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Features and Advantages of the Recurrent Approximation of Retention Times in Reversed-Phase High-Performance Liquid Chromatography

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Abstract: Background: Recurrent approximation of retention parameters in reversed-phase HPLC is effective for revealing anomalies difficult to detect otherwise, namely, the reversible hydration of analytes. This was demonstrated previously for restricted sets of analytes with acetonitrile–water eluents. Expanding the number of analytes and eluents seems to be a topical problem. Two kinds of derivatives of aromatic carbonyl compounds were characterized: unsubstituted hydrazones and oximes. **Methods:** If analyte demonstrates no anomalies in dependences of retention times vs. concentration of organic modifier, the recurrent approximations of these dependences are linear. To explain the features of recurrent approximations, the numerical experiments were proposed and considered. The artificial shifting of one, two, or more points allows modeling the different kinds of deviations of approximations from linearity. **Results:** It was shown that hydrazones are the class of analytes having no anomalies of retention parameters. On the contrary, several anomalies were detected for oximes. Downward deviations of points in the plots of recurrent approximations of retention times are the signs of reversible hydration. This effect for methanol–water eluents was detected for the first time. Some of oximes underwent hydrolysis. **Conclusion:** Recurrent approximation of retention times allows detecting chemical transformations of analytes during RP HPLC analysis.

Keywords: Reversed-phase HPLC; retention times; recurrent approximation; detection of anomalies; chemical transformations of analytes

1. Introduction

One of the general approaches in reversed-phase high-performance liquid chromatography (RP HPLC) for characterizing both analytes and sorption materials in chromatographic columns is based on the dependences of the retention parameters (retention times t_R, retention volumes, retention factors, etc.) on the content of the organic component in an eluent (C, volume fraction of the organic modifier) [1–6]. As opposed to gas chromatography, in RP HPLC it is accepted that there is no single universal equation for dependences $t_R(C)$. Several functions were proposed in the literature for approximation of these dependencies, namely, hyperbolic correlation (Scott–Kuchera approach [7]), logarithmic dependence (Soczewinski–Wachtmeister equation [8,9]), log–log model (Snyder–Soczewinski equation [8,10]), the second-degree polynomial (proposed by Schoenmakers [11]), and many others. All these relationships are functions of retention parameters at various eluent compositions (at different ratios of organic modifier and water). If the sorption mechanism or chemical nature of an analyte remains the same within the whole range of eluent composition variations, all the equations provide more or less accurate linearization of the dependences $k'(C)$, where k' is retention factor [$k' = (t_R - t_0)/t_0$], and t_0 is hold-up retention time. However, if the sorption mechanism or analyte speciation depends on the eluent composition, anomalies appear in approximation of the retention data. Such anomalies in $k'(C)$ dependences (appearance of minima instead of monotonic variations) are observed for retention parameters of several pyridinium oximes [12], some 4-aminoquinoline derivatives [5], etc.

The variations of the chemical nature of analytes in an eluent can be caused by the following factors:

i.   Shifts of the prototropic equilibria. Such shifts may be due to variations of the concentration of an organic modifier in an eluent (an increase in its concentration causes an increase in pH):

$$B \ + \ H^+ \ \longleftarrow \ [BH]^+ \tag{1}$$
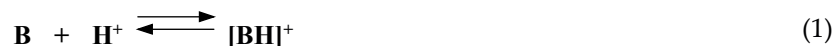
ii.   Reversible shifts of the equilibrium between non-hydrated and hydrated forms of analytes in an eluent. Such shift seems to be most noticeable when hydrolysis constants are close to unity ($K_{hydr} \approx 1$) [13,14]. Unlike inorganic salts, organic analytes preferably form monohydrates:

$$X \ + \ H_2O \ \longleftarrow \ X \times H_2O \tag{2}$$

iii.   The last case seems to be not a common one, but cannot be ruled out. It is the possibility of partial or complete irreversible hydrolysis of analytes during chromatographic runs. An example is the hydrolysis of unsubstituted hydrazones of some carbonyl compounds [15] in acidic eluents at pH < 7. Such processes seem to be most likely for compounds that are formed by condensation reactions and, hence, are most susceptible to the hydrolysis, namely, acetals, ketals, esters, hydrazones, oximes, etc. Even slightly acidic medium of an eluent promotes hydrolysis of such analytes.

However, up to now, most of chromatographic retention anomalies in RP HPLC were usually attributed to changes in sorption mechanisms rather than in properties of analytes.

In this connection, it is important to note that, instead of several equations [7–11], most of the $t_R(C)$ dependences in RP HPLC can be approximated by the single so-called recurrent relations [16,17]. This kind of relations is well known in mathematics, but is rarely used in chemistry:

$$t_R(C + \Delta C) = a t_R(C) + b, \tag{3}$$

where ΔC = const is a constant increment of the organic component concentration in an eluent (in our work, we used $\Delta C = 5$ or 10% *v/v*).

Principal features of linear recurrent relations (3) have been considered in several previous papers (see, e.g., [16,17] and references therein), and there is no need to consider them in detail once again. Recurrent regressions are applied to functions of integer or equidistant values of an argument. They have several unusual mathematical features. First, recurrences unite the properties of arithmetic (at $a \equiv 1$ and $b \neq 0$) and geometric (at $0 < a \neq 1$ and $b \equiv 0$) progressions, which accounts for their unique approximating ability. The applicability of recurrences to equidistant values of the argument extends their applications to properties that depend on pressure, temperature, or concentrations of sample constituents. Examples of the latter two variables are the dependences of t_R on temperature in gas chromatography and on the content of organic modifier in an eluent in RP HPLC. It is noteworthy that using the recurrence relations in chromatography does not require preliminary determination or calculation of the hold-up time (t_0). Another feature that seems to be important for plotting recurrent dependences is that the values of arguments are not represented in such plots; every point is fixed by two "neighboring" values of functions. Hence, the variations of any value in the initial data set lead to variations of two adjacent points on the plots.

When applied to retention parameters in RP HPLC, recurrence relations (3) are characterized by correlation coefficients $R > 0.999$ for analytes that show no anomalies of their chemical nature or no variations of the sorption mechanism at different contents of an organic modifier in an eluent. This fact can be illustrated by the retention data for acetophenone, the first reference compound for determining retention indices (RI) in RP HPLC. The plot in Figure 1a represents the initial approximately hyperbolical dependence of acetophenone retention times, t_R, on the concentration of methanol in an eluent, C. The set of $t_R(C)$ data is indicated in the caption to Figure 1. As the retention of acetophenone on C_{18} silica gel in RP HPLC is characterized by no anomalies, the recurrent approximation of these data at Figure 1b is linear with the correlation coefficient $R = 0.99999$. Other parameters of linear regression are listed in the caption to the Figure 1, as well.
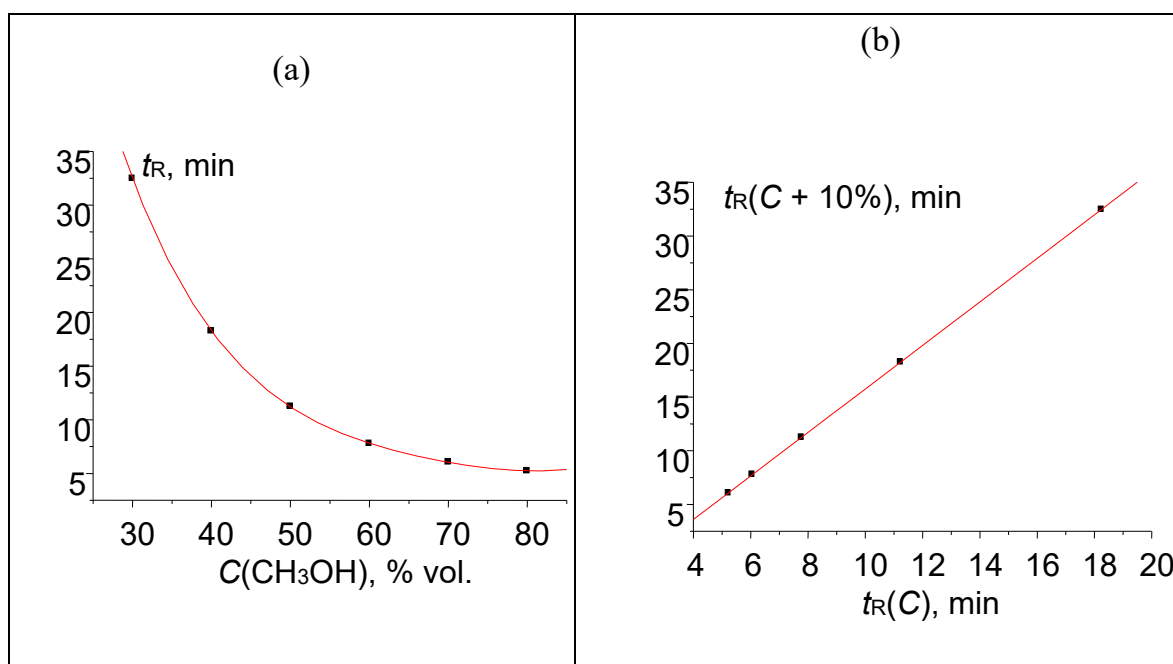
Figure 1. (a) Plot of the acetophenone retention time (min) *vs*. methanol concentration in the eluent, $t_R(C)$: 32.464(30), 18.244(40), 11.230(50), 7.774(60), 6.052(70), and 5.226(80); (b) recurrent approximation of these data: $t_R(C + 10\%) = at_R(C) + b$. Parameters of the linear regression: $a = 0.4932 \pm 0.0005$, $b = 2.231 \pm 0.008$, $R = 0.99999$, $S_0 = 0.01$.

