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Chromatographic and spectroscopic analysis of bound and unbound phenolic acids in *Lagenaria breviflora* fruit

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Abstract: The fruit pulp of *Lagenaria breviflora* Robert (Cucurbitaceae), used in Nigeria as an anti-bacterial and anti-fertility drug, was found to contain phenolic acids. Isolation and characterization of these compounds was based on column chromatography, TLC, PC, UV, IR and GC–MS. While *p*-hydroxybenzoic and vanillic acids were found to occur as free and bound acids in the pulp, ferulic acid was found to occur only as an ester. An optimized HPLC procedure for the quantitative analysis of these acids was developed, featuring short retention times, high sensitivity and excellent resolution. The concentration of these phenols in the fruit mesocarp was established.

Keywords: Lagenaria breviflora; *Cucurbitaceae; phenolic acids; spectroscopy; HPLC; GC–MS.*

Introduction

Lagenaria breviflora Robert (Cucurbitaceae) fruit is used in Nigerian traditional medicine and has been reported to possess anti-bacterial [1] and anti-fertility [2] activities. Although phenolics and saponins were detected as the main chemical components in the fruit [3], the chemistry of the plant has not been studied in detail and its active constituents have not been reported. Earlier workers on the HPLC analysis of phenolic acids recorded on a single chromatogram, either as authentic standards [4] or as present in plant extracts [5], have employed gradient elution which featured poor base-line separation and would be expected to be unreliable for quantitative measurement. An assay procedure [6] using a steel column packed with 5-μm Spherisorb-ODS and two separate solvent systems, for the benzoic and cinnamic acid derivatives, led to greater quantitative reliability but with retention times of up to 30 min. In the present work, the phenolic acid content has been identified by spectroscopic and chromatographic methods, and then quantified by a rapid HPLC procedure.

Experimental

Materials and equipment

Sephadex anion exchanger and cation exchanger (Pharmacia AB, Uppsala, Sweden) slurry-packed into 140 × 15 mm glass columns; *p*-hydroxybenzoic acid (PA), vanillic acid (VA), ferulic acid (FA), *m*-hydroxybenzoic acid (MA) and anisic acid (AA), were purchased from Sigma Ltd (UK); Perkin–Elmer UV–vis spectrophotometer (Lamda 5; 1 cm matched silica cells) (Perkin–Elmer, Beaconsfield, UK) and Perkin–Elmer model 297 IR spectrophotometer (liquid film $CHCl_3$) and GC–MS (Ribermag R 1010 B) with electron impact at 70 eV, were used.

All solvents used were of analytical grade (BDH Chemicals Ltd, Poole, UK); pyridine (Fluorochem Ltd, Glossop, Derbyshire, UK). N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) both obtained from Pierce Chemicals Co. (Rockford, IL, USA) were used for silylation of the phenolic acids prior to GC analysis.

Plant material

The fruit of *Lagenaria breviflora* Robert (Cucurbitaceae) was collected from Ile-Ife, Nigeria and authenticated with the herbarium specimen kept at the Forest Research Institute of Nigeria, Ibadan. The fresh fruit was separated into epicarp, mesocarp (pulp) and seed. The pulp was dried at 65°C and powdered before use.

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Extraction of unbound phenolic acids

