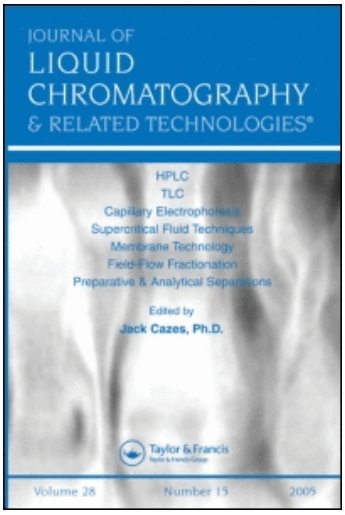

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 13 March 2000

To cite this Article Chervenkova, K. and Nikolova-Damyanova, B.(2000) 'THE ELUTION PATTERN OF IRIDOID GLUCOSIDES FROM *GALIUM* SPECIES ON C-18 REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 23: 5, 741 — 753
To link to this Article: DOI: 10.1081/JLC-100101486
URL: http://dx.doi.org/10.1081/JLC-100101486

J. LIQ. CHROM. & REL. TECHNOL., 23(5), 741–753 (2000)

THE ELUTION PATTERN OF IRIDOID GLUCOSIDES FROM *GALIUM* SPECIES ON C-18 REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The retention and separation of ten iridoid glucosides typical for the plants of genus *Galium* (Rubiaceae) were examined by RP-HPLC on octadecyl (ODS) column with water-methanol mobile phases. The compounds have the same basic carbon skeleton with a glucose moiety at C-1 but differ by the type, the number, and the position of the additional functional groups. In general, the retention increased with the increasing total hydrophobicity of the molecule, but was substantially affected by the different combinations of free and esterified hydroxyl and/or carboxyl groups. The water-based mobile phase, the high polarity and the bulky structure of the iridoid glucosides examined suggested that the solute-mobile phase interactions dominated in the retention mechanism. Water-methanol (85:15, v/v) was found to be a suitable mobile phase for the fractionation of the iridoid glucosides into acidic and non-acidic components on a single column.

Two ODS columns connected in a series and a stepwise gradient of water-methanol-phosphoric acid (95:5:0.02, v/v/v) to 100

% methanol over 60 min were required for a reliable "fingerprint" analysis of iridoid glucosides in *Galium* plant extracts.

INTRODUCTION

Iridoid glucosides are a large group of monoterpenoids with partially hydrogenated cyclopenta[c]pyrane system and a glucose moiety attached to C-1 in the pyrane ring (see Figure 1). They are minor components in the polar extracts of some Dicotyledone plants. There is a constant interest in the examination of iridoid glucosides since many compounds have shown substantial biological activity, e.g.: hypotensive, anti-inflammatory, antifungal, antibacterial, etc.[1-3] It appeared also that different plant species might have a different and specific iridoid composition, and this feature was supposed to be of use for the chemotaxonomy and evolution studies.[4,5]

The plant extract, respectively treated to eliminate the accompanying non-iridoid compounds, is usually comprised of a complex mixture of iridoid glucosides that differ in structure and quantity. The phytochemical examination requires an adequate analytical assay with appropriate approaches for resolution of the iridoid mixture with three main tasks: (i) a "fingerprint" presentation of the composition, (ii) a quantitative estimation and (iii) separation and isolation of individual compounds for determination of the structure and examination of biological activity.

Reversed-phase high performance liquid chromatography (RP-HPLC) is the most widely explored separation technique so far. The reported procedures[1,3,4,6-14] are directed mostly to the isolation of individual components. Appropriate resolution and quantification were achieved in a few cases only; mostly with mixtures of relatively simple composition or in cases where the components were relatively nonpolar. The main analytical problem was the high polarity of iridoid glucosides and the great variety of closely related structures. The elution pattern of such compounds in RP-HPLC had not been systematically investigated and no attempts were made to relate the retention to the structure.

The present work deals with the RP-HPLC of a group of iridoid glucosides isolated from several species of the genus *Galium* (Rubiaceae), collected in Bulgaria. The selected compounds have the same carbon skeleton which enabled the examination of the effect of the different functional groups on their retention. An octadecylsilane (ODS) column and elution with two of the most often used mobile phases: water-methanol and water-methanol-acid were employed. Conditions for satisfactory "fingerprint" resolution of these components in extracts of *Galium* species are proposed.