If any of the above-mentioned factors (variations of sorption mechanisms and/or chemical nature of analytes) takes place, the deviations of recurrent dependences (3) from the linearity can be expected. This is due to the fact that the linearization "ability" of recurrences is significant but not infinite; any changes in the ratio of two (or more) coexisting forms of analyte lead to the distortions of the linearity of the recurrent dependences.

The deviations of recurrent dependences from linearity were detected for the first time for such complex organic compounds as synthetic drugs [18] with acetonitrile–water eluents. The subsequent analysis of the causes of this anomaly showed that it is due to the presence of highly polar sulfonamide groups $-SO_2-NRR'$ in analyte molecules. As most of sulfonamides form hydrates in the crystalline state, it was assumed that the cause of retention anomalies is the reversible formation of hydrates in an eluent at high water content. To confirm this assumption, a series of N-substituted arenesulfonamides $ArSO_2NRR'$ were synthesized and characterized; all of them exhibited the similar deviations. It should be emphasized that this effect for arenesulfonamides was observed for the water–acetonitrile eluents and but practically was not observed for the water–methanol eluents [19]. This was attributed to the preferable formation of more stable methanol hydrates. The free energy of methanol hydration was estimated experimentally at -5.1 kcal mol^{-1} [20]; it exceeds the hydration energies of the majority of organic compounds.

Further verification of the concept for hydrate detection in RP HPLC using recurrent approximation of retention times requires considering the following tasks:

- So far as the recurrent approximation still remains to be a non-standard unusual form of data processing, a special consideration of its features and anomalies is required;

- So far as the anomalies of recurrent approximation have previously been observed for acetonitrile–water eluents only, it seems reasonable to find compounds (or classes of compounds) for which these anomalies are observed in methanol–water systems.

With these aims in mind, we examined the features of recurrent approximation of retention parameters in methanol–water eluents for such compounds as unsubstituted hydrazones (I) and oximes (II) of aromatic carbonyl compounds:
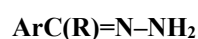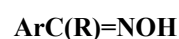
$$\mathbf{ArC(R)=N-NH_2} \qquad\qquad (I) \qquad\qquad \mathbf{ArC(R)=NOH} \qquad\qquad (II)$$
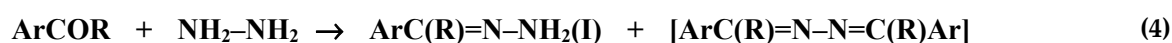
It is interesting that oximes are believed to be more resistant to hydrolysis than hydrazones [21]. Literature data on the formation of hydrates of organic compounds of both these classes are very limited. Formation of hydrates is confirmed for 2-acetyl-3-hydroxybenzo(b)thiophene hydrazone (CAS no. 194783-95-4, information from Merck), 1,3-diphenyl-1H-pyrazole-4-carboxaldehyde 4-nitrophenylhydrazone (CAS no. 1173017-26-9, Merck), and cyclohexanone hydrazone (melting point 102–104°C, no CAS). According to information from ChemSpider, the formation of hydrate (1 : 1) is confirmed for *bis*(3-fluorobenzylidene)hydrazine (it is not a hydrazone but the structurally related azine). Within the group of oximes, the hydrate formation is confirmed for 4-hydroxybenzaldehyde oxime [22,23], 3-(2-mercaptoethyl)-1,3-benzothiazol-2(3H)-one oxime (CAS no. 1173016-19-7), and some more complex compounds.

This study is aimed at detecting retention anomalies for a series of unsubstituted hydrazones and oximes of aromatic carbonyl compounds by considering recurrent approximations of their net retention times, relative optical densities at different wavelengths, $A_{rel} = A(254)/A(220)$, and retention indices (RI) together with their concentration coefficients, $d\mathrm{RI}/dC$.

## 2. Matherial & Methods

Synthesis of hydrazones and oximes. All hydrazones were synthesized from hydrazine hydrate (99%, Lenreaktiv) and corresponding aromatic carbonyl compounds: *p*-methylbenzaldehyde (chemical grade, Reakhim, Moscow), acetophenone, propiophenone, butyrophenone (all from Sigma–Aldrich Rus LLC), and *p*-methylacetophenone (Reakhim, Moscow). The reaction of carbonyl compounds (100 μL of liquid or approx. 100 mg of solid substances) with 1 mL of hydrazine hydrate was carried out at ambient temperature in a solution with 1 mL of isopropyl alcohol (chemical grade, Kriokhrom, St. Petersburg) for homogenization of reaction mixtures. The large molar excess of hydrazine (approx. 25–30) was taken to prevent the formation of azines as the principal by-products:

$$\mathrm{ArCOR} \; + \; \mathrm{NH_2\text{–}NH_2} \; \rightarrow \; \mathrm{ArC(R)\!=\!N\text{–}NH_2(I)} \; + \; [\mathrm{ArC(R)\!=\!N\text{–}N\!=\!C(R)Ar}] \tag{4}$$

All hydrazones were analyzed directly in the reaction mixtures diluted with the mobile phase in the 1 : 100 ratio.

All the oximes were synthesized from hydroxylamine sulfate (Reakhim, Moscow) and corresponding aromatic carbonyl compounds in the presence of a base. The carbonyl precursors were benzaldehydes [2-methyl- (Lancaster, UK), 4-methyl-, 2-hydroxy- (Aldrich, USA), 4-hydroxy- and 2-methoxy- (Fluka, UK), 4-methoxy- (Kiev, former USSR), 3-hydroxy-4-methoxy- (vanillin, Ferak Berlin, Germany), 4-hydroxy-3-methoxy- (isovanillin, Janssens Chimica, Belgium), and 3,4-dimethoxy- (veratric aldehyde, Acros, Belgium)] and alkyl aryl ketones $C_6H_5COC_nH_{2n+1}$ with $n = 1$–3 [acetophenone, propiophenone, and butyrophenone (all from Sigma–Aldrich Rus LLC)]. The same ketones were used as reference components for calculating the hold-up time and determining the retention indices:

$$\mathrm{ArCOR + NH_2OH{\times}H_2SO_4 + 2NaOH} \; \rightarrow \; \mathrm{ArCR\!=\!NOH\ (II) + Na_2SO_4 + 2H_2O} \tag{5}$$
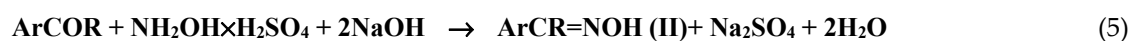
The reactions of carbonyl compounds (100 μL of liquid or approx. 100 mg of solid substances, 0.65–0.80 mM) with approx. 100 mg of hydroxylamine sulfate (0.80 mM) and approx. 40 mg of sodium hydroxide (1 mM, Reakhim, Moscow) were carried out in 2–4 mL of isopropyl alcohol (chemical grade, Kriokhrom, St. Petersburg). The reaction mixtures were kept at ambient temperature for approx. 24 h with intermittent stirring to complete the reactions, which was followed by dilution with the mobile phase by a factor of 10^3.

All oximes as well as hydrazones were characterized directly in reaction mixtures (without isolation). This is permitted if their formation can be considered as a derivatization reaction [24].