A 0.12 kg mass of powdered fruit pulp of *L. breviflora* was defatted with petroleum spirit and extracted by Soxhlet apparatus, successively, with *ca* 250 ml ethyl acetate (Extract No. 1) and then with *ca* 250 ml methanol (Extract No. 2). The ethyl acetate extract (No. 1) was evaporated to dryness, taken up in water, and purified by chromatography on cationic and anionic Sephadex columns in series with water, followed by 0.1 N HCl as eluents according to previous methods [7] to yield an acid fraction. This fraction was partitioned three times with *ca* 30 ml ethyl acetate and the combined ethyl acetate portions evaporated to dryness and redissolved in 20 ml methanol (F2). A 10-ml aliquot of this solution was further purified by preparative thin-layer chromatography (preparative TLC) on 1.0 mm layers of silica gel GF 254 (Merck AG, Darmstadt, Germany) with chloroform–glacial acetic acid (9:1, v/v) giving compounds PA and VA. These were selectively detected by UV irradiation at 254 nm, followed by Barton's (ferric chloride/potassium ferricyanide) spray reagent and then recovered into methanol. Analytical paper chromatography (PC) on Whatman No. 1 with water as eluent and spectroscopy (UV, IR, GC–MS) was used to confirm the identities against reference standards, followed by quantitation by HPLC.

Ester-bound phenolic acids

A 50-ml aliquot of the methanol extract (No. 2) was evaporated to dryness and hydrolysed with 25 ml aqueous 2 M NaOH for 4 h at room temperature in an atmosphere of nitrogen. The mixture was neutralized with 2 M HCl and shaken three times with 20 ml ethyl acetate. The combined organic layer was evaporated to dryness, redissolved in water and subjected to ion-exchange column chromatography [7] as for Extract No. 1 above to obtain a similar fraction coded F3 (in methanol). On PTLC, compounds PA and VA were obtained, together with another compound FA, not present in Extract No. 1. These bands were recovered in methanol and identified as described above.

Gas–liquid chromatography/mass spectrometry (GC–MS) of fraction F2 or F3

A preliminary separation for fraction F2 or F3 was developed on a Pye-Unicam gas chromatograph GCV (Philips Ltd, Cambridge, UK) equipped with a glass column (1.5 × 4 mm i.d.) packed with 5% OV-101 on Chromosorb G in order to establish suitable conditions for GC–MS. Trimethylsilyl derivatives of the phenolic acids were prepared by evaporation of a 5-ml aliquot of fraction F2 or F3 to dryness which was redissolved in 100 μl dry pyridine, mixed with 200 μl BSTFA containing 1% TMCS and then heated at 90°C for 30 min. The GC method employed temperature programming from 163 to 280°C at 10°C min^{-1} with the FID detector maintained at 300°C and column nitrogen flow at 35 ml min^{-1}.

GC–MS was carried out on a Varian 3700 gas chromatograph equipped with a fused silica capillary OV-101 column (50 × 0.22 mm i.d.). The temperature gradient was extended from 120 to 300°C at 9°C min^{-1}; helium gas at the inlet pressure of 2 bar with an estimated linear gas velocity of 26.7 cm s^{-1} was used. Separation was followed by mass spectrometry (Ribermag R 1010 B) and an on-line library facility was used to characterize the phenolic acids as their trimethylsilyl derivatives.

HPLC

A suitable dilution of fraction F2 or F3 of the PTLC-purified phenolic acids (see above) was injected on a "Guardpak" precolumn (3 × 3 mm i.d.; 10 μm; Waters Assoc., MA, USA), coupled to a steel analytical column (25 cm × 4.6 mm i.d.) packed with 5-μm Spherisorb 5-ODS (Phase Separations Ltd, Deeside, Clwyd, UK). The HPLC system consisted of a high pressure pump (Altex, Berkeley, CA, USA) delivering mobile phase at 1.8 ml min^{-1}, a 20 μl loop valve injector (Rheodyne Inc., CA, USA), a variable wavelength UV detector (LDC Spectromonitor 3000, Milton Roy, Stone, Staffordshire, UK) at 254 nm (0.1 a.u.f.s.) and a Perkin–Elmer 56 (Beaconsfield, UK) recorder. Elution was carried out isocratically with 0.01 M sodium acetate (pH 4.5)–methanol (60:40, % v/v; elution system 2) for FA and VA, with anisic acid as internal standard. The organic modifier content and pH were optimized to separate the phenolic acids. The relative standard deviation (RSD) was estimated from 10 replicates of 3.75 μg ml^{-1} authentic PA sample. The percentage recovery of PA was assessed, in duplicate, by mixing together equal volumes of 2.5 μg ml^{-1} authentic PA and PTLC-purified extract containing an accurately known amount (*ca* 5.0 μg ml^{-1})

