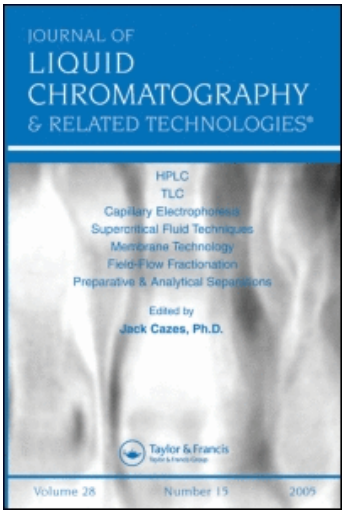

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Retention Indices as the Most Reproducible Retention Parameters in Reversed Phase HPLC. Calculation for Hydrophilic Phenolic Compounds Using Reference n-Alkyl Phenyl Ketones

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Retention Indices as the Most Reproducible Retention Parameters in Reversed Phase HPLC. Calculation for Hydrophilic Phenolic Compounds Using Reference n-Alkyl Phenyl Ketones

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Abstract: A comparison of various presentation forms of retention parameters measured in multi-step gradient elution regimes in reversed phase HPLC indicates that retention indices in the scale of *n*-alkyl phenyl ketones (so-called Smith′s RI system) have the maximal reproducibility. However, the determination of these parameters for most hydrophilic compounds eluted before the first reference acetophenone (RI = 800) implies their calculation by extrapolation, that can lead to the high irreproducibility of results, especially in regimes with initial isocratic stages followed by fast gradient elution. This fact explains the necessity of elaboration of a new calculation method based on the extrapolation of retention concentrations, which provides the appropriate interlaboratory coincidence of RI values at least within the range 600–800 i.u. Using this algorithm, retention indices for about 60 phenolic compounds have been determined, that permits us to identify them in complex

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mixtures of natural plant's extractive substances using HPLC without corresponding reference samples.

Keywords: Organic compounds of phenol series, Reversed-phase HPLC, Retention indices, Reference *n*-alkyl aryl ketones, Extrapolation, Interlaboratory reproducibility

INTRODUCTION

Many natural plant extractive substances belong to the series of phenol and polyphenol compounds, including simplest alkyl phenols, esters of hydroxylated arenecarboxylic acids, flavonoids, antocyanidines, tanins, lignanes, and so on. Most of compounds of these groups indicate various kinds of biological activity, like antioxidant, antiradical, hepatoprotective, anti-inflammatory, anticancerogenic, etc. (see, e.g.,[1–10]). In spite of wide application of these compounds in medicinal practice, the mechanisms of their action *in vitro* and *in vivo* still remain unknown. One of the most important analytical problems for these objects seems to be the absence of standards needed for their chromatographic identification. Hence, the success in characterization of natural phenolic compounds implies the development of reliable methods of their identification first of all.

Reversed phase high performance liquid chromatography (RP HPLC) (such as LC-MS hyphenated techniques) seems to be the preferable analytical method for semi- and non-volatile compounds of these series. The "traditional" application of HPLC implies the comparison of analytical parameters of unknown compounds with those of reference samples, namely raw retention times (t_R), relative retention times ($t_{R,rel} = t_R/t_{R,stand}$), retention factors (capacity coefficients, $k' = (t_R - t_0)/t_0$, where t_0 is hold-up time), their logarithms ($\log k'$), etc. However, all of them cannot be considered as the interlaboratory constants of analytes, but rather present their properties at fixed conditions of chromatographic separation (depending on the parameters of LC column, composition of eluent, and its flow rate, etc.), and, hence, cannot be classified as reference data. Undoubtedly, any additional (spectral) parameters are useful in HPLC identification, namely not only complete UV-VIS spectra registered by diode array detectors (DAD),[11] but even relative absorbencies at fixed wavelengths $A_{rel} = A(\lambda_1)/A(\lambda_2) = P(\lambda_1)/P(\lambda_2)$.[12] Their determination requires the calculation of peak areas or height ratios after duplication of analyses at various wavelengths (at the equal injected samples). Nevertheless, the problem of invariant presentation of retention parameters in HPLC seems extremely important at present.

By analogy with gas chromatography (GC), retention indices (RI) seem to be the most reproducible form of retention data presentation:

$$RI_x = RI_n + (RI_{n+1} - RI_n) \times [f(t_{R,x}) - f(t_{R,n}))]/[f(t_{R,n+1}) - f(t_{R,n})] \quad (1)$$

where $t_{R,x}$, $t_{R,n}$ and $t_{R,n+1}$ are retention times of analyte and reference components usually corresponding to the following inequality: $t_{R,n} < t_{R,x} < t_{R,n+1}$ (calculation by interpolation).

An application of any RI system requires at first the choice of the set of reference compounds. In GC most of them are based on the easily available *n*-alkanes, C_nH_{2n+2} with postulated values $RI_n = 100n_C$. In RP HPLC, the most "popular" RI system implies the use of the series on *n*-alkyl phenyl ketones, $PhCOC_nH_{2n+2}$ (so-called Smith's RI system[13,14]) having the same postulated RI values $RI_n = 100n_C$. Other propositions are based on the sets of *n*-alkyl benzenes, 1-nitroalkanes,[15] etc., but at present they have not received a spread application.

A special problem is the choice of function $f(t_R)$ needed for calculation of RIs with relationship (1). In isocratic regimes of HPLC elution the following general regularity is fulfilled:

$$\log(t_R - t_0) = an_C + b \text{ or (equivalent)} \quad RI = a' \log(t_R - t_0) + b' \quad (2)$$

Hence, the function $f(t_R)$ should be log $(t_R - t_0)$, that is the ground of Kovats RI system.[16] In various regimes of gradient elution (analogous to the temperature programming in GC) the system of linear RI is preferable $[f(t_R) = t_R]$.[17] The last type of this function is the generalization of both previous ones into the so-called lin-log RI system.[18,19]:

$$f(t_R) = t_R + q \log(t_R - t_0) \quad (3)$$

The variable parameter q can be calculated using t_R data for at least three reference compounds, for instance (the simplest case) three consecutively eluted standards:

$$q = (t_{R,n-1} + t_{R,n+1} - 2t_R)/(2 \log t'_{R,n} - \log t'_{R,n-1} - \log t'_{R,n+1}) \quad (4)$$

In general, any sets of reference compounds can be used for calculation of this auxiliary parameter.[20]

The most important difference in application of RIs in GC and HPLC is connected with the RI value of the first reference compound. If we consider the simplest *n*-alkane (methane CH_4), only a few (preferably inorganic) compounds are characterized by $RI < 100$, when their calculation using formula (1) needs extrapolation. For example, the list of these compounds for polymer sorbent Porapak Q is restricted by components of air (N_2, O_2 with $RI \approx 50 \pm 15$), CO (60 ± 6), Ar (62 ± 11), NO (80 ± 16), and CF_4 (83 ± 9).[21] All other multitudes of possible analytes having $RI > 100$ can be characterized by RIs calculated with formula (1) using interpolation at the appropriate choice of reference *n*-alkanes.