Conditions of HPLC analysis. Chromatographic analyses of reaction mixtures were performed using a Shimadzu LC-20 Prominence liquid chromatograph equipped with a diode-array detector and Phenomenex C18 columns 250 mm long and 4.6 mm i. d. with a sorbent particle size of 5 μm. Water–methanol mobile phases were used in several isocratic modes with 5 or 10% concentration steps of the organic component at an eluent flow rate of 1.0 mL min^{-1} and column temperature of

30°C. No acidic substances were added to the eluent. The samples were injected using an SIL-20A/AC autosampler; the sample volume was 20 μL. To prepare eluents, we used deionized water (resistivity 18.2 MΩcm) prepared using a Milli-Q device (Millipore, USA), acetonitrile (HPLC-gradient grade, Panreac, Spain), and methanol (analytical grade, Kriokhrom, St. Petersburg).The pH values of the eluent were 6.2–6.3. The number of replicate injections of each sample was 2–3. The interinjection variations of the retention times of the target analytes in all the cases did not exceed 0.01–0.02 min.

Data processing. All hydrazones and oximes were characterized by logarithmic (Kovats) retention indices under the conditions of isocratic elution [25]:

$$RI_x = RI_n + (RI_{n+k} - RI_n)\,[\log(t_{R,x}') - \log(t_{R,n}')] \,/\, [\log(t_{R,n+k}') - \log(t_{R,n}')], \tag{6}$$

where t_R', $t_{R,n}'$, and $t_{R,n+k}'$ are the adjusted retention times of the target analyte and reference n-alkyl phenyl ketones $C_6H_5COC_nH_{2n+1}$, $RI_n = 100n_C$ (n_C is the total number of carbon atoms in a molecule) with the closest retention times, $t_R' = t_R - t_0$, $k \geq 1$.

The retention times of the theoretically nonsorbed component (hold-up time, t_0) were evaluated from the net retention times of three reference n-alkyl phenyl ketones using Peterson and Hirsch equation [26]:

$$t_0 \approx (t_{R,1}t_{R,3} - t_{R,2}^2) \,/\, (t_{R,1} + t_{R,3} - 2t_{R,2}) \tag{7}$$

The data were statistically processed using Excel software (Microsoft Office, 2010). ORIGIN software (versions 4.1 and 8.1) was used for calculating the parameters of recurrences and plotting the regression equations.

Allhydrazones and oximes were characterized by relative optical densities at different wavelengths:

$$A_{rel} = A(\lambda_1) \,/\, A(\lambda_2) \approx S(\lambda_1) \,/\, S(\lambda_2) \tag{8}$$

where λ_1 was selected as 254 nm and λ_2, as 220 nm.

The A_{rel} values were used for controlling the completeness of the transformation of carbonyl compounds to oximes. In addition, they are informative for revealing possible changes in the chemical nature of analytes at different concentrations of an organic modifier in an eluent.

3. Results &Discussion

Identifying anomalies of recurrent approximation by numerical experiments. So far as recurrent approximation of retention parameters in RP HPLC is still a nonstandard method of data processing, a special consideration of its features seems to be desirable. Let us start the discussion with the experimental dependences $t_R(C)$ and corresponding recurrent approximation for acetophenone, presented in Figure 1 above. Nonlinear dependence $t_R(C)$ in RP HPLC (a) can be approximated by various functions (hyperbolic, exponential, polynomial, etc.) with the comparable accuracy. The curves of approximation with 4th degree polynomials are shown for all the cases considered below. This approach is similar to the proposition of R. Kaiser [27] to use 5th degree polynomials for approximating most of chromatographic dependences. As for the recurrent approximation (b), it is a practically straight line (correlation coefficient $R = 0.99999$) due to the absence of any retention anomalies for acetophenone[1].

The numerical experiments discussed below consist in the artificial distortions of some t_R values in the initial data set. To optimize the discussion, let us restrict these distortions by shifting the maximal t_R values only, which correspond to the maximal content of water in an eluent.

The first kind of distortion is a hypothetical increase in the value of 32.464 min by two minutes (for methanol content $C = 30\ \% \ v/v$), namely 32.464 → 34.464 min. Such deviation has practically no effect on the shape of dependence $t_R(C)$, as can be seen in Figure 2(a). It means that one cannot detect

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[1] It is interesting that n-alkyl phenyl ketones having no retention anomalies in RP HPLC were chosen, maybe intuitively, as the reference compounds for the determination of retention indices in the mid-1980s.

such anomaly visually by considering the "raw" retention data. Moreover, the applications of the known mathematical methods of data processing (e.g., numerical differentiation, or finite-differences method [28,29]) do not allow us to detect anomalous values and flag them. At the same time, recurrent approximation of retention times immediately allows us to find the anomalous rightmost point on the plot; it deviates downwards from the regression line.
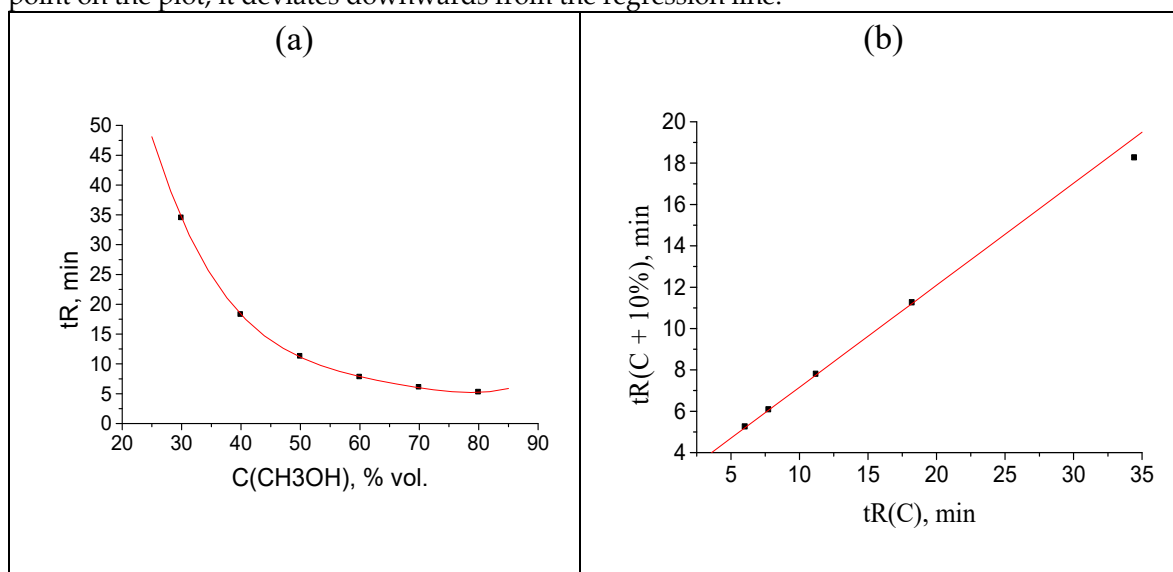
Figure 2. (a) Plot of the acetophenone retention time (one point is shifted: $32.464 \rightarrow 34.464$ min) *vs.* methanol concentration in the eluent, $t_R(C)$; (b) recurrent approximation of these data: $t_R(C + 10\%) = at_R(C) + b$. Parameters of the linear regression (without outlying point): $a = 0.4932 \pm 0.0005$, $b = 2.231 \pm 0.008$, $R = 0.99999$, $S_0 = 0.01$.

