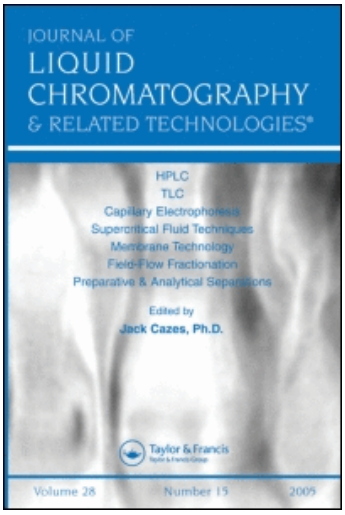

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENOLIC ACIDS ISOLATED FROM PLANTS

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ABSTRACT

A simple and rapid method is described for the estimation by high-performance liquid chromatography of *cis* and *trans* isomers of the substituted cinnamic acids, p-coumaric (p-CA) and ferulic (FA), using p-anisic acid (p-AA) as internal standard. Chromatographic separation and quantification were performed on a reversed-phase Nova-Pak C_{18} column with isocratic elution water-n-butanol-acetic acid (98:1.5:0.5). A flow-rate of 1.5 ml/min, a column temperature of 35°C and detection at 270 nm were employed. As little as 175ng/ml for p-CA and 63 ng/ml for FA can be estimated by this procedure with a 20 μl injection. Application of this method are illustrated by estimation of these acids in extracts, prepared with 1 M sodium hydroxide of barley straw, mixed grass hay and alfalfa hay.

INTRODUCTION

The substituted cinnamic acids p-coumaric and ferulic are widely distributed in plants. These acids crosslink lignin to structural

carbohydrates of plant cell walls [1] and lignin is known to depress cell wall digestibility presumably by reducing access to the structural carbohydrates by anaerobic bacteria. Because ruminants consume vast quantities of forage and thus depend on microbial fermentation of cell walls for energy [2-3], the analysis of phenolic monomers content of forages may allow to increase the appreciation of the effects of these acids on animal nutrition.

Several workers have proposed methods for the determination of phenolic acids. Some procedures are based on the separation of the trimethylsilylether (TMS) derivatives of the substituted cinnamic acids by GLC [4-7]. Other authors have developed procedures for the determination of these acids in biological samples, plants and soil by HPLC [8-10]. In this work we describe a simple and rapid RP-HPLC method for the simultaneous determination of *cis* and *trans* isomers of p-coumaric and ferulic acids in forages for the study of ruminant metabolism.

EXPERIMENTAL

Chemicals and Reagents

p-*trans*-CA, *trans*-FA and p-AA were obtained from Fluka Chemie (Buchs, Switzerland), n-butanol of HPLC grade from Carlo Erba (Milan, Italy). Water was previously distilled and purified with a Milli-Q system purchased from Millipore (Bedford, MA, USA). Other chemicals were of the highest purity commercialy available.

Equipment

HPLC analyses were performed with a Waters Model 600E system equiped with a loop injector Waters U6K, a Waters Model 484 UV-detector and a Waters Model 745B integrator.

Standard Solutions

Stock solutions of p-*trans*-CA, *trans*-FA and p-AA (1 mg/ml) were prepared in methanol and kept at 4°C up to one month. All manipulations of *trans*-phenolic acids were carried out in "white" fluorescent light to prevent isomerization.

Calibration graphs were constructed and were linear over the concentration range investigated, 5 to 200 μg/ml.

The *cis*-isomers were prepared by leaving the *trans*-isomers solutions exposed to light.

The quantification were achieved by regression analysis of the peak areas of each compound against concentration .Triplicate injection were made. For calculations it is assumed that the *cis*-isomers have the same response factor as the *trans*-isomers.

Sample preparation

All forage samples were dried at 35°C for 48 h and ground through a 1mm screen. 100 mg of sample were accurately weighed and 5 ml 1 M NaOH were added to completely soak the sample. The mixture was shaken under nitrogen, leaved at room temperature overnight in the dark and filtered (No 1 porosity glass sinter). 0.5 ml of p-AA solution (1 mg/ml in methanol), the internal standard, were added and the solution was acidified with 6 M hydrochloric acid to pH < 1. Solution was extracted with ethyl acetate (3 x 5 ml). The organic phase was then separated and dried in a rotary evaporator from Büchi (Flawil, Switzerland). The residue was dissolved in 5 ml of mobile phase. The solution was filtred through a 0.45 μm filter and 20 μl were injected on to the HPLC column.