In RP HPLC, the situation is principally different. The first reference component from a series of *n*-alkyl phenyl ketones, acetophenone, is a highly hydrophobic substance ($\log P = 1.66 \pm 0.06$.[22,23]). Hence, there is a lot of more hydrophilic organic compounds, which should be eluted

before acetophenone. This means that the problem of RI calculation by extrapolation outside the range restricted by reference compounds is typical for HPLC data processing. There is no problem with RI extrapolation in isocratic regimes, owing to the existence of the general regularity (2): experimental determination of retention times for two reference components [$t_R(1)$ and $t_R(2)$], and hold-up time t_0 permits us to calculate any RI values within the interval $t_0 < t_{R,x} \leq t_R(2)$ and even more (by extrapolation "up"). Numerous organic compounds are characterized by values $RI < 800$ (see, e.g.,[14]) presented without any discussions of their calculation parameters. Most of them have been determined just at isocratic conditions.

Unfortunately, this simplest mode of calculations is inapplicable at any regimes of gradient elution, especially in multi-step regimes combining isocratic and gradient stages, because of the absence of appropriate function $RI(t_R)$ at the range $t_0 < t_{R,x} < t_R(1)$.

The present work is devoted to elaboration of the new approach to RI (HPLC) calculation for hydrophilic organic compounds eluted before the first reference component (acetophenone), under gradient regimes, with an initial isocratic period often used in analytical practice. Quality control of this method was performed by comparison of interlaboratory data and its application in RI determinations for some natural plant extractive compounds of the phenol and polyphenol series.

EXPERIMENTAL

The following HPLC eluents and chemicals have been used. Organic solvents: acetonitrile (HPLC grade, "Kriokhrom", St. Petersburg, Russia), methanol (for HPLC, Merck, Germany); orthophosphoric acid (85%, HPLC grade, Sigma); water (triply distilled, purified using Millipore filtering system, Milli-P QG, Waters).

The primary solutions of characterized compounds were prepared by dissolving their exact amounts in methanol. The samples for HPLC analyses were prepared by additional dilution of primary solution by acetonitrile.

The following phenolic compounds were taken for characterization:

- *monofunctional phenols* (HPLC grade, Sigma): phenol, 2-methylphenol (*o*-cresol), 3-methylphenol (*m*-cresol), 2,3-dimethylphenol (*o*-xylenol), 2,5-dimethylphenol (*p*-xylenol), 3,5-dimethylphenol (*symm-m*-xylenol);
- *polyfunctional phenols:* 1,2-dihydroxybenzene (pyrocatechol), 1,3-dihydroxybenzene (resorcinol), 1,4-dihydroxybenzene (hydroquinone), 5-metylresorcinol (orcinol), 1,3,5-trihydroxybenzene (phloroglucinol), 1,2,3-trihydroxybenzene (pyrogallol), 3,4,5-trihydroxybenzoic acid propyl ester (propyl gallate);
- *hydroxybenzoic acids, aldehydes and ketones* (isolated from natural raw material and purified in Georgian Agricultural Institute, Georgia):

1-acetyl-4-hydroxy-3-methoxybenzene (acetovanillone), 1-acetyl-4-hydroxy-3,5-dimethoxybenzene (acetosyringone), 4-hydroxy-3-methoxybenzaldehyde (vanillin), 4-hydroxy-3-methoxybenzoic (vanillic) acid, 4-hydroxy-3,5-dymethoxybenzoic (syringic) acid, 3,4-dihydroxybenzaldehyde (protocatechuic aldehyde), 3,4-dihydroxybenzoic (protocatechuic) acid, 3,4,5-trihydroxybenzoic (gallic) acid, ellagic acid;

- *cinnamic and hydroxycinnamic acids and aldehydes* (isolated from natural raw material and purified in Georgian Agricultural Institute, Georgia): 3-phenyl-2-propen-1-ol (cinnamic alcohol), 3-phenyl-2-propenoic (cinnamic) acid, 3-(4-hydroxy-3-metoxyphenyl)-2-propen-1-ol (coniferyl alcohol), 3-(4,5-dihydroxyphenyl)-2-propenoic (caffeic) acid, (Z) and (E)-3-(3-hydroxy-5-metoxyphenyl)-2-propenoic (ferulic) acid;
- *stilbenes*: 4-hydroxystilbene (99%, Lancaster), 3,5,4′-trihydroxystilbene (*trans*-resveratrol, synthesized by K.E.Polunin and X.-G. Schmaltz;[24]);
- *flavonoids* (HPLC grade, Fluka): 3,5,7,4′,5′-pentahydroxyflavan (catechol and *epi*-catechol), 3,5,7-trihydroxyanthocyanidine (pelargonidin) chloride (isolated from natural raw material and purified in Ukraine Botany Institute, Kiev, Ukraine), 3′,4′,5,7-tetrahydroxyflavon (luteolin), 2′,3,4′,5,7-pentahydroxyflavon (morin), 3,3′,4′,5,7-pentahydroxyflavon (quercetin), 3′,4′,5,7-tetrahydroxyflavon-3-rutinoside [rutin, Rut = β-D-Glcp ← (6)-α-L-Rha], 3,3′,4′,5,7-pentahydroxyflavanon (dihydroquercetin, synonymous taxifolin), 4′,5,7-trihydroxyflavanon (naringenin);
- *some related compounds, having no phenolic OH groups:* benzaldehyde, benzoic acid, cinnamic aldehyde, coumarin, 2,4-dimethoxybenzaldehyde, 3,4-dimethoxy (veratric) aldehyde, 2,3,4-trimethoxybenzaldehyde;
- *other compounds* (from various sources) used as reference samples in analytical practice of interregional center "Adaptation": 4-hydroxybenzaldehyde, α-naphthol, 5-methyl-2-(1-methylethyl)phenol (thymol), 7-hydroxycoumarin (umbelliferon and its acetate), 6,7-dihydroxycoumarin (esquletin), anthron, 7-hydroxyflavon, 7-hydroxy-4′-methoxyisoflavon (formononetin), 3′,5,7-trihydroxyisoflavon (genistein), 5,7-dihydroxy-4′-methoxyisoflavon (biochanin A), 3, 4′,5,7-tetrahydroxyflavon (kaempferol), 3,3′,4′,7-tetrahydroxyflavon (fisetin), 3,3′,4′,5,5′,7-hexahydroxyflavon (miricetin), luteolin-7-glucoside (cinarosid).

