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Journal of Chromatography B, 855 (2007) 276–279
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Determination of ellagic acid in oak leaves and in sheep ruminal fluid by ion-pair RP-HPLC

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Received 29 January 2007; accepted 26 April 2007
Available online 10 May 2007

Abstract

An isocratic ion-pair high-performance liquid chromatography (IP-RP-HPLC) method with UV detection was developed to identify and quantify ellagic acid (EA). This phenolic compound is widely distributed in the plants and is often present in the diet of ruminants. The method was validated and validation parameters were: linearity range 5–100 mg/L; correlation coefficient, 0.9995; mean recoveries (99.94 and 101.07%) and detection limit 1.4 mg/L. Method was applied for the determination of ellagic acid in oak leaves and in ruminal fluid from to a vitro ruminal system. The proposed method proved to be rapid and accurate and can be successfully used in ruminant nutrition studies.

Keywords: Ellagic acid; Oak leaves; Ruminal fluid; Ion-pair reversed-phase liquid chromatography

1. Introduction

Tannins are poliphenolic compounds that can be bonded to the proteins in a wide pH interval, forming a stable complex that impedes the access of the microbial enzymes of the rumen and avoiding their degradation [1]. Natural hydrolysable tannins (HTs), ellagitannin and gallotannin, occur in wood, bark, leaves and fruits and they show different nutritional, ecological and medicinal effects. The tannin effects are very variable depending on the animal species that consume them. HTs are toxic to ruminants. Tannin toxicity from HTs may occur in animals fed oak (*Quercus* spp.) and several tropical tree legumes (e.g. *Terminalia oblongata* and *Clidema hirta*) [2]. Microbial metabolism and gastric digestion convert HTs into absorbable low molecular weight metabolites. Some of these compounds are toxic. Currently, more attention is focused mainly on intestinal microflora biodegradation of tannins especially ellagitannins which can contribute to the definition of their bioavailability for both, human beings and ruminants [3]. Although ellagitannins are more difficult to be degradated than gallotannins, numerous studies are still in progress to determine their degradation.

Ellagitannins are the primary source of ellagic acid (EA). This compound is a dietary hydroxybenzoic acid, which may occur in the free form in plants. There are apparently conflicting claims for beneficial and toxic effects caused by ellagitannins and EA in various animal species including rodents and ruminants. In order to clarify the studies on the toxicity of this metabolite, a reliable method for determining EA in biological materials needs to be developed. A few numbers of spectrophotometric methods have been presented for the quantification of EA [4]. According to a literature review, RP-HPLC with UV detection is the most widespread chromatographic technique for determinations of this compound in plant-derived foods [5–8] and biological samples [9–11]. In this study we have explored the possibility of using the capacity of the EA of forming a "like paired-ion" with the sulfonic acid to develop an optimized ion-pair RP-HPLC method for the determination of EA in oak leaves and in ruminal fluid, which contributes to the ruminant nutrition studies.

2. Experimental

2.1. Chemicals

Ellagic acid and octanesulfonic acid were purchased from Sigma (St. Louis, MO, USA). Methanol HPLC-grade and the

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doi:10.1016/j.jchromb.2007.04.042

other chemicals were obtained by Merck (Barcelona, Spain). Buffers were prepared using ultrapure water (Milli-RO 15 reagent-grade water system, Millipore, MA, USA).

2.2. Standard solutions

Stock solution of EA (1000 mg/L) was prepared by dissolving EA in a mixture methanol:acetonitrile (1:1). The stock solution was stable for at least two months at −18 °C. This stock solution was diluted with deionized water to prepare working standard solution of 25 mg/L. Each day, a 20 μL aliquot was used to check all conditions of the HPLC procedure. Calibration curve was obtained by the analysis of ten concentrations ranging from 5 to 100 mg/L of EA standard solution. Quantification was achieved by regression analysis of the peak areas against concentration. Triplicate injections of each concentration were made.