Hence, the following rule can be formulated: the downward deviation of the rightmost point on the plot of the recurrent approximation of retention times from the regression line is the sign of the anomalous increase in the maximal t_R value within the data set above its "normal" value. Using the same logical scheme of numerical experiment, if we decrease the value of 32.464 min by two minutes ($\rightarrow$ 30.464 min), the corresponding point appears to be shifted upwards from the regression line, as shown in Figure 3(b). Similarly to the previous case, no visually detected anomalies are observed in the $t_R(C)$ plot (Figure 3a).
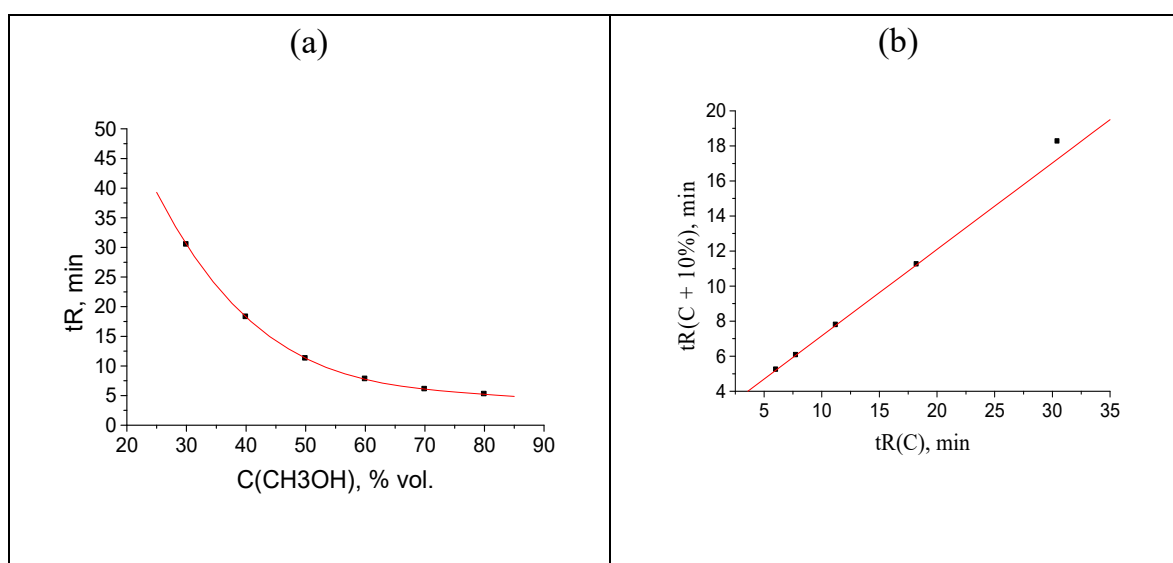
Figure 3. (a) Plot of the acetophenone retention time (one point is shifted: $32.464 \rightarrow 30.464$ min) *vs.* methanol concentration in the eluent, $t_R(C)$; (b) recurrent approximation of these data: $t_R(C + 10\%) =$

$at_R(C) + b$. Parameters of the linear regression (without outlying point) are the same as in the caption to Figure 2.

A more complex case for visual perception using recurrent approximation is a simultaneous shift of two points from their "normal" values (see caption to Figure 1). Let us increase two t_R values, namely, 18.244 → 20.244 and 32.464 → 34.464 min. This case corresponds to rather unusual shift of two points on the plot of recurrent data approximation from the regression line: the rightmost point appears to be located above the regression line, whereas the previous one is located below it, as illustrated in Figure 4.
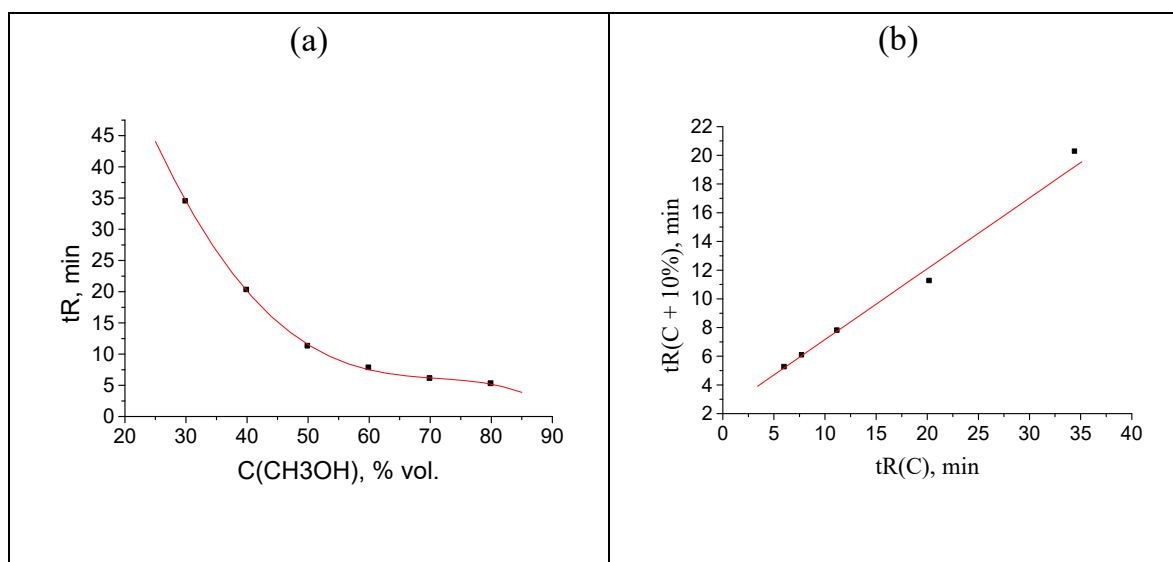
Figure 4. (a) Plot of the acetophenone retention time (two points are shifted: 18.244 → 20.244, 32.464 → 34.464 min) *vs*. methanol concentration in the eluent, $t_R(C)$; (b) recurrent approximation of these data: $t_R(C + 10\%) = at_R(C) + b$.

The downward shifts of two retention times (e.g., 18.244 → 16.244 and 32.464 → 30.464 min) expectedly lead to opposite deviations of two points on the plot (Figure 5b) relative to the case shown in Figure 4.
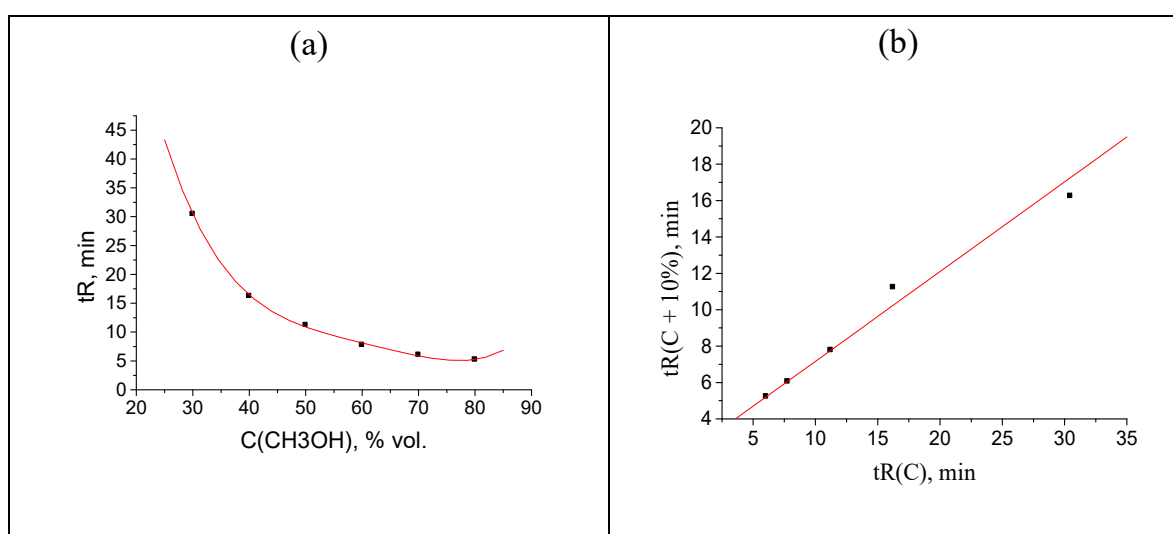
Figure 5. (a) Plot of the acetophenone retention time (two points are shifted: 18.244 → 16.244, 32.464 → 30.464 min) *vs*. methanol concentration in the eluent, $t_R(C)$; (b) recurrent approximation of these data: $t_R(C + 10\%) = at_R(C) + b$.