of PA and then estimating the total PA in the mixture. Calibration graphs, based on peak-height ratios, with five dilutions in duplicate for each compound, were constructed in the range: 0–12.5 μg ml^{-1} (PA) and 0–12.5 μg ml^{-1} (VA) with MA as internal standard (at 75 μg ml^{-1}); and 0–25 μg ml^{-1} (FA), with AA as internal standard (at 12.5 μg ml^{-1}).

Results and Discussion

For the separation of phenolic acids in plants, HPLC is widely represented in the literature [4, 5] and has tended to displace GC which requires pre-derivatization [8]. The TLC, PC and the spectroscopic data for identifying the phenolic acids in this work are presented in Table 1 and were found to be identical to those of authentic samples. PC and UV fluorescence at 336 nm, was used to differentiate between VA (non-fluorescent) and FA (blue fluorescence); similarly, between PA (non-fluorescent) and *p*-coumaric acid (blue fluorescence) which possessed very close R_f values on both TLC and PC. The MS fragmentation patterns of the compounds are fully documented in the literature [9, 10].

Effect of organic modifier concentration and pH

Figure 1 shows the effect of eluent composition on the capacity factors of the authentic benzoic acid derivatives, using the reversed-phase HPLC system. For all eluent compositions, MA was well separated while the resolution of PA and VA decreased as the eluent composition approached 25% v/v methanol. It was found that 18% v/v methanol gave the best separation. The capacity factors varied inversely with the pH for all the compounds tested (Fig. 2). This is because at low pH values, the acids exist in un-ionized forms and are more strongly retained resulting in higher k' values. At high pH values, they exist mostly in ionized forms and are retained less (low k' values). Therefore, as regards the capacity factor (Fig. 2) it appeared that pH 3.0, 4.0 or 4.5 could be used. However, as regards

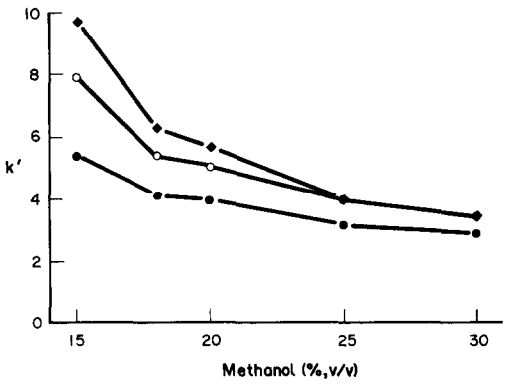

Figure 1
Effect of mobile phase composition at pH 3.0 on capacity factors of phenolic acids. ● MA; ○ PA; ■ VA.

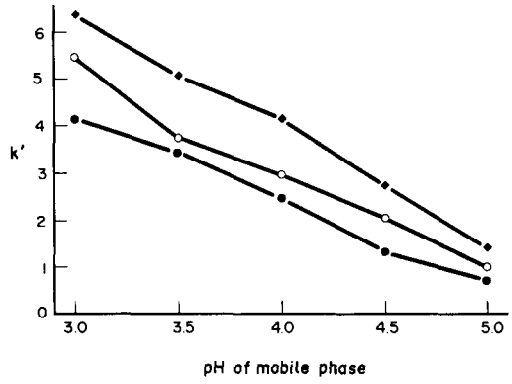

Figure 2
Effect of pH on capacity factors of phenolic acids at 18% methanol. ● MA; ○ PA; ■ VA.

