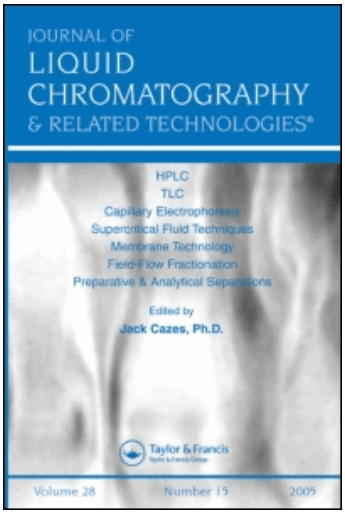

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RETENTION OF IRIDOID GLUCOSIDES ON OCTADECYLSILANE AND DIOL COLUMNS

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ABSTRACT

The retention behavior of ten iridoid glucosides on reversed octadecylsilane and normal diol stationary phases is compared. To consider in quantitative terms the experimental results, structure-retention relationships have been studied. When free rotation around σ-bonds in the solute molecule during the chromatographic process was taken into account, increased accuracy of calculated retention was obtained. The values of positive, respectively negative contribution of the same functional groups to the solute molecule retention on each column were calculated.

INTRODUCTION

Iridoid glucosides are minor components in the polar extracts of some Dicotyledone plants. They belong to a large group of monoterpenoids with partially hydrogenated cyclopenta[c]pyrane system with a glucose moiety attached to C-1 in the pyrane ring and free or substituted hydroxyl and carboxyl groups in the aglycone. 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 often have a specific iridoid composition, and

this feature was supposed to be of use for chemotaxonomical and evolution studies.[4,5]

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the most widely explored analytical technique so far and many procedures have been offered. They are limited mostly to the resolution of simple mixtures of few compounds or to isolation and purification of individual compounds.[1,3,4,16-14]

The main problems in RP-HPLC analysis are connected with the high hydrophilicity of most of them. The combination of free and substituted hydroxyl and carboxyl functional groups results in a weak retention on RP columns, in diminished selectivity and in formation of mixed peaks. This has recently been illustrated.[15] No satisfactory separation of ten iridoids glucosides, isolated from plants of genus *Galium*, on a reversed-phase octadecylsilane (ODS) column has been achieved under various chromatographic conditions. It appeared that iridoid glucosides, especially those with free carboxyl group, were weakly retained and poorly resolved.

No substantial improvement was achieved by changing the mobile phase pH and composition, the elution mode or by addition of an ion-pair agent. All acidic iridoids, monotropein especially, have k' values bellow 1.0 and hence their retention is without practical meaning. Besides, at no conditions, were resolution of 6-O-acetylscandoside and deacethylasperulosidic acid methyl ester achieved.[15]

The task of the present work is to study the retention of the same iridoid glucosides on a normal (diol) stationary phase and to estimate, in quantitative terms, the impact of the structure on the retention on both ODS and diol columns by using computing methods. These methods allow the connection of molecule structure descriptors with the experimental retention. The achieved accuracy can serve as criterion for the successful selection of molecule descriptors. The selected descriptors will help for explanation of the reasons for the observed retention and their magnitude will probably show the direction of necessary changes in chromatographic condition. Besides, from the chromatography point of view, the calculated k' values can serve for peak identification with a minimum number of certified reference materials.

EXPERIMENTAL

Materials and Reference Substances

HPLC-grade acetonitrile and tetrahydrofuran (E. Merck, Darmstadt, Germany) were used.

The reference iridoid glucosides were isolated from different *Galium* species, purified and characterized by spectral methods (MS, ^{1}H, and ^{13}C NMR).[16] Stock solutions in methanol were prepared from the solid substances with concentrations in the range 0.40 - 0.90 mg/mL. The structures of the iridoid glucosides under study are shown on Figure 1. Compounds **2**, **4**-**7**, **9**, and **10** have the same basic carbon skeleton and the same position of the functional groups at C-4, C-6 and C-8 (see compound **2** for the numbering of carbon atoms in the skeleton), but differ by the number, the type, and the stereochemistry of these groups.

Compound **1**, monotropein, differs by the positions of the double bond and the hydroxyl group in the cyclopentane ring. Compounds **3** and **8** are the lactones of compounds **2** and **9**, respectively.

HPLC

The chromatographic analyses were performed on a Perkin Elmer Series 2 liquid chromatograph equipped with a LC-75 multi-wavelength UV detector (Perkin Elmer Corporation, Norwalk, Connecticut, USA), and a Rheodyne 7125 sample loop injector (20 μL) (Supelco, Gland, Switzerland). The chromatograms were registered with a computing integrator Chromatopac C-R3A (Shimadzu Corporation, Kyoto, Japan).