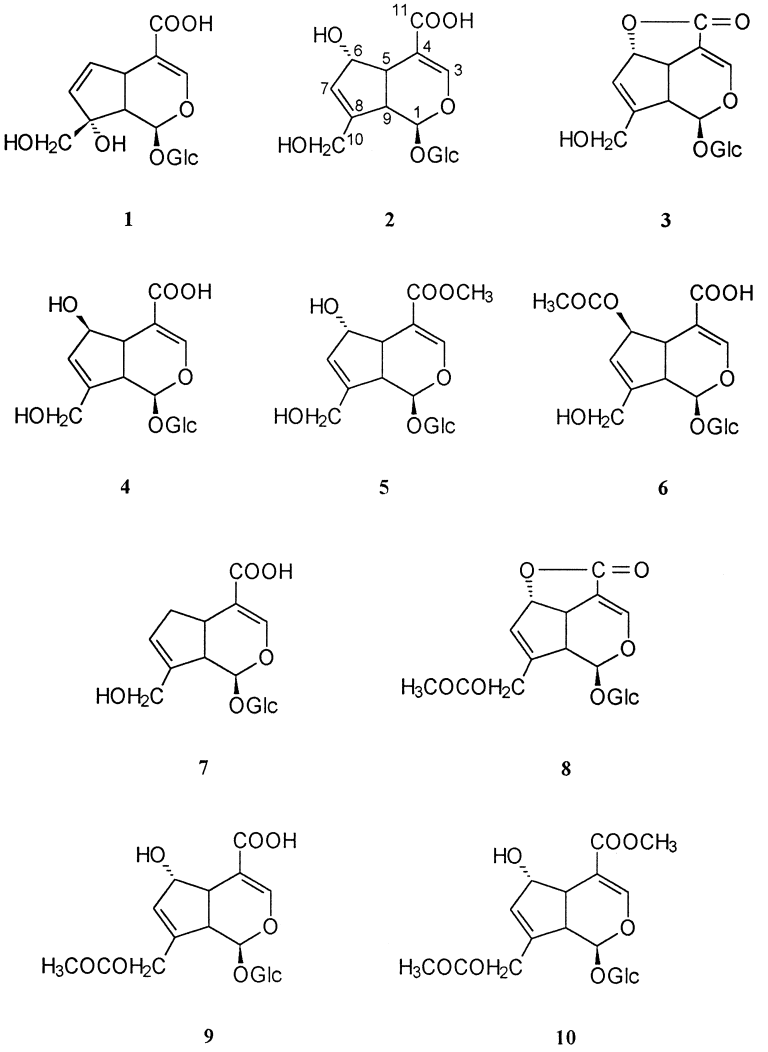

Figure 1. Structures of the examined iridoid glucosides, Glc denotes the glucose moiety; 1, Monotropein; 2, Deacetylasperulosidic acid; 3, Deacetylasperuloside; 4, Scandoside; 5, Deacetylasperulosidic acid methyl ester; 6, 6-O-Acetylscandoside; 7, Geniposidic acid; 8, Asperuloside; 9, Asperulosidic acid; 10, Daphylloside.

EXPERIMENTAL

Materials and Reference Substances

All solvents were either analytical- or HPLC-grade. Deionized water was used to prepare the mobile phases. Phosphoric acid (85%) was purchased from Merck (E. Merck, Darmstadt, Germany).

The reference iridoid glucosides and the plant extracts were kindly provided by Ms. M. Mitova from the Institute of Organic Chemistry, Bulgarian Academy of Sciences. The reference compounds were isolated from different *Galium* species, purified and characterized by spectral methods (MS, ^{1}H and ^{13}C NMR) as described by Mitova et al.[15] Solutions in methanol were prepared from the solid substances with concentrations in the range 0.40 - 0.90 mg/mL.

Prior to the RP-HPLC the crude plant extracts were purified as described[16] and re-dissolved in methanol-water (1:1, v/v).