Table 1
Chromatographic and spectroscopic data of the phenolic acids isolated from *L. breviflora* fruit

	p-Hydroxybenzoic acid	Vanillic acid	Ferulic acid
TLC (R_f)	0.48	0.68	0.75
PC (R_f)	0.89	0.86	0.23, 0.77*
GC (t_R, min)	2.0	3.0	5.0
MS (M^+, m/z)†	282	312	338
UV, MeOH (nm)	253, 224	296, 259 218	319, 294 213
UV, MeOH + NaOH (nm)	274, 210	302	346, 207
IR, $CHCl_3$ (cm^{-1})	3400, 1720 1610, 1590	3475, 1670 1590, 1520	3450, 1680 1600, 1500

*These represent two *cis–trans* isomers not resolved by other methods.
†As trimethylsilyl derivatives.

resolution (Fig. 3), pH 4.5 or 5.0 was favoured because the separation of the adjacent peak in each pair was reasonably equal at these pH values. Thus, the optimum conditions for eluent system 1 were: 18%, v/v methanol at pH 4.5.

Quantitative performance

Linear calibration graphs with correlation coefficients (n = 5) of 0.9999 (PA), 0.9997 (VA) and 0.9997 (FA) were obtained. The corresponding regression equations were: for PA, $y = 0.113x - 0.003$; for VA, $y = 0.066x + 0.006$; for FA, $y = 0.044x - 0.001$. The column efficiency under the optimized conditions was *ca* 16,000 plates metre. RSD values (n = 10) were: for PA, 1.16%; for VA, 1.24%; and for FA, 1.30%. The minimum detectable amounts were: 0.31 ng (PA); 0.85 ng (VA) and 1.8 ng (FA) (at signal-to-noise ratio of 3; n = 4). For PA, 96% recovery was obtained.

Unbound phenolic acids (present in Extract No. 1)

Eluent system 1 was used for free PA and VA which eluted in the order of their polarity, as is typical for reversed-phase systems. They were separated with short retention times (t_R) and low capacity factors (k'), suitable α values and high resolution (Rs) (Table 2; Fig. 3). FA was not detected in eluent system 1, because it was highly retained under the isocratic conditions of this assay. Since the unbound VA was in trace amounts only in this plant, it could not be assayed in its natural ratio to PA [Fig. 4(a)], hence preparative TLC was required. Thus, both acids were quantified after preparative TLC by mixing each in a suitable proportion to give on-scale peaks in a single chromatogram [Fig. 4(b)]. Table 2 shows that PA and VA were present in the fruit as free acids at concentrations of 342.5 and 18.5 μg g^{-1}, respectively, while FA was not present in the free form. The high quantities of the unbound phenolic acids found in this fruit may have implications for the antibacterial properties already reported [1].

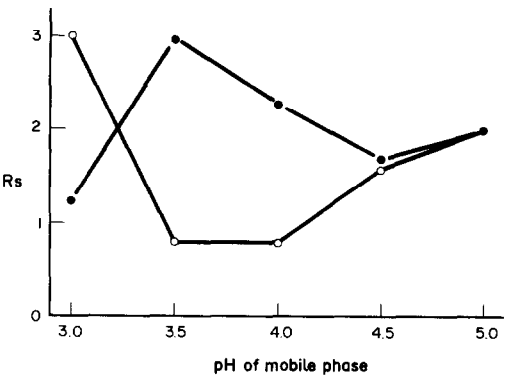

Figure 3
Effect of pH on resolution of phenolic acids at 18% methanol. ● MA/PA pair; ○ PA/VA pair.

Ester-bound phenolic acids (present in Extract No. 2)

Eluent system 2 gave excellent separation (Fig. 5, Table 2) of the ester bound VA and FA. PA co-eluted with VA in this system and was assayed (after preparative LTC) with eluent system 1 as described above. In fact, it was found possible to quantify VA by either system with closely similar results: 29.3 μg ml^{-1}, with eluent system 1; 28.0 μg g^{-1} with eluent system 2. FA (54.2 μg ml^{-1}) existed only in the bound form, while the PA content (51.0 μg g^{-1}) was much lower in the bound form than in the free form.