The chromatographic conditions on ODS column are described elsewhere.[15] The experiments on normal-phase were carried out at room temperature on a Hibar LiChrospher 100 DIOL (10 μm) stainless-steel column (25 x 0.4 cm I. D.) with a preliminary optimized mobile phase of acetonitrile-tetrahydrofuran (98:2, v/v). Flow rate was 1.0 mL/min.

The examined iridoid glucosides have a maximum absorbency in relatively narrow range (232 - 239 nm).[17,18] A working wavelength of 233 nm was chosen for this work since, at this wavelength all reference compounds were detected with good sensitivity.

The hold-up time (3.0 ± 0.1) was measured by the negative peak of an air bubble. Retention times were determined as the mean of three parallel measurements with a standard deviation not exceeding 0.1. Injection volume was 10 μL. The k' values of the iridoid glucosides on both ODS and diol columns are shown in Table 1.

The separation on the diol column is depicted on Figure 2 and the corresponding k' values are also given in Table 1. It is evident that the k' value of the first eluting component from the diol column (compound **8**) was about five

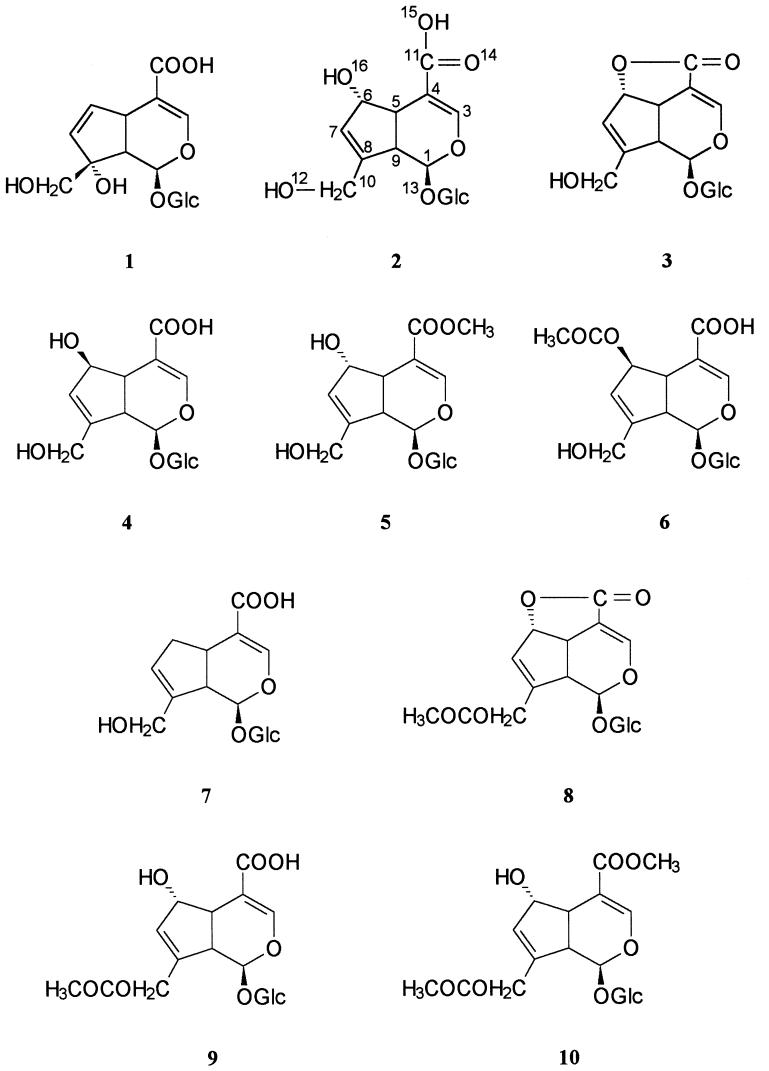

Figure 1. Structures of the examined iridoid glucosides, Glc denotes the glucose moiety; 1, Monotropein; 2, Deacetylasperulosidic acid; 3, Deacetylasperuloside; 4, Scandoside; 5, Deacetylasperulosidic acid methyl ester; 6, 6-O-Acetylscandoside; 7, Geniposidic acid; 8, Asperuloside; 9, Asperulosidic acid;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

Table 1

Comparison of the Experimental and Calculated with Equations (2) and (3) k' Values of Studied Iridoid Glucosides on Both ODS and Diol Stationary Phases

Compound No.	k'_{exp} ODS	k'_{calc} ODS	k'_{exp} Diol	k'_{calc} Diol
1	0.17	-0.15	7.75	7.85
2	0.25	0.30	12.70	12.75
3	0.49	1.00	1.93	2.59
4	0.70	0.69	8.50	7.88
5	1.43	1.25	4.43	4.50
6	1.50	1.66	3.95	4.48
7	1.61	1.70	5.46	5.35
8	3.49	3.33	0.93	0.25
9	3.98	3.82	3.95	3.80
10	13.60	13.63	1.27	1.39

times higher than the k' value of the first eluting peak (compound **1**) on the ODS column.