These unusual graphical representations of distorted sets of retention times are accounted for just by specific properties of recurrent regressions. After these simplest examples (when t_R values of one or two points were distorted), let us consider the example when four minimal t_R values are increased by two minutes each. A graphical manifestation of such distortions is illustrated in Figure 6. The rest of nondistorted t_R values correspond to the rest of the initial data set (straight fragment in Figure 6a), when four distorted t_R values (three points) form a new straight fragment (Figure 6b). Similar cases are observed for recurrent approximation of the temperature dependence of solubility of inorganic salts in water [30]. Let us consider the case when, at high temperatures, the salts exist in the non-hydrated form in solutions, and at low temperatures, in hydrated forms. In this case, the recurrent data approximation consists of two straight linear fragments. Hence, two different linear sections in the recurrent plots of $t_R(C)$ dependences are the sign of the coexistence and/or interconversion of two chemical forms of analytes.
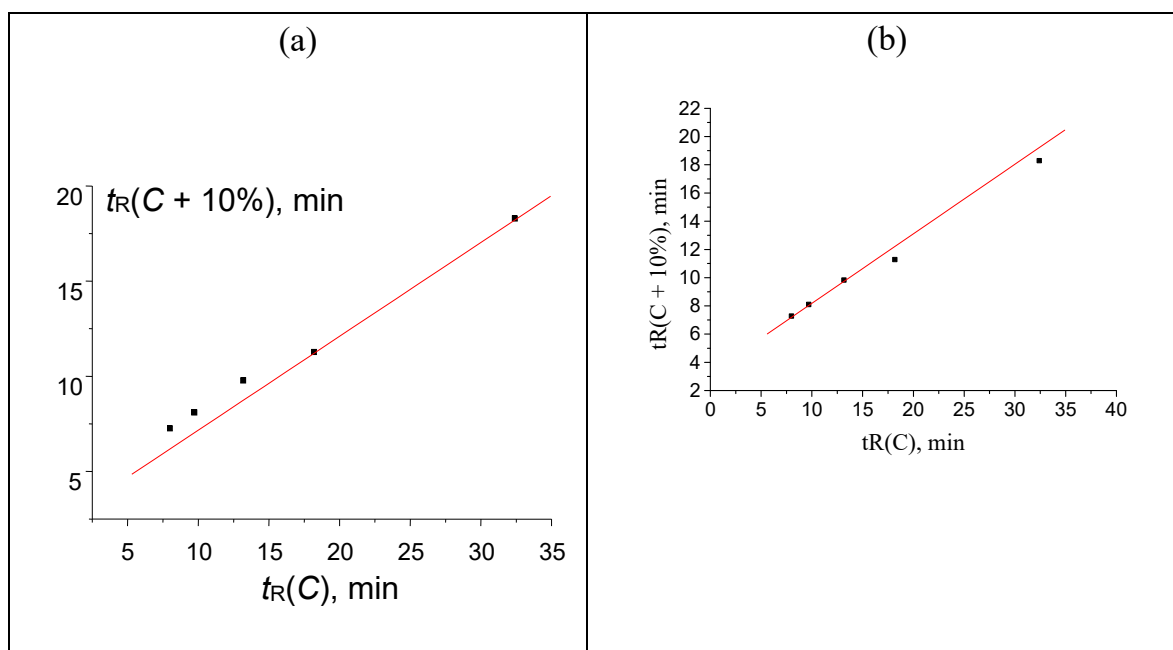
Figure 6. Recurrent approximation of acetophenone retention times (four points are shifted: $5.226 \rightarrow 7.226$, $6.052 \rightarrow 8.052$, $7.774 \rightarrow 9.774$, $11.230 \rightarrow 13.230$ min), $t_R(C + 10\%) = at_R(C) + b$:(a) linear regression line for the subset of nondistorted data and (b) the same for the subset of distorted points.

To summarize the results of these numerical experiments, it should be noted that most the cases mentioned above were found among recurrent approximations of retention times of any analytes under RP HPLC conditions considered below.

Revealing compounds capable of chemical transformations during RP HPLC separation. As noted in the Introduction, there is a class of organic compounds capable of reversible hydrate formation under RP HPLC conditions: it is N-substituted arenesulfonamides. The nonlinearity of recurrent approximations of their retention times appeared to be visually detectable only when acetonitrile–water eluents were used; the analogous dependences for methanol–water eluents turned out to be practically linear. Hence, a question arises: is the linearity of the recurrent dependences a property of the methanol–water eluents, or are there analytes that form hydrates even in the presence of methanol? To answer this question, two series of derivatives of aromatic carbonyl compounds were synthesized: unsubstituted hydrazones ArC(R)=N-NH$_2$ and oximes ArRC(R)=N-OH. The choice of these derivatives is governed by the literature data on the existence of hydrated forms for some of them (see Introduction).

All compounds (hydrazones, oximes, and initial aromatic aldehydes and ketones) were characterized with net retention times, parameters of their recurrent approximations, retention indices (RI), concentration coefficients of retention indices, dRI/dC (primarily for oximes), relative

optical densities $A_{rel} = A(254)/A(220)$, and (for hydrazones and oximes) differences between their RIs and initial carbonyl compounds, ΔRI.

   Unsubstituted hydrazones (of three aldehydes and four ketones) show no anomalies of retention and auxiliary parameters at the methanol content in the eluent within the range 50–85% with the step of 5%.Thus, considering such analytes is not of immediate interest. Nevertheless, the "condensed" analytical data for hydrazones are presented in Table 1 just as an example of the class of organic compounds having no retention anomalies in RP HPLC. This table, along with molecular weights (MW) of hydrazones, includes such "compressed" analytical data as correlation coefficients of recurrent approximation of retention times (R), concentration coefficients of retention indices (dRI/dC) with standard deviations $\pm s_{dRI/dC}$, average values of relative optical densities, $<A_{rel}>$, and average values of differences between RIs of hydrazones and initial carbonyl compounds.

Table 1. Some "compressed" analytical data for unsubstituted hydrazones of aromatic carbonyl compounds at the methanol content C(CH$_3$OH) in the eluent in the range 50–85% v/v: molecular weights (MW), correlation coefficients of recurrent approximation of retention times (R), concentration coefficients of retention indices (dRI/dC) with standard deviations, $s_{dRI/dC}$, average values of relative optical densities within the same range of C(CH$_3$OH), $<A_{rel}>$, and average values of RI differences between hydrazones and initial carbonyl compounds, ΔRI.

Initial carbonyl compound	MW of hydrazone	R	dRI/dC $\pm s_{dRI/dC}$	$<A_{rel}>$	ΔRI
Acetophenone	134	0.9995	-0.1 ± 0.1	1.16 ± 0.09	-64 ± 2
2-Methylbenzaldehyde	134	0.9996	-0.6 ± 0.1	0.60 ± 0.04	-143 ± 6
4-Methylbenzaldehyde	134	0.9997	0.11 ± 0.06	0.98 ± 0.07	-155 ± 4
4-Methylacetophenone	148	0.995	4.3 ± 1.0	1.18 ± 0.03	-70 ± 22
Propiophenone	148	0.99992	2.3 ± 0.5	1.13 ± 0.10	-74 ± 16
Butyrophenone	162	0.99995	1.6 ± 0.4	1.16 ± 0.09	-103 ± 11
2-Hydroxybenzaldehyde	136	0.9997	-0.6 ± 0.1	0.44 ± 0.03	-98 ± 14

   High values of correlation coefficients (R) confirm the linearity of recurrent approximations of retention times of hydrazones, so there is no need to illustrate this fact with plots (in all cases, they are approximately linear). The similarity of A_{rel} values may be used (i) as an indicator of the constancy of a chromophore in analyte molecules, (ii) as a criterion of neglecting any interactions of analytes with eluent components, and (iii) as an additional criterion for identification of analytes in RP HPLC [31]. All $<A_{rel}>$ values are close to each other for all analytes except 2-hydroxybenzaldehyde hydrazone (due to the different chromophore type; oxygen atom of hydroxyl group is conjugated with aromatic system) and 2-methylbenzaldehyde hydrazone (weaker conjugation of aromatic system and CH=N fragment due to steric hindrance produced by the methyl substituent in *ortho*-position).

   The concentration coefficients of retention indices, dRI/$dC \pm s_{dRI/dC}$, are slightly different for hydrazones of aldehydes (from –0.6 to 0.11) and of ketones (from –0.1 to 2.3). The similar differences between derivatives of aldehydes and ketones are observed in ΔRI values: –132 ± 30 for hydrazones of aldehydes and –78 ± 17 for hydrazones of ketones. Both these facts may be of analytical interest for distinguishing the chemical nature of carbonyl compounds.

   As compared to unsubstituted hydrazones, the data for *oximes of aromatic carbonyl compounds* (nine aldehydes and three ketones) exhibit a noticeable number of retention anomalies in RP HPLC. Therefore, their more detailed consideration is necessary. The information on chromatographic parameters of oximes of aromatic carbonyl compounds is summarized in Table 2.

Table 2. Some analytical parameters of oximes of aryl-substituted aromatic carbonyl compounds at different content of methanol in the eluent (C), retention times (t_R), retention indices (RI) and values of dRI/dC, relative optical densities $A_{rel} = A(254/220)$ as a function of C, abd differences ΔRI = RI(oxime) – RI(carbonyl compound). Anomalous values are marked in bold with the indication of the deviation direction (upwards ↑ or downwards ↓). The dash means that the measurement at this concentration was not carried out.