Although gradient elution would eliminate the need for two different eluent systems, this

Table 2
Retention characteristics and content of phenolic acids in *L. breviflora* fruit by HPLC

Compound	HPLC system	t_R (min)	k'	α	Rs	μg g^{-1}*
Unbound acids (Extract F2):						
m-Hydroxybenzoic (MA)	1	3.4	1.4	1.4	1.6	—
p-Hydroxybenzoic (PA)	1	4.2	2.0	1.4	1.7	342.5
Vanillic (VA)	1	5.2	2.7			18.5
Ester bound acids (Extract F3):						
p-Hydroxybenzoic (PA)	1	4.2	2.0	1.1	3.0	51.0
Vanillic (VA)	2	2.0	1.1	2.1	3.3	28.0
Ferulic (FA)	2	3.2	1.3			54.2
Anisic (AA)	2	5.2	2.7			—

*Calculated with respect to dry weight of fruit pulp.

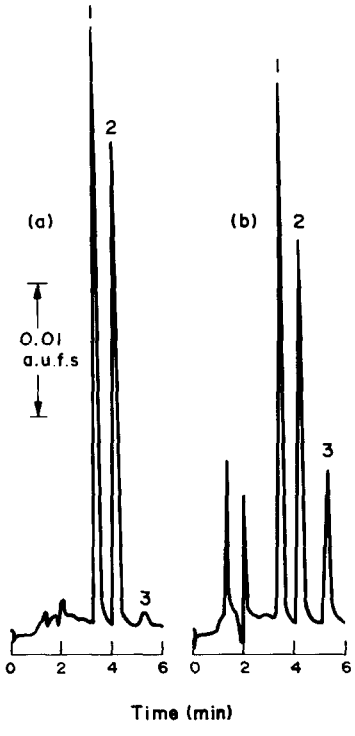

Figure 4
HPLC of unbound phenolic acids in extract No. 1, Eluent System 1: (1) MA (i.s.); (2) PA; (3) VA. (a) Fraction F2 containing (2) and (3) in their natural proportions. (b) Mixture formed by addition of (2) and (3) isolated from fraction F2 by PTLC.

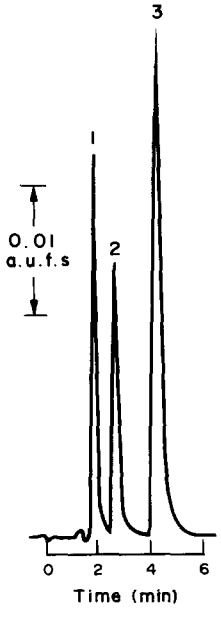

Figure 5
HPLC of bound phenolic acids in hydrolysed extract No. 2 following PTLC of fraction F3. Eluent System 2: (1) VA; (2) FA; (3) AA (i.s.).

is generally considered to be inadvisable due to the tendency of base-line drift leading to lack of quantitative precision. Therefore the benzoic and cinnamic acid derivatives in plant extracts, possessing different structural and polarity characteristics, are better quantified by using the two different HPLC solvent systems proposed here, as suggested by other workers [6].

It should be noted that the long retention times (up to 30 min) reported earlier [6] have been reduced in the present work to less than 6 min (Figs 4 and 5). Although such a resolution was not required in the present work, it was observed that the *cis*- and *trans*-ferulic acid (FA) were not separated by our method in contrast to the earlier work by Hartley and Buchan [6].

The relative difficulty in isolating the bound forms of these acids for structural studies may be due largely to the microgram (or nanogram) quantities [5] present in plant samples. However, it would appear feasible to isolate PA and FA esters from a large quantity of *L. breviflora* fruit for structural analysis in the alcohol extract and for subsequent screening of the ester for biological activity. Work in this direction is continuing in the authors' laboratories.

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