Reversed phase HPLC analyses (regime I) were carried out using Agilent 1100 HPLC chromatograph (Agilent Technologies, USA) equipped with diode-array detection (DAD) and steel column 100 × 2.1 mm with Hypersil ODS (3 μm) and precolumn, 20 × 2.1 mm with Hypersil ODS (5 μm). The injected volume was 10 mkl (Microsyringe Hamilton, Reno, USA). Eluents: acetonitrile and water solution of phosphoric acid with pH 3.5; eluent flow 300 mkl × min^{-1}. The following fast gradient regime of elution was used: 0–3 min–isocratic 5% MeCN; 3–18 min 5→100 % MeCN; 18–20 min–isocratic 100% MeCN; 20–25 min–100→5% MeCN; 25–30 min–isocratic 5% MeCN. Detection wavelengths were 240, 264, 280, 334. and 513 nm.

All analytes have been characterized by λ_{max} values in complete UV-spectra registered with DAD.

Regime (II) of HPLC analyses was realized on a HPLC chromatograph, Beckman System Gold with UV detector, the column Luna C18 (150 × 4.6 mm, 5 μm) and precolumn (20 × 4.6 mm) with the same sorbent. Eluent: acetonitrile (the same grade as that in regime I) and 0.03% water solution of trifluoroacetic acid (pH 2.8–3.0), eluent flow 1.0 mL/min. Initial acetonitrile concentration was 10% (without isocratic step), ramp 1%/min up to 90%, or until the last peak has been eluted. Injected volume 20 mkl, detection wavelengths 220 and 254 nm. Data processing has been provided by software GOLDV402.

The set of reference *n*-alkyl phenyl ketones $PhCOC_nH_{2n+1}$ (from acetophenone, RI = 800, to enantophenone, RI = 1300, Tetra-Elsiko, Moscow, Russia) was used in the determination of retention indices. Within RI range 800–1000 i.u., all compounds were characterized by lin-log retention indices calculated using standard procedure.[18–20] Two algorithms were used for calculation of the RI values for compounds with $t_R < t_R(R1)$ (R1 = acetophenone, RI = 800) by extrapolation. One of them [used in regime (II)] has been proposed previously[25,26] for slow gradient elution without initial isocratic stages, while the second one [used in regime (I)] is developed specially for data processing measured in stepwise elution regimes, including initial isocratic stages followed by fast gradients. The simplest QBasic programs for RI calculation by both of these algorithms are presented in appendices 1, 2.

DISCUSSION

The optimization of HPLC separation conditions for determination of various phenolic compounds in plant extracts (so-called observed or prospective analyses) lead us to the choice of fast gradient elution regime (5→100% CH_3CN at 15 min) preceded by an initial isocratic step (5% CH_3CN during 3 min). Of course, owing to objective reasons, all these conditions are not absolutely fixed and may differ from one laboratory to another, including the HPLC instrument and the type of LC column. It is difficult to foresee all possible variations of them in the model experiments at one analytical laboratory. Nevertheless, we have modeled the influence of variations of gradient elution regimes (duration of initial isocratic stages and ramps), temperatures of LC column (from 20 to 70°C), and eluent flow (from 0.25 to 0.35 mL/min) on the reproducibility of various retention parameters.

Table 1 presents the results of statistical processing of net retention times (t_R), retention factors (log k′) and retention indices (RI) of reference *n*-alkyl phenyl ketones $PhCOC_nH_{2n+1}$ ($1 \le n \le 6$) measured in seven randomly selected regimes of HPLC elution.

These data illustrate that reproducibility (relative standard deviations) of net retention times varies within 9–14%, appears slightly better for retention

Table 1. Comparison of the reproducibility of absolute retention times (t_R), retention factors (capacity coefficients, log k′) and retention indices (RI) of n-alkyl phenyl ketones at different conditions of chromatographic analyses (N = 7)

Reference *n*-alkyl phenyl ketones [from acetophenone (C_8) to enantophenone (C_{13})]					
C_8	C_9	C_{10}	C_{11}	C_{12}	C_{13}
Absolute retention times (t_R)					
11.3 ± 1.4 (12.7 %)[a]	13.0 ± 1.5 (11.3 %)	14.2 ± 1.9 (13.5 %)	15.4 ± 2.1 (13.8 %)	16.6 ± 1.4 (8.6 %)	17.5 ± 1.6 (9.2 %)
Retention factors (log k′)					
0.93 ± 0.05 (5.4 %)	1.00 ± 0.05 (5.0 %)	1.04 ± 0.06 (5.8 %)	1.08 ± 0.06 (5.6 %)	1.12 ± 0.06 (5.4 %)	1.15 ± 0.07 (6.1 %)
Retention indices (RI)					
—	898 ± 8 (0.9 %)	1000 ± 4 (0.4 %)	1100 ± 5 (0.5 %)	1203 ± 10 (0.8 %)	—

[a]Relative standard deviations of corresponding chromatographic parameters are presented in parentheses.

factors (5–6%), and less than 1% for RIs. Absolute RI standard deviations for reference compounds C_9–C_{12} are 4–12 i.u. The data for the first and for the last *n*-alkyl phenyl ketones within series are omitted, because they cannot be calculated by interpolation using a general relationship (1). The RI evaluation for acetophenone by extrapolation gives appropriate average value, but indicates inappropriate standard deviation (803 ± 35). This example illustrates that just RIs possess the maximal reproducibility among other retention parameters not only in GC, but in RP HPLC also. Hence, they can be used as interlaboratory invariants and should be collected in RI databases.