RP-HPLC

The isocratic elution was performed on a Perkin Elmer series 2 liquid chromatograph equipped with a Rheodyne 7125 sample loop injector (20 μL) (Supelco, Gland, Switzerland) and a LC-75 variable wavelength UV detector (Perkin Elmer Corporation, Norwalk, Connecticut, USA). The chromatograms were registered with a computing integrator Chromatopac C-R3A (Shimadzu Corporation, Kyoto, Japan). The experiments were carried out at room temperature (21 ± 1°C) on a Whatman Partisil ODS-3 (10 μm) stainless-steel column (25 x 0.46 cm I. D.) with mobile phases water-methanol (85:15, v/v) and water-methanol-phosphoric acid (85:15:0.02, v/v/v) at a flow rate of 1.0 mL/min.

The gradient elution was performed using two ISCO model 2350 pumps and V^4 variable wavelength detector (ISCO Inc., Lincoln, Nebraska, USA). The chromatograms were registered by ISCO Chemresearch version 2.3 software for IBM-PC. The experiments were carried out at room temperature (21 ± 1°C) on two Whatman Partisil ODS-3 (10 μm) stainless-steel columns (25 x 0.46 cm I. D.) connected in a series with mobile phases: A, water-methanol-phosphoric acid (95:5:0.015, v/v/v) and B, methanol, and a gradient from 0 to 45% B over 45 min, to 60% B over 5 min, and to 100% B over 10 min. The flow rate was 0.8 mL/min.

The reference iridoid glucosides reportedly have a maximum absorbance in relatively narrow range (232-239 nm).[17,18] A working wavelength of 233 nm was chosen in the present work, since all reference compounds were detected with good sensitivity. The hold-up time (3.5 ± 0.05 min) was measured by the negative peak of an air bubble. Retention times were determined as the

mean of three parallel measurements with a maximum standard deviation not exceeding 0.1. Injection volume was 10 μL.

RESULTS AND DISCUSSION

The reference compounds used in the present work are shown in Figure 1. The number assigned to each compound is arbitrary and corresponds to the elution order established in this study. Compounds **2-10** have the same basic carbon skeleton and the same position of the functional groups, at C-1, C-4, C-6 and C-10 (see compound **2** on Figure 1 for the numbering of the carbon atoms in the skeleton), but differ by the number, the type, and the stereochemistry of these groups. These compounds are often denoted as geniposide-type iridoids. Monotropein, 1, is included in the examination since it was constantly present in the extracts of *Galium* species although it does not belong to the same structural group as **2-10**. Monotropein differs by the positions of the double bond and of the hydroxyl groups in the cyclopentane ring (Figure 1). In the iridoid chemistry compounds with a free carboxyl group at C-4 (as in **1**, **2**, **4**, **6**, **7** and **9**) are often denoted as iridoid acids.[7] In order to simplify the discussion on retention in RP-HPLC, the presence of the sugar moiety was not considered and the compounds were separated arbitrarily into three types based on the character of the other functional groups as follows: type I, compounds **1**, **2**, **4**, and **7** with a free carboxyl group and, one (in **7**) or two free hydroxyl groups in addition; type II, compounds **6** and **9** with a free carboxyl group and one free and one acetylated hydroxyl groups; type III, the non-acidic compounds **3**, **5**, **8** and **10** which are either methyl esters (**5** and **10**) or lactones (**3** and **8**).

Two-component water-based mobile phases with methanol and, occasionally, with acetonitrile or isopropanol as modifiers, were employed so far for the resolution of iridoid glucoside mixtures by RP-HPLC on ODS columns.[1,3,4,6,7,9] Since, in most phases, the elution of compounds with free -COOH group often resulted in peak splitting or tailing, the addition of a small amount of formic acid as a third component was suggested.[7]

The presence of formic acid (pH of the mobile phase was about 3), was supposed to keep the free -COOH group in a fully protonated form, resulting in better shaped peaks. Table 1 presents the k' values obtained and Figures 2 and 3 show the resolution achieved with water-methanol (85:15, v/v) and water-methanol-phosphoric acid (85:15:0.02, v/v/v), respectively, under isocratic elution on an ODS column. The methanol proportion was chosen so that the most strongly retained compound, **10**, eluted for not longer than 50 minutes.