Chromatographic Conditions

RP-HPLC separations were carried out on a Nova-Pak C_{18} column (150 mm x 3.9 I.D.; 4-μm particles) (Waters). The mobile phase was

water-n-butanol-acetic acid (98:1.5:0.5). The flow rate and column temperature were 1.5 ml/min and 35°C respectively. The detection wavelength was set at 270 nm.

RESULTS AND DISCUSSION

Method Performance

The nature and the percentage of organic modifier of the mobile phase, and column temperature were studied to determine their influence on system selectivity thus stablishing the best conditions for separation.

The solvents studied were methanol, ethanol, iso-propanol and n-butanol. Table 1 shows the variations of the retention time with the organic solvent.

On the basis of these results we decided to select n-butanol as the organic modifier. As Fig.1A shows the retention time of species in the chromatographic system decreased when the n-butanol content increased, as expected.

The effects of column temperature (25-50°C) on the capacity factors are shown in the Fig. 1B. In these experiments the n-butanol content of the mobile phase was kept constant and equal to 1.2% .

The results of this study allow us to select for the determination of phenolic acids the conditions specified under Experimental.

Chromatograms of standards and the barley straw sample are shown in Fig.2. Under these chromatographic conditions there were no other endogenous forage components that can interfere with the peaks of the analytes.

The retention times were ca. 4.5 min for p-*trans*-CA and 5.4 min for *trans*-FA. The retention time of I.S was ca. 12.0 min. The retention times of *cis*-CA and *cis*-FA were ca. 5.1 min and 6.8 min respectively.

TABLE 1

Effect of the Organic Modifier on the Retention Time

Organic Solvent	Retention Time (min) P-CA	FA
methanol	50.8	93.2
ethanol	36.7	54.9
iso-propanol	21.2	35.6
n-butanol	6.5	8.1

* Organic modifier porcentage = 1.2%

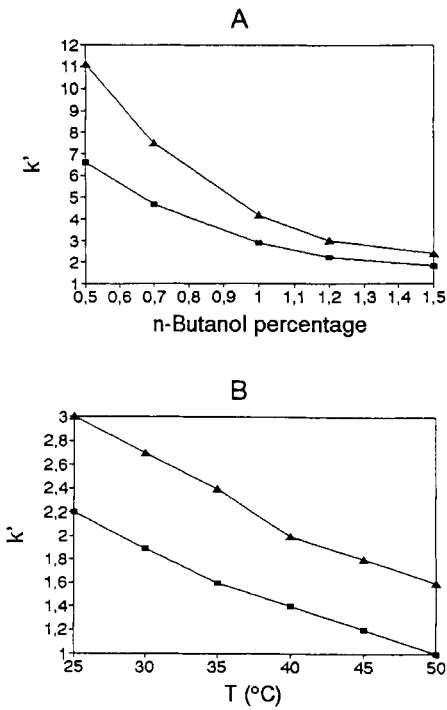

FIGURE 1. Optimization of the analitical conditions. (A) Relation between capacity factors (k') and n-butanol concentration, column temperature 35°C . (B) Effect of column temperature on k' values. Mobile phase contained 1.2% of n-butanol.
Symbols. ▲ = p-*trans*-CA; ■ = *trans*-FA.

