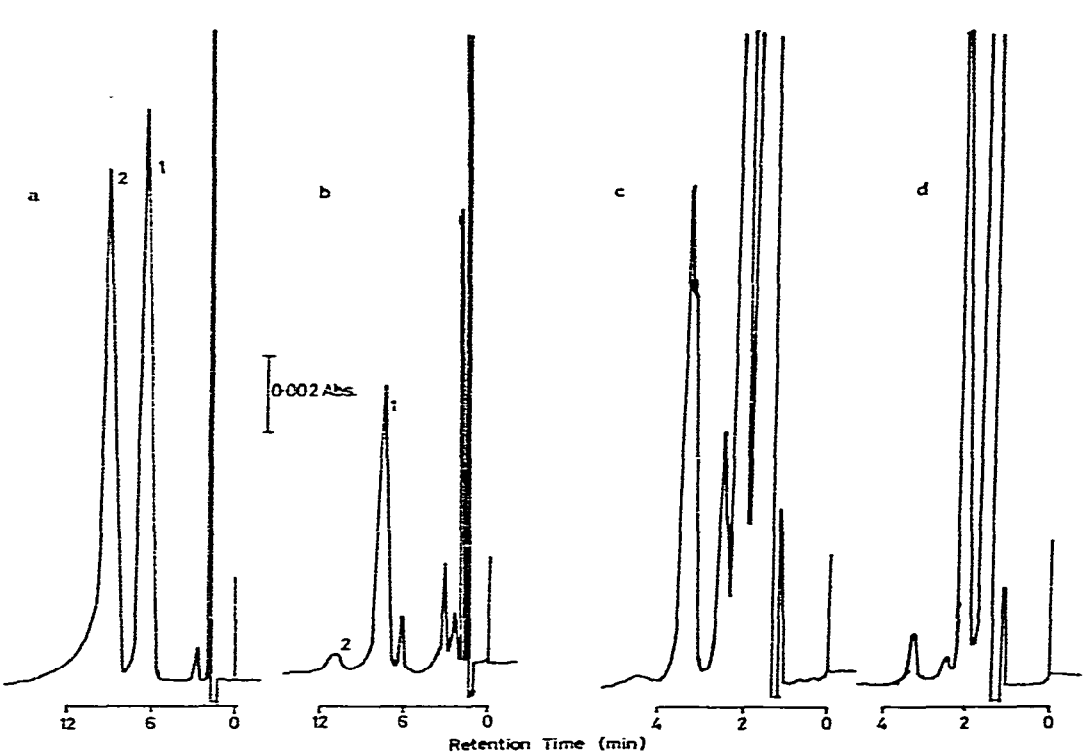

Fig. 2. Typical chromatograms of (a) a standard solution of mimosine and 3-hydroxy-4(1*H*)-pyridone; (b) a *Leucaena* leaf meal (LLM) extract; and serum samples from chicks fed (c) an LLM diet and (d) a diet without LLM. Peaks: 1 = mimosine; 2 = DHP. Flow-rate of pH 2.25 buffer, 1.8 cm^3 min^{-1}; detector 269 nm and 0.02 a.u.f.s.

TABLE I

MIMOSINE CONTENT OF *LEUCAENA LEUCOCEPHALA*, *LEUCAENA* SEEDS AND EXCRETA DETERMINED USING IEC AND HPLC

Sample	*Mimosine concentration (g kg^{-1} dry matter)*		*Percentage R.S.D.*	*HPLC / IEC*
	IEC	*HPLC (± S.D.)*		
Leucaena leaf meal (sun-dried, pelleted) "Peru" cultivar *ex* Malawi 1979	24.27	24.73 (±0.692)*	2.80	1.019
Leucaena leaf meal (sun-dried, unpelleted) "Peru" cultivar *ex* Malawi 1977	10.26	10.42 (±0.260)**	2.50	1.016
Leucaena leaf meal (sun-dried, unpelleted) "Peru" cultivar *ex* Malawi 1979	23.28	23.76 (±0.254)***	1.07	1.021
Leucaena seeds *ex* Mexico 1981	67.54	67.95 (±0.350)§	0.52	1.006
Leucaena seeds *ex* Mexico 1981	73.19	73.93 (±0.052)§	0.07	1.010
Chick excreta (from chicks fed *Leucaena* diets)	3.63	3.43 (±0.027)§	0.79	0.945
Chick excreta (from chicks fed *Leucaena* diets)	1.11	1.01 (±0.007)§	0.69	0.910

* Mean of duplicate analyses of three samples.
** Mean of duplicate analyses of six samples.
*** Mean of four analyses of two samples (two at 278 nm and two at 269 nm).
§ Mean of triplicate analyses of one sample.