Computing Method

The calculations were performed by the OASIS software.[19-21] Because all compounds have glucose moiety attached to C-1, its influence on retention was assumed to be equal for all compounds, therefore, the glucose moiety has been replaced in the calculations by -OCH_3 group. Only the descriptors of the aglycone moiety have been calculated. On account of the limited number of compounds, the number of descriptors included in the equation was limited to be not more than three, according to the statistical demands for lack of occasional regression.[22]

The following model for creation of regression equations, proposed,[23] has been used:

$$R = b_0 + b_1 B + \Sigma b_i T_i \qquad (1)$$

where **R** is the retention (the capacity factor, **k'**, in HPLC), $\mathbf{b_0}$ is the intercept, $\mathbf{b_1}$ and $\mathbf{b_i}$ are parameter estimations; **B** is a descriptor with basic contribution to the experimental retention, which allows to calculate k' close to the

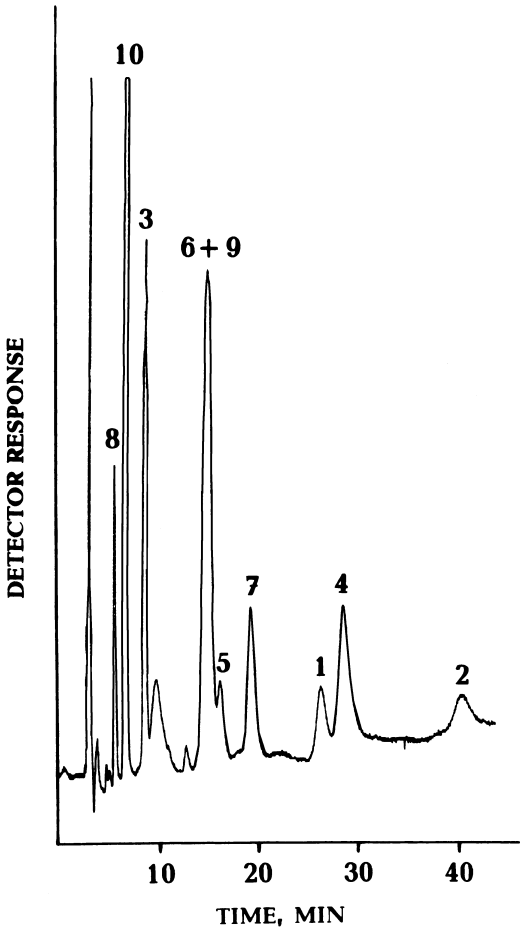

Figure 2. Chromatogram of the examined iridoid glucosides: Hibar LiChrospher 100 DIOL column; mobile phase acetonitrile - tetrahydrofuran (98:2, v/v); flow rate: 1.0 mL/min.

experimental k' values. $\mathbf{T}_i$ are tuning descriptors, which ensure better fit between both experimental and calculated k' values. The descriptor values are calculated for free state of the solute molecule ("classical" approach).

It has been supposed, further, that more accurate results could be obtained if the proposals given[24-26] had been followed. The authors assumed, that due to the released interaction energy several conformational changes in the solute

molecule can occur during the sorption process. To distinguish the newly obtained structures, arising in/on the stationary phase from the conformers of molecule in free state, the former had been called "rotamers".

Because the higher the accuracy, the more reliable the interpretation of the obtained regression equations, corresponding "rotamers" have been constructed by rotation around σ-bonds of substituents. The calculations of descriptor values, therefore, include not only the structures of conformers with the lowest energy, as it is commonly accepted, but also structures which are not in the global minimum, but are in a local minimum.

Forty six rotamers of investigated compounds have been constructed. A total of 71 structure descriptors (quantumchemical, topological, and geometrical indices), as well as some physicochemical properties and some indicator variables have been used in the regressions.

RESULTS AND DISCUSSION

Of interest is the method of estimating quantitatively the contribution of both global and local molecule features on retention. Molecular mass, molecular refraction, connectivity index, hydrophobicity (e.g. as logP), etc., are global descriptors. Atom charges, acceptor or donor delocalization energies etc., as well as, defined molecule fragments (e.g. functional groups), are local descriptors.[27]

Usually, the global descriptors are basic contributors to the rough calculated retention value. Local descriptors tune this rough value to the experimental retention. One of the questions in the present study is to determine which iridoid features contribute to the obtained retention value.