General formula (1) permits us to calculate RI(HPLC) by interpolation only for compounds eluted not earlier than 11.6 min (retention time of the first reference component). The calculation of these parameters at $t_R < 11.6$ min is a problem of special consideration.

The Algorithm of RI Calculation for Hydrophilic Compounds by Extrapolation of Retention Time Squares

The only known algorithm for determination of RI <800 in RP HPLC with gradient elution has been proposed ten years ago.[25,26] So far as the evaluation of parameter q (formula 4) needed for calculation lin-log RIs is based on the retention times of three reference compounds, it is necessary to choose three points covering the target range of retention times. The first of them obviously should be the retention time of acetophenone (RI = 800), the second t_R was

taken for butyrophenone (RI = 1000), and the third is the point t_0, corresponding to the hypothetical component having RI = 0. Insofar as the term log ($t_R - t_0$) presents in equation (1), when $t_R \to 0$ this logarithm tends to $-\infty$, that is inadmissible in any method of RI calculations. To prevent this uncertainty, retention times of this hypothetical component with RI = 0 were taken to be equal $t_0 + \delta$, where δ is a small non-zero addend (in practice it can be chosen much less than 10^{-3}).

The second feature of this algorithm is the necessity of additional verification of the quality of RI evaluation. Between reference compounds C_8 and C_{10} another one (propiophenone) is placed. Its RI calculation using data for ($t_0 + \delta$), $t_R(C_8)$, and $t_R(C_{10})$ should give the postulated value 900, with the reasonable deviation not more than 0.1–1.0 i.u. If this deviation exceeds 1 i.u., it indicates non-optimal choice of artificial addend δ and the necessity to change it (typically by decreasing in 10^n times). The choice of optimal δ value can be fulfilled by numerical methods.

The third feature of this algorithm is the additional mathematical transformation of retention times. There are a lot of HPLC elution regimes when the ratios $[t_R(C_{10}) - t_R(C_8)]/[t_R(C_8) - t_0]$ are less than 1 (it is typical, for example, at low initial content of organic solvent in eluent and relatively fast gradients). If the "distance" $[t_R(C_8) - t_0]$ is more than $[t_R(C_{10}) - t_R(C_8)]$ it means that conditions of RI calculation by extrapolation are not optimal. To improve this situation it was proposed to increase this ratio by conversion of retention times into their squares, because $[t_R^2(C_{10}) - t_R^2(C_8)]/[t_R^2(C_8) - t_0] \gg [t_R(C_{10}) - t_R(C_8)]/[t_R(C_8) - t_0]$. Initially, it was confirmed that this t_R transformation has no significant influence on the lin-log RIs values.[26]

So far, as lin-log RI system is predestinated for any regimes of chromatographic elution, this method of calculation for RIs less than 800 can be used not only in gradient, but isocratic regimes as well. Some examples of this algorithm application for hydrophilic organic compounds eluted before acetophenone are presented in Table 2.

All RI values for gradient regimes are compared with corresponding values at isocratic conditions, when Equation (2) provides the maximal precision of extrapolation. As it can be seen from these data, there is an appropriate coincidence between RIs determined in various elution regimes. Obviously, RI reproducibility in the range RI < 500 is worse than that for the range $600 <$ RI < 800 and, of course, for RI > 800 (calculation by interpolation). Nevertheless, in all cases, the differences |RI(isocratic)–RI(gradient)| do not exceed standard deviations s_{RI} (caused by instrumental factors) in each of these regimes.

The discussed above algorithm permitted us to start the calculation of RI values for hydrophilic organic compounds eluted before the first reference component in HPLC at gradient elution. However, the lin-log RI system indicates the maximal precision only for processing of data measured at stationary (stepless) regimes, i.e., temperature programming with constant ramp in GC, or analogous gradient elution without isocratic stages in HPLC. Particu-

Table 2. The comparison of retention indices RI(II) of some hydrophilic organic compounds calculated by extrapolation of retention time squares in various regimes of HPLC gradient elution (C and C_0—concentration of acetonitrile; %, R—ramp, %/min)

	Isocratic, C = 20			Isocratic, C = 30			Gradient, $C_0 = 10$, R = 1		Gradient (A), $C_0 = 20$, R = 1		Average RI values		
Compound	t_R (min)	RI (eq.2)	RI (II)	t_R (min)	RI (eq.2)	RI (II)	t_R (min)	RI (II)	t_R (min)	RI (II)	Gradient	Isocratic	Reproducibility in regime A
Hold-up time	2.07			1.96			2.07		2.04				
p-Aminobenzosulfamide	2.61	470	472	2.37	470	456	2.87	482	2.60	464	477 ± 12	464 ± 8	473 ± 19
Furfurol	3.40	586	587	2.95	603	595	4.56	608	3.47	590	592 ± 14	594 ± 8	585 ± 10
5-Methylfurfurol	4.63	670	671	3.49	668	664	6.82	682	4.63	671	671 ± 8	668 ± 5	660 ± 10
Benzaldehyde	—	—	—	—	—	—	10.92	763	7.24	770	768 ± 5	774 ± 5	766 ± 5
$PhCOCH_3$	9.13	800	800	5.58	800	800	13.35	800	8.33	800	—	—	—
Ethyl *p*-aminobenzoate	11.22	833	833	—	—	—	16.23	841	9.51	828	832 ± 9	828 ± 7	829 ± 4
$PhCOC_2H_5$	17.48	900	900	8.85	900	900	20.49	900	12.95	900	—	—	—
$PhCOC_3H_7$ —	35.94	1000		1000	14.81	1000	1000	27.37	1000	18.22	1000	—	—

larly, so far as the problem of RI extrapolation into the area $t_0 < t_R < t_R(C_8)$ is under consideration, no initial isocratic regimes are permitted. This restriction seems to be unjustified in many cases, as it is in our conditions for fast observed analyses of natural phenolic compounds (see Experimental). It is better to elaborate the optimal methods of data processing for various experimental conditions than to operate *vice versa.* Hence, there are objective grounds to improve the method of RI(HPLC) extrapolation.