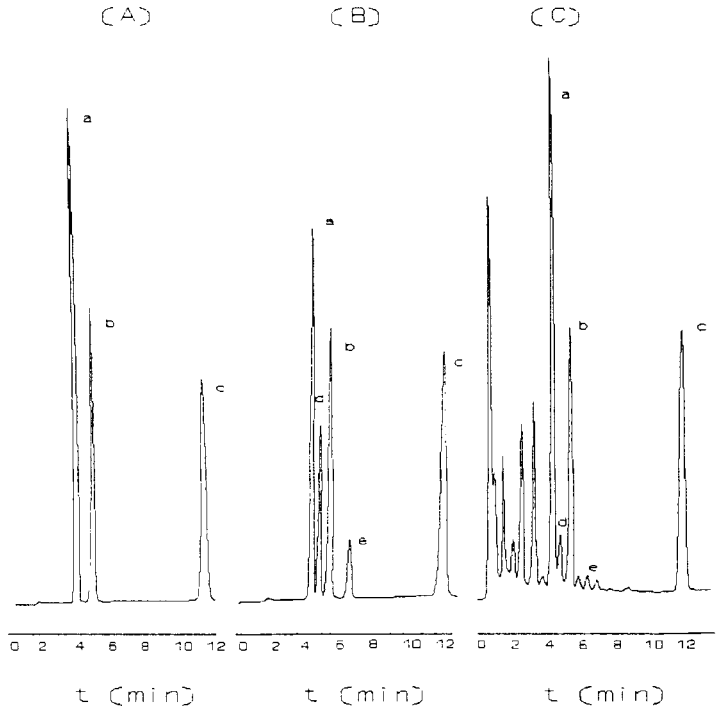

FIGURE 2. Chromatographic separation. (A)-Standard solution of p-*trans*-CA (a) and *trans*-FA (b). (B)- Standard solution of p-*trans*-CA (a), *trans*-FA (b), p-*cis*-CA (d) and *cis*-FA (e). (C) barley straw sample. I.S. was p-AA (c).

Linearity

Linearity was checked by measuring different concentrations in the range (5-200)μg/ml for p-*trans*-CA and *trans*-FA. In all cases the concentration of internal standard was 100 μg/ml. Linear relationships between the peak areas and the concentrations tested were found. The average slopes and y-intercepts of the calibration curve equations are shown in Table 2.

TABLE 2

Calibration Curves and Standard Addition Method.

Calibration Curves

Acid	Linear regression equation	R^2	N	Concentration Range (μg/ml)
CA	$y = 6.81\ 10^{-3}\ x + 3.35\ 10^{-2}$	0.9992	8	5-200
FA	$y = 4.69\ 10^{-3}\ x - 1.64\ 10^{-2}$	0.9995	8	5-200

Standard Addition Curves

Acid	Linear regression equation	R^2	N	Conc. Added Range(μg/ml)
CA	$y = 6.65\ 10^{-3}\ x + 3.14\ 10^{-1}$	0.9966	5	2.5-20
FA	$y = 4.75\ 10^{-3}\ x + 1.53\ 10^{-1}$	0.9999	5	2.5-20

N = number of concentrations

The standard addition method was used to check for chemical interferences in the quantitation of different acids. The equations calculated are shown in Table 2.

The slopes found of the calibration and standard addition curves were similar for each compound. Statistical analysis by the t-test showed that the slope values are not statistically significant ($t = 0.033$, $p = 0.97$ for p-CA and $t = 0.0027$, $p = 0.98$ for FA).

Analytical Recovery, Precision and Accuracy

Analytical recovery was evaluated by assaying forage samples spiked with different amounts of each acid ranging from (2.5-20 μg/ml)

TABLE 3

Inter-day Precision and Accuracy

Acid	Conc.Added (μg/ml)	Conc.Found (μg/ml;mean ± SD; n = 5)	CV(%)	RE(%)
CA	2.5	2.56 ± 0.10	3.9	2.4
	5	5.16 ± 0.08	1.6	3.2
	10	10.17 ± 0.15	1.5	1.7
	15	15.07 ± 0.07	0.4	0.5
	20	20.38 ± 0.12	0.6	1.9
FA	2.5	2.42 ± 0.02	0.8	3.2
	5	4.86 ± 0.11	2.3	2.8
	10	9.79 ± 0.16	1.6	2.1
	15	14.76 ± 0.08	0.5	1.6
	20	19.68 ± 0.13	0.7	1.6

for p-CA and FA. Replicate analyses (n = 5) at each concentration were made. The mean recoveries for p-CA and FA were: 101.96 ± 1.02 (CV = 1.0%) and 97.79 ± 0.67 (CV = 0.7%) in barley straw, 98.35 ± 1.12 (CV = 1.1%) and 96.87 ± 2.02 (CV = 2.1%) in alfalfa hay and 102.13 ± 2.12 (CV = 2.1%) and 97.76 ± 1.88 (CV = 1.9%) in mixed grass hay.

The inter-day precision and accuracy were assessed by analyzing forage samples containing different concentrations of each metabolite, five times per day during one week. The results for barley straw are shown in Table 3. Similar results were obtained for mixed grass hay and alfalfa hay.

The limit of detection was 175 ng/ml for p-CA and 63 ng/ml for FA with a 20 μl injection. The limit of detection was determined from the calibration curves acording to the method described by Miller and Miller [11].

CONCLUSION

An RP-HPLC system has been developed for the determination of *cis* and *trans* isomers of the p-coumaric and ferulic acids. The method is accurate, precise, rapid and easy to perform, and should be applicable to studies of forage digestibility in ruminants.

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