2.3. Sample preparation

Oak leaves were collected from northern Spain in May, 2005, when the concentration of tannins is very high. The leaves were frozen and stored at −20 °C and processed according to Makkar [12]. Dried (finely ground) oak leaves (≈200 mg) are taken in a glass beaker and 10 mL of aqueous acetone (70%) are added. The beaker is suspended in an ultrasonic water bath for 20 min at room temperature. The contents are centrifuged for 10 min at 3000 × *g* at 4 °C. The supernatant is collected and keep it on ice (extract A). One millilitre of extract A was evaporated to dryness under vacuum below 40 °C. The concentrate was diluted to 1 mL with water, and then filtered through a 0.45 μm filter before the HPLC injection (20 μL).

Ruminal fluid samples were obtained as follows: oak leaves (≈500 mg), milled to pass a 1 mm screen, were incubated in 125 mL sealed serum bottles at 39 °C with 40 mL phosphate/bicarbonate buffer (pH ≈ 7) and 10 mL of rumen inoculum, obtained from cannulated sheep. Fermentation was ended after 24 h by swirling the bottles on ice. 10 mL of ruminal fluid, in this way obtained, were centrifuged for 30 min at 3000 × *g* at room temperature according to Froidmont [13]. The supernatant was cooled on ice. An aliquot of 2 mL is taken and 1 mL of trichloroacetic acid (15%) was added. This mixture was subjected for centrifugation (4000 × *g*) for 30 min at room temperature. A portion (20 μL) was injected into the HPLC after filtration (45 μm).

2.4. Apparatus and chromatographic conditions

HPLC analyses were performed on a Waters Model (Mildford, MA, USA) 600-E instrument equipped with a Waters Model 717 plus injector and a 484 UV detector. The detection wavelength was set at 360 nm. The column used was a Symmetry C18 (250 nm × 4.6 nm I.D., 5 μm) (Waters, Mildford, MA, USA). Quantification was based on integration of the peak areas using Borwin 1.5 software (JMBS Development).

EA was eluted isocratically at a flow rate of 0.7 mL/min using mobile phase consisting of 10 mM phosphate buffer pH 4 with 3 mM 1-octanesulfonic acid, sodium salt, mixed with methanol (50%, v/v). Before use the mobile phase was vacuum filtered through a 0.45 μm membrane filter (Millipore, MA, USA). The chromatographic experiments were carried out at 40 °C and the sample injection volume was 20 μL. Identification of the compound was performed by means of retention time and UV spectra, as well as by spiking the samples with the standard. The purity of EA was tested by comparison of the peak areas obtained at wavelengths 280 and 360 nm.

3. Results and discussion

3.1. Optimization of chromatographic conditions

For the ion-pair HPLC method development, the main chromatographic conditions to be optimized were: pH, buffer and counter-ion concentration, column temperature, percentage organic modifier and flow rate.

EA is a poliphenolic compound and it has the capacity of forming hydrogen bonds with other molecules for the interaction between the hydrogen of the phenolic acid and the basic center (negative charge) of the molecule. Hence, to low pH EA can form a "like paired-ion" with the sulfonic acids, which would increase its affinity for the stationary phase. Consequently, the pH must be adjusted to values low enough to avoid the deprotonation of EA. In this work we studied the variation on the EA retention time with the pH of the mobile phase ranged from 3.0 to 6.5. The retention time increased when the pH was lowered from 4.5 to 3.0 due to the formation of "ionic-pair." In this work we decided to adopt pH 4.0.

The effect of molarity of the mobile phase on the retention time, tested between 10 and 50 mM, seems to be negligible. A buffer concentration of 10 mM was selected to the subsequent experiments. Increasing the percentage of methanol in the mobile phase allowed a more rapid elution of EA. Similar studies were carried out with acetonitrile as organic modifier. On the basis of these studies we decided to select methanol (50%, v/v) in this determination. The flow rate was maintained in 0.7 mL/min during the analysis.