Substituent in phenyl ring	MW of oxime	Analytical parameter	Content of methanol in the eluent, C, % v/v						Comments
			30	40	50	60	70	80	
2-Methyl	135	t_R, min	31.262	21.736	14.261	8.869	**6.399↑**	**5.220↑**	Two straight sub-lines in the plot (see comments in the text); $R_1 = 0.9997$, $R_2 = 0.9997$
		RI	796	826	846	837	825	799	No anomalies (thereafter marked as **NA**); dRI/dC = -1.5 ± 0.3 (R = -0.968)
		A_{rel}	-	0.93	1.18	1.23	0.96	1.17	NA; $<A_{rel}>$ = 1.09 ± 0.14
		A_{rel} (ald)	-	-	4.60	4.77	4.79	4.27	NA; A_{rel} values for oxime and aldehyde are statistically different
		ΔRI	-	-	-24	-41	-60	-77	NA; RI values for oxime and aldehyde are statistically different
4-Methyl	135	t_R, min	-	**28.953↓**	15.001	9.459	6.636	5.320	The value t_R(40)-t_R(50) is below the regression line; R = 0.9998
		RI	-	**806↓**	855	855	842	813	The value RI(40) is less than others; dRI/dC = -1.4 ± 0.5 (R = -0.906)

Compound	No.	Parameter	C1	C2	C3	C4	C5	C6	Comments
		A_{rel}	-	**0.83↓**	1.82	1.78	1.07	1.86	The value A(40) is less than others; $\langle A_{rel}\rangle$ = 1.6 ± 0.4
		A_{rel} (ald)	**-**	**-**	3.63	3.93	3.39	3.20	NA
		ΔRI	-	-	-2	-2	-16	-33	RI values are close to the RI values for aldehyde
2-Hydroxy	137	t_R, min	27.586	16.432	10.798	7.738	6.166	5.266	R = 0.9998
		RI	780	785	792	799	808	805	NA; Average RI value is 795 ± 11
		A_{rel}	0.88	0.86	0.80	0.72	0.82	0.77	$\langle A_{rel}\rangle$ = 0.80 ± 0.06
		A_{rel} (ald)	0.92	0.91	0.84	0.61	0.60	0.59	A_{rel} values for oxime and aldehyde are close to each other
		ΔRI	-2	0	+2	+1	+6	+7	RI values for oxime and aldehyde are close to each other
4-Hydroxy	137	t_R, min	-	18.913	10.817	7.772	6.699	5.981	R = 1.0000
		RI	747	798	792	800	846	886	dRI/dC = 3.3 ± 0.6 (R = -0.969)
		A_{rel}	-	-	0.02	0.07	0.07	0.04	$\langle A_{rel}\rangle$ = 0.04 ± 0.02
		A_{rel} (ald)	-	-	0.04	0.04	0.04	0.04	The values RI and A_{rel} are close to the values for aldehyde
		ΔRI	-	-	41	+4	0	0	
2-Methoxy	151	t_R, min	39.031	18.916	11.151	7.460	5.700	4.853	R = 0.9998
		RI	822	805	799	788	772	746	NA; dRI/dC = -1.4 ± 0.1 (R = -0.978)
		A_{rel}	0.75	0.71	0.76	0.67	0.76	0.54	NA; $\langle A_{rel}\rangle$ = 0.70 ± 0.08
		A_{rel} (ald)	-	-	0.08	0.08	0.08	0.05	NA
		ΔRI	-	-	-103	-119	-136	-146	NA
4-Methoxy		t_R, min	30.999	15.673	9.448	6.647	5.340	4.610	R = 0.9993
		RI	794	778	765	753	741	708	NA; dRI/dC = -

Compound	#	Parameter	C1	C2	C3	C4	C5	C6	Notes
	151								1.31 ± 0.04 ($R = -0.998$)
		A_{rel}	1.23	1.24	1.34	1.29	1.28	**0.52↓**	$<A_{rel}> = 1.28 \pm 0.04$
		A_{rel} (ald)	0.52	0.49	0.52	0.49	0.54	0.63	NA
		ΔRI	-8	-15	-25	-36	-48	-74	NA
4-Hydroxy-3-methoxy (Vanillin)	167	t_R, min	11.444	7.250	5.476	4.698	4.286	4.077	$R = 0.9994$
		RI	672	655	643	635	623	611	NA; $d\mathrm{RI}/dC = -1.17 \pm 0.06$ ($R = -0.995$)
		A_{rel}	0.52	0.54	0.54	0.53	0.55	0.54	NA; $<A_{rel}> = 0.54 \pm 0.01$
		A_{rel} (ald)	**0.33↑**	0.04	0.05	0.04	0.04	0.04	Anomaly in A_{rel} value for initial aldehyde
		ΔRI	-27	-52	-106	-162	-223	-276	NA
3-Hydroxy-4-methoxy (Isovanillin)	167	t_R, min	13.344	7.884	5.700	4.786	4.332	4.094	$R = 0.9993$
		RI	691	669	653	642	630	614	NA; $d\mathrm{RI}/dC = -1.47 \pm 0.09$ ($R = -0.993$)
		A_{rel}	0.54	0.55	0.53	0.53	0.53	0.52	NA; $<A_{rel}> = 0.53 \pm 0.01$
		A_{rel} (ald)	0.05	0.04	0.05	0.05	0.05	0.04	NA
		ΔRI	-55	-47	-97	-154	-216	-272	NA
3,4-Dimethoxy	181	t_R, min	**21.929↓**	11.074	7.174	5.534	4.747	4.374	The value $t_R(30)$-$t_R(40)$ is below the regression line; $R = 0.9995$
		RI	753	724	707	694	680	668	NA; $d\mathrm{RI}/dC = -1.63 \pm 0.14$ ($R = -0.985$)
		A_{rel}	0.58	0.34	0.59	0.62	0.60	0.57	NA; $<A_{rel}> = 0.55 \pm 0.10$
		A_{rel} (ald)	-	-	0.04	0.04	0.04	0.04	NA
		ΔRI	-7	-15	-43	-103	-165	-219	NA
Acetophenone	135	t_R, min	**37.194↓**	18.494	11.428	7.852	6.176	5.264	The value $t_R(30)$-$t_R(40)$ is below the regression line; $R = 0.9998$

Compound	m/z								Notes
		RI	816	802	803	803	810	800	NA; Average RI value is 795 ± 11
		A_{rel}	3.70	3.51	3.31	2.12	2.21	1.97	NA; $<A_{rel}> = 2.8 \pm 0.8$ or $<A_{rel}> = aC + b$, $a = -0.04 \pm 0.01$, $b = 5.0 \pm 0.4$
		A_{rel} (ketone)	-	-	3.34	2.85	2.78	2.53	NA
		ΔRI	16	2	3	3	10	0	The values RI and A_{rel} are practically the same as values for initial ketone
Propiophenone	149	t_R	67.65↓	30.02↑	20.86	11.306	7.50	5.719	The value $t_R(40)$-$t_R(50)$ is upwards, while the value $t_R(30)$-$t_R(40)$ – downwards the regression line; $R = 0.9993$
		RI	887	872	915	902	893	862	NA; dRI/dC = -1.7 ± 0.3 ($R = -0.962$)
		A_{rel}	-	-	1.14	1.16	1.18	0.99	$<A_{rel}> = 1.12 \pm 0.09$
		A_{rel} (ketone)	-	-	1.98	2.56	2.29	1.82	NA
		ΔRI	-13	-28	15	2	-7	-38	NA
Butyrophenone	163	t_R	-	-	36.86	17.89	9.49	6.515	$R = 0.998$
		RI	-	968	1015	1015	987	948	NA; dRI/dC = -2.3 ± 0.6 ($R = -0.932$)
		A_{rel}	-	-	1.09	1.12	1.14	1.13	NA; $<A_{rel}> = 1.12 \pm 0.02$
		A_{rel} (ketone)	-	-	2.78	2.76	2.52	2.37	NA
		ΔRI	-	-32	15	15	-13	-52	NA

For each oxime, this table includes the net retention times at the methanol concentrations (C) within the range 30–80% v/v with 10% step (in some cases, these ranges were reduced, as indicated in Table 2), retention indices, relative optical densities $A_{rel} = A(254)/A(220)$, corresponding A_{rel} values for the initial carbonyl compounds (for confirming the completeness of their conversion), and differences between the retention indices of the oximes and carbonyl precursors, ΔRI. The "integrated" analytical parameters include the correlation coefficients of recurrent approximation of retention times (R) and concentration coefficients of retention indices, dRI/dC. If the dependence of A_{rel} on C is clearly expressed, the parameters of linear regression $A_{rel} = aC + b$ are presented; otherwise, the average values <A_{rel}> with the corresponding standard deviations are given. If no anomalies are observed within the sets of analytical parameters at different methanol contents, they are marked with symbol NA (No Anomalies).

