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Note

Analytical and preparative high-performance liquid chromatographic systems for the separation of an anomeric mixture of 4-O-(D-glucopyranosyl)gallic acid

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Phenolic compounds are widespread in nature and, although they mostly occur as glycosides, the physiological activity is normally released by the aglyca as in the case of juglone [1]. In contrast to this common rule, the turgormediated leaf movement of *Mimosa pudica* L. is controlled by a sulphated glucoside of gallic acid, 4-O-(β-D-glucopyranosyl-6-sulphate)gallic acid, which we named PLMF 1 (periodic leaf movement factor) [2]. This compound has been isolated from fourteen higher plants which exhibit nyctinastic movements [3].

For our investigations of the structure–activity relationship of PLMF 1, for leaf-closing activity on leaves of *Mimosa pudica* L., we had to synthesize the PLMF 1 anomeric compound, *i.e.*, 4-O-(α-D-glucopyranosyl-6-sulphate)gallic acid [4]. As it was not possible to obtain the pure compound by stereoselective glucosidation, the problem was to separate an anomeric mixture containing the α- and β-anomers in a ratio of 1:1.

Separation of anomers is usually performed by fractional crystallization, but unfortunately this method failed in this instance. With the medium-pressure liquid chromatographic (MPLC) systems developed for the purification of synthesized PLMF 1 and the free glucosides [5], the anomers could not be separated even on different stationary phases and using several different mobile phases.

In this paper we report the high-performance liquid chromatographic (HPLC) systems with which we finally succeeded in separating the anomeric glucosides of gallic acid and their sulphated derivatives on both analytical and preparative scales.

EXPERIMENTAL

Chemicals

3,5-Diacetyl 4-O-(D-2′,3′,4′,6′-tetra-O-benzylglucopyranosyl)methylgallate was

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synthesized [6] using the trichloroacetimidate method described by Schmidt [7]. Cleavage of the protecting groups was carried out in two steps: first, debenzylation by hydrogenolysis with hydrogen at room temperature and atmospheric pressure using Pd/C (10%) as catalyst, and second, cleavage of the acetyl groups and the methyl group with $Ba(OH)_2$ in concentrated aqueous solution in an inert gas atmosphere to give finally the α- and β-anomers of 4-O-(D-glucopyranosyl)gallic acid in a ratio of 1:1. The separated glucosides were sulphated using an SO_3–pyridine complex in absolute dimethylformamide as sulphating agent [8].

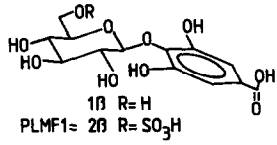
Water was purified by deionization on ion-exchange columns and passage through a Milli-Q water purification system. Methanol was donated by BASF (Ludwigshafen, F.R.G.) and was purified by rectification. Trifluoroacetic acid (TFA) for spectroscopy (Uvasol) was purchased from Merck (Darmstadt, F.R.G.).

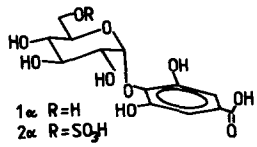
HPLC systems

The chromatographic system for analytical separations consisted of an LDC ConstaMetric III HPLC pump and a Philips PU 4020 variable-wavelength absorbance detector. Chromatograms were recorded on a Merck/Hitachi Model D 2000 integrator. Columns were 250 mm × 8 mm I.D., stainless steel, laboratory-packed with Nucleosil C_{18} (5 μm, 100 Å) purchased from Macherey-Nagel (Düren, F.R.G.). The chromatographic system for separations on a preparative scale consisted of a Model HD-2-200 HPLC pump (Besta, Heidelberg, F.R.G.) and an LDC Spectro-Monitor III variable-wavelength absorbance detector. The column, purchased from Macherey-Nagel, was 250 mm × 20 mm I.D., stainless steel, packed with Nucleosil C_{18} (7 μm, 100 Å) coupled with a precolumn of 30 mm × 16 mm I.D., stainless steel, packed with Nucleosil C_{18} (5 μm, 100 Å).

RESULTS AND DISCUSSION

4-O-(α-D-Glucopyranosyl)gallic acid (**1α**), 4-O-(β-D-glucopyranosyl)gallic acid (**1β**) and their sulphated derivatives (**2α** and **2β**) were separated by reversed-phase





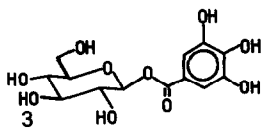


TABLE I

REVERSED-PHASE SEPARATION OF THE ANOMERIC GLUCOSIDES OF GALLIC ACID

Eluents: I = methanol–water (15:85, v/v) containing 2 m*M* TFA; II = methanol–water (12:88, v/v) containing 2 m*M* TFA; III = methanol–water (10:90, v/v) containing 3 m*M* TFA; IV = methanol–water (12:88, v/v) containing 3 m*M* TFA. a, Analytical system, flow-rate 3 ml/min; b, preparative system, flow-rate 16 ml/min.