TABLE II

RECOVERY OF 3-HYDROXY-4(1*H*)-PYRIDONE (DHP) FROM *LEUCAENA* LEAF MEAL (LLM) AND CHICK EXCRETA (MEASURED AT 269 nm) USING HPLC

Sample	*DHP content by analysis (g kg^{-1} dry matter)*	*Expected DHP content (g kg^{-1} dry matter)*	*Percentage recovery (± S.D.)*	*Percentage R.S.D.*
LLM (sun-dried, unpelleted)* "Peru" cultivar *ex* Malawi 1977 + DHP	3.084	3.069	100.5 (± 2.6)	2.59
LLM (sun-dried, unpelleted)** "Peru" cultivar *ex* Malawi 1977 + DHP	8.484	9.024	98.1 (± 5.4)	5.49
LLM (sun-dried, unpelleted)** "Peru" cultivar *ex* Malawi 1977 + DHP	5.475	5.912	92.6 (± 5.2)	5.64
Excreta (obtained from chicks fed on LLM-free diet)** + DHP	5.197	4.974	104.5 (± 1.3)	1.28

* DHP solution was added to three LLM extracts prior to analysis.

** Crystalline DHP was added to powdered sample prior to extraction. Three samples were taken for extraction of DHP.

R.S.D. values for recovery of DHP from LLM are fairly high, averaging 5.57%. Recovery of DHP from excreta, however, is slightly higher than expected although precision of analysis is good.

Recovery of mimosine and DHP from chick serum (Table III) shows that substantial losses of both mimosine and DHP occur during sample preparation. This is a problem which has been reported[39,41] for analysis of compounds in blood and was not unexpected. Recovery of mimosine was highest when SSA was used as the precipitant with serum levels of mimosine and DHP of 62.5 and 125 nmol cm^{-3}, respectively. Precision of analysis was fairly low however. The use of SSA as the precipitant yielded only *ca.* 58% recovery of DHP at a concentration of 125 nmol cm^{-3}, although precision was fairly good. It was observed that when SSA was used as the precipitant, peak broadening and eventually splitting occurred after the analysis of twelve samples. This condition remained even when standard solutions were subsequently loaded. Removal and replacement of the top 1–2 mm of column packing resolved the problem, indicating that perhaps some proteinaceous material had been adsorbed on to the top of the column. Another possible cause may have been due to adsorption of some SSA on to the column. This possibility, although remote because of the hydrophilic groups on the benzene ring of SSA causing lack of retention, would appear to be confirmed by the fact that peak splitting slowly decreased as the number of injections of a standard solution of mimosine and DHP increased. PTA was used as the preferred precipitant since peak broadening or splitting did not accompany its use. As can be seen (Table III) a reversal in recovery is produced when PTA was used to precipitate the protein from serum containing the same concentrations of mimosine and DHP as those used for SSA precipitation. Precipitation of protein from serum samples containing half the concentration of mimosine and DHP produced an increase in recovery of mimosine but a slight reduction in recovery of DHP. R.S.D. values for the recovery of DHP are fairly consistent but are quite high and variable for mimosine. The combined molar recoveries of both mimosine and DHP are almost identical for both SSA- and PTA-treated serum, although the values obtained for the

TABLE III

RECOVERY OF MIMOSINE AND 3-HYDROXY-4(1*H*)-PYRIDONE (DHP) FROM CHICK SERUM USING HPLC

Serum sample	*Mimosine concentration (nmol cm^{-3})*	*Mimosine recovery (%) (±S.D.)*	*Percentage R.S.D.*	*DHP concentration (nmol cm^{-3})*	*DHP recovery (%) (±S.D.)*	*Percentage R.S.D.*	*Combined recovery (%) of DHP and mimosine*
A*	62.5	81.3 (± 5.5)	6.77	125.0	57.7 (± 1.6)	2.77	69.5
B**	62.5	54.9 (± 1.7)	3.10	125.0	81.8 (± 2.1)	2.57	68.4
C**	31.25	71.1 (± 4.5)	6.33	62.5	75.5 (± 1.2)	1.59	73.3

* Values are the means of triplicate analyses of three samples; sulphosalicylic acid used as protein precipitant.
** Values are the means of duplicate analyses of three samples; phosphotungstic acid used as protein precipitant.

lower concentrations of mimosine and DHP is slightly higher than those for the higher concentrations.