The Algorithm of RI Calculation by Extrapolation of Retention Concentrations

The essence of the new approach for RI(HPLC) extrapolation is to draw the analogy between GC analyses with initial isothermal periods followed by linear temperature programming, and those of HPLC with initial isocratic regimes followed by gradient elution. It is known, that in the original publication of Van den Dool and Kratz,[17] introducing the system of linear RIs in chromatography, not retention times, but so-called retention temperatures (T_R) have been proposed for RI calculation with formula (1) with $f(t_R) = T_R$. Later, T_R values in this RI system have been replaced by t_R owing to the higher precision of their measurement and direct proportionality of both of these parameters at linear temperature programming, i.e. $T_R = T_0 + r\ t_R$, where T_0 is the initial temperature of GC process and r–ramp, grad/min. Nevertheless, the approach based on T_R values remains important for any stepwise temperature regimes, but the dependence $T_R(t_R)$ becomes more complex.[27] For instance, if the GC regime includes initial isotherm (temperature T_1, duration t_1), followed by ramp r up to temperature T_2 at the moment t_2, the calculation of retention temperatures needs use of three equations system [but the dependence $T_R(t_R)$ remains smooth]:

$$\begin{aligned} T_R(t_R \le t_1) &= T_1 \\ T_R(t_1 \le t_R \le t_2) &= T_1 + r(t_R - t_1)^2/(2t_R) \\ T_R(t_R \ge t_2) &= T_2 + (t_2 - t_1)(T_2 - T_1)/(2t_R) - t_2(T_2 - T_1)/t_R \end{aligned} \tag{5}$$

The last relationship indicates that at $t_R > t_2$ T_R values asymptotically tend to the limit T_2, but theoretically cannot reach it. Following use of these T_R values in formula (1) provides an appropriate precision of RI calculation at multi-step temperature programming in GC. One can believe, that a similar approach can be expanded on RP HPLC with multi-step gradient elution regimes. For this purpose, by analogy with retention temperatures, it is necessary to introduce the term "retention concentrations" (C_R) keeping in mind the variable content of organic solvent in eluent.

In this case all relationships (5) remain to be correct in HPLC, but should be re-written with other variables. If the regime of analysis includes initial isocratic period with concentration of organic solvent in the eluent C_1

(duration t_1), followed by linear gradient $C_1 \rightarrow C_2$ at the time $t_1 \rightarrow t_2$ with ramp $R = (C_2 - C_1)/(t_2 - t_1)$, and final isocratic step at $t_R > t_2$ with $C_2 = \text{const}$, thus:

$$\begin{aligned} C_R(t_R \leq t_1) &= C_1 \\ C_R(t_1 \leq t_R \leq t_2) &= C_1 + R(t_R - t_1)^2/(2t_R) \\ C_R(t_R \geq t_2) &= C_2 + (t_2 - t_1)(C_2 - C_1)/(2t_R) - t_2(C_2 - C_1)/t_R \end{aligned} \quad (6)$$

After using these variables, the target relationship for calculation of linear RIs should be presented in the following form:

$$RI_x = RI_n + (RI_{n+1} - RI_n) \times (C_{R,x} - C_{R,1})/(C_{R,2} - C_{R,1}) \quad (7)$$

where $C_{R,x}$, $C_{R,1}$, and $C_{R,2}$—retention concentrations for analyte and reference *n*-alkyl phenyl ketones.

This algorithm seems to be more convenient in comparison with that previously proposed,[25,26] because retention times not three, but only two reference components are needed for calculations. Unfortunately, it cannot be used principally at $t_R < t_1$ when $C_R = C_1 = \text{const}$. It cannot be recommended also for retention times processing within the range $t_1 < t_R < \approx (3t_{R,1} - 2t_{R,2})$ owing to the large errors of extrapolation. In our conditions (see experimental) it means that this method is absolutely inapplicable at $t_R < 3$ min, and seems to be suitable only for approximate RI estimations at $3 < t_R < \approx 7.8$ min. However, only a few most hydrophilic compounds are eluted in this t_R range, namely phloroglucinol (1.98 min), hydroquinone (2.34), resorcinol (4.18), and pyrocatechol (5.29). This fact, no doubt, explains the necessity of further development of RI calculation methods by extrapolation.

Advantages of this approach are the most striking for processing of data measured in regimes with initial isocratic steps and fast gradients, i.e., just in those conditions when the previously proposed method[25,26] seems inapplicable.

Hence, the information needed in RI estimation for compounds eluted before first reference component includes the indication of initial and final concentrations of organic solvent in the eluent (C_1 and C_2), the durations of initial isocratic step (t_1) and gradient ($t_2 - t_1$) and, of course, retention times of analyte and two reference components (preferably acetophenone, RI = 800, and propiophenone, RI = 900). The simplest QBasic program for RI calculation by this method is presented in Appendix 2.

For example, the set of these data for orcinol (5-methyl resorcinol) is (all t_R values are presented in minutes):

$$C_1 = 5\%, t_1 = 3, C_2 = 100\%, t_2 = 18,$$
$$t_{R,1} = 11.602, t_{R,2} = 13.509, t_{R,x} = 8.392$$

Hence, $RI = 637.9 \approx 638$. Determination of the RI of the same compound in another elution regime, namely:

$$C_1 = 5\%, t_1 = 0, C_2 = 100\%, t_2 = 20,$$
$$t_{R,1} = 9.731, t_{R,2} = 12.095, t_{R,x} = 6.336$$

using the same algorithm gives the value 656. The previously evaluated average reference RI value for orcinol, calculated by statistical processing of all available literature and experimental data (taken from the collection of corresponding author) is 649 ± 22. All initial data before averaging have been calculated using previously the proposed algorithm.[25,26] The coincidence of these RI values means that the new and previous extrapolation methods give the comparable results, that is the simultaneous confirmation of the reliability of both of them. Obviously, in the area $RI < 600$ the precision of extrapolation by both methods visibly decreases.

It should be noted, that not only the methods of RI calculation are responsible for low RI reproducibility for most of hydrophilic organic compounds. Especially among these compounds, there is a lot of substances indicating strong dependencies dRI/dC of different signs.[14,26] Some of them are nonlinear, that makes their mathematical processing highly difficult. For illustration of this fact, the plots of functions $RI = f(C)$ for 1-nitropropane ($dRI/dC > 0$) and caffeine ($dRI/dC < 0$) are presented on Figures 1 and 2, respectively. Just these dependencies seem to be the main restriction of RI reproducibility in RP HPLC.