It is logical to relate the experimental k' on ODS column directly to the hydrophobicity of the analyte. The attempt failed to be suitable. Also, the other studied global descriptors did not give encouraging results.

The attention was directed, therefore, to find local descriptors (functional groups) which contribute substantially to the retention on both ODS and diol stationary phases.

Relationships on ODS Column

The equation obtained with descriptor values calculated at minimum energy of iridoid molecules (minimum of calculated heat of formation, CHF_{min}) had

correlation coefficient, $r^2 = 0.6974$ and variance, $v = 7.39$. Evidently the "classical" QSRR approach was not practical in this case.

There were several statistically equal equations, obtained after adding several rotamers for every iridoid. Two examples are depicted on Figure 3. The

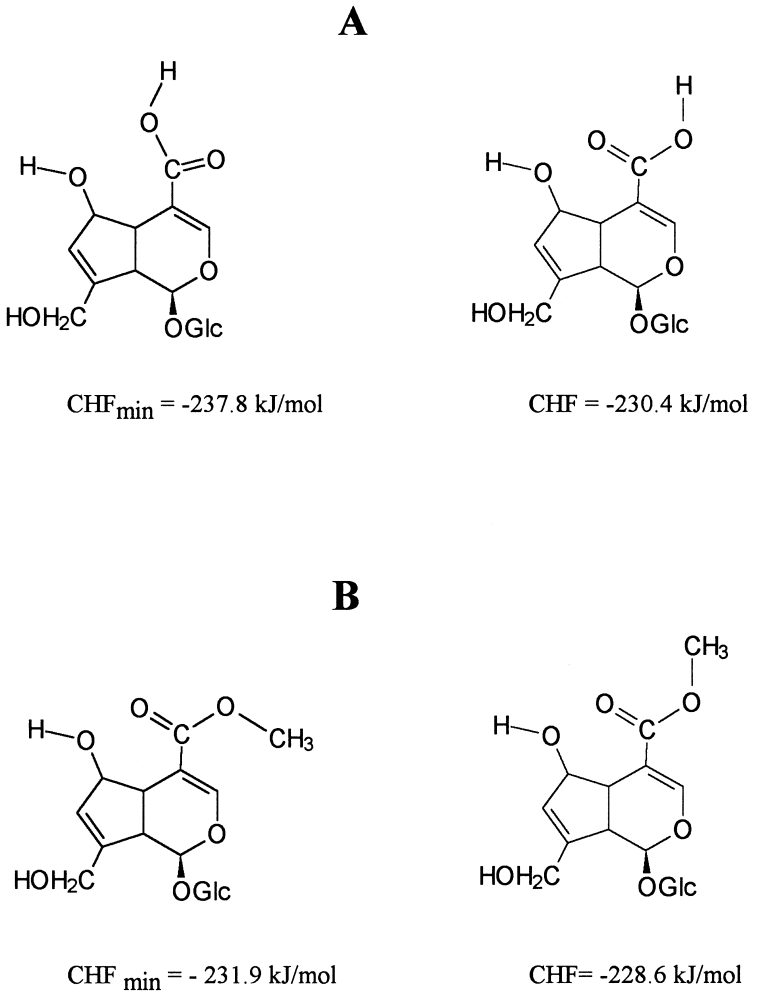

Figure 3. Comparison of conformers at CHF_{min} with preferred rotamers at higher energy; A: on ODS column; B: on diol column.

most accurate, i.e. giving minimal discrepancy between k'_{exp} and k'_{calc} is the equation:

$$k' = 212.2(\pm 9.5) + 176(\pm 5)\ Q^{[12]} - 422(\pm 24)\ Q^{[11]}$$

$$+ 9.1(\pm 0.8)\ \text{pop HOMO}^{[14]} \qquad (2)$$

with correlation coefficient, $r^2 = 0.9967$ and variance, $v = 0.0803$. The experimental and the calculated k' values of the iridoid glucosides are shown in Table 1. The coefficients of individual correlation (ir) of descriptors and of interrelation (r_i) between the descriptors are given in Table 2. Equation (2) was tested for reliability with "leave-one-out" approach and show no occasional regression.

$Q^{[12]}$ stands for the electron charge of the hydroxyl oxygen atom at C-10, i.e., it accounts for the effect of $-CH_2OH$ group. $Q^{[11]}$ stands for the electron charge of carboxylic C-11. Pop $HOMO^{[14]}$ stands for the electron population on the highest occupied molecular orbital of the carbonyl oxygen atom at C-11. Evidently, the carboxylic functional group is presented by two local descriptors, which do not interrelate. $Q^{[12]}$ has a negative value while the value of $Q^{[11]}$ is positive.