Isocratic Elution with Water-Methanol (85:15, v/v)

Under these experimental conditions the elution order appeared to be: **1** = **2** ≅ **4** = **6** = **7** ≅ **9** < 3 < **5** << **8** <<< **10**. Compounds **1**, **2**, **4**, **6**, and **7** behaved

Table 1

The Capacity Factor (k') Values of Compounds 1-10 on a Whatman Partisil ODS-3 Column*

No.	Compound	k' Mobile Phase I	Mobile Phase II
1	Monotropein	0	0.1
2	Deactylasperulosidic acid	0	0.2
3	Deacetylasperuloside	0.4	0.4
4	Scandoside	0	0.6
5	Deacetylasperulosidic acid methyl ester	1.2	1.2
6	6-O-Acetylscandoside	0	1.2
7	Geniposidic acid	0	1.3
8	Asperuloside	2.8	2.8
9	Asperulosidic acid	0.2	3.2
10	Daphylloside	11.0	11.1

* **I** water-methanol (85:15, v/v) and **II**: water-methanol-phosphoric acid (85:15:0.0, v/v/v) at a flow rate of 1.0 mL/min.)

as non-retained solutes (Table 1). All compounds with a free carboxyl group (type I, **1**, **2**, **4**, **7** and type II, **6** and **9**), eluted as a single peak with two shoulders (Figure 2). Despite the absence of an acid in the mobile phase, the peak was well-shaped, probably because the methanol proportion in the mobile phase employed in this work was higher than those used previously.[7] By spiking the injected mixture with the respective compounds it was found that compounds **1** and **2** form the first shoulder, **4**, **6**, and **7** followed and **9** eluted last. As shown above, compounds **6** and **9** differed by the position of the acetylated -OH group in the cyclopentane ring. The acetylated -OH at C-10 in **9** led to a weak but measurable increase of k' , while **6** (acetylated -OH at C-6) was practically not retained (see Table 1). Note also, that **6** formed a mixed peak with **4** (a free -OH group at C-6 with the same stereochemistry) and **7** (no -OH at C-6). The non-acidic iridoid glucosides **3**, **5**, **8** and **10** had a k' of reasonable high values and were well resolved from the other components and from each other. The elution order logically followed the gradual decrease in their polarity.

The comparison between **3** and **8**, and between **5** and **10**, showed very clearly the effect of the functional groups on the retention. In each pair the earlier eluting compound had a free hydroxyl group at C-10, while this group was acetylated in the compound retained stronger. The result was about a six-fold increase of the respective k' values (Table 1). Logically, the bulky tricyclic **3**

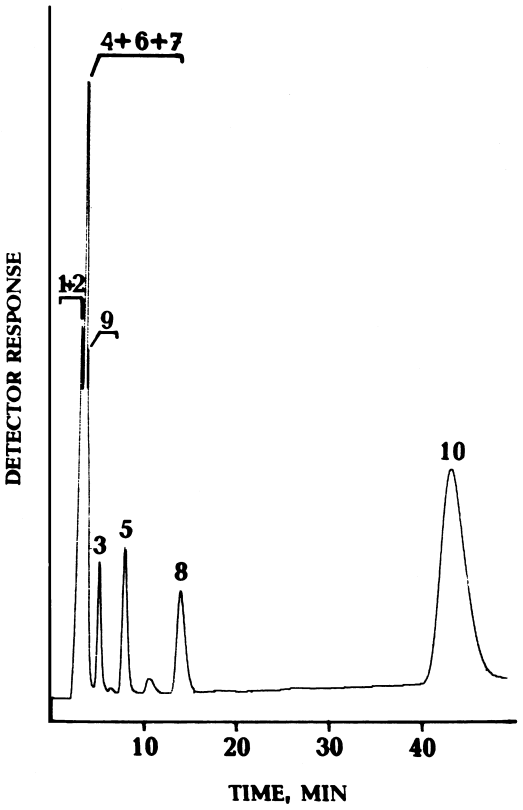

Figure 2. Chromatogram of the standard mixture of iridoid glucosides 1 - 10. Conditions: Partisil Whatman ODS-3 (10 μm), 25x0.46 cm I. D column; mobile phase: water:methanol (85:15, v/v); flow rate: 1.0 mL/min; UV detection at 233 nm.

and **8** (lactone ring) eluted ahead of **5** and **10** despite the same type and position of the other functional groups (Figure 1).