The anomalous values of t_R, RI, and A_{rel} in Table 2 are marked in bold. If these outliers exceed the average values for other data (upward deviations from the regression lines), they are additionally marked with symbol "↑", and in the case of downward deviations, with symbol "↓". For example, the value of A_{rel} for 4-methylbenzaldehyde oxime (0.83↓) at $C(CH_3OH) = 40\%$ is smaller than all other values (within the range 1.07–1.86, average value 1.6 ± 0.4).

At first glance, oximes seem to be more hydrophilic than their carbonyl precursors because of the presence of so-called active hydrogen atom in the OH group. However, comparison of the ΔRI values for these compounds does not confirm this conclusion: The average differences ΔRI = RI(oxime) – RI(carbonyl compound) do not statistically significantly differ from zero and are equal to –20 ± 28 (aldehyde oximes, cf. –132± 30 for aldehyde hydrazones) and –6 ± 21 (ketone oximes, cf. –78 ± 17 for ketone hydrazones). These inconsistency between different polarity and/or hydrophilicity/hydrophobicity characteristics is a known fact, well commented in the review by Katritzky et al. [32]. Some oximes demonstrate extremely large variations of ΔRI values with increasing methanol content of the eluent from 30 to 80%. For instance, for vanillin oxime these variations are from –27 to –276; for isovanillin oxime, from –55 to –272; and for 3,4-dimethoxybenzaldehyde oxime, from –7 to –219 i.u. However, this may result from the presence of other functional groups in the molecules. The additional heuristic value of these large negative ΔRI values is that they confirm the conversion of aromatic carbonyl compounds into the corresponding oximes. At the same time, this fact makes any algorithms of RI precalculation in RP HPLC considerably less reliable; this inconstancy restricts the applicability of additive schemes to evaluating retention indices.

Similar discrepancies can be traced for other polarity characteristics. If we compare such "classical" variables as dipole moments (μ) and dielectric permittivity (ε), both of them are higher for carbonyl compounds. For instance, for benzaldehyde μ = 2.9 D and ε = 17.8, whereas for benzaldoxime μ = 0.9 D and ε = 3.8. On the other hand, the values of the hydrophobicity factor log$P$ are comparable for the carbonyl compounds and their oximes:

Carbonyl compound	log$P$	Mutual relation	log$P$ of oxime
Benzaldehyde	1.46 ± 0.02*	≈	1.49
2-Hydroxybenzaldehyde	1.83 ± 0.19	≈	1.88
4-Hydroxybenzaldehyde	1.3	>	1.2
4-Methoxybenzaldehyde	1.7	>	1.5
Acetophenone	1.70 ± 0.09	<	1.88
Propiophenone	2.23 ± 0.05	≈	2.27

*) The values indicated with standard deviations are calculated using ACD software. The averaged value of the differences of log$P$ values of oximes and carbonyl compounds is –0.01 ± 0.18; i.e., it is statistically insignificant.

As shown recently, the more polar is the analyte, the lower is the concentration coefficient of retention indices, dRI/dC [33]. Hence, these coefficients can be recommended as another polarity characteristic. So far as the coefficients dRI/dC for aromatic oximes (from –1.2 to –1.6) appeared to be

lower than for their carbonyl precursors (from –0.3 to 4.6) and in most cases had even the opposite sign, this polarity criterion is opposite to the previous one.

Joint consideration of the A_{rel} and ΔRI values is of interest to distinguish initial carbonyl compounds and their oximes. In other words, there parameters allow us to control both the completeness of the carbonyl $\rightarrow$ oxime derivatization or the possible hydrolysis of oximes in an eluent. The A_{rel} and ΔRI values for carbonyls and oximes statistically significantly differ for nine compounds of twelve. Only three analytes appeared to be "suspicious" in accordance with these criteria: oximes of 2-hydroxy- and 4-hydroxybenzaldehydes and acetophenone oxime (Table 2). For example, the average difference of A_{rel} values for 2-hydroxybenzaldehyde oxime and corresponding aldehyde itself is 0.06 ± 0.12. In combination with the average ΔRI value of 2 ± 4 only, we must conclude that we deal with the same analyte (no conversion of the initial carbonyl compounds to oxime or hydrolysis of the oxime). The second example is 4-hydroxybenzaldehyde oxime, for which $<\Delta A_{rel}> = 0.02 \pm 0.02$ and ΔRI(60–80% CH_3OH) = 1 ± 2 i.u. (index units). The last suspicious analyte is acetophenone oxime, for which $<\Delta A_{rel}> = -0.5 \pm 0.3$, but ΔRI(30-80% CH_3OH) = 6 ± 6 i.u. The anomalous properties of acetophenone oxime are confirmed by strong dependence $A_{rel} = aC + b$ ($a = -0.04 \pm 0.01$, $b = 5.0 \pm 0.4$). In the case of 4-methylbenzaldehyde oxime, RI values are close to each other, but A_{rel} values are statistically different.

Several causes of this anomaly should be considered. The first version is no reaction between the "problematic" carbonyl compounds and hydroxylamine. The second possible cause is rapid hydrolysis of oximes at the moment of dissolving the reaction mixtures (basic media) in a large amount of a water-containing eluent. Finally (third cause), the hydrolysis of oximes seems to be possible during separation within the chromatographic column. This process was observed for unsubstituted hydrazones of aromatic aldehydes separated using eluents containing acidic additives [15]. Hence, the most likely cause for the anomalies observed for the three oximes is the second factor: rapid hydrolysis of oximes during dissolution of reaction mixtures in the eluent. All the dRI/dC values determined for such "problematic" oximes were excluded from further consideration.

Anomalies in recurrent approximation of retention times of oximes. The results of numerical experiments (see above) confirm the existence of several kinds of anomalies in recurrent approximation of retention times. The most common anomaly appears to be the downward deviation of the points corresponding to the highest water content in an eluent from the regression lines. Such deviations suggest the conversion of analytes to more hydrophilic forms, in particular, reversible formation of hydrates. Among the compounds considered in this work, such behavior has been confirmed for oximes of 4-methyl-, 2-methoxy-, and 3,4-dimethoxybenzaldehyde oximes. The recurrent approximation of retention times for 2-methoxybenzaldehyde oxime is plotted in Figure 7 as an example. It is completely analogous to the plot of numerical experiment results, shown in Figure 2:
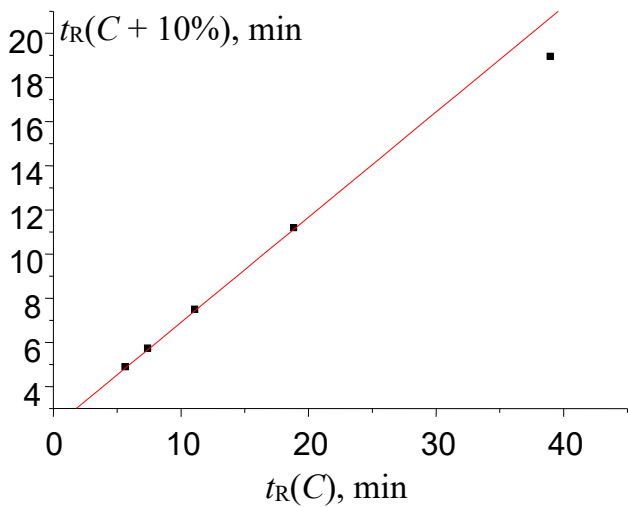
Figure 7. The plot illustrating the anomaly (the point below the regression line) in the recurrent approximation of retention times for 2-methoxybenzaldehyde oxime. Parameters of the linear regression (without outlier): $a = 0.4763 \pm 0.0006$, $b = 2.144 \pm 0.007$, $R = 1.000$, $S_0 = 0.006$.

It should be noted that the downward deviations of points from regression lines due to formation of hydrates were observed previously for acetonitrile–water eluents only. Just oximes of aromatic carbonyl compounds demonstrate similar deviations with the methanol–water eluents. Hence, this effect (deviations caused by hydrate formation) is general and it is not a specific property of acetonitrile-containing eluents.