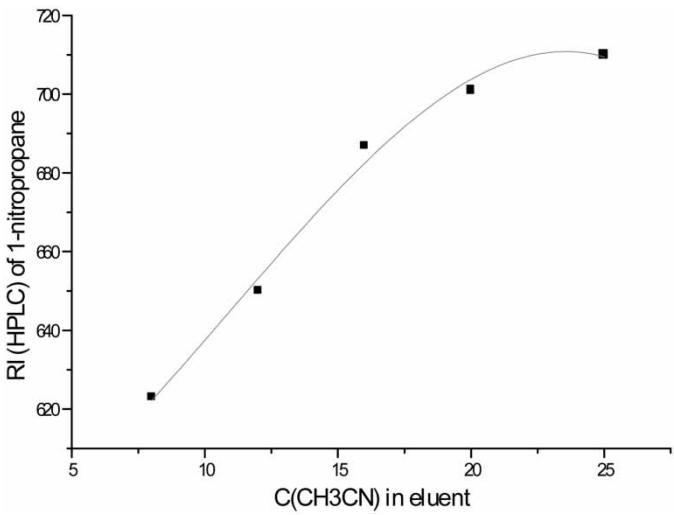


Figure 1. Plot of the dependence of RI(C) for 1-nitropropane (C is the concentration of acetonitrile in the eluent). $dRI/dC > 0$.

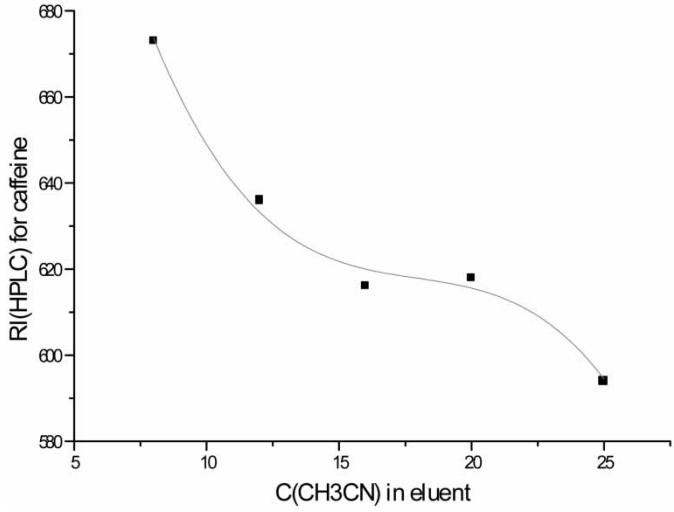


Figure 2. Plot of the dependence of RI(C) for caffeine (C is the concentration of acetonitrile in the eluent). $dRI/dC < 0$.

Some of these anomalies may by caused by objective differences in retention mechanisms of organic compounds in RP HPLC. At low content of organic solvent in the eluent (approx. <5-10% for various solvents) the hydrophobic surface of C_{18} silica gel can be non-moistened by eluent. Moreover, some part of micro pores of this sorbent can be filled with gas (usually air), that leads to the realization not of liquid-liquid partition regime of chromatographic separation, but so-called liquid-gas-solid (LGS) chromatography.[28] Hence, the unknown (in general case) limit of sorbent moistening in RP HPLC seems another restriction of RI calculation for hydrophilic compounds by extrapolation.

Nevertheless, in spite of these "inconvenient" properties of RI(HPLC), more than 60 compounds of phenol and polyphenol series have been characterized by these analytical parameters measured independently in two different laboratories using various algorithms of RI extrapolation [the values RI(I) are obtained with newly proposed method, whilst RI(II)—using extrapolation of retention time squares]. All RI values are additionally compared with previously existed reference data (if available). The list of these compounds is presented in Table 3 in the ascending order of their molecular weights.

All objects characterized by appropriate coincidence between various RI values are marked by word "accepted" in the last column of this table. First determined data are marked by "new value". Each specific case was labeled with symbols (A, B, C, etc.) and shortly commented in the text below.

Table 3. Comparison of retention indices of some phenolic compounds measured at different conditions of reversed phase HPLC analyses and calculated using various algorithms [RI(I)—by extrapolation of retention concentrations; RI(II)—by extrapolation of retention time squares (previously known algorithm)]

Compound	Number of OH groups	MW	RI(I)	RI(II)	Available reference RI data	Comments (see text for details)
Phenol	1	94	659		686 ± 16	Accepted
Benzaldehyde	none	106	758		777 ± 8	Accepted
o-Cresol	1	108	791		775 ± 17	Accepted
m-Cresol	1	108	784		760 ± 15	Accepted
Hydroquinone	2	110	<500	605 ± 4; 630 ± 4	593 ± 22	A
Resorcinol	2	110	*464*[a]	*628 ± 4*	*549 ± 71*	B
Pyrocatechol	2	110	*500*	*648 ± 5*	*568 ± 14*	C
o-Xylenol	1	122	860		860	Accepted
p-Xylenol	1	122	866		864	Accepted
sym-m-Xylenol	1	122	854		857	Accepted
4-Hydroxybenzaldehyde	1	122		*677*	566 ± 28	D
Benzoic acid	1	122	745	750 ± 2	750 ± 7; 633 ± 27	E
Orcinol	1	124	638		649 ± 22	Accepted
Phloroglucinol	3	126	<500	*582*	482 ± 30	F
Pyrogallol	3	126	*784*	*596 ± 9*		G
Cinnamic aldehyde	none	132	853	868 ± 8		Accepted
Cinnamic alcohol	1	134		802 ± 2	803 ± 3	Accepted
Salicylic acid	2	138		*581*		H
Protocatechuic aldehyde	2	138	805			New value

α-Naphthol	1	144		911 ± 2	925 ± 31	Accepted
Coumarin	none	146		777 ± 3	791 ± 13	Accepted
Cinnamic acid	1	148	826	827 ± 5	816 ± 14	Accepted
Thymol	1	150		1017 ± 3	1031 ± 8	Accepted
Vanillin	1	152	769	693	702 ± 22	Accepted
Protocatechuic acid	3	154	806			Accepted
Umbelliferon	1	162		712	728 ± 9	Accepted
Veratric aldehyde	none	166		760 ± 11		New value
2,4-Dimethoxybenzaldehyde	none	166	817			New value
Acetovanillon	1	166	705		685 ± 10	Accepted
Vanillic acid	2	168	790			New value
Gallic acid	4	170	803	593 ± 5	804; 510	I
Esquletin	2	178		654 ± 3		New value
Caffeic acid	3	180	656	664 ± 2		Accepted
Veratric acid	1	182		719 ± 2		New value
Coniferyl alcohol	2	182	791			New value
Anthron	1 (enol)	194		1041 ± 3		New value
(*Z*)-Ferulic acid	2	196	718			New value
(*E*)-Ferulic acid	2	196	726			New value
4-Hydroxystilbene	1	196	1044			New value
Acetosyringone	1	196	715			New value
2,3,4-Trimethoxybenzaldehyde	none			1081 ± 3		New value
Syringic acid	2	198	658			New value
Umbelliferon acetate	none	204		832	792 ± 5	Accepted
(*E*)-Resveratrol	3	228	715			New value
7-Hydroxyflavone	1	238		870 ± 2		New value

(*continued*)

Table 3. Continued.