Compound	Retention time (min)[a]			
	Ia	IIa	IIIa	IVb
1α	11.0 ± 0.2	13.0 ± 0.3	17.0 ± 0.5	20.4 ± 0.6
1β	13.3 ± 0.3	16.2 ± 0.4	20.9 ± 0.6	26.3 ± 0.7
2α		10.0 ± 0.5	11.3 ± 0.2	
2β		11.8 ± 0.6	14.0 ± 0.3	

[a] Mean values ± standard error.

chromatography. The retention times of these substances obtained with different chromatographic systems are presented in Table I. In all instances the α-anomer elutes first (Fig. 1). Complete separation of the anomers **1**α and **1**β on a prepparative scale was achieved within 30 min. The amount of substance separated with this sys-

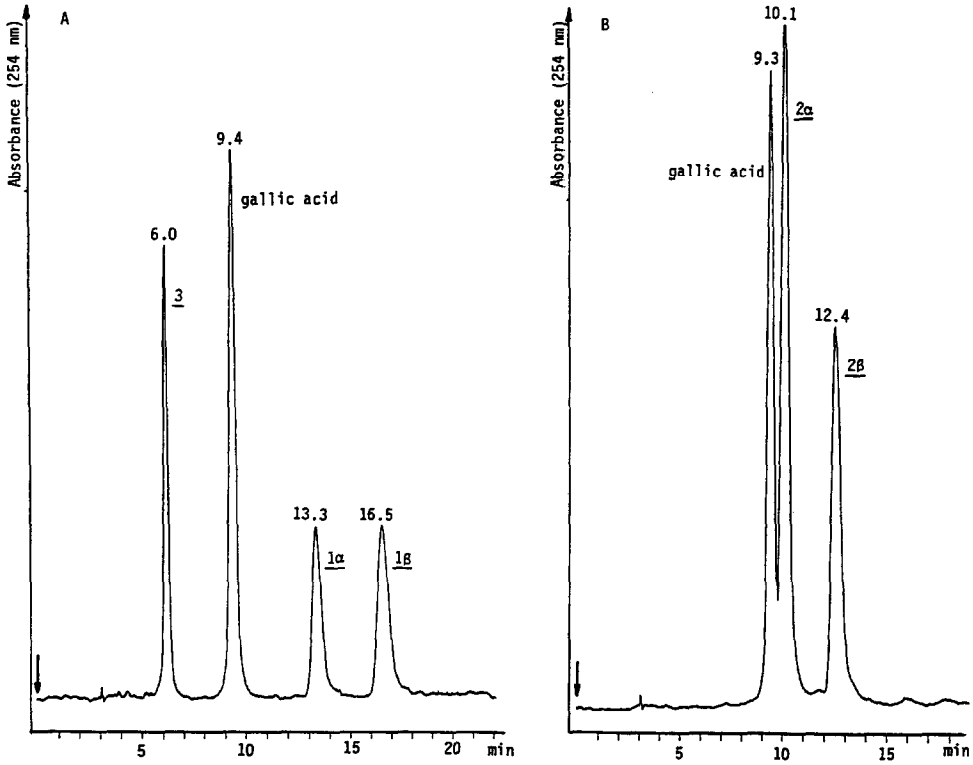


Fig. 1. Typical analytical separation of (A) compounds **3**, **1**α and **1**β (B) and compounds **2**α and **2**β. Gallic acid was added as an internal standard in both runs. Eluent, methanol–water (12:88, v/v) containing 2mM TFA; flow-rate, 3 ml/min; column, 250 mm × 8 mm I.D., Nucleosil C_{18} (5 μm, 100 Å).

tem was usually 15 mg per run. During the scale-up of the analytical system, a column of 250 mm × 16 mm I.D. packed with Nucleosil C_{18} (5 μm, 100 Å) was used. On this column 3 mg were separated per run using methanol–water (12:88, v/v), containing 2 m*M* TFA as eluent at a flow-rate of 10 ml/min (results not shown).

Of major importance was the incorporation of TFA in the eluent, as it provides some advantages over glacial acetic acid. Only small amounts of TFA (1–3 m*M*) were needed to obtain a high resolution. It also prevented tailing of peaks eluting with higher retention times. Further, TFA is more convenient to use for preparative separations, as it is easier to evaporate than glacial acetic acid.

Increasing the TFA content in a given mobile phase in 1 m*M* steps resulted in an increase in the retention time of the acidic compounds owing to greater protonation of their acid groups [9]. This influence could be demonstrated by comparing **1**β with its isomer 1-galloyl-β-D-glucopyranose (**3**), in which β-D-glucose is connected to the carboxylic group of gallic acid [10,11]. As **1**β possesses a carboxyl group, its dissociation can be suppressed by the addition of TFA, which shifts the equilibrium in the direction of the less polar undissociated acid having a longer retention time on the reversed-phase column. In the isomeric compound **3** the carboxyl group is esterified with β-D-glucose, thus largely eliminating the influence of TFA on the retention time of this substance. Whereas the retention time of **1**β was 16.2 ± 0.4 min with solvent system IIa, 1-galloyl-β-D-glucopyranose eluted after 5.8 ± 0.3 min using the same system (Fig. 1A) [6]. As illustrated by the retention times of the sulphated glucosides **2**α and **2**β with system IIIA, the separation of strongly acidic compounds is also possible.

The HPLC systems presented here are helpful for the separation and purification of glucosides of phenolic acids. Scale-up from an analytical scale to the described preparative separation is possible without any problems. The preparative separation of the anomeric glucosides of gallic acid yields pure compounds that can be used without further purification.

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