Another kind of transformations of analytes that may affect the linearity of recurrent approximations of retention times may be shifts of the prototropic equilibria. However, in the case of oximes, these processes can be neglected, because their pK_a values strongly exceed pH of the eluent (approx. 6.2–6.3):

Oxime of	pK_a
Benzaldehyde	11.3 ± 0.1
2-Hydroxybenzaldehyde	9.1 ± 0.1*
2-Methoxybenzaldehyde	11.9
Acetophenone	11.4 ± 0.1

*) Lower pK_a value for 2-hydroxybenzaldehyde oxime is caused by the presence of phenolic hydroxyl group.

If the downward deviations of points in the plots of recurrent t_R approximations mean the reversible formation of more hydrophilic hydrated forms of analytes, then, following the same logics, the upward deviations of points should mean the transformation of analytes into *more hydrophobic forms*. It should be noted that no examples of upward deviations of points in the plots of recurrent approximations of retention times (cf. Figure 3) are observed for oximes, because hydrates cannot be more hydrophobic than the initial analytes. Maybe examples of such deviations will be found in the future for other organic compounds.

Instead of that, rare anomaly was found for propiophenone oxime (Table 2). The plot of recurrent approximation of its retention times has two anomalous points; one of them (not the rightmost point, but the previous one also), namely, the point $t_R(40) - t_R(50)$, is located above the regression line, whereas the rightmost point $t_R(30) - t_R(40)$ is located below the regression line (Figure 8).
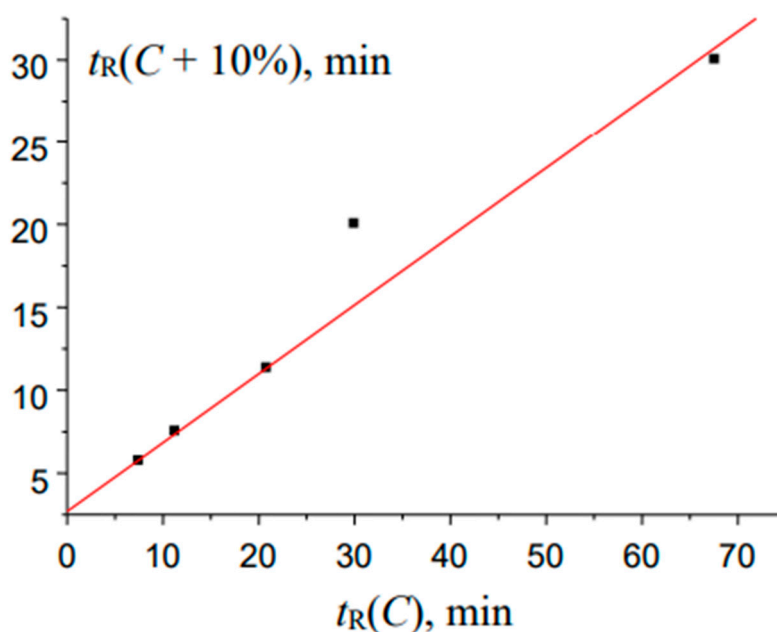
Figure 8. The plot illustrating the anomalous locations of two points in the recurrent approximation of retention times for propiophenone oxime. Parameters of the linear regression (without two outliers): $a = 0.41 \pm 0.02$, $b = 2.144 \pm 0.007$, $R = 0.9993$, $S_0 = 0.15$.

This case is rather similar to one of the numerical experiment results presented in Figure 6. It means that not one, but two maximal values of retention times (at two minimal contents of methanol in the eluent, 30 and 40%) are underestimated. Thus, it is the same effect as those for other oximes (one point is distorted), but more pronounced (two points are distorted). In other words, the hydration of propiophenone oxime is observed at the lower content of water in the eluent than for some other oximes.

Finally, the interesting anomaly of recurrent approximation of retention times is observed for 2-methylbenzaldehyde oxime; the plot of this dependence is shown in Figure 9.
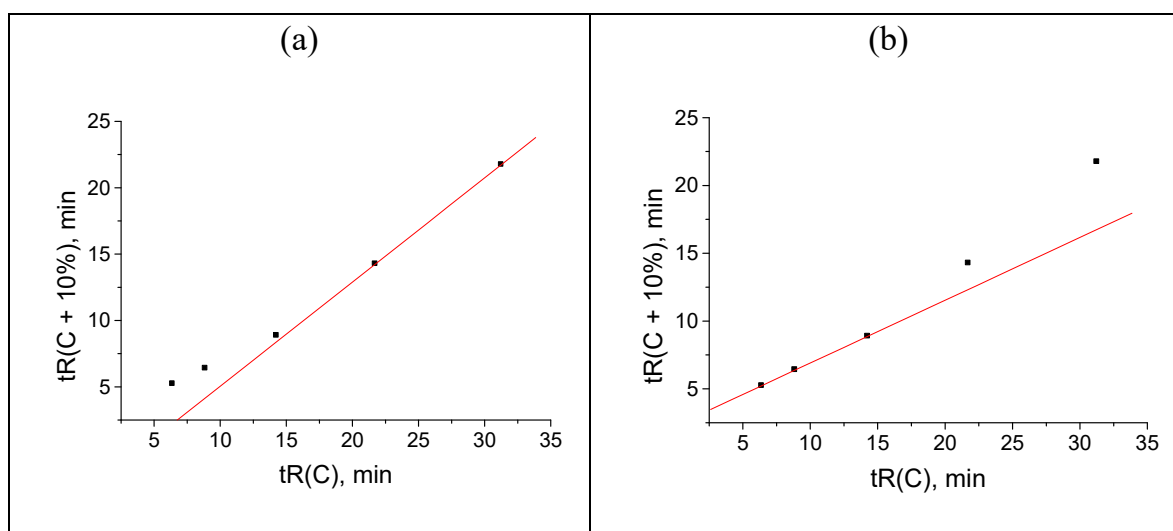
Figure 9. The plot illustrating the anomalous locations of points in the recurrent approximation of the retention times for 2-methylbenzaldehyde oxime (two subsets of points corresponding to two straight-line segments): (a) maximal t_R values for eluents with high water content and (b) minimal t_R values for eluents with lower water content.

Instead of a straight line, or a straight line with one or two outlying points, the plot consists of two straight segments. One of them (a) is formed by two points corresponding to the maximal t_R values for eluents with maximal water content, and the second one (b), by three points, t_R values for eluents with lower water content. Keeping in mind the analogy with the temperature dependence of the solubility of inorganic salts in water [30], this pattern may be caused by the existence of two relatively stable forms of this oxime. At present, it is still difficult to establish what exactly these forms are. The boundary between the hydrated forms of analytes and any products with chemically bound water can be quite blurred. In any case, this kind of anomalies of recurrent approximations of retention times in RP HPLC requires further consideration.

4. Conclusion

Recurrent approximation of retention parameters in RP HPLC proved to be useful in identifying small anomalies in their values that are difficult to detect otherwise, namely, in confirming the reversible hydration of analytes. Before starting this work, these potentialities of recurrent approximations were revealed for a restricted set of organic compounds with acetonitrile–water eluents only. Hence, expansion of the range of chemically different analytes and characterization of methanol–water eluents seem to be topical problems. In this connection, two kinds of derivatives of aromatic carbonyl compounds were characterized: unsubstituted hydrazones and oximes.

Theoretical consideration of the possible anomalies (one, two, or more retention times deviated from their "normal" values) appeared to be useful in their explanation. The downward deviations of points corresponding to the highest water content from the regression lines mean the reversible conversion of analytes to more hydrophilic forms, most often, their hydration. The deviations of two points in opposite directions from regression line suggest the same but more pronounced effect. The "limiting" case is when the plots of recurrent approximation consist of two straight-line segments. By analogy with the temperature dependence of the solubility of inorganic salts in water (when hydrated and non-hydrated forms are observed), this can be attributed to the existence of two different chemical forms of analytes.

Hydrazones of aromatic carbonyl compounds, in contrast to the corresponding oximes, have no anomalies of their retention parameters in RP HPLC. On the contrary, several kinds of anomalies were revealed for oximes. First, some oximes undergo hydrolysis; most likely it happens at the moment of dissolving the reaction mixtures in the methanol–water eluent. The downward deviations of points in the plots of recurrent approximation of retention times of several oximes can be interpreted as the sign of their reversible hydration. For methanol–water eluents, this effect was detected for the first time.

Conflicts of Interest: The authors declare no conflict of interest.

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