Compound	Number of OH groups	MW	RI(I)	RI(II)	Available reference RI data	Comments (see text for details)
Pelargonidine (cation)	3	255	844			New value
Formononetin	1	268		986 ± 4		New value
Genistein	3	270		848 ± 3	846	Accepted
Naringenin	3	272	850	856 ± 3	853 ± 5	Accepted
Biochanin A	2	284		892 ± 2		New value
Luteolin	4	286	812	812 ± 4	832 ± 14	Accepted
Kaempferol	4	286		867 ± 3		New value
Fisetin	4	286		768 ± 5		New value
(+)-Catechol	5	290	636	646		Accepted
(−)-*epi*-Catechol	5	290	660	669		Accepted
Ellagic acid	4	302	1260			New value
Quercetin	5	302	805	818 ± 2	817 ± 6	Accepted
Morin	5	302	793	777	794 ± 17	Accepted
Taxifolin (Dihydroquercetin)	5	304	731	736 ± 3		Accepted
Miricetin	6	322		770 ± 4		New value
Cinarosid	7	448		723 ± 3		New value
Rutin (Quercetin-3-rutinoside)	10	712	704	704 ± 2	712 ± 13	Accepted

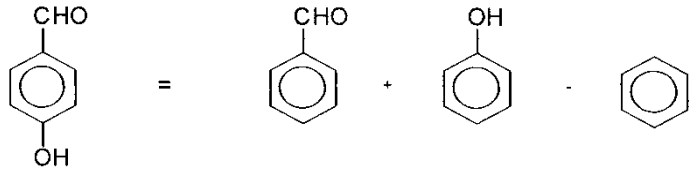
[a]Unaccepted RI values are printed in italics (see text for comments).

Quality Control of RI(HPLC) Data

A. The value RI(I) for hydroquinone cannot be calculated by new proposed method owing to small retention time of this compound ($2.34 < 3$ min), but the application of previous algorithm has no restriction [RI(II)]. Some of hydroquinone samples indicate two peaks on the chromatograms, that is observed in our case. Second of them with RI 630 ± 4 belongs to the complex organic compound—quinhydrone $C_6H_4(OH)_2 \times C_6H_4O_2$—formed from benzoquinone (the product of easy oxidation of hydroquinone by oxygen from air) and hydroquinone itself. At the same time, the RI value of the first peak (605 ± 4) is in a good accordance with previously determined average RI value (593 ± 22) and should be accepted as correct new datum.

Unfortunately, the risk of similar oxidation should be taken into account during analyses of other polyphenols. Maybe, just this reason explains the bad coincidence of interlaboratory data for most of them, namely resorcinol (B), pyrocatechol (C), phloroglucinol (F), and pyrogallol (G). The problem becomes more severe owing to small retention times of these analytes and, hence, high uncertainties of RI evaluation by both considered methods. In cases B and C all three RI values are strongly different and no one of them can be accepted. For phloroglucinol (F) the non-calculated RI(I) value (<500) principally corresponds to the average reference RI 482 ± 30, but RI(II) is inappropriate. The value RI(I) = 784 for pyrogallol seems absolutely impossible, but the reason of its appearance remains unknown. Hence, all these data need additional experimental verification.

D. Experimentally measured RI(II) value for 4-hydroxybenzaldehyde (677) strongly differs from available average reference data (566 ± 28). In similar cases (for not very complex organic compounds having no innermolecular hydrogen bonds), RI values can be approximately evaluated using known data for structural analogues and simplest additive schemes. For instance, RI of 4-hydroxybenzaldehyde can be predicted (by analogy with the method discussed in ref [29]) using following structural transformations of molecules:



$$\mathrm{RI(HPLC)} = \mathrm{X} = (777 \pm 8) + (686 \pm 16) - (932 \pm 17)$$

It gives $\mathrm{X} = 531 \pm 25$, that means "old" average reference RI value 566 ± 28 seems more reliable than "new" RI = 677. The reason of this discrepancy may be in easy oxidation of this aldehyde (e.g., during long time

storage) by oxygen from air resulting in formation of 4-hydroxybenzoic acid. Undoubtedly, this assumption needs extra verification.

E. All three RI values for benzoic acid (pKa = 4.20) are in good accordance with each other. However, if the eluent with pH > 4.5 is used, this acid is eluted as an anion, having another RI value, namely 633 ± 27. At pH ≈ pKa, or when the buffer capacity of eluent is too small, any RI values within the range 630 < RI < 750 can be obtained.

H. The same previous problem exists for salicylic acid, but, moreover, this compound indicates strong dRI/dC < 0 dependence.[26] Thus, the new single RI value cannot entirely characterize this dependence.

I. Observed differences in RI values for gallic acid are similar to those for benzoic acid. The highest values RI(I) = 803 and 804 (average reference datum) obviously belong to the H-form of this acid, while the minimal (another reference datum 510) to the corresponding anion.

Possibly, the suspicious RI(I) values for protocatechuic aldehyde (805) and corresponding acid (806) need the extra verification. However, small RI differences for arenecarboxylic acids and arenecarboxaldehydes are confirmed by numerously measured reliable data for benzaldehyde (758, 750 ± 8) and benzoic acid (745, 750 ± 2, 750 ± 7), also indicating small differences.

Keeping in mind these above mentioned comments, all "suspicious" RI values in Table 3 are printed in italics. Most of the compounds are characterized by reliable data, which can be used for supplementing RI databases and applied for identification of phenolic compounds in various samples.