A study was conducted into the influence of the counter-ion concentration (3–10 mM) on the retention time. We observed a nonlinear relationship between retention time of EA and octanesulfonic concentration. Consequently, a 3.0 mM octanesulfonic acid concentration was selected.

We studied determination of EA between room temperature and 45 °C. The effect on analysis time is irrelevant. We select 40 °C in order to increase solute mass transfer and minimize the pressure drop across the column.

The chromatograms resulting from the injection of pure standards, oak leaves and ruminal fluid samples under the chromatographic conditions finally adopted are presented in Figs. 1 and 2. The retention time for EA was 6.4 min. A high

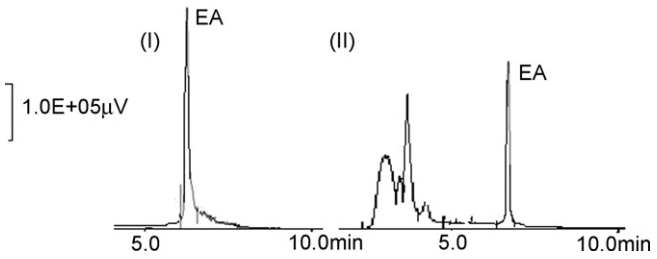

Fig. 1. Chromatograms of (I) EA standard and (II) oak leaves sample.

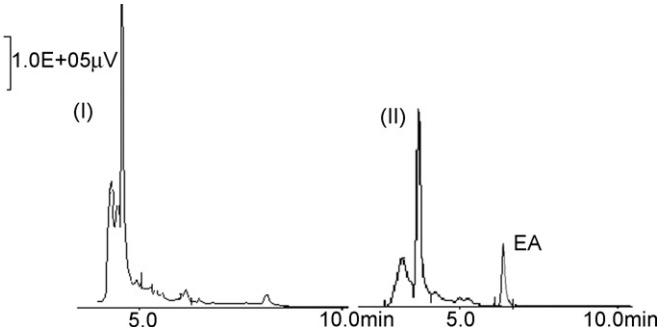

Fig. 2. Chromatograms of (I) blank ruminal fluid and (II) ruminal fluid after administration of oak leaves.

reproducibility in the retention time was obtained with relative standard deviation (RSD) less than 3%.

3.2. Validation

Calibration curve was generated by the analysis of triplicates of ten points in the range from 5 to 100 mg/L of EA standard solutions. The calibration graph showed a good fitting to a linear model between the peak areas and analyte concentrations with the regression coefficient >0.999. The equation calculated was: $y = (0.0549 \pm 0.0002)x - (0.0430 \pm 0.0111)$; SD = 0.0250. The linearity of the calibration graph was also checked with two different statistical tests: linearity and proportionality tests. For the linearity test, the linearity of the method was confirmed by showing that the response factor RSD (0.55%) and slope RSD (1.04%) values were lower than 5 and 2%, respectively. The value obtained from the Fisher test (ANOVA) ($F_{exp} = 1.942$) was lower that the tabulate one ($F_{tab} = 2.447$; $\alpha = 0.05$). In the proportionality test, it was demonstrated that the intercept was not statistically different from 0 ($t_{exp} = 1.529 < t_{tab} = 2.306$; $\alpha = 0.05$). These values indicate that the proposed method presents a good linearity.

The detection limit was 1.4 mg/L with a 20 μL injection. This was determined from the calibration curve according to the method described by Miller and Miller [14].

Recovery experiments and standard addition method were performed in order to study the accuracy of the method. The analytical recoveries were determined by triplicate analyses of ruminal fluid or oak leaves spiked with standards at concentrations ranging from 5 to 30 mg/L. The mean recoveries were 99.94 ± 3.29; RSD = 3.30% and 101.07 ± 2.24; RSD = 2.22%, for ruminal fluid and oak leaves respectively (Table 1).