Taking into account the parametric estimation signs, these two descriptors contribute to diminish the retention. Pop $HOMO^{[14]}$ has a negligible average contribution (see equation 2 and Table 3) and its sign changes depending on the respective structure, resulting in significant tuning effect on the calculated k' values.

Equation (2) allowed:

i) to predict correctly the elution order of all compounds but one, which fell in the region of $k' < 1$ and, hence, can not be exactly assessed quantitatively;

Table 2

Individual Correlation, ir, and Interrelations, r_i of Parameters of Equations (2) and (3)

Parameter ODS	ir	r_i $Q^{[12]}$	$Q^{[11]}$	Parameter Diol	ir	r_i $Q^{[15]}$	n_{CH3}
$Q^{[12]}$	0.835	---	0.055	$Q^{[15]}$	0.677	---	0.146
$Q^{[11]}$	0.261	0.055	---	popLUMO$^{[10]}$	0.003	0.077	0.220
popHOMO$^{[14]}$	0.315	0.371	0.426	n_{CH3}	0.405	0.146	---

Table 3

Average Individual Absolute Contribution of Any Descriptor to the Experimental Retention Value (in %) Excluding Intercept

Descriptor ODS	Contribution	Descriptor Diol	Contribution
$Q^{[12]}$	about 29	$Q^{[15]}$	about 77
$Q^{[11]}$	about 71	popLUMO$^{[10]}$	varies
popHOMO$^{[14]}$	± correction	n_{CH3}	about 12

ii) to explain the reason for the weak retention on ODS column - the valuable descriptors are connected with the -COOH functional group and their contribution diminishes the possible retention.

Relationships on Diol Column

The equation obtained with the descriptor values calculated at minimum energy of iridoid molecules had correlation coefficient, $r^2 = 0.829$ and variance, $v = 3.46$. Evidently, this equation was also with no practical meaning in the studied case.

When considering several rotamers for each iridoid, only one equation was obtained which was statistically correct and satisfactory exact (i.e. giving minimum discrepancies between k'_{exp} and k'_{calc}):

$$k' = -19(\pm 2) - 72(\pm 4)\, Q^{[15]} - 3(\pm 0.3)\, n_{CH3} - 399(\pm 43)\, \text{pop LUMO}^{[10]} \quad (3)$$

with $r^2 = 0.9866$ and $v = 0.273$. The experimentally obtained and the calculated k' values are shown in Table 1. The coefficients of individual correlation (ir) of descriptors and of interrelation (r_i) between the descriptors are given in Table 2. Equation (3) was tested for reliability with "leave-one-out" approach and show no occasional regression.

Here, $Q^{[15]}$ stands for the electron charge of hydroxyl oxygen atom in -COOH, popLUMO[10] stands for the electron population of the lowest unoccupied molecular orbital at C-10 (i.e. it accounts for the -CH_2OH group) and n_{CH3} accounts for the total number of substituted functional groups. $Q^{[15]}$ has a negative sign. Taking into account the signs, $Q^{[15]}$ has a positive contribution to the retention, pop LUMO[10] has tuning effect, while n_{CH3} has a negative contribution.

The contribution of $Q^{[15]}$, i.e. of the -COOH group was about five times higher than that of pop LUMO[10] (Table 3) which means that as with the ODS column, the -COOH group again plays a marked role. On the diol column the elution order was exactly predicted without exceptions. It can be expected, that with a more effective column compounds **6** and **9** can be separated.

A very good agreement between the experimental and predicted k' values both on ODS and diol columns were achieved. It is considered, that these results are due to the application of the new approach, which includes rotamer structures. If descriptor values have been calculated at minimum energy of iridoid molecule only, no clear distinguishing of functional group contribution can be found.

CONCLUSION

The good agreement between the experimental and calculated k' values shows that the QSRR calculations using rotamers is helpful for better understanding of the impact of the functional groups in the iridoid molecule on the retention. Certainly, experienced chromatographers can assume, that the presence of -COOH and -CH_2OH groups decreases the solute retention on the ODS column and increases the retention on the diol stationary phase. It is not possible, however, to estimate the magnitude of this effect.

Here, for the first time, a quantitative estimation of functional group contribution to the retention is given for iridoids. It was also confirmed, that to achieve better accuracy, creation of structures with higher than global minimum energy must be taken into account. The results revealed, that these might indeed take place in the practice and should not be neglected.

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