Taking into account the impact of the functional groups on the retention the elution order was:

[(-COOH)(- OH at C8) (- CH_2OH)] = [(-COOH) (-OH in α-position at C-6) (-CH_2OH)] ≅ [(-COOH) (-OH in β-position at C-6) (-CH_2OH)] = [(-COOH) (-$OCOCH_3$) (-CH_2OH)] = [(-COOH) (-CH_2OH)] ≅ [(-COOH) (-OH) (-CH_2OCOCH_3) < [(LACTONE RING) (-CH_2OH)] < [(-$COOCH_3$)

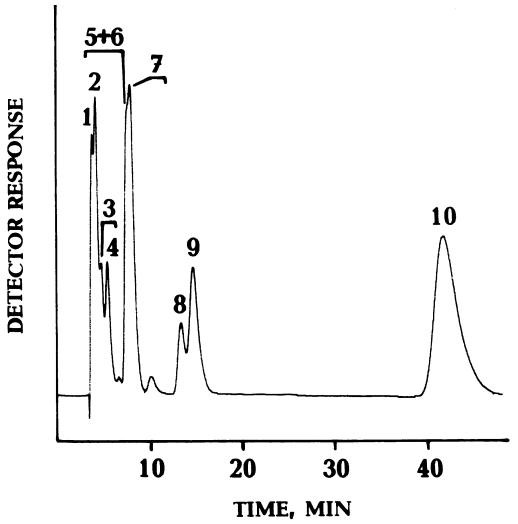

Figure 3. Chromatogram of the standard mixture of glucoiridoids 1 - 10. Mobile phase: methanol:water:phosphoric acid (85:15:0.020, v/v/v); other conditions as in Figure 1.

(-OH) ($-CH_2OH$)] << [(LACTONE RING) ($-CH_2OCOCH_3$)] <<< [($-COOCH_3$)(-OH)($-CH_2OCOCH_3$)].

Thus, while water-methanol solvent system provided satisfactory resolution of non-acidic iridoid glucosides, it cannot be used for the resolution of the very interesting group of iridoid acids. However, this solvent system can be successfully employed for a fractionation of the multi-component mixtures into acidic and non-acidic components.

Isocratic Elution with Water-Methanol-Phosphoric Acid (85:15:0.02)

As seen from Table 1, in the presence of acid, the k' values of compounds **1**, **2**, **4**, **6**, **7** and **9** increased while those of the **3**, **5**, **8** and **10** remained constant. The elution order changed to **1** < **2** < **3** < **4** << **5** = **6** = **7** << **8** < **9** <<< **10**. The chromatogram on Figure 3 reveals that despite the low k' values (<1) a partial resolution of **1**, **2**, **3** and **4** was achieved. Moreover, the chromatographic system was able to differentiate between the diastereomers with different stereochemistry at C-6. Compound **2**, with -OH in α-position, eluted ahead of **4** with -OH in β-position. The two compounds were fully resolved with a resolution factor of 1.2. The retention of compounds **6**, **7**, and **9** was also substantially

affected; the k' of **6** increased from 0 to 1.2, the k' of **7** from 0 to 1.3, and the k' of **9** from 0.2 to 3.2. Unfortunately, as a result of the increased retention of **6** and **7**, a mixed peak with compound **5** (k' = 1.2, not affected by the presence of the acid) was formed. Evidently under isocratic elution the absence of -OH group at C-6 (as in **7**), the presence of an acetylated -OH group at C-6 (as in **6**) and the methylated -COOH group at C-4 (as in **5**) had the same effect on the retention. Considering the role of the functional groups, the elution pattern in acidic aqueous methanol can be presented as:

[(-COOH) (-OH at C8) ($-CH_2OH$)] < [(-COOH) (-OH in α-position at C-6) ($-CH_2OH$)] < [(LACTONE RING)($-CH_2OH$)] < [(-COOH)(-OH in β-position at C-6) ($-CH_2OH$)] < [($-COOCH_3$) (-OH) ($-CH_2OH$)] = [(-COOH) ($-OCOCH_3$) ($-CH_2OH$)] = (-COOH) ($-CH_2OH$)] << [(LACTONE RING) ($-CH_2OCOCH_3$)] < [(-COOH)(-OH)($-CH_2OCOCH_3$)] <<< [($-COOCH_3$)(-OH)($-CH_2OCOCH_3$)].