The determination of reliable RI data in RP HPLC is the first step in formation of comprehensive evaluated RI collections, that is necessary for increasing of the information content of these analytical data.

CONCLUSION

The progress of contemporary HPLC methods seems impossible without application of a retention index concept. Keeping in mind that when many hydrophilic organic substances are analyzed by HPLC techniques, the problem of RI determination for these compounds, when eluted before the first reference component of Smith's RI system (which is based on the series of *n*-alkyl phenyl ketones) seems very a propos for any gradient elution regimes.

Two algorithms of RI calculation in these conditions are compared. One of them has been proposed previously and implies the extrapolation of retention time squares using the linear-logarithmic indices system. It provides the appropriate precision of results in stepless elution regimes with slow gradients. The second approach is proposed in the present paper and is based on the extrapolation of retention concentrations. It is better applicable to regimes with initial isocratic stages, followed by fast gradients. The results of RI calculation for sets of hydrophilic phenolic compounds indicate their high interlaboratory reproducibility.

Nevertheless, two compared algorithms are only the particular cases of HPLC data processing. Neither one of them can be considered as the general solution of the problem of RI determination within the range between t_0 (hold-up time) and retention time of the first reference component in gradient elution regimes. This fact implies the need for further consideration of this problem.

APPENDIX 1

QBasic program for calculation of linear-logarithmic retention indices

```
REM: PROGRAM
PRINT: "Calculation lin-log retention indices"
PRINT "*****************************************": PRINT
PRINT "input dead time (zero or nothing if you don't know this parameter"
PRINT "t (0) = ";: INPUT t: PRINT
PRINT "input the numbers of carbon atoms and the retention times of";
PRINT "three reference n-alkanes": PRINT "C(1), t(1)";: INPUT C(1), t(1)
PRINT "C(2), t(2)";: INPUT C(2), t(2): PRINT "C(3), t(3)";: INPUT C(3), t(3)
t(1) = t(1)-t: t(2) = t(2)-t: t(3) = t(3)-t: PRINT
Q = (C(3)-C(2)) * t(1) + (C(2)-C(1)) * t(3)-(C(3) - C(1)) * t(2)
P = (C(3) - C(2)) * LOG (t(!) + (C(2)-C(1)) * LOG (t(3)) - (C(3) - C(1)) *
LOG (t(2))
Q = - .01 * INT (100 * Q/P + .5): R(1) = t(1) + Q * LOG(t(1))
R(2) = t(2) + Q * LOG(t(2))
PRINT "the value of q for regime being used is"; Q: PRINT
2: PRINT "input retention time of analyte (zero or nothing for exit)": PRINT
"t(X) = "; INPUT X: IF X = 0 then 1
X = X-t: X = X + Q * LOG(X)
I = C(1) + (C(2)-C(1)) * (X-R(1))/(R(2)-R(1)): I = 100 * I: PRINT
PRINT "RETENTION INDEX is";.1 * INT(10 * I + .5): PRINT: GOTO 2
1: PRINT "END OF CALCULATIONS": PRINT: END
```

APPENDIX 2

Program for calculation of retention indices for hydrophilic compounds having retention times less than those for the first reference component by extrapolation of retention concentrations

```
REM: PROGRAM FOR CALCULATION RI(HPLC) < 800
REM: FOR ONE-STEP ISOCRATIC REGIME FOLLOWED BY FAST
GRADIENT"
PRINT: PRINT "************************************": PRINT
```

```
PRINT "CALCULATION OF RI(HPLC) BY EXTRAPOLATION OF";
PRINT "RETENTON CONCENTRATIONS": PRINT
PRINT "INPUT INITIAL CONCENTRATION OF ORGANIC SOLVENT,
C1 (%)";
PRINT "AND INITIAL ISOCRATIC TIME, T(MIN)";: INPUT C1, T1:
PRINT
PRINT "INPUT FINAL CONCENTRATION OF ORGANIC SOLVENT, C2
(%)";
PRINT "AND TIME OF FINAL ISOCRATIC REGIME, T(MIN)";: INPUT
C2, T2
PRINT: R = (C2 - C1)/(T2 - T1)
PRINT "Input retention time of the first reference compound, RI = 800";:
INPUT R1
PRINT
PRINT "Input retention time of the second reference compound, RI = 900";:
INPUT R2
PRINT: L = 3*R1 − 2*R2: PRINT: IF L < 0 THEN 1
PRINT "WARNINGS !"
PRINT "1. DO NOT USE THIS PROGRAM FOR PROCESSING";
PRINT "OF DATA LESS THEN"; T1; "MIN": PRINT
PRINT "2. APPLICATION OF THIS PROGRAM FOR RET. TIMES";
PRINT "LESS THEN";.1*INT(10*L + .5); "MIN"
PRINT "LEADS TO HIGH UNCERTAINTY OF RESULTS": PRINT:
GOTO 2
1: PRINT "CHOOSE ANOTHER ALGORITHM FOR RI CALCULATION":
GOTO 12
2: PRINT "INPUT RET. TIME OF ANALYTE, RT(X,MIN)";
INPUT X: IF X > T1 THEN 3
C = C1: GOTO 5
3: IF X > T2 THEN 4
C = C1 + R*(X - T1)^2/(2*X): GOTO 5
4: C = C2 + (T2 − T1)*(C2 − C1)/(2*X) - T2*(C2 − C1)/X
5: IF R1 > T1 THEN 6
L1 = C1: GOTO 8
6: IF R1 > T2 THEN 7
L1 = C1 + R*(R1 − T1)^2/(2*R1): GOTO 8
7: L1 = C2 + (T2 − T1)*(C2 − C1)/(2*R1) − T2*(C2 − C1)/R1
8: IF R2 > T1 THEN 9
L2 = C1: GOTO 11
9: IF R2 > T2 THEN 10
L2 = C1 + R*(R2 − T1)^2/(2*R2): GOTO 11
10: L2 = C2 + (T2 − T1)*(C2 − C1)/(2*R2) − T2*(C2 − C1)/R2
11: PRINT: I = 800 + 100*(C − L1)/(L2 − L1): PRINT
PRINT "RI(X) = ";.1*INT(10*I + .5): PRINT
PRINT "REPEAT (# 0) ?";: INPUT G: IF G = 0 THEN 12:
```

GOTO 2
12: PRINT "END OF CALCULATIONS": END

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