Ion-pairing of mimosine and DHP with the precipitants could have been partially responsible for the low recoveries. If ion-pairing had occurred then it seemed likely that some mimosine and DHP would be eluted simultaneously with either SSA or PTA, both of which were retained only slightly. This premise was tested by adding SSA or PTA to a standard solution of mimosine and DHP (0.125 m*M* and 0.25 m*M*, respectively, in 0.1 *M* HCl) in the same ratio as for precipitation of the protein from serum (*i.e.* 2 cm^3 of standard plus 0.5 cm^3 of precipitant). Analysis of these sep-pak pretreated standards confirmed that losses occurred, possibly by ion-pairing. Recoveries of 85.7% (± 0.7) and 87.9% (±1.9) for mimosine and DHP, respectively, were obtained from triplicate analysis of an SSA treated standard. Analysis of a PTA-treated standard showed that recoveries of 78.1% (± 0.4) and 85.6% (± 0.6) were obtained for mimosine and DHP, respectively. It would appear, therefore, that precipitation of protein from serum using either SSA or PTA causes loss of mimosine by at least two mechanisms: adsorption of mimosine and DHP to the protein, and non-retention of both during chromatography due to ion-pairing with the precipitant. No loss of mimosine or DHP was detected when standard solutions (without added precipitant) were treated with Sep-Pak cartridges prior to analysis. The re-use of Sep-Pak cartridges for LLM and excreta extracts produced cleaner samples than those which had been passed through unused cartridges, without loss of mimosine or DHP.

Recovery of 100.3% (± 1.6) has been reported for mimosine added to ovine plasma (in the range 0.5–1.25 μmol cm^{-3}) when analysed using IEC[30]. It appears, however, that this recovery was obtained from samples with mimosine added after protein precipitation with SSA. Losses during protein precipitation would therefore not have been accounted for. We have not yet been able to detect mimosine or DHP in the serum of chicks fed LLM, although levels of up to 0.4 μmol of mimosine cm^{-3} of ovine plasma have been reported for sheep fed diets containing mimosine[9]. Our inability to detect mimosine or DHP in chick serum may be due to a variety of factors, not least of which may be that mimosine or DHP do not enter the blood system. This possibility is likely since it has been reported that a high proportion of mimosine ingested by the chick is excreted[22]. It is also possible that mimosine and DHP, if present in the blood, is bound/adsorbed to protein and thus lost during serum preparation. Different sample preparation techniques, such as hydrolysis of whole blood or ultra-filtration, may yield information on mimosine and DHP in chick blood. Another possibility is that the "known addition" technique could be used to estimate mimosine and DHP in blood and, perhaps, urine. Further work is continuing on this aspect of sample preparation.

A recent report has shown that mimosine and DHP have been separated in 0.1 *M* HCl extracts of *leucaena* and in ruminant urine using RP-IP-HPLC[35]. The authors reported that, when using orthophosphoric acid as the ion-pairing agent, resolution of DHP and DHP-glycoside was not possible. They subsequently resorted to hydrolysis of the urine to convert the DHP-glycoside to DHP. Retention times for mimosine and DHP were of a similar order to those obtained by us although we used a higher effluent flow-rate. No recoveries for DHP or mimosine were given although negligible levels of mimosine were detected in urine. The very low mimosine levels, compared to DHP, appear to be in direct contrast to work with chicks[22,40] (unpub-

lished data) and sheep[9] which has shown that substantial amounts of mimosine and DHP were excreted.

A total of *ca.* 900 samples consisting of LLM, excreta and serum have been analysed in this laboratory, using the same column, since the work presented here was carried out. During these analyses the top 2–3 mm of column packing has been changed five times and, on two occasions, the column was washed with aqueous methanol (70% v/v). The column efficiencies before (901 $\pm$ 25; $n = 4$) and after (996 $\pm$ 34; $n = 4$) the analyses of the 900 samples were almost identical. On both occasions efficiency was determined on two separate days, using the mimosine peak. The long column life indicates that sample preparation was good.

The current study shows that the use of RP-IP-HPLC, using sodium octyl sulphate as the ion-pairing agent, provides a rapid, accurate and precise technique for the estimation of mimosine and DHP in LLM and excreta with minimal sample preparation, using a column of medium efficiency ($N \approx 4000$ measured using naphthalene). Although analysis of both mimosine and DHP in serum by this method gives low recoveries and variable precision, it seems feasible that the technique can be developed with further study and used for the estimation of mimosine, DHP, other metabolites and related analogues in ruminant and non-ruminant blood.

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