The water-methanol-phosphoric acid mobile phase was promising in the efforts to improve the resolution of the iridoid glucosides under examination. The efficiency of a gradient elution was examined next with the aim to improve the separation of compounds **1** to **4** and to resolve compounds **5**, **6**, and **7**. Preliminary experiments with different linear and stepwise gradients on a single column did not lead to any substantial improvement, however. Better results were achieved on two ODS columns connected in series, with a stepwise gradient from water-methanol-phosphoric acid (95:5:0.015, v/v/v) to 100% methanol over 60 min., as is demonstrated on Figure 4 for the mixture of reference compounds.

These conditions provided satisfactory separation of all components, but not of compounds **5** and **6** which still formed a mixed peak only partially resolved from **7**. Thus, despite the gradient elution mode and the elongation of the elution path, the present system was not able to differentiate between a solute with a free -OH in α-position (**5**) and acetylated -OH group in β-position (**6**) at C-6. On the other hand, compounds **2** and **4** (eluting in this order), which differed by the stereochemistry of the free -OH groups at the same carbon atom (α- and β-, respectively), were fully resolved.

Evidently, substitutions in the functional groups which decreased the overall polarity of the molecule are the most important but evidently not the single factor affecting the retention and resolution of iridoids in RP-HPLC. Other factors, like stereochemistry, intra-molecular interactions may have also a significant impact. Also, the use of RP-HPLC for the separation of iridoid glucosides has certain features that must be taken into account in the further efforts to improve the resolution. The solutes are highly polar compounds with a sugar moiety and up to three additional functional groups of different polarity. The RP-HPLC separation requires water-rich mobile phases.

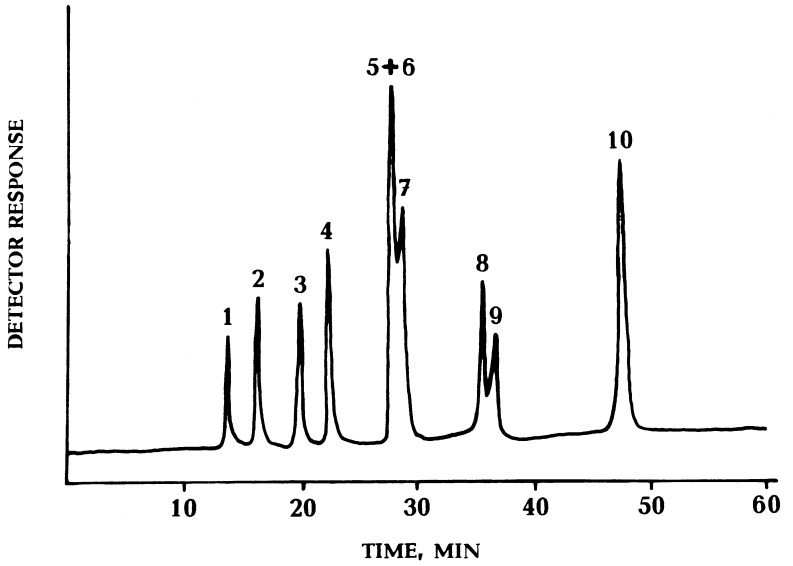

Figure 4. Chromatogram of the standard mixture of iridoid glucosides 1 - 10. Conditions: two Partisil Whatman ODS-3 (10 μm), 25x0.46 cm I. D. columns connected in a series; mobile phases: A, water:methanol:phosphoric acid (95:5:0.015, v/v/v); B, methanol, gradient: from 0 to 45% B over 45 min, to 60% B over 5 min, and to 100% B over 10 min; flow rate: 0.8 ml/min; UV detection at 233 nm.

It has been supposed that due to the interaction between the mobile and stationary phases, the grafted C18 chains fold on the top of each other, resulting in a more rigid structure of the stationary phase. The retention mechanism is eventually of a partition type.[19]

Because of the bulky structure and the high polarity of the iridoid molecules, stronger interactions between the solutes and the mobile phase solvents can be expected which supposedly govern the retention and resolution. The result is the observed relatively weak retention of the most polar components on octadecyl columns and the relatively low selectivity of separation. Because of the complexity of the interactions it was not possible to give a quantitative evaluation of the impact of the functional groups on the retention at this stage.