The standard addition method (standard plus ruminal fluid or oak leaves) was used to check for chemical interferences in the determination of EA. The equations calculated were: $y = (0.0557 \pm 0.0073)x + (0.5091 \pm 0.0840)$, (SD = 0.0108) and $y = (0.0562 \pm 0.0007)\ x + (0.7280 \pm 0.0075)$, (SD = 0.0097) for the ruminal fluid and oak leaves, respectively. The slopes for the calibration curve (0.0549) and standard addition graphs (0.0557, for ruminal fluid) and (0.0562, for oak

Table 1
Recoveries of EA from ruminal fluid and oak leaves

Sample	Added (mg/L)	Found (mg/L) ($n = 3$)	Recovery (%)
Ruminal Fluid	5	4.81 ± 0.13	96.26
	15	15.39 ± 1.12	102.61
	30	30.29 ± 0.32	100.95
Oak Leaves	5	4.92 ± 0.09	98.49
	15	15.32 ± 0.21	102.15
	30	30.77 ± 0.08	102.57

Ruminal fluid: mean recovery 99.94 ± 3.29; RSD = 3.30%. Oak leaves: mean recovery 101.07 ± 2.24; RSD = 2.22%.

Table 2
Intra and inter-day precision and accuracy of the ellagic acid determination in ruminal fluid

Added (mg/L)	Found ± SD (mg/L)	RSD intra-day ($n = 5$)	RE (%)	Found ± SD (mg/L)	RSD inter-day ($n = 3$)	RE (%)
5	5.25 ± 0.10	1.90	5.00	4.63 ± 0.18	3.88	−7.40
15	15.17 ± 0.22	1.45	1.13	14.52 ± 0.33	2.27	−3.20
30	29.63 ± 0.73	2.46	−1.23	30.31 ± 0.21	0.69	1.03
35	35.02 ± 0.69	1.97	−0.06	34.84 ± 0.38	1.09	−0.46
40	39.97 ± 0.83	2.08	−0.08	40.26 ± 1.01	2.51	0.65

Table 3
Intra and inter-day precision and accuracy of the ellagic acid determination in oak leaves

Added (mg/L)	Found ± SD (mg/L)	RSD intra-day ($n = 5$)	RE (%)	Found ± SD (mg/L)	RSD inter-day ($n = 3$)	RE (%)
10	9.85 ± 0.24	2.44	−1.50	9.59 ± 0.20	2.12	4.10
20	20.08 ± 0.06	0.28	0.40	20.17 ± 0.31	1.54	0.85
30	30.25 ± 0.31	0.83	−1.23	30.02 ± 0.56	1.86	0.07
40	40.22 ± 0.09	0.22	0.55	40.13 ± 0.50	1.24	0.33
50	48.97 ± 0.63	1.29	−2.06	50.14 ± 0.37	0.73	0.28

leaves) were similar. The values obtained from the Welch test ($\alpha = 0.05$) ($t_{exp} = 0.645 < t_f = 3.182$, for ruminal fluid) and ($t_{exp} = 0.835 < t_f = 3.182$, for oak leaves) indicated that the slopes values are not statistically different. All these results thus testify to the accuracy of the proposed method.

Intra and inter-day precision were assessed by analysing five times per day and three times per day for a week respectively, fluid ruminal samples spiked at five concentrations, (Table 2). The results were similar using oak leaves samples (Table 3).

4. Conclusion

The new ion-pair RP-HPLC method developed has been found to be suitable and effective for the rapid analysis of ellagic acid in ruminal fluid and in oak leaves samples. The described method has been shown to be linear, precise, accurate and specific. Thus, this method is reliable for the determination of ellagic acid in both biological samples.

Acknowledgements

We express our thanks to Dra. Frutos of the CSIC (Estación Agrícola Experimental de León) for their contributions. This study was supported by a Grant from M.E.C. (AGL-2004-06076-C02-02/GAN).

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