The gradient elution on two ODS columns was found to be a suitable compromise for a "fingerprint" analysis of plant extract as is demonstrated with the examples shown on Figure 5. The resolution achieved in the present work is

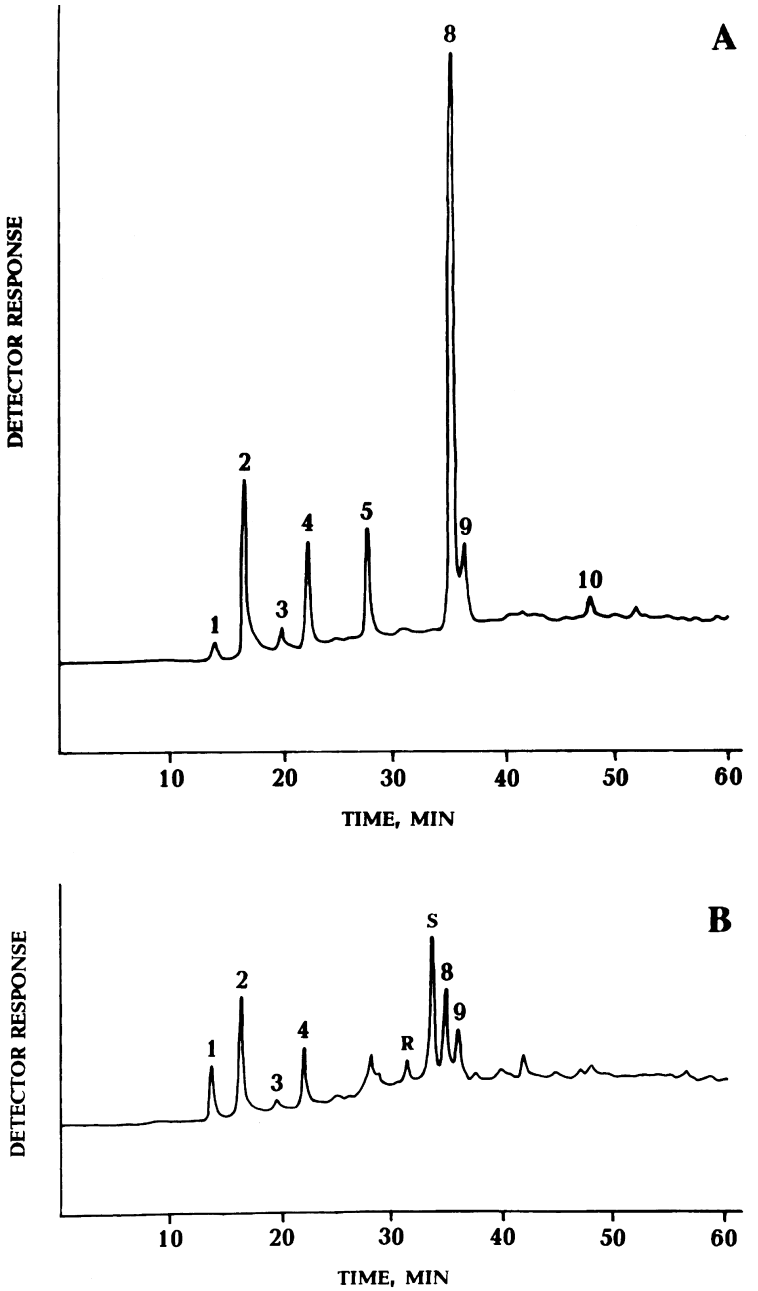

Figure 5. Chromatogram of plant extracts of *Galium macedonicum* (A) and *G. album* ssp. *album* (B). Conditions as in Figure 4.

better than those reported previously for iridoid glucosides of *Galium* species in that more components have been resolved to a satisfactory extent.

We assume, however, that the question whether resolution of mixed peaks can be achieved and whether RP-HPLC is, in general, the best method for analysis of iridoid glucosides remains open.

ACKNOWLEDGMENTS

The financial support of the Bulgarian National Foundation for Scientific Research (contract X-513) is gratefully acknowledged. Thanks are also due to Miss M. Mitova from the Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, for kindly providing the iridoid substances and to Prof. S. Popov from the same Institute for the helpful discussion.

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Received May 6, 1999 Author's Revisions October 18, 1999
Accepted May 20, 1999 